Extraction, Characterization and Antioxidant Activity of Fucoidan from New Zealand *Undaria pinnatifida* (Harvey) Suringar

by

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Extraction, Characterization and Antioxidant Activity of Fucoidan from New Zealand *Undaria pinnatifida* (Harvey) Suringar

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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.

Name…….

Signed……

Date……...

Dedication

I wish to dedicate this thesis to the memory of my late mother; my inspiration, Connie Mak. She took care of me when I was young, and I didn't appreciate what she gave me and how much she loved me until she faced terminal illness with astonishing courage and strength right to the end. She taught me to cherish life even to the last second. Completing this degree meant a huge amount to me because she never had the chances that I have been given. Thank you for raising me as a child and making sure I wasn't a troublemaker.

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I would like to convey thanks to Wakatu Corporation for providing us resources during our study by means of allowing us to harvest seaweed from their mussel farms.

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Abstract

The New Zealand government has recently allowed the harvest of *Undaria pinnatifida* (*U. pinnatifida*), an invasive alga, from human-made structures as it has potential commercial value. *U. pinnatifida* is a rich source of fucoidan, which has anticancer effects and can act as an antioxidant and anticancer agent. With the vast amounts of seaweed resources available in New Zealand, it was important to develop methods for extraction and purification of fucoidan from *U. pinnatifida* in order to further investigate its beneficial pharmaceutical properties.

The first part of this thesis involved the study of different methods of fucoidan extraction. This was followed by characterization of fractionated fucoidan, and investigation of its antioxidant activity. *U. pinnatifida* was harvested from mussel farms in the Marlborough Sounds, New Zealand. Three different fucoidan extraction techniques that included calcium chloride, hydrochloric acid and water extraction were used. The calcium chloride extraction gave the best quality fucoidan in terms of its yield, sulphate and fucose content while extraction with deionized water gave the least protein contamination out of the three methods. The calcium chloride extraction method was subsequently used for the extraction of fucoidan. It was also found that the sporophyll part of the alga contained more fucoidan than the blade.

As algae are subject to seasonal variations, monthly changes in the fucoidan content and composition of New Zealand *U. pinnatifida* were investigated. Crude fucoidan was extracted from the sporophyll of *U. pinnatifida* collected from July to October (from three different mussel farms). Fucoidan content increased significantly from July to September (25.4-26.3 to 57.3-70.0% dry weight) as sporulation occurred. At the same time, sulphate content increased significantly from 5.6-5.9 to 13.7-16.4% dry weight in sporophyll-derived fucoidan, and uronic acid increased from 1.4-2.1 to 2.1-3.6% dry weight in blade-derived fucoidan. These changes were probably related to the alga maturity and sporophyll synthesis.

The antioxidant activities of the fucoidan fractions were further investigated. Prior to this, fucoidan was separated into different fractions by means of ion-exchange

chromatography. The fractions were tested for antioxidant activity using the DPPH and CUPRAC assays. Sulphate content in the three fractions isolated, F1, F2 and F3, were 6.96, 22.78 and 25.19%, respectively. The sulphate to fucose ratio also increased from F1 to F3 while the fucoidan fractions showed an increase in reducing ability towards both DPPH radicals and copper ions indicating a relationship between sulphate:fucose ratio with antioxidant activity. The molecular weight of the fractions was also determined. Crude fucoidan had the highest molecular mass of 1350 KDa with the strongest reducing ability. F1 had a molecular weight of 1067 KDa and the last two fractions, F2 and F3, had a mass of around 840 KDa. Results showed that the chemical composition and molecular weight greatly influenced the degree of bioactivity in fucoidan.

1 Introduction

1.1 Problem background

U. pinnatifida is a type of brown seaweed with high nutritional value. It is rich in calcium, iron, iodine, protein, vitamins and beta-carotene (McHugh, 2003; Raffo, Eyras, & Iribarne, 2009). *U. pinnatifida* is native to many parts of the Northern Hemisphere including Japan, Korea and China where it is primarily cultivated for human use. It is found in miso soup and occasionally consumed as a secondary ingredient in tofu salads or as a salted snack (Watanabe, 2006). Japan was the main producer of seaweed (Silva, Woodfield, Cohen, Harris, & Goddard, 2002) but now China has become the major producer of seaweed (Werner, Clarke, & Kraan, 2006) but with popularity increasing around the world, there are now French producers (Silva et al., 2002).

Over the past few decades, *U. pinnatifida* has been accidentally introduced into the French Mediterranean, Argentina, Italy, Australia, the European Atlantic, the United States of America and New Zealand (Silva et al., 2002; Uwai et al., 2006). *U. pinnatifida* arrived in New Zealand in the late 1980s. As it was classed as an unwanted organism, it was illegal to harvest (Hay & Luckens, 1987). Therefore, little research has been carried out on *U. pinnatifida* grown in New Zealand. *U. pinnatifida* can now be found throughout most New Zealand coastal waters on the east coast. Given that it is here to stay and its high commercial value, MAF Biosecurity has recently approved the harvesting of *U. pinnatifida* from man-made structures such as mussel farms (Stuart, 2004).

U. pinnatifida is a rich source of bioactive components, including the polysaccharide fucoidan and the colour pigment fucoxanthin (Fitton, Irhimeh, & Falk, 2007). Fucoidan is a sulphated polysaccharide which gives seaweed its slippery texture (Li, Lu, Wei, & Zhao, 2008). It is found in the cell wall of several types of brown seaweed and protects them from harsh environmental conditions. Fucoidan has recently been reported to possess a wide range of bioactivities including antioxidant and antiviral properties, weight-loss effects and blood-thinning properties (Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008).

Fucoidan is widely available from certain types of seaweed and other sea creatures such as the sea cucumber, therefore increasing types of fucoidan have been extracted and studied in recent years to investigate their potential in the drug and food industry (Li, Lu et al., 2008). This compound is gaining popularity among researchers and consumers as it has been proven to support a number of bodily functions. As a result, several fucoidan structures have been elucidated but there are many more yet to be discovered and analyzed (Li, Lu et al., 2008).

1.2 Purpose of the study

Fucoidan extracted from brown seaweed have been extensively studied in the past and interest in its pharmaceutical properties is growing. Research on fucoidan has so far been carried out in Japan, Korea, France, Australia, and the United States using local and imported *U. pinnatifida* but fucoidan has never been extracted from *U. pinnatifida* grown in New Zealand waters. Furthermore there is little information on the fucoidan composition and its yield in the blades and sporophylls of *U. pinnatifida*. Techniques used to extract fucoidan have a major impact in the composition and yield of fucoidan.

Other factors that may influence fucoidan content and composition include geographic location and harvest season. Previous research suggests that spring is *U. pinnatifida's* peak harvesting season and during this period sporulation of the alga occurs. This is then followed by the degeneration of the blades. Within this period, the maximum amount of fucoidan is found (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2009). However, there is a lack of research to indicate if seasonal variations in the composition and content of fucoidan extracted from the New Zealand *U. pinnatifida* exist. Additionally, variations in the fucoidan content from *U. pinnatifida* harvested from different locations in New Zealand are also unknown.

Hence in the present study, the following aspects of *U. pinnatifida* were investigated: (1) the effect of three extraction techniques on fucoidan composition and content to see how variable the fucoidans may be using different extraction methods; (2) monthly variations of fucoidan during the sporulation of *U. pinnatifida* to see what effect maturation of this alga may have on the content and composition of its fucoidan; (3) characterization of fucoidan fractions by purification of crude fucoidan using ion-

exchange chromatography; (4) antioxidant activity of crude fucoidan and its fractions. With the vast amounts of seaweed resources available in New Zealand, it is important to determine the chemical composition of fucoidan derived from New Zealand *U. pinnatifida* in order to further investigate its beneficial pharmaceutical properties.

1.3 Overview

Chapter 2 describes the biology and reproduction of *U. pinnatifida.* This is followed by a discussion of its status as an unwanted organism and previous research programmes on its distribution and management in New Zealand. In addition, this chapter introduces the bioactive compound, fucoidan. Fucoidan structure, its major bioactive properties, and commonly used fucoidan extraction methods and previous research are outlined.

Chapter 3 compares the three extraction techniques commonly used to extract fucoidan to find the method which produces the best quality fucoidan based in terms of percentage yield, fucose content, sulphate content and uronic acid content. Using the best extraction method, fucoidan was examined in a more detailed manner in Chapters 4 and 5.

Chapter 4 describes the changes in fucoidan content over four months during the peak harvesting period of *U. pinnatifida* in terms of yield and quality. This chapter also explores differences between fucoidan derived from two different locations.

Chapter 5 describes the purification of fucoidan into separate fractions that were subjected to further characterisation. This chapter also examines the antioxidant activity of fucoidan and its fractions using two antioxidant assays.

Chapter 6 describes two additional experiments that assessed the efficiency of the extraction method, and if a commercial *U. pinnatifida* product on the market shelf contained fucoidan. Finally in Chapter 7, the results from Chapters 3 to 6 are summarized and the different aspects of the thesis were considered as a contribution to the existing literature about fucoidan. Limitations in the study and potential future research work were also discussed.

2 Review of the literature

2.1 Introduction

Floating around the waters of the ocean are large amounts of microscopic phytoplankton which is the origin of the ocean's food chain. Along the edges of the ocean lies a much larger organism known as seaweeds. Seaweeds are a group of algae that inhabit the sea or brackish water. They have the ability to use sunlight as an energy source to convert water and carbon dioxide into carbohydrates. Seaweed has evolved into a diverse group of photosynthetic organisms. Each species are grouped according to their size, cell structure, morphology, pigments, polysaccharides, ecology, and the habitat they grow in. Roughly 36,000 known species of algae have been classified, representing an estimated 17% of all algal species. This is a measure of the lack of knowledge we have of algae despite the important role they play on earth (Chopin & Sawhney, 2008).

U. pinnatifida, also known as 'wakame', is a type of brown seaweed which is widely used throughout Japan, China, and Korea as a popular food source. The rate at which *U. pinnatifida* was harvested in certain regions, mainly along the East China Sea coast, eventually superseded the rate at which it grows in its natural habitat, so rope cultivation was introduced in 1955 (Tseng, 1981). *U. pinnatifida*, which is often used in miso soups, sushi and salads is a rich source of calcium, iron, protein, iodine, magnesium and zinc. Besides that, *U. pinnatifida* also shows antioxidant activity, antiviral properties, anti-obesity properties and anti-cancer activity (Hayashi et al., 2008).

U. pinnatifida can reach an overall length of 1-3 metres and consists of a spiral-shaped sporophyll, the reproductive organ of the seaweed, and a midrib which forms the blade and stipe of the seaweed as shown in Figure 1. *U. pinnatifida* is an opportunistic seaweed with the ability to grow and colonise both natural and artificial substrates, from rocky reefs and mudstones to plastic bottles and ropes. It can tolerate from very low amounts to extremely high amounts of sunlight but faces difficulties growing in areas with a large fresh water input (Verlaque, 2007).

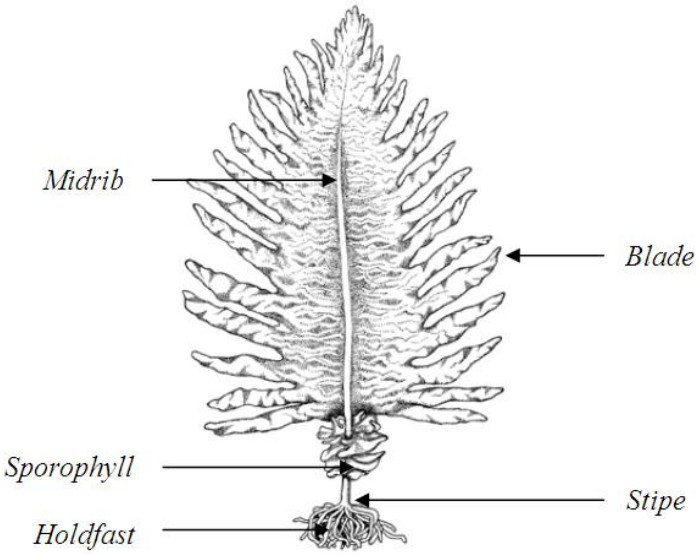

Figure 1 Structure of *U. pinnatifida*. Adapted from "Guide to marine invaders in the gulf of maine" by S. Lonhart (2011, July 22). Retrieved from http://www.mass.gov/czm/invasives/docs/potentialinvaders/u_pinnatifida.pdf

Spring is the main growth period for *U. pinnatifida*. Sporophytes grow rapidly from end of winter through to spring and degenerate during late summer and autumn. In Asia, during the coldest temperatures in winter, sporophytes are not present. On the other hand, the *U. pinnatifida* sporophytes in New Zealand are present throughout the year as winter temperatures are not cold enough to stop their growth, even though New Zealand has a narrower annual temperature range and much cooler summer and spring temperatures (Parsons, 1994).

2.1.1 Biology of *U. pinnatifida*

U. pinnatifida has an annual life cycle, characterised by sporophyte and gametophyte stages (Stuart, 2004). The sporophyll from a mature *U. pinnatifida* can produce millions of spores that drift with the oceans current until they attach onto a suitable surface. The settled spores then germinate into male and female gametophytes, which produce eggs and sperm. A new sporophyte is produced through sexual reproduction when an egg is fertilised, and the life cycle repeats again when the seaweed matures

(Figure 2). *U. pinnatifida* gametophytes can survive being dormant for at least three years, a real problem for places where *U. pinnatifida* is considered a pest (MOF, 2001).

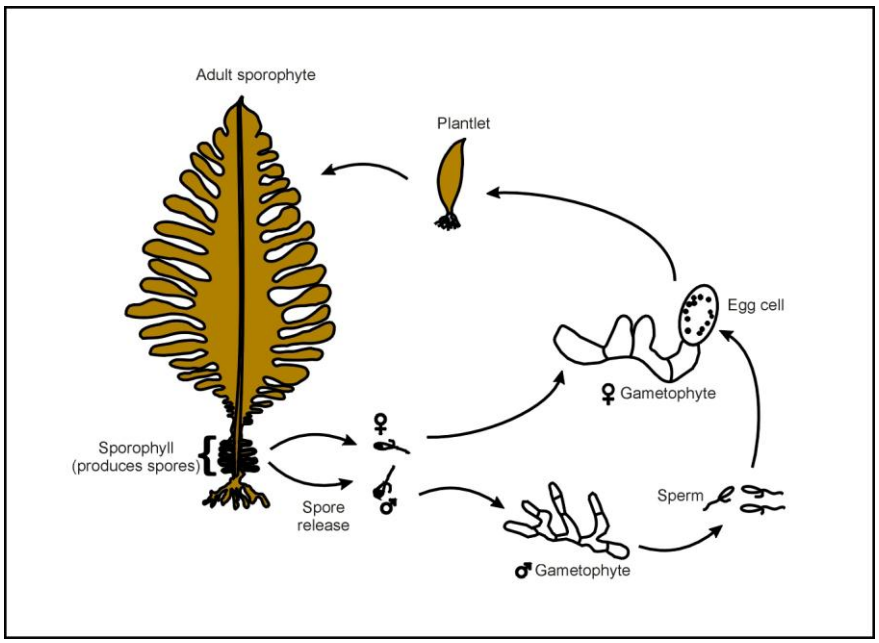

Figure 2 The life cycle of *U. pinnatifida*. Adapted from "Options for a National Pest Management Strategy for the Asian Kelp, Undaria: A Discussion Paper" by J. Sinner, B. Forrest, M. Dodgshun, T. Brown and W. Gibbs, 2000.

2.1.2 Distribution of *U. pinnatifida* in New Zealand

U. pinnatifida is native to Korea, Japan, and China, and has spread to France, Australia, Spain, North and South America, and New Zealand (MOF, 2001). *U. pinnatifida* is now found in 12 countries over four continents, including New Zealand with its first ever sighting in 1987 at Wellington Harbour (Hay & Luckens, 1987). Now after nearly a quarter of a century, *U. pinnatifida* has spread throughout New Zealand's coastal waters, as shown in Figure 3.

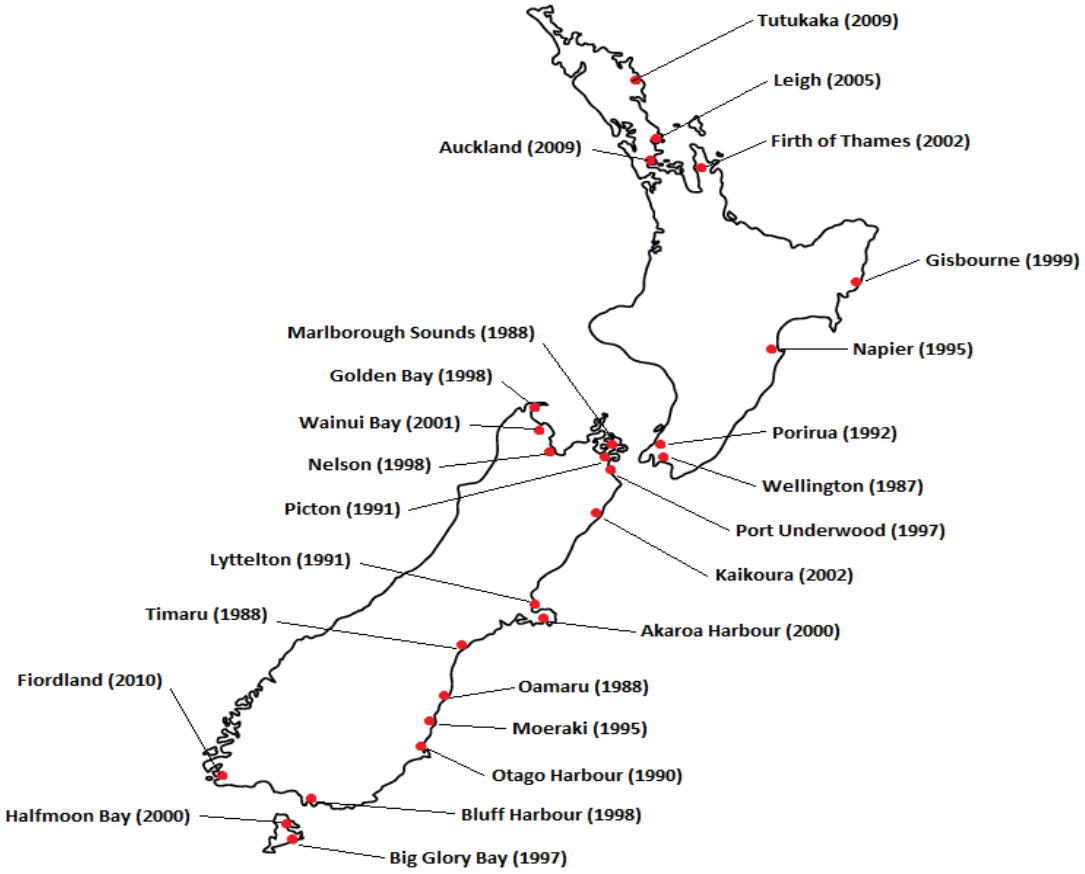

Figure 3 Distribution of *U. pinnatifida* in New Zealand, and year it was first sighted (Carter, 2010; Stuart, 2004).

Spores of *U. pinnatifida* can disperse over hundreds of metres, while whole sporophytes can spread up to a few kilometres. Previous field observations suggested that *U. pinnatifida* can spread from 50 m up to 10 km per annum, whether the dispersal was from natural sources or from human-mediated vectors such as hull fouling or marine farming (Stuart, 2004). It was thought that *U. pinnatifida* was introduced into New Zealand by international shipping in ballast water, and has since spread around our coastal waters by natural sources and by marine equipments or vessels. *U. pinnatifida* was considered an unwanted organism under the Biosecurity Act 1993 (MAF, 2010; MOF, 2001).

2.1.3 Impact of *U. pinnatifida* in New Zealand

U. pinnatifida has a rapid growth rate which may alter the structure and balance of the ecosystem they invade, especially in places where no other seaweeds are growing. However, its impact is not fully understood and is expected to vary depending on the location they are invading (Morelissen, 2012). A potential impact which is most likely to occur is the displacement of coralline algae that directly influence the recruitment of paua. Displacement of other native macroalgal communities and decreased sub-canopy sessile diversity are also likely to occur (MOF, 2001). *U. pinnatifida*, although not scientifically proven to cause direct harm to mussels, are considered a nuisance to mussel farmers as it infests commercial mussel lines, as shown in Figure 4 (Moore, 2011).

Figure 4 Mark Allsopp, Wakatu Research Development Manager looks at an *U. pinnatifida*-infested mussel line, Marlborough Sounds.

U. pinnatifida will also have a substantial impact on the economy. Raffo, et al., (2009) suggest that the inter-tidal and sub-tidal accumulation of *U. pinnatifida* could interfere with beach uses, as well as the practice of snorkelling and scuba diving around the infected area. Costs may increase when harvesting farmed products as well as cleaning and maintaining vessels and ports. A heavier workload on the community may also be needed to eradicate and control the spread of this pest. However, more research is required before we can fully understand the environmental and economical impacts of *U. pinnatifida* in New Zealand (MOF, 2001).

2.2 Management of *U. pinnatifida* in New Zealand

Since its arrival in 1987, *U. pinnatifida* has made New Zealand one of its many habitats around the globe. Eradication programs were applied to control the spread of this founding population but eradication was not achieved even though the spread of *U. pinnatifida* had been reduced (Hunt, Chadderton, Stuart, Cooper, & Carruthers, 2009). Now nearly 25 years later, management programmes have been applied and soon after changed due to the ability of *U. pinnatifida* to cope and reproduce in all sorts of areas and temperatures.

2.2.1 Management history

The Department of Conservation (DoC) and The Ministry of Fisheries (MFish) in New Zealand have worked together closely in managing *U. pinnatifida* since April 1997. In March 2000, *U. pinnatifida* was considered 'unwanted' under the Biosecurity Act 1993. As a result, these management agencies considered the impacts of *U. pinnatifida* on native species and high conservation value areas. They also assessed the risks of transporting farm equipment which could spread *U. pinnatifida* to other locations, and the effects of marine farms providing additional substrates for *U. pinnatifida* to grow on (MOF, 2001; Stuart, 2004).

Little attempt have been made to remove *U. pinnatifida* from infected areas in New Zealand until the implementation of the 2004 policy put in place by the Ministry of

Agriculture and Forestry (MAF) which allowed limited commercial harvest of *U. pinnatifida*. This policy allowed *U. pinnatifida* to be harvested when it is part of a control programme or as a by-catch of some sort of activity such as mussel farming. This policy was soon replaced by a new policy implemented in 2010 (MAF, 2010).

2.2.2 Current management

In 2009, the government reviewed the 2004 policy as the spread of *U. pinnatifida* continued strongly. A new policy came into action from April 2010 and allowed greater commercial use of *U. pinnatifida*. Under this new policy, *U. pinnatifida* still remained an unwanted organism under the Biosecurity Act 1993. This ensured a national oversight to this problem and constrained harvesting to ensure that any activity does not suddenly increase *U. pinnatifida's* impact to the ecosystem. The scope of this new policy allowed harvesting of *U. pinnatifida* in: heavily infested areas, artificial structures, and beach shores but prohibited harvesting on natural surfaces (MAF, 2010).

The reasons behind the plan of allowing *U. pinnatifida* to be harvested from artificial sources and not from natural surfaces was that the removal of *U. pinnatifida* from natural sources could remove or destroy native species growing in that area and eventually provide more space for *U. pinnatifida* to grow on. However, harvesting on natural surfaces was allowed when a control programme is in place. *U. pinnatifida* growing on artificial structures or have been beach cast can be harvested as it is unlikely to lead to a proliferation of *U. pinnatifida* (MAF, 2010). Table 1 outlines how the 2010 policy differs from the 2004 policy.

Table 1 Comparison of the previous and current policy for *U. pinnatifida* (MAF, 2010).

ACTIVITY	2004 Policy	2010 Policy
Harvesting when part of a control programme for *U. pinnatifida*	Yes	Yes
Harvesting as a by-catch of another activity	Yes	Yes
Harvesting from natural surfaces	No	No
Harvesting from artificial structures	No	Yes
Harvesting as a beach cast *U. pinnatifida*	No	Yes
Farming in heavily infested farming areas with *U. pinnatifida*	No	Yes

2.3 Previous research in New Zealand

Since *U. pinnatifida's* first discovery in New Zealand during 1987, programmes have been established to explain its impacts on ecosystems and the extent of its distribution. The DoC, the Cawthron Institute, the MFish, and a number of universities across New Zealand have taken part in implementing measures to control the spread of this pest (Stuart, 2004). However, no research has been carried out on the chemical composition and bioactive components of *U. pinnatifida* grown in New Zealand.

2.3.1 Department of Conservation (DoC)

The first science project investigated by the DoC in 1992 measured and assessed the ecological impact of *U. pinnatifida* on native marine fauna, and the abundance and means of *U. pinnatifida* distribution. The report also discussed the seasonality of the sporophytes (Parsons, 1994). In 1997, *U. pinnatifida* was discovered in Big Glory Bay and a programme was implemented to monitor the sporophyte stage. Divers removed

U. pinnatifida manually to help reduce the sporophyte, but elimination was not achieved (Stuart, 2004).

DoC also carried out experiments on eliminating *U. pinnatifida* attached to floating structures. A treatment that involved heating *U. pinnatifida* at 60^{o}C water for five seconds caused 100% mortality of gametophytes. This technique was soon applied to benthic populations of *U. pinnatifida* in the Chatham Islands, Halfmoon Bay, and Stewart Island. In 1999, a vessel monitoring programme was put in place to evaluate the risk of hull fouling on the dispersal of *U. pinnatifida*. Two years of data was collected from this programme and reported to the MFish (Stuart, 2004).

2.3.2 Cawthron Institute

The Cawthron Institute completed a number of research programmes including a risk-assessment model which estimated the link between transport and establishment of *U. pinnatifida*. They discovered ways to reduce invasion rates of non-native species and means of improving the management of native species. An assessment was also carried out to determine the different pathways *U. pinnatifida* may spread by vessels and marine farming activities. The Cawthron Institute also assessed ways on killing *U. pinnatifida* on marine equipments and seed mussels (Sinner et al., 2000).

2.3.3 Ministry of Fisheries (MFish)

In 2002, a policy toward *U. pinnatifida* was created by the MFish to slow the spread of *U. pinnatifida*. These steps comprised:

- Implementation of vector managing programmes
- Education of marine stakeholder organizations on how to avoid the spread of *U. pinnatifida*
- Support of research to minimize translocation events
- Support of developing treatment methods and learning material

The vessel monitoring programme developed by the DoC was used by the MFish on the *Seafresh 1*, a vessel fouled with *U. pinnatifida* in Hanson Bay. Sections of the vessel

were placed in a 70^{o}C water box for 15 minutes. A total of 524 *U. pinnatifida* sporophytes were removed from the vessel in 2001, and no sporophytes were found on the vessel for the next two years with monthly inspections (Stuart, 2004).

2.3.4 Universities

A number of universities have already undertaken research on *U. pinnatifida* found in New Zealand. The University of Otago has investigated the physiology and ecology of *U. pinnatifida* and worked with the Cawthron Institute in understanding the dispersal characteristics of the algae. The Victoria University of Wellington monitored the spread of *U. pinnatifida* at Island Bay and Wellington, while the University of Canterbury worked on the impacts of *U. pinnatifida* on native flora in shallow waters and identifying factors which could affect the survival of *U. pinnatifida* (Stuart, 2004).

Auckland University of Technology is also making a considerable contribution to the study of *U. pinnatifida*, just after the government restrictions were lifted which allowed it to be harvested from human-made structures i.e. mussel farms. Phycologist Dr. Lindsey White, analytical chemist Dr. John Robertson, food technologist Dr. Nazimah Hamid and pharmacologist Dr. Jun Lu have set up eight separate Master of Science thesis projects that commenced in 2011. These projects looked at the chemical properties of *U. pinnatifida* including fatty acid composition, protein content, and heavy metal concentration, as well as sensory evaluation comparing *U. pinnatifida* grown in New Zealand with other *U. pinnatifida* products grown elsewhere. Two bioactive compounds namely, fucoxanthin and fucoidan were also investigated.

2.4 Fucoidan: A sulphated fucan

2.4.1 Introduction

U. pinnatifida and many other types of seaweed are known to be rich sources of bioactive compounds. The term ‘bioactive compound’ is defined as a substance, which at low concentrations, may be harmful or beneficial to living organisms (Arunkumar, Sivakumar, & Rengasamy, 2010). Bioactive compounds from seaweed polysaccharides

usually have one or more of the following properties: anticoagulant, antithrombotic, antivirus, antitumor, immunomodulatory, antioxidant, and anti-inflammatory (Li, Lu et al., 2008). These properties give seaweed great potential as a food supplement or for the extraction of these bioactive compounds. Many of these properties are generated by the polysaccharide fucoidan (Li, Lu et al., 2008).

The number of published articles on fucoidan-related topics has increased dramatically since the first publication in 1913 (Kylin, 1913). Specifically, this increase took place in the last 5-10 years (Figure 5). Recent interest on fucoidan had focused primarily on the antitumour, anticoagulant, and antioxidant activities, as well as activities against liver and urinary system failures (Ale, Mikkelsen, & Meyer, 2011). As more scientists continue to explore this unique polysaccharide, more of its biological health benefits are being discovered (Hayashi et al., 2008).

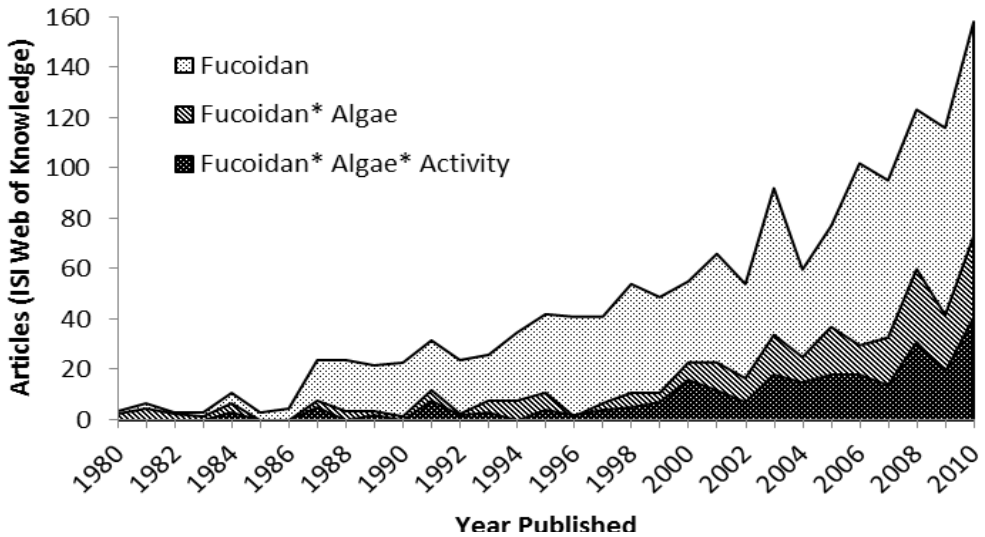

Figure 5 Number of published articles on fucoidan-related work from 1980-2010. Numbers were recorded using the ISI Web of Knowledge search engine with the following keywords: Fucoidan; Fucoidan*Algae; Fucoidan*Algae*Activity (Ale et al., 2011).

2.4.2 Structure

Fucoidan is a natural polysaccharide made essentially of sulphated L-fucose residues. Also known as sulphated fucan, it was first extracted in 1913 from brown algae (Kylin,

1913). Fucoidan is present in the cell walls of brown algae and other animal species, including the sea cucumber and sea urchin. Particularly high amounts of fucoidan are found in *U. pinnatifida* (Bilan et al., 2002; Irhimeh, 2005). Though many studies on identifying the structural properties of fucoidan have been carried out, the structure still remains uncertain due to the absence of strict regularity and the numerous components that make up fucoidan as a whole (Zvyagintseva et al., 2003). Figure 6 shows the general structure of fucoidan but the chemical composition and structure of fucoidan varies with species (Hayashi et al., 2008).

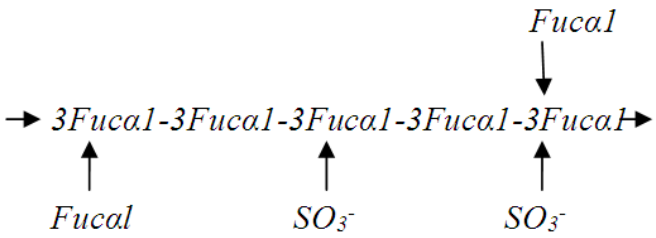

Figure 6 Pankter model of fucoidan (Source: Li, Lu et al., 2008).

Most fucoidans have very complex chemical composition and only little regularity in the structural components is known present (Rioux, Turgeon, & Beaulieu, 2007). Fucoidan largely contains sulphated L-fucose residues. Hence fucose is the primary sugar in fucoidan. Sulphate groups also represent a large component of fucoidan and the biological activities of fucoidan is strongly related to its sulphate content (Yang, Chung, Shin et al., 2008). Besides fucose and sulphate, other monosaccharides (glucose, mannose, galactose, xylose, etc), uronic acids, and even protein are present in detectable amounts. All these compounds have increased the difficulty in structural elucidation of fucoidan (Li, Lu et al., 2008).

2.4.3 Extracting fucoidan

Various extraction methods have been studied and employed in industry to produce and preserve high quality fucoidan. The precise structure of fucoidan is still being debated, mainly due to difficulty in extraction and purification (Marais & Jacob, 2001). Most extraction methods tend to extract fucoidan as a multicomponent crude form of

fucoidan, commonly called crude fucoidan (Eluvakkal, Sivakumar, & Arunkumar, 2010). In order to obtain purified fucoidan, ion-exchange chromatography or gel filtration can be applied to crude fucoidan. Raw seaweeds are usually extracted with acid/base solutions as the solvent (Bilan et al., 2002; Yang, Chung, Shin et al., 2008) but water is now frequently used to extract crude fucoidan as it can maintain the stability of the molecular weight and overall charge of the polysaccharide (Li, et al., 2008). Using water as the solvent is critical in producing high quality fucoidan; in addition it ensures that the extracted material retains its natural bioactivity (McNally, 2007).

Extraction with acidic solvents such as hydrochloric acid (HCl) give higher yields of crude fucoidan (Kawamoto et al., 2006) but could result in the extraction of undesirable products such as alginic acid and metals (Hagiwara, 2010) and may cause degradation of fucose chains (Ale et al., 2011). The use of salts like calcium chloride ($CaCl_2$) are effective solvents in removing insoluble components which could affect the purity of the fucoidan (Kawamoto et al., 2006; Umeda, Kihara, Ikai, & Kato, 2003) but in turn can lower the yield of crude fucoidan (Hagiwara, 2010). High quality fucoidan should contain less than 0.1% of contaminated proteins. Hence protein content determination can be carried out to estimate the purity of fucoidan and the effectiveness of the extraction process (Hayakawa & Nagamine, 2009).

Chemical composition of fucoidan varies according to the season, geographic location, species, and maturity of the plant (Rioux et al., 2007). Fucoidan is a sulphated fucan and the regularity of the structural characteristics of fucoidan is minimal. Fucoidan is mainly composed of fucose, sulphate, uronic acid, and small quantities of monosaccharides. The composition will vary between species and the extraction technique used to extract the fucoidan also have a large impact on the determination of the final structure of fucoidan (Rioux et al., 2007). The method used to extract fucoidan may result in fucoidans that vary in chemical composition and structure. As Ponce *et al.* reported, fucoidan extracted at room temperature and at $70^{o}C$ had completely different chemical compositions (Ponce, Pujol, & Damonte, 2003).

Companies experienced in producing pure fucoidan such as Umi No Shizuku® (http://www.k-fucoidan.com) claim that the colour of fucoidan is directly related to the amount of fucoidan in the powder. If the powder contained very small amounts of

fucoidan, the colour should be close to white or creamy. However, a powder that contained high amounts of fucoidan should be close to a dark brown colour (Tachikawa, 2010). This was supported by the Yaizu Suisankagaku Industry® (http://www.yskf.jp) who also produced fucoidan of a brown colour. Other companies however such as CD Biosciences Inc® (http://www.creative-biomart.com) and Xi'an Day Natural Tech Ltd® (http://www.daynatural.com) declare that their fucoidan have a white visual appearance and not brown, while Haewon Biotech Inc® (http://www.fucoidan.co.kr) claimed that their fucoidan have appearances ranging from white to brown. Therefore, the perception of how fucoidan should look like is still being debated.

2.4.4 Health benefits of fucoidan

Research on fucoidan has so far been carried out in Japan, Korea, France, Australia, China, and the United States. Studies have indicated that fucoidan is non-toxic, non-allergenic, and has no negative effects on the human body once consumed (Shibata, Takagi, & Nagaoka, 2000). This statement is further supported by the fact that nutraceutical and food supplements containing fucoidan have been marketed for a number of years with no known adverse effects (Choi et al., 2010). No toxicological changes were observed when rats were orally administered with up to 1000 mg/Kg body weight per day of fucoidan for 28 days, but when the dose was increased to 2000 mg/Kg body weight per day of fucoidan, the plasma ALT level, a biomarker of liver injury was increased indicating that the consumption of fucoidan up to 1000 mg/Kg body weight per day was safe in rodents (Chung et al., 2010).

Fucoidan is known to exhibit a wide variety of biological activities. Among them are: anticoagulant, antioxidant, antiviral, antithrombic, and anticancer activities (Li, Lu et al., 2008). Many researchers have targeted the anticoagulant, anticancer, and antioxidant activities of fucoidan as being the most important activities in fucoidan. The effectiveness of these activities are related to the chemical composition of fucoidan (Synytsya et al., 2010). Seaweed polysaccharides are usually heterogeneous and branched; it may contain monosaccharide components with acetyl groups and the amount of sulfation is irregular (Bilan et al., 2002). As mentioned earlier, the structural complexity of fucoidan may vary from species to species, depending on the extraction method. For that reason, each type of fucoidan that may have unique structural features

and possess varied bioactivities, could potentially be a new drug (Eluvakkal et al., 2010).

The precise structure and backbone of fucoidans have been extensively studied for some time, but debate about its actual arrangement is still ongoing due to its complex structure. A reason for that is because fucoidan is difficult to extract in its pure form. Crude fucoidan is a polysaccharide made up of a complex mixture of fucose, sulphate, and low uronic acid, to a low sulphated fucan polysaccharide with high uronic acid content (Marais & Jacob, 2001). Some examples of the composition of crude fucoidan are summarized in Table 2 (Lee, Lim, Lee, & Park, 2006; Li, Wei, Sun, & Xu, 2006; Ly, Buu, Nhut, Thinh, & Van, 2005; Mabeau, Kloareg, & Joseleau, 1990; Ponce et al., 2003; Usov, Smirnova, & Klochkova, 2005; Wang, Zhang, Zhang, & Li, 2007; Yang, Chung, & You, 2008).

Table 2 Yield and chemical composition of fucoidan extracted from different species of seaweed.

Species	Yield*	Uronic acid♠	Fucose♥	Sulphate♣	Protein♦
Pelvetia canaliculata[a]	61.2	28.1	13.1	11.6	11.0
Fucus vesiculosus[a]	52.2	28.2	9.7	6.9	11.1
Sargassum muticum[a]	51.8	27.9	3.2	5.0	19.4
Laminaria digitata[a]	41.0	46.4	2.3	3.7	11.9
Laminaria japonica[b]	-	1.9	29.1	33.0	1.3
Alaria fistulosa (blade)[c]	13.2	-	2.1	-	-
A. fistulosa (sporophyll)[c]	58.7	-	4.2	-	-
Sargassum swartzii[d]	-	6.7	-	23.5	-
Hizikia fusiforme[e]	-	19.4	18.6	11.8	1.7
Adenocystis utricularis[f]	2.9	10.0	-	24.0	3.0
U. pinnatifida pinnatifida f. *typica* [g]	1.1	-	-	-	-
U. pinnatifida f. *distans*[g]	2.1	-	-	-	-
*U. pinnatifida (*Samcheok*)*[g]	3.8	-	-	-	-
*U. pinnatifida (*sporophyll*)*[h]	8.8	-	-	41.5	2.8
Cladosiphon okamuranus[i]	-	9.9	39.6	16.9	-
Sophora wightii [j]	71.5	-	17.3	8.9	-
Dictyota dichotoma [j]	67.2	-	16.9	7.8	-
Turbinaria decurrens [j]	57.2	-	15.5	6.4	-

*Dry weight, in percent of dry alga weight.
♠Uronic acid content was measured using the carbazole-sulphuric acid method (Bitter & Muir, 1962).
♥Fucose content was measured using the cysteine-sulphuric acid method (Dische & Shettles, 1948).
♣Sulphate content was measured using the barium chloride assay (Dodgson & Price, 1962).
♦Protein content was measured using the Bradford assay (Bradford, 1976).
[a](Mabeau et al., 1990); [b](Wang et al., 2007); [c](Usov et al., 2005); [d](Ly et al., 2005); [e](Li et al., 2006); [f](Ponce et al., 2003); [g](Lee et al., 2006); [h](Yang, Chung, & You, 2008), [i](Shimizu et al., 2005), [j](Eluvakkal et al., 2010).

Crude fucoidan can be purified into fractions using ion-exchange chromatography, a technique which separates molecules based on the overall charge of the molecule. As fucoidans generally have an overall negative charge due to their sulphate groups, they can bind with anion exchangers, which contain positively charged functional groups such as diethylaminoethyl (DEAE) (Chotigeat, Tongsupa, Supamataya, & Phongdara, 2004; Huang & Lam, 2011). Table 3 shows the chemical composition of fucoidan fractions obtained after subjecting crude fucoidan from *S. swartzii* to DEAE Sephadex A-25 (Ly et al., 2005). Table 4 shows the fractionation of crude fucoidan from *U. pinnatifida* using DEAE Sephadex A-25 (Skriptsova et al., 2009).

Table 3 Composition and yield of crude fucoidan fractions from *S. swartzii* (Ly et al., 2005).

Fraction	NaCl eluted	Yield %	Sulphate %	Uronic acid %	Neutral monosaccharide* (%)						
					Fucose	Mannose	Rhamnose	Arabinose	Galactose	Glucose	Xylose
Total	-	-	23.5	6.7	54	3.9	2.1	4.4	28.7	2.8	1.8
F1	0.0 M	2.0	5.6	15.0	nd	nd	nd	nd	nd	nd	nd
F2	1.0M	20.2	14.6	13.1	49.5	4.7	2.7	7.5	29.4	3.0	2.7
F3	2.0M	33.3	18.4	5.5	56.0	3.0	1.9	5.2	28.9	3.2	1.9
F4	2.5M	26.2	28.0	7.6	55.6	3.6	3.3	4.1	27.9	2.8	2.4
F5	3.5M	16.0	42.3	1.9	57.1	4.0	2.0	4.2	27.4	2.0	0.9

*Neutral monosaccharide content was measured using a HPLC system
nd – not determined

Table 4 Crude fucoidan fractions obtained from *U. pinnatifida* (Skriptsova et al., 2009).

Fraction	NaCl eluted	Yield %	Sulphate %	Uronic acid %	Neutral monosaccharide* (%)				
					Fucose	Mannose	Galactose	Glucose	Xylose
F1	1.0M	31.7	14	2.0	58.49	9.05	28.68	1.97	1.81
F2	2.0M	29.7	29	-	52.38	1.02	46.59	0.0	0.0

*Neutral monosaccharide content was measured using a HPLC system

2.4.4.1 Anticoagulant activity

The anticoagulant activity of fucoidan is by far the most widely studied. Many studies showed that the sulphate content, molecular weight, and sugar composition may be related to the anticoagulant activity of fucoidan (Colliec et al., 1991; W. Kim, J et al., 2007; Li, Lu et al., 2008; Li, Rui, & Xin, 2008). In general, the higher content of sulphate, the higher anticoagulant activity. Conversely, the anticoagulant activity gradually decreased up to a point where the sulphate content was too high. This was

proven with the use of oversulfated fucoidans prepared by chemical sulfation of natural fucoidan where highly sulphated fucoidan showed an increase in anticoagulant activity up to a certain degree of sulfation that then gradually decreased anticoagulant activity (Li, Lu et al., 2008)

Molecular weight of fucoidan was closely related to the anticoagulant action of fucoidan. Fucoidan requires a sugar-chain long enough to bind the thrombin, so a certain minimum molecular weight is required to attain anticoagulant activity. Fucoidan extracted from *Lessonia vadosa*, which had high anticoagulant activity possessed a molecular weight of 320,000 Da (Chandia & Matsuhiro, 2008). A smaller fucoidan fraction with a molecular weight of 32,000 Da showed weak anticoagulant activity.

Some studies have also showed that the anticoagulant activity may be related to the sugar composition of fucoidan (Nishino, Yokohama, & Dobahi, 1989). Li, Lu et al., (2008) speculated that it was not the sugars that affected anticoagulant activity but rather the sulphate groups attached to those sugars. Uronic acid composition although not necessary for anticoagulant activity, could improve the anticoagulant action by enhancing the sugar chain's flexibility (Li, Rui et al., 2008).

2.4.4.2 Anticancer activity

Recent work on fucoidan has targeted its anti-carcinogenic properties. Cho et al., (2011) showed that fucoidan had cytotoxic effects against CCL39 cancer cells (Cho, Lee, & You, 2011). In Japan, fucoidan administered to lymphoblastoid cancer cells wiped out the cells within 72 hours of incubation (Ohigashi, Yamaguchi, Umezaki, & Koshimizu, 1992). In addition to being completely safe, fucoidan destroyed cancer cells specifically and did not attack normal cells. Consequently, fucoidan produced no side-effects unlike chemotherapy drugs (Tachikawa, 2003). Tachikawa (2003) also stated that fucoidan used three mechanisms to destroy cancer cells: induced apoptosis (self-destruction), immunity boost, and prevention of angiogenesis which cuts off the nutrient supply to the cancer cells.

Fucoidan's anticancer properties were further supported by a study that compared the breast cancer rates in Japan with other developed nations (Teas, Zhang, & Muga, 2006).

There was a correlation where women who consumed miso soup more than six times a week were reported to have half the risk of breast cancer compared to women who did not. They concluded that the brown seaweed components such as fucoidan may inhibit cancer cell formation (Teas et al., 2006). Figure 7 shows the breast cancer rates across the world in 2008. Cancer rates were excessively high throughout the United States, Australasia, and many parts of Europe, but relatively low in most parts of Asia where seaweed consumption is considerably high (Laurence, 2008).

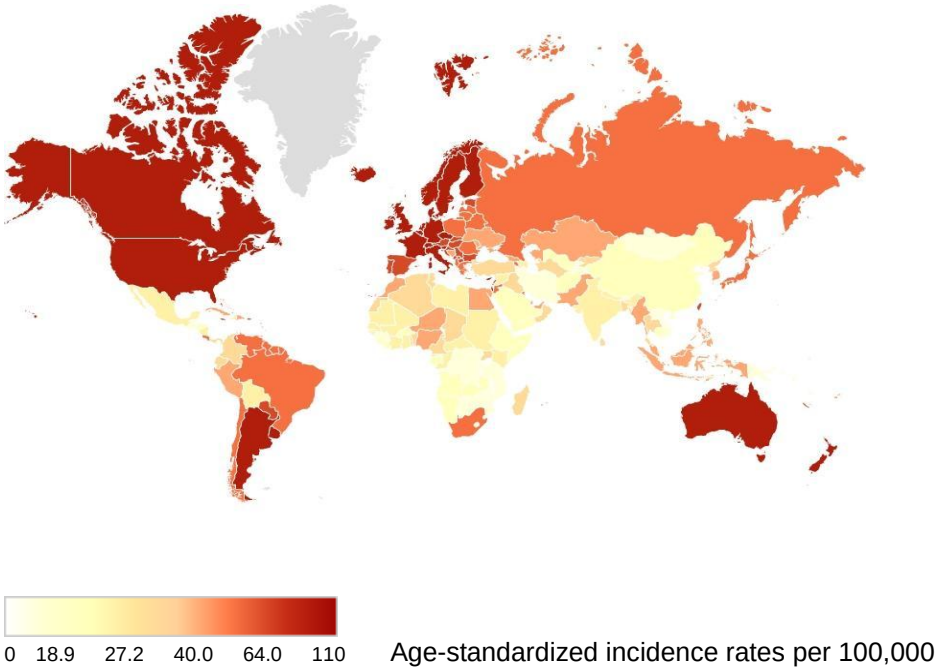

Figure 7 Breast cancer rates across the world in 2008 (Ferlay et al., 2008).

2.4.4.3 Antioxidant activity

Nearly all organisms are able to repair oxidative damage in their body and the negative effects that free radicals have on our bodies are well recognized. Antioxidants can delay or prevent the oxidative stresses applied on our organs. Fucoidan has, in recent years demonstrated total antioxidant capacity/antioxidant capacity (TAC/AOC) (Wang et al., 2007). Unlike synthetic antioxidants, fucoidan is a natural antioxidant and has large potential for avoiding or delaying free radical-mediated illnesses (Li, Lu et al., 2008).

Reactive oxygen species (ROS) are generated continuously in our biological systems through metabolism and environmental sources. Even though our body has its own natural defensive mechanisms against ROS, it cannot prevent the damage entirely. Antioxidants are substances that can prevent these radical reactions by forming stable free radicals out of ROS (Wang, Zhang, Zhang, Zhang, & Li, 2009). Commonly used antioxidants such as butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) are now restricted by legislation as they are suspected to produce toxic side effects and are possible carcinogens. As a result, the food and pharmaceutical industries are considering the use of natural antioxidants which have no side effects and are nontoxic to humans (Wang et al., 2007).

Fucoidans have a much higher scavenging activity than vitamin C and κ-carrageenan (Wang et al., 2007). However scavenging effects were not as strong in large molecular weight fucoidan compared to low molecular weight fucoidan. The ratio of fucose content/sulphate was also a useful indicator of the antioxidant activity in fucoidan (Wang et al., 2007). Ruperez et. al. (2002) demonstrated that fucose could be the key to the radical scavenging ability of fucoidan and proposed that it could be potentially used as a natural antioxidant by the food industry (Ruperez, Ahrazem, & Leal, 2002).

Several techniques are commonly used to estimate the efficiency of antioxidants. The DPPH assay was one of the best-known methods to assess antioxidant activity due to its simplicity and accuracy (Szabo, Iditoiu, Chambre, & Lupea, 2007). 1, 1-diphenyl-2-picylhydrazyl (DPPH), a stable nitrogen radical, has a spare electron delocalising around the whole molecule. This delocalization causes the molecule to appear as a dark purple colour. When DPPH is mixed into solution and a hydrogen atom donor (H-A) is added, a non-radical form of DPPH (DPPH-A) is formed, which is pale yellow in colour. The decolourization of the purple DPPH radical to yellow is an indication of the scavenging activity of the antioxidant (Szabo et al., 2007).

$$\underset{Purple}{(DPPH*)} + (H-A) \rightarrow \underset{Yellow}{DPPH-A} + (H*)$$

The drawbacks of this technique include the loss of DPPH colour through mechanisms like radical reaction, reduction, and steric accessibility. Antioxidants that react quickly with peroxyl radicals involved in lipid peroxidation may not react to DPPH due to steric inaccessibility (Prior, Wu, & Schaich, 2005). Most DPPH assays have a reaction time

of 30 min, but shorter times have been used, such as 5 min (Lebeau et al., 2000), and 10 min (Schwarz et al., 2001). The reaction time highly depended on the substrate used; therefore, the best technique was to follow the reaction to completion (Molyneux, 2004). All these complications can lead to imprecise estimations of AOC (Prior et al., 2005).

The CUPRAC (Cupric Reducing Antioxidant Capacity) assay of total antioxidant capacity has been effectively applied to antioxidants in plants, human serum, and hydroxyl radical scavengers (Guclu, Ozyurek, Bektasoglu, & Apak, 2010). This method is based on the reduction of the copper ion Cu(II) to Cu(I) where absorbance is recorded at the maximum absorption wavelength of 450 nm. This is a result of the redox reaction of the CUPRAC reagent, Cu(II)-neocuproine ($Cu(Nc)_2^{2+}$), with an antioxidant (A-OH), to form the CUPRAC chromophore, Cu(I)-neocuproine ($Cu(Nc)_2^{+}$) chelate (Guclu et al., 2010).

$$Cu(Nc)_2^{2+} + (A-OH) \rightarrow Cu(Nc)_2^{+} + (A-O)^{-} + H^{+}$$

The use of iron in this assay rather than copper ions, is referred to as the ferric reducing antioxidant power (FRAP) assay. However, copper ions have a lower redox potential than iron, giving the CUPRAC assay less interference from sugars, and other common interfering substances. The copper reaction also responds faster than iron, which consequently give the CUPRAC assay a shorter completion time than the FRAP assay (Prior et al., 2005). Complex mixtures still required a 30-60 min reaction time, and therefore have similar problems to the DPPH assay in terms of selecting an appropriate reaction time with complex molecules (Prior et al., 2005).

2.4.5 Variations in fucoidan composition

As mentioned earlier, fucoidan content, chemical composition, and its structural characteristics vary in relation to the seaweed species, season of harvest, and maturity of the plant (Skriptsova et al., 2009). Maximum amounts of fucoidan can be found in the sporophylls compared to the blade, but their chemical compositions are quite similar within a given species (Usov et al., 2005). Moreover, a study established a correlation between seasonality and fucoidan content, and reported that fucoidan content was highest during the reproductive stages of *U. pinnatifida* (Skriptsova et al., 2009).

During this period, the physiological and biochemical processes of the alga varies, and the structure and chemical composition of fucoidan certainly convoy with such changes (Usov et al., 2005).

Environmental conditions may also have an effect on the chemical composition and structural characteristics of seaweed. Therefore, these changes are likely to effect the chemical composition of fucoidan in a similar manner (Mamatha, Namitha, Senthil, Smitha, & Ravishankar, 2006). Seaweed grows especially well in clean ocean water, particularly in shallow areas where the amount of sunlight is relatively high. Seaweed also prefers environments which are sheltered in some way from strong ocean swells, as it may damage its structures and make it difficult for spore's attachment to surfaces. Conversely, seaweed obtains nutrients from the water, so moderate water movement is essential but not too slow to cause silting. Seaweed grown in areas with a large fresh water input may also slow down growth and can eventually kill the alga over time (Tiroba, 2007). Excessive fluctuations in the water temperature and salinity may also affect the growth of the seaweed (Tiroba, 2007).

3 Comparison of three extraction techniques of fucoidan

3.1 Introduction

As stated above, fucoidan can be extracted using a variety of methods that use different solvents to solubilise fucoidan from the cell walls of seaweed. Different techniques of fucoidan extraction may lead to extraction of a completely different fucoidan with distinct chemical properties (Li, Lu et al., 2008). Difficulty in extracting good-quality fucoidan has limited the knowledge obtained on the structural characteristics of pure fucoidan (Marais & Jacob, 2001). Different manufacturers have their own specific technique to extract fucoidan. Three extracting solvents normally used to extract fucoidan are deionized water, hydrochloric acid (HCl) and calcium chloride ($CaCl_2$), with each method claiming to have its own advantages over other conventional extraction methods (Bilan et al., 2002; Li, Lu et al., 2008; Yang, Chung, Shin et al., 2008). This chapter will compare three fucoidan extraction methods based on the extracting solvents: $CaCl_2$, HCl and water.

3.2 Methods

3.2.1 Harvesting

U. pinnatifida was harvested during November from Great Barrier Island, New Zealand (36^o 11.355'S, 175^o 18.922'E). Seaweed was removed from selected mussel-harvesting lines by hand, making sure that nearby mussels were not damaged or removed during the process. Seaweed was rinsed with sea water to remove epibionts and placed into re-sealable plastic bags, frozen, packaged in polystyrene containers and sent to AUT University the following morning.

3.2.2 Drying

Frozen seaweed was thawed and washed under running tap water. Visible debris was carefully removed with slight agitation, while the holdfast and any degraded areas of the seaweed were removed by hand. The sporophyll was separated from the blade and laid separately onto metal trays covered with tinfoil as shown in Figure 8. Seaweed was then dried in an oven (Sanyo Convection Oven MOV-112F) at 60^{o}C to constant weight with regular turning to prevent the alga from sticking.

Sporophyll and blade were milled separately with a food blender (Krups 75 blender) and sieved through a metal sieve with pore size of 600 microns (Endecotts Ltd) to obtain a fine powder. Milled seaweed was transferred into glass beakers, weighed and recorded. Beakers were transferred into the oven and dried at 60^{o}C overnight and reweighed the next morning. A homogenous sample of dry seaweed powder was achieved when the weight of the powder remained constant.

Figure 8 The blade (left) and sporophyll (right) prior to drying.

3.2.3 Fucoidan extraction

3.2.3.1 Method I - Water extraction

The water extraction method from Yang et al. (2008) was employed to extract fucoidan from *U. pinnatifida*. Four replicate samples of dried *U. pinnatifida* blade/sporophyll (10 g) were treated with 200 mL of 85% ethanol (BDH Laboratories) with constant mechanical stirring. The mixture was stirred for at least 12 hours at room temperature to destroy and/or remove colour pigments, proteins, and other unwanted material. The ethanol mixture was drained off and the *U. pinnatifida* was washed with acetone (BDH Laboratories). The residue was recovered by centrifugation at 1800 x *g* for 10 minutes (Eppendorf Centrifuge 5810R V3.1). The supernatant was removed and the residue dried overnight at room temperature. Deionised water (25 mL) was added to the dried and treated biomass (5 g) and kept at 65^{o}C for one hour with mechanical stirring. The extract was centrifuged for 10 minutes at 18500 x *g* and the supernatant was collected and measured. This solution was mixed with 1% $CaCl_2$ (BDH Laboratories) (1:1) and stored at 4^{o}C overnight to precipitate the alginic acid.

The solution was then centrifuged at 18500 x *g* for 10 minutes and 99% ethanol was added into the supernatant to obtain a final ethanol concentration of 30%. The solution was left at 4^{o}C for 4 hours and subjected to centrifugation again at 18500 x *g* for 10 minutes to remove the unwanted impurities. The supernatant was collected and 99% ethanol added until a total ethanol concentration of 70% was achieved. This solution was stored at 4^{o}C overnight to precipitate fucoidan. Fucoidan was recovered by centrifuging the solution at 18500 x *g* for 10 minutes, followed by washing with ethanol and acetone then left to dry at room temperature. The percentage yield of fucoidan was calculated by dividing the weight of fucoidan by the weight of dried biomass after treatment with 85% ethanol. Dried fucoidan was ground using a mortar and pestle apparatus and stored in small glass vials until needed.

3.2.3.2 Method II – Acid extraction

Acid extraction was performed using the method of Lee et al. (2004) in which HCl was used to extract fucoidan. Dried *U. pinnatifida* (10 g) was transferred into a round-bottom flask and refluxed in 100 mL ethanol for two hours at 80^{o}C to remove fat and

colour pigments. The solution was centrifuged at 1800 x *g* for 10 minutes and the supernatant removed, and then the defatted alga was left to dry overnight at room temperature. The defatted alga (5 g) was extracted with 100 mL of 0.15M HCl (RCI Labscan Ltd) for two hours at 65^{o}C with constant mechanical stirring. After centrifugation at 18500 x *g* for 10 minutes, the supernatant was neutralized with 3M NaOH to prevent any acidic damage to the fucoidan structure during storage. A pH meter (Radiometer Copenhagen PHM201) was used to assess the pH of the solution with mechanical stirring while 3M NaOH was added drop wise until pH 7 was achieved. The neutralized extract was stored at 4^{o}C overnight.

Four volumes of absolute ethanol were added into the extract and the solution was stored overnight at 4^{o}C to precipitate fucoidan. Fucoidan was recovered by centrifuging the solution at 18500 x *g* for 10 minutes. The precipitate was removed from the centrifuge tubes, washed with ethanol and then dried to constant weight. A mortar and pestle was then used to grind the fucoidan that was stored in glass vials until needed.

3.2.3.3 Method III – Salt extraction

A $CaCl_2$ extraction method was utilised from Bilan et al. (2002). Ten grams of milled *U. pinnatifida* was pre-treated with 100 mL of methanol (Thermofisher), chloroform (Scharlau Chemie), and water (4:2:1) with mechanical stirring overnight to remove fat, protein and colour pigments. The solution was filtered through Whatman's filter paper (90 mm GF/D), washed with acetone and dried at room temperature. *U. pinnatifida* was heated in 100 mL of 2% $CaCl_2$ (BDH Laboratories) in an 85^{o}C water bath for 5 hours, with regular stirring, to extract water-soluble polysaccharides. The solution was filtered again and mixed with 30 mL of 10% Cetavlon (Sigma) and left to precipitate out fucoidan at 4^{o}C overnight.

The solution was again centrifuged at 18500 x *g* for 15 minutes and the supernatant was removed. The precipitate was washed with water, and stirred with 50 mL of 20% ethanolic sodium iodide (BDH Laboratories) solution for 3 days at room temperature to remove and decompose any cetavlon residue. The solution was centrifuged at 18500 x *g* for 15 minutes, and the precipitate was washed with ethanol to remove sodium iodide and lyophilized (Christ LOC 1-M Alpha 2-4, Martin Christ, Osterode am Harz, Germany) to give crude fucoidan.

3.2.4 Fucoidan composition

3.2.4.1 Fucose content

Free fucose was determined in fucoidan by the cysteine-sulphuric acid method for methyl pentoses (Dische & Shettles, 1948). Four replicate samples were prepared in different concentrations with deionized water ranging from 20-200 µg/mL. Commercial L-fucose (Sigma) was used as the standard.

Each sample solution (1 mL) was placed into separate test tubes and cooled in an ice water bath. 4.5 ml of sulphuric acid (H_2SO_4) (J.T Baker Ltd) reagent (prepared by adding six volumes of concentrated sulphuric acid with one volume of water) was added into each tube and mixed. Tubes were warmed in a 25^{o}C water bath for 3-4 minutes, and then placed into a boiling water bath for 3 minutes. Tubes were then cooled under running tap water and 0.1 mL cysteine hydrochloride solution (5% cysteine hydrochloride in deionized water) was added to each tube and mixed. Absorbance was read at 396 nm and 427 nm, after zeroing the spectrophotometer (Ultrospec 2100) with a water blank treated in the same manner. Absorbance values were calculated by using the following equation: Absorbance = (A396 nm – A427 nm). This corrects for the presence of hexoses (Dische & Shettles, 1948).

3.2.4.2 Sulphate content

The measurement of sulphate in the fucoidan was based on the barium sulphate ($BaSO_4$) determination using barium chloride ($BaCl_2$) (Dodgson & Price, 1962), whereby sulphate content was estimated turbidimetrically as $BaSO_4$.

The conditioning reagent was prepared by mixing 50 mL glycerol (Scientific Supplies Ltd), 30 mL concentrated hydrochloric acid (RCI Labscan Ltd), 300 mL deionized water, and 100 mL isopropyl alcohol (Scientific Supplies Ltd), and sodium chloride (Ajax Finechem NZ Ltd) into a large beaker with mechanical stirring overnight. Four replicate samples were prepared by weighing 15 mg of dried fucoidan into separate closed test tubes containing 5 mL 4 M HCl. Samples were subjected to acid hydrolysis for 2 hours at 100^{o}C. A solution of potassium sulphate standards with concentrations ranging from 200-1000 µg/mL of sulphate was prepared.

Sample solution was added into a 100 mL conical flask containing 15 mL of deionised water. Conditioning reagent (5 mL) was added and stirred mechanically at a constant speed. $BaCl_2$ from BDH Chemicals Ltd. (0.3 g) was added, stirred for exactly 1 minute and then left standing for 4-6 minutes to allow the $BaSO_4$ precipitate to form. Absorbance was measured at 420 nm using a spectrophotometer (Ultrospec 2100) after zeroing with a water blank that was treated in the same manner.

3.2.4.3 Uronic acid content

The method of Bitter & Muir (1962) was used to estimate the uronic acid content in fucoidan. This was a modification of the original procedure developed by Dische (1947). The modified procedure was reported to have less interference, a more stable colour formation, and reacted faster (Bitter & Muir, 1962).

Tetraborate acid reagent (0.025 M) was prepared by dissolving 0.503g of sodium tetraborate (May and Baker Ltd) into 100 mL of concentrated sulphuric acid (J.T. Baker Ltd) and stirring the solution mechanically overnight. A 0.125% carbazole reagent was prepared by mixing 0.125 g of carbazole (Sigma Aldrich) with 100 mL of absolute ethanol in a brown glass bottle and stored at 4^oC until needed. Screw-cap tubes were filled with 3 mL of tetraborate acid reagent and cooled in an ice water bath. Four replicate samples with a concentration of 1 mg/mL dissolved in deionised water saturated with benzoic acid (BDH Chemicals Ltd) was carefully added (0.5 mL) to the acid and the tubes were closed. The tubes were shaken vigorously with constant cooling in the ice water bath for 5-10 seconds. Tubes were then heated for 10 minutes in a boiling water bath and then cooled to room temperature. Carbazole reagent (0.1 mL) was added to each tube and heated for a further 15 minutes in the boiling water bath, and cooled to room temperature. Absorbance was measured at 530 nm after zeroing the spectrophotometer (Ultrospec 2100) with a water blank treated in the same way. D-glucuronic acid (Sigma Aldrich) was used as the standard.

3.2.4.4 Protein content

The Bradford protein assay (Bradford, 1976) was utilised in this study to determine the amount of contaminated protein present in crude fucoidan. It is based on the dye,

Coomassie Brilliant Blue G-250, which changed initially from red to blue due to protein binding. The protein content was then estimated spectrophotometrically.

Bradford reagent was made by dissolving 100 mg of Coomassie brilliant blue G-250 (Sigma Aldrich) into 50 mL of 95% ethanol, followed by the addition of 100 mL 85% (w/v) phosphoric acid (BDH Chemicals Ltd). This mixture was transferred into a one litre volumetric flask and diluted to the graduation mark with deionized water. Four replicate samples with a concentration of 20 mg/mL dissolved in water was added into 1.6 mL Bradford reagent in a test tube. Samples were incubated for 15 minutes at room temperature and the absorbance was measured at 595 nm after zeroing the spectrophotometer (Ultrospec 2100) with a water blank treated in the same way. Bovine albumin (ICP Bio Ltd) was used as the standard.

3.2.5 Statistical analysis

Minitab® (Version 15), analysis of variance (ANOVA) was carried out to test for differences between extraction techniques and compostion of the extracted fucoidan. Where significant differences occurred, Tukey's HSD was employed to examine where that effect occurred.

3.3 Results

3.3.1 Extraction

There were significant differences in the fucoidan content extracted using the three techniques (p = 0.000) (Figure 9). There was also a significant difference between the fucoidan content in the sporophyll and blade (p = 0.000). Treatment of *U. pinnatifida* with water as the extracting solvent at 65^{o}C gave a yield of 10.74% ± 0.134SE fucoidan in the sporophyll, and provided the highest yield (p = 0.000) out of the three methods when extracting fucoidan from the blade (4.78% ± 0.045SE).

The method of Bilan et al. (2002), where $CaCl_2$ was used as the extracting solvent at 85^{o}C gave the lowest yield of blade-derived fucoidan at 2.78% ± 0.17SE (p = 0.000). In contrast, the same technique provided the highest yield of sporophyll-derived fucoidan

(p = 0.000). Acid extraction using the method of Lee et al. (2004) gave the lowest percentage yield (p = 0.000) from the sporophyll and only slightly higher yields in the blade when compared to the $CaCl_2$ extraction.

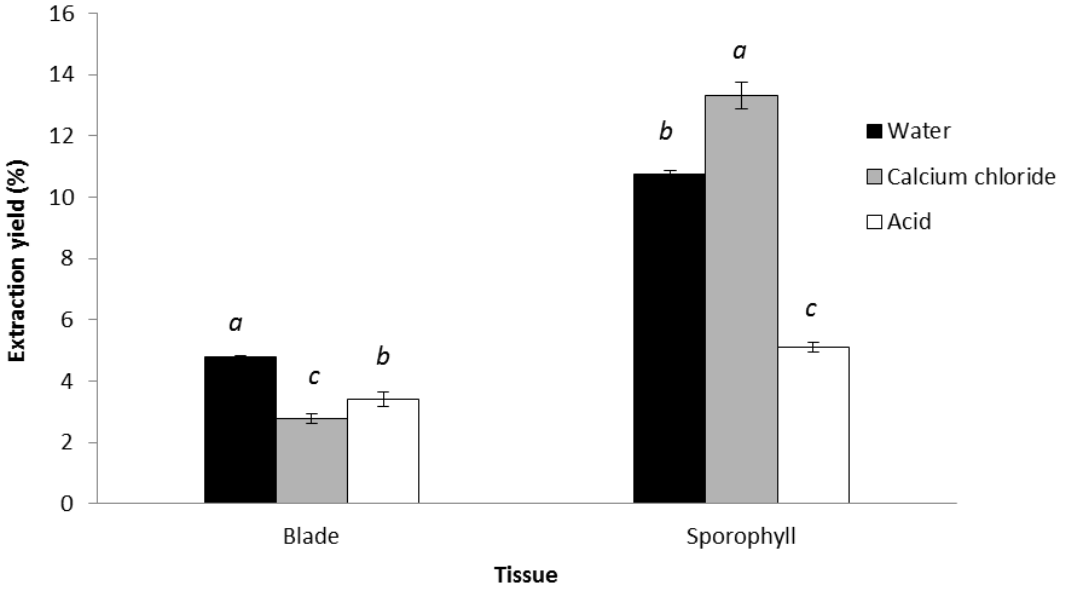

Figure 9 Crude fucoidan content of *U. pinnatifida* with three extraction techniques. Mean ± SE (n = 4). Different superscript letters indicate significant differences at p < 0.05 by one-way ANOVA. Same superscript letters indicate no significant differences. Blade and sporophyll were examined separately using Tukey's HSD.

Apart from the yield, the colour of fucoidan was also crucial when selecting an extraction method that produces good quality fucoidan. Colour of the fucoidans extracted using the three methods are shown in Figure 10. Water extraction gave the whitest coloured fucoidan from both the blade and sporophyll, while the acid extraction gave a brownish powder from the blade and a pale white powder from the sporophyll. The $CaCl_2$ extraction resulted in the darkest powder for both the blade and sporophyll. Colour of the blade-derived fucoidan using this extraction technique gave the closest resemblance to the commercial fucoidan powder purchased from Sigma. Although fucoidan from the sporophyll using $CaCl_2$ extraction was not as dark, it was still the darkest brown powder compared to other sporophyll-derived fucoidans (Figure 10).

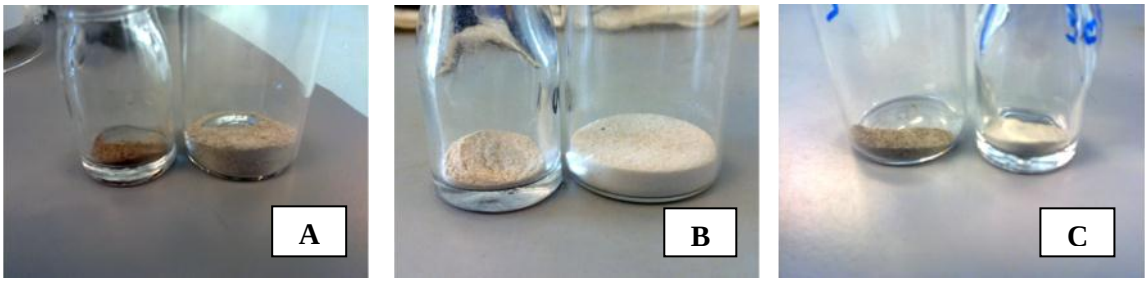

Figure 10 The colour of fucoidan extracted using $CaCl_2$ extraction (A), water extraction (B), and acid extraction (C). (Left vial = blade-derived fucoidan; right vial = sporophyll-derived fucoidan).

3.3.2 Constituents of crude fucoidan

Chemical composition of the fucoidan derived from the three extraction methods are shown in Table 5. Extraction of seaweed with 2% $CaCl_2$ revealed, with Tukey's HSD, the largest percentage of fucose and sulphate content in both the blade and sporophyll (p = 0.000). This extraction also gave significant differences in the uronic acid content (p = 0.002), that had the highest uronic acid content in blade, but the lowest uronic acid content in the sporophyll. Protein content was also the highest among the three methods (p = 0.000).

Water extraction was considered the best of the three methods employed in producing uncontaminated fucoidan, due to the low protein content in both the sporophyll and blade-derived fucoidan. However, the fucose and sulphate content were no better when compared with the $CaCl_2$ extraction, but the uronic acid content in the sporophyll was slightly higher than that from the $CaCl_2$ extraction.

Fucose (p = 0.000) and sulphate content (p = 0.000) from fucoidan extracted from the blade using HCl was significantly lower than the other two methods. Nevertheless, uronic acid content in sporophyll-derived fucoidan using HCl was the highest (p = 0.000) when compared to the other two methods. The amount of protein from HCl extraction was significantly lower for both the blade (p = 0.000) and sporophyll (p = 0.000) than that of the $CaCl_2$ extraction but significantly higher than the water extraction.

Table 5 Results (in percent of dry weight) from the chemical analysis of fucoidan derived from three extraction methods. Mean ± SE, $n = 4$ (B = blade, S = sporophyll).

Extraction method	Fucose	Sulphate	Uronic acid	Protein
$CaCl_2$ - B	16.37 ± 0.29	28.01 ± 0.09	4.91 ± 0.03	0.193 ± 0.001
$CaCl_2$ - S	14.96 ± 0.11	34.55 ± 0.61	2.38 ± 0.02	0.340 ± 0.005
Water - B	9.52 ± 0.25	27.88 ± 0.03	2.09 ± 0.12	0.092 ± 0.006
Water - S	12.69 ± 0.05	24.48 ± 0.02	2.77 ± 0.04	0.059 ± 0.002
Acid – B	4.26 ± 0.31	22.09 ± 0.68	3.75 ± 0.04	0.161 ± 0.006
Acid - S	8.57 ± 0.09	14.41 ± 0.16	4.42 ± 0.02	0.231 ± 0.004

3.4 Discussion

In this study, the brown seaweed *U. pinnatifida* was treated with different extracting solvents. All three procedures required the use of alcohol as a pre-treatment in order to remove mannitol, lipids, salts, and colour pigments, which may interfere with the extraction process (Bilan et al., 2002; Ponce et al., 2003). The alga was then treated with three solvents commonly used to extract fucoidan: deionized water, HCl solution and $CaCl_2$ solution.

Obvious differences were found in relation to the percentage yields of fucoidan using the three extraction methods: (1) As proven previously by Usov et al. (2005), the sporophyll contained a much higher quantity of fucoidan than the blade; (2) $CaCl_2$ extraction gave the highest yield of fucoidan in the sporophyll but conversely the lowest in the blade; and (3) this study supported the fact that the yield of fucoidan differed depending on the type of extraction technique used.

The acid extraction yield in our study contrasts with results reported by Kawamoto, et al. (2006), who stated that the use of acidic solvents to extract fucoidan would provide high yields of fucoidan (Kawamoto et al., 2006). However this was not proven in their study as purified water was used as the extracting solvent instead. They stated that extracting fucoidan using water can produce fucoidans cloest to 'native' fucoidan (Kawamoto et al., 2006). Conversely in this study, acid extraction yielded the smallest amount of fucoidan out of all the three methods of extraction for both the blade and sporophyll from *U. pinnatifida*. A maximum amount of crude fucoidan from *U. pinnatifida's* sporophyll was found using the $CaCl_2$ extraction as opposed to the blade, which had the lowest yield out of all blade extractions. Water extraction produced the highest yield of blade-derived fucoidan

Unlike yield, constituents of crude fucoidan such as fucose, sulphate, protein, and uronic acid content were significantly higher from the $CaCl_2$ extraction than the other two methods. Fucose, the major sugar constituent of fucoidan, was found to be the highest in blade fucoidan using the $CaCl_2$ extraction, followed by the sporophyll using the same technique. The lowest fucose content was found in the acid extraction of both blade and sporophyll. It is worth noting that the sugar rhamnose is also a methyl pentose (or a deoxy-hexose) and may have also contributed to the total amount of fucose estimated in the fucoidan, even if present as a minor component of fucoidan.

Of the three extraction methods tested, $CaCl_2$ extraction yielded the highest sulphate content in both the blade and sporophyll, followed by water extraction and then acid extraction. The presence of sulphate groups in fucoidan is vitally important as it is related to fucoidan's bioactive properties (Yang, et al., 2008). As for protein content, the higher the protein content, the more contaminated the crude fucoidan was. Water extraction yielded fucoidan that was almost devoid of protein contamination. Although the $CaCl_2$ extraction, especially in the sporophyll, yielded fucoidan with more protein contamination, this value was not high in terms of crude fucoidan. Pure fucoidan should have a protein content below 0.1% (Hayakawa & Nagamine, 2009).

Visual inspection of our fucoidans showed that the colour of blade-derived fucoidan was noticeably darker than those obtained from the sporophyll. With reference to Tachikawa (2010), a good quality fucoidan should have a dark brown appearance. In this research, the $CaCl_2$ extraction yielded the darkest brown fucoidan from the blade,

and resembled the colour of fucoidan purchased from Sigma Aldrich. In addition, this method also gave the darkest brown sporophyll-derived fucoidan. Nevertheless, fucoidan-producing companies such as CD Biosciences Inc® and Xi'an Day Natural Tech Ltd® claim that fucoidans have a white creamy appearance, and not brown. Hence the debate on how fucoidan should look like is still ongoing. In my opinion, the colour of fucoidan should not be the only main characteristic to indicate good quality fucoidan.

In comparison with the yields obtained from previous studies (Table 2), fucoidan yield from New Zealand *U. pinnatifida* using the $CaCl_2$ extraction was higher than other studies that used *U. pinnatifida* grown in Korea (Lee et al., 2006; Yang, Chung, & You, 2008). However, the highest yield obtained in this chapter was less than the yield reported from studies using other species of alga (Table 2). The yields of some species of alga reported were over 50% dry weight, whereas the highest yield in *U. pinnatifida* in our study was only 13.32% ± 0.45SE dry weight.

Fucoidan derived from the $CaCl_2$ extraction gave the highest protein content in both blade and the sporophyll. Although this may be the highest protein values out of the three methods tested, it was still considerably low compared to the protein values reported in Table 2, with the lowest protein content of 1.7% reported in fucoidan derived from *H. fusiforme*, and the highest of 19.4% in fucoidan derived from *S. muticum* (Li et al., 2006; Mabeau et al., 1990).

The fucose, sulphate, and uronic acid content of fucoidan obtained from the $CaCl_2$ extraction were comparable to the values reported in Table 2. This supports the idea that the fucoidan extracted using the $CaCl_2$ method of Bilan et al. (2002) was good quality. However, apart from the the type of fucoidan extraction carried out, the season in which the alga was harvested, location and habitat in which the alga was grown in, and the species of alga investigated, would all have an effect on the content and chemical composition of fucoidan and should be further investigated.

3.5 Conclusion

In this study, the fucose, sulphate, and uronic acid content of crude fucoidan extracted from *U. pinnatifida* grown around the coastal waters of New Zealand were comparable to other published studies. The yield and constituents of fucoidan varied with the type

of extraction method used. Of the three methods tested, $CaCl_2$ extraction appeared to be the best method to extract good-quality fucoidan that had the highest yield, as well as highest fucose and sulphate content, possibly due to the nature of $CaCl_2$ where it reacts with sugar-chain polymers and link them together. Calcium-extracted fucoidan also was the darkest brown in colour, characteristic of a good-quality fucoidan.

4 Monthly changes in fucoidan content and composition

4.1 Introduction

In spite of the increase in awareness regarding brown seaweed as a rich source of fucoidan, changes in fucoidan content on a monthly basis with varying seaweed maturity is lacking (Skriptsova et al., 2009). Several studies have reported that variations in the content and composition of fucoidan are affected by the season in which the alga was collected (Honya, Mori, Anzai, Araki, & Nishizawa, 1999), and plant maturity (Zvyagintseva et al., 2003). There is also a correlation between fucoidan content and seasonality, with maximum fucoidan content obtained during the reproductive stages of the alga (Honya et al., 1999). As shown in Figure 2, *U. pinnatifida's* reproductive cycle involved several reproductive stages. During this period, the physical and biochemical state of the alga changed (Skriptsova et al., 2009). Therefore, it was important to determine the optimum time to harvest *U. pinnatifida* that yielded the highest amount of fucoidan.

4.2 Materials and methods

4.2.1 Sample collection and drying

U. pinnatifida was harvested from Port Underwood and Pelorus Sound situated in the Marlborough Sounds near the top of the South Island (Figure 11). *U. pinnatifida* was harvested at three selected mussel farms (sites 106, 253, and 327) in Port Underwood on a monthly basis from June 2011 through to October 2011, and in Pelorus Sound (sites 233, 122, and 353), from August 2011 to October 2011.

Seaweed was removed from the mussel lines by hand and rinsed to remove epibionts. The holdfast was completely removed from the line, preventing them from growing back at the same area. The blades of the seaweeds were separated from the sporophyll

on the boat, and each sample was placed in labelled bags. These samples were then frozen overnight prior to being air freighted to Vitaco Limited, a freeze-drying plant in Auckland, to be lyophilized in bulk within 48 hours of frozen storage. Dry weights of the freeze dried samples were recorded prior to the samples being milled using a coffee grinder (Breville CG2B Coffee 'n' Spice Grinder) and sieved using a 600 micron sieve. The powdered samples were then stored in 200 mL PET bottles and kept in a cupboard at room temperature prior to analysis.

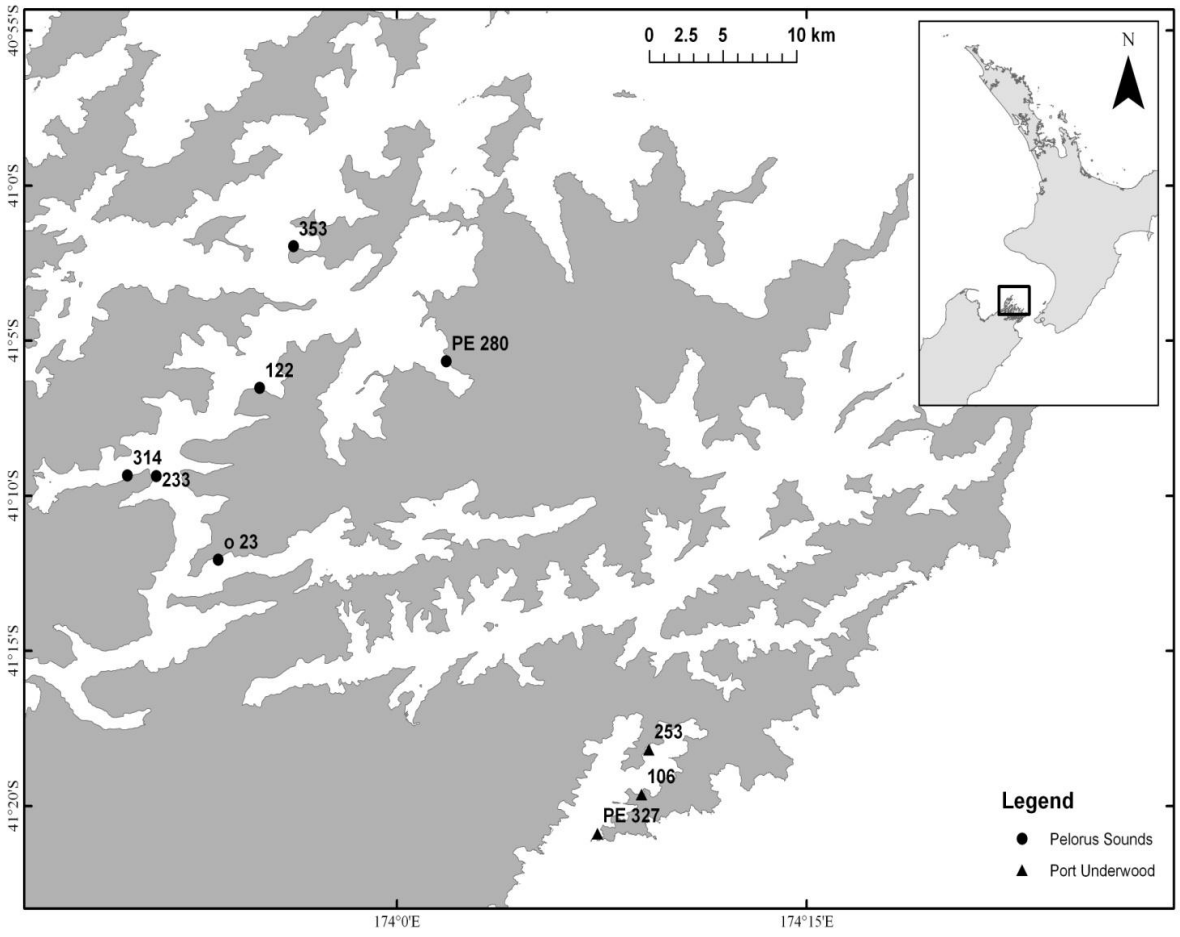

Figure 11 Mussel farming sites in the Marlborough Sounds, New Zealand.

4.2.2 Fucoidan extraction

Based on the results from Chapter 3, fucoidan was extracted using the $CaCl_2$ extraction method from Bilan et al. (2002) to examine differences in fucoidan content between mussel farms across the growing season of *U. pinnatifida*. Fucoidan was extracted from individual alga to account for any variation between individuals grown in the same area.

Three individual algae (blade and sporophyll) were extracted from each farm. Table 6 summarizes the month and farm from which *U. pinnatifida* was harvested. It also shows the harvests used to extract fucoidan in this study. Three individual assays were carried out using fucoidan derived from three individual algae; thus, fucoidan from each alga was considered a replicate.

Table 6 The month and farm where *U. pinnatifida* was harvested and extracted for fucoidan (√ = extracted for fucoidan, × = harvested but not extracted for fucoidan).

Month	**Port Underwood**			**Pelorus Sound**		
	106	**253**	**327**	**233**	**122**	**353**
June	×	×	×	-	-	-
July	√	×	√	-	-	-
August	√	×	√	×	×	×
September	√	×	√	×	×	×
October	√	×	√	×	×	√
November	×	×	-	-	-	-

4.2.3 Fucoidan composition

Extracted fucoidan was analyzed for its fucose, sulphate, uronic acid and protein content using the same methods as described in Chapter 3.

4.2.4 Statistical analysis

Using Minitab® (Version 16), ANOVA was carried out to test for differences in the yield of fucoidan between months, farms, and also the composition of fucoidan. Where significant differences occurred, Tukey's HSD was employed to examine where that effect occurred.

4.3 Results

4.3.1 Monthly changes in crude fucoidan content and composition

Monthly yields of crude fucoidan isolated from *U. pinnatifida* from farms 106 and 327 are shown in Figure 12. Yield of blade-derived fucoidan from farm 106 increased from July to September nearly four-fold from 3.56 to 13.71% dry weight (p = 0.000) and decreased down to 5.98% in October. Blade-derived fucoidan from farm 327 started with a similar yield with 3.35% dry weight in July but there was no significant difference between months (p = 0.183). Fucoidan from sporophyll in farm 106 increased more than two-fold during July to September from 26.34 to 57.28% dry weight (p = 0.001), and decreased slightly in October to 47.42%. A similar pattern was observed with sporophyll-derived fucoidan from farm 327 that showed an increase in yield from July to September from 25.38 to 69.98% dry weight (p = 0.000), that then decreased to 59.30% in October.

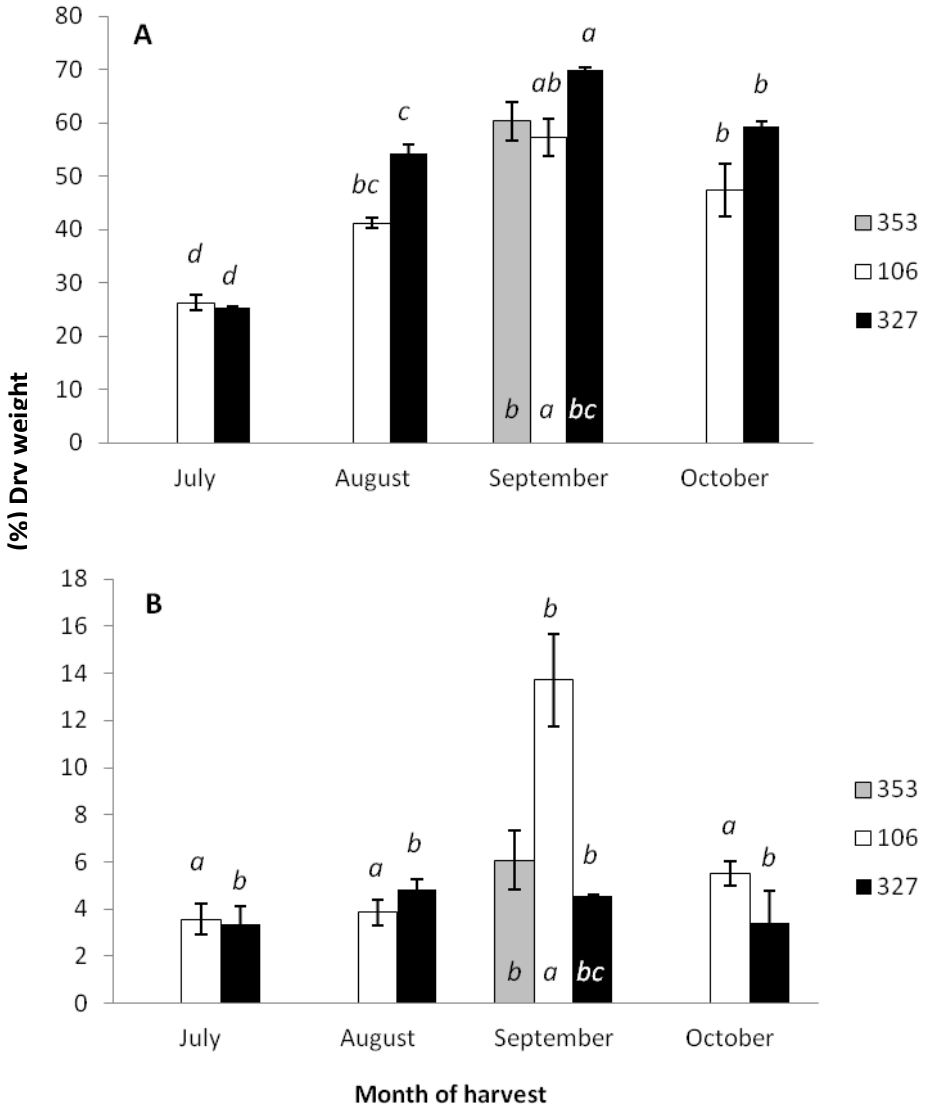

Figure 12 Monthly variation in the percentage yield of crude fucoidan from *U. pinnatifida* sporophyll (A) and blade (B) (106/327 = farm number). Mean ± SE (n = 3). Both farms were examined separately. Different superscript letters indicate significant differences at $p < 0.05$ by Tukey's test. Subscript letters examine differences between farms in September. Same superscript letters indicate no significant differences. Note differences in scale on y axis.

Constituents in fucoidan were significantly affected by the month of collection (Figure 13). The monthly content of sulphate in sporophyll-derived fucoidan showed a similar trend to the percentage yield of fucoidan, where the sulphate content increased from July to September, and then declined in October. Sporophyll-derived fucoidan from Farm 106 had increased sulphate content of more than two-fold from July (5.63%) to September (13.75%) (p = 0.000). Similarly sporophyll-derived fucoidan from farm 327

also showed an increase of more than two-fold during July to September from 5.92 to 16.39% dry weight (p = 0.000). Blade derived fucoidan showed a different trend. The sulphate content of blade-derived fucoidan from farm 327 remained constant from July to October (p = 0.052). However sulphate content of blade-derived fucoidan from farm 106 increased significantly in September and then decreased in October (p = 0.000).

Uronic acid content in fucoidan was also affected by the month of harvest. Blade-derived fucoidan from both farms 106 and 327 showed a similar trend in uronic acid content changes to that of sporophyll-derived fucoidan yield. An increase in uronic acid content from July to September was observed for farms 106 (p = 0.000) and 327 (p = 0.000). A maximum amount of uronic acid was found in blade derived fucoidan obtained in September from farm 327 that constituted 3.66% dry weight. As for fucoidan in the sporophyll, an opposite trend was found in the uronic acid content from farm 106, which showed a decrease from 0.89% dry weight in July to 0.62% dry weight in October (p = 0.000). Similarly sporophyll derived fucoidan from farm 327, showed a decrease in uronic acid content in from 1.01 to 0.82% dry weight over the same period. Uronic acid content in the blade appeared to be more affected by monthly changes in the reproductive stage of *U. pinnatifida*. Protein content of the fucoidans throughout the four months of harvest however was all below 1% dry weight (data shown in appendix).

Fucose composition of fucoidan was significantly affected by the month of harvest. Investigation of the fucose composition of fucoidan revealed that blade-derived fucoidan from both farms 327 and 106 had a decreased fucose content from July to October. Fucose yield decreased from 12.23 to 7.43% dry weight (p = 0.004) and 13.21 to 8.31% dry weight (p = 0.031) in blade-derived fucoidan from farms 106 and 327, respectively. The amount of fucose in sporophyll-derived fucoidan remained unchanged throughout the four months of harvest in both farms 106 (p = 0.091) and 327 (p = 0.176).

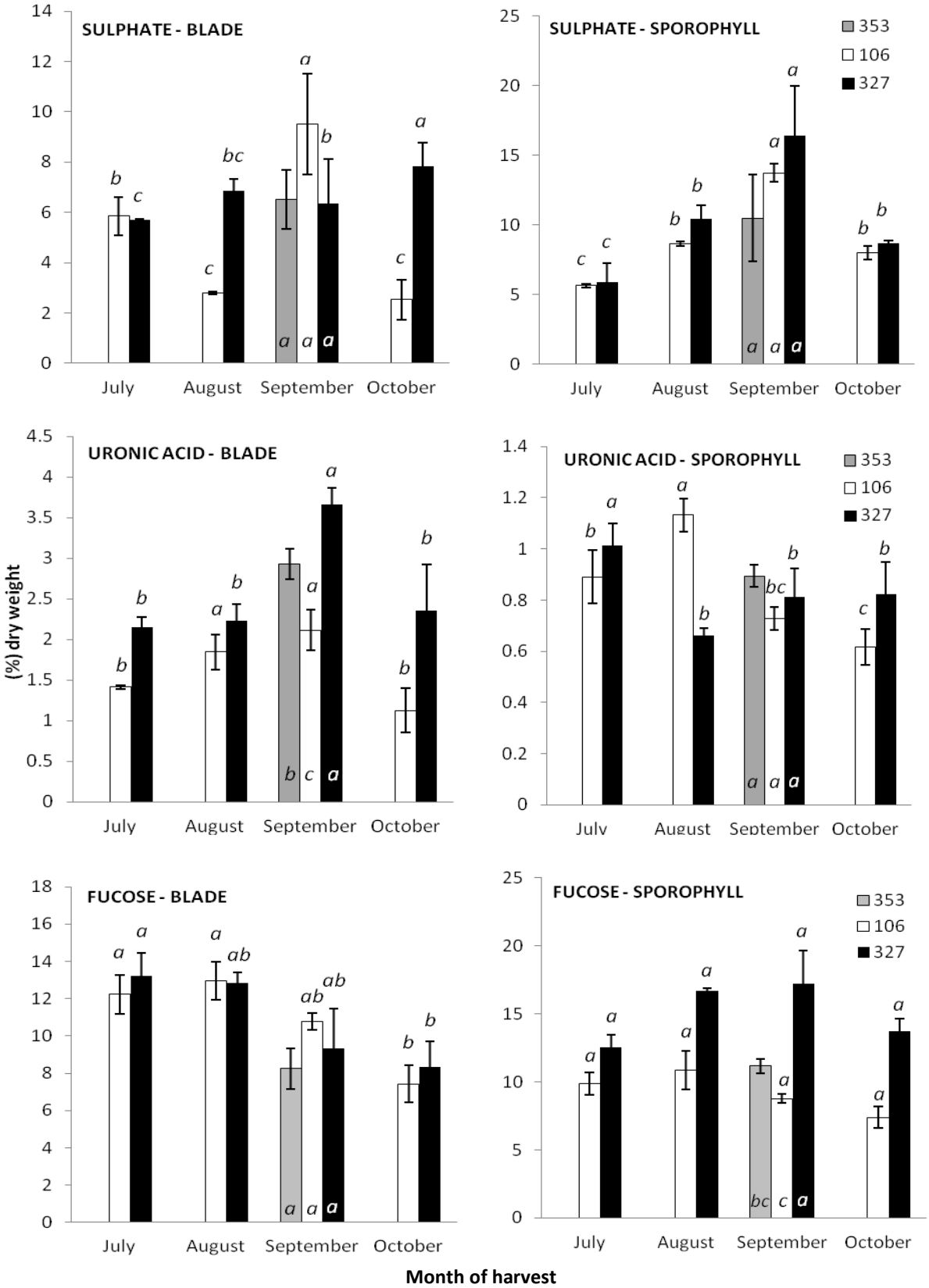

Figure 13 Monthly variations in the sulphate, uronic acid and fucose content in fucoidan (106/327/353 = farm number). Mean ± SE (n = 3). Farms were examined separately. Different superscript letters indicate significant differences at $p < 0.05$ by Tukey's test. Subscript letters examine differences between farms in September. Note differences in scale on y axis.

4.3.2 Differences in crude fucoidan content between farms

Extraction yields of fucoidan in the September harvest from farms 106 (Port Underwood), 327 (Port Underwood) and 353 (Pelorus Sound) are shown in Figure 12. Significant differences were found in the yields of sporophyll-derived fucoidan between farm 353 in Pelorus Sound and farm 106 in Port Underwood (p = 0.033). Farms 106 and 327 also showed significant differences in the yields of fucoidan from both the sporophyll (p = 0.003) and blade (p = 0.001) even though both farms are situated in Port Underwood (refer to Figure 11). In addition, blade-derived fucoidan from farm 353 was significantly different to farm 106 (p = 0.010) but not 327 (p = 0.290).

As shown in Figure 13, no significant differences were observed between the sulphate content of fucoidan extracted from the sporophyll from all three farms (p = 0.079). Blade-derived fucoidan from all three farms also showed no significant differences in its sulphate content (p = 0.726). Uronic acid content in blade-derived fucoidan from all three farms showed significant differences (p = 0.000), with farm 327 having the highest amount (3.65% ± 0.209SE dry weight). The content of uronic acid in sporophyll-derived fucoidan was relatively similar between farms, with all less than 1% dry weight. Blade-derived fucoidan also showed similar fucose content between farms (p = 0.512). However significant differences were found in sporophyll-derived fucoidan between farms (p = 0.028).

4.4 Discussion

The variations in the results obtained indicated that crude fucoidan extracted from *U. pinnatifida* was heterogeneous with respect to the sulphate contents, uronic acid content and the fucose concentration. Variations in chemical composition have also been reported for fucoidan from other species of algae (Duarte, Cardoso, Noseda, & Cerezo, 2001; Ly et al., 2005). The results also indicated that the reproductive stages of *U. pinnatifida* may influence the monthly changes in fucoidan content in terms of yield. Sporogenesis of *U. pinnatifida* in the Marlborough Sounds begins roughly at the end of winter and the start of spring in July when the water temperature begins to increase (Parsons, 1994). In this study, over the months of June and July 2011, the algae started

developing sporophylls that may result in physiological and chemical changes, including the degradation of blade and photosynthetic pigments (Honya et al., 1999; Nimura & Mizuta, 2001), the increase in nucleic acids (Nimura & Mizuta, 2001), the decrease in alginic acid (Skriptsova et al., 2009), and the maturation of sporophyll (Honya et al., 1999).

Fucoidan yield increased two-fold in the sporophyll from farm 106 from July to September, while those from farm 327 increased three-fold over the same period. Similarly, *U. pinnatifida* grown in Japan as well as other species of brown algae peak in fucoidan yield just before the summer (Honya et al., 1999; Skriptsova et al., 2009; Zvyagintseva et al., 2003). Among the farms and months investigated in this study, a maximum yield of 69.98% crude fucoidan was recorded in *U. pinnatifida* from the Marlborough Sounds. Of the 14 different species of seaweed listed in Table 2, this maximum yield was not far from the highest yield reported for *S. wightii*, which was 71.50% dry weight. Nevertheless, the maximum yield of crude fucoidan in this study was significantly higher than the yields obtained from other studies using *U. pinnatifida* and much higher than the yield obtained in Chapter 3 (13.32%), where three fucoidan extraction techniques were compared using *U. pinnatifida* harvested from Great Barrier Island.

Sulphate content varied between months, indicating that sporogenesis of *U. pinnatifida* may directly affect the amount of sulphate groups in the polysaccharide, especially in the sporophyll. The sulphate content in sporophyll-derived fucoidan when the seaweed matured, reached a maximum of 13.75% (farm 106) and 16.39% (farm 327) dry weight in September. These results were in accordance with Honya et al. (1999) which showed a similar trend in the changes of fucoidan sulphate content from *L. japonica,* where the molar ratio of sulphate increased as the alga matured. The increase in sulphate content was proportional to the percentage yield of sporophyll-derived fucoidan over the July-September harvest that may be attributed to endogenous changes of *U. pinnatifida* due to the formation and maturation of sporophylls, as previously stated by Skriptsova et al. (2009). Inconsistency in the sulphate content of blade-derived fucoidan suggests that the majority of the endogenous changes of *U. pinnatifida* during sporogenesis occur in the reproductive region of the alga. Similarly a previous study also showed that the sulphate content in blade-derived fucoidan from *L. japonica*, was stable during its growing season (Honya et al., 1999).

Monthly changes in the uronic acid content of fucoidan may also be affected by the sporogenesis of *U. pinnatifida*. The yield of uronic acid increased to a maximum in September for both farms 106 (2.12% ± 0.25SE) and 327 (3.66% ± 0.21SE). Much like the yield and sulphate content in sporophyll-derived fucoidan, the uronic acid content in blade-derived fucoidan decreased in October. However the uronic acid content in sporophyll-derived fucoidan from both farms 327 and 106 showed a decrease in uronic acid content from July to October. Although no publications have examined the monthly changes in uronic acid content, it can be postulated that the decrease in uronic acid content from sporophyll-derived fucoidan may be the result of sporophyll development, and the increase in uronic acid from blade-derived fucoidan may be associated with blade degradation (Honya et al., 1999; Skriptsova et al., 2009).

Fucose content also varied when *U. pinnatifida* matured. Significant differences were found in blade-derived fucoidan from both farms, where there was a decrease in fucose content from July to October. Much like the increase in uronic acid content in the blade was likely to be caused from blade degradation; degradation of the blade may also be responsible for the decline in fucose content in blade-derived fucoidan. As for the fucose content in sporophyll-derived fucoidan, no significant changes were detected over the four months of harvest, implying that sporogenesis of *U. pinnatifida* had little effect on the fucose concentration in sporophyll-derived fucoidan. Similarly Skriptsova, et al. (2008) reported that *U. pinnatifida* collected in Peter the Great Bay (Sea of Japan) also showed no significant changes in the fucose content of sporophyll-derived fucoidan during sporogenesis. Further work is needed before we can claim correlation between the chemical compositions of fucoidan with sporulation of *U. pinnatifida*.

Besides sulphate, uronic acid and fucose, protein was also present in detectable amounts during the four month harvesting period. All values were well below 1% dry weight, which was acceptable as this value related back to the efficiency of the extraction technique, and indicated little protein contamination. Fucoidan has a innate tendency to retain salts and impurities such as protein, even after several washes with alcohol (Schweiger, 1962). Thus, it cannot be ascertained whether these small amounts of protein were actually contaminants, or part of the fucoidan structure. A study suggested that pure fucoidan should have a protein content of less than 0.1% dry weight

(Hayakawa & Nagamine, 2009). Therefore, the protein content of fucoidan reported from other studies (see Table 2) and our study, suggested that the fucoidan was crude.

Comparing between farms, it was found that farm 327 from Port Underwood and farm 353 from Pelorus Sound had a similar yield of fucoidan compared to farm 106 from Port Underwood. Sulphate and fucose content in sporophyll-derived fucoidan from different farms were significantly different, but not for blade derived fucoidan. Conversely, uronic acid content of blade-derived fucoidan was significantly different between farms, but not for sporophyll derived fucoidan. These results suggest that *U. pinnatifida* from the Marlborough Sounds that grew in different locations underwent sporulation at around the same time due to the similar fucoidan yields in both farms 327 and 353. It also suggests that the endogenous changes in the alga during sporogenesis are much more likely to affect the composition of fucoidan than environmental factors. This was due to similar fucoidan yield, sulphate and fucose content from both farms 353 (Pelorus Sound) and 327 (Port Underwood) even though the farms were approximately 40 kilometres apart. A previous study reported that the composition of fucoidan was more affected by the life cycle stage of the alga rather than environmental aspects (Skriptsova et al., 2009).

It was important to note that *U. pinnatifida* harvested in the Pelorus Sounds were much smaller in comparison to those in Port Underwood. This limited the study to only a comparison of *U. pinnatifida* in September for samples obtained from Pelorus Sound and Port Underwood rather than a comparison over four months due to the small size of the plants from Pelorus Sound that is necessary for reproducible fucoidan extractions. A possible explanation of this, as mentioned in section 2.4.5, may be the amount of fresh water concentration in the sea water. Ocean with a high degree of fresh water input are capable of slowing down the growth and even killing the alga grown in that area (Tiroba, 2007). The farms at Pelorus Sound was situated further from the open ocean than Port Underwood and was mainly surrounded land, while Port Underwood was situated near the ocean (refer to Figure 11). Hence it may be postulated that Pelorus Sounds have a higher fresh water input than Port Underwood, leading to a slower growth rate of *U. pinnatifida* in the Pelorus Sound. This, however, did not significantly affect the yield and chemical composition of fucoidan from Pelorus Sounds.

4.5 Conclusion

In the present work, the monthly changes in fucoidan content and composition of *U. pinnatifida* in the Marlborough Sounds, New Zealand was investigated. This study showed that sporogenesis of this alga began around the end of July due to an observed increase in fucoidan content, and reached maximum maturity at around September. Over this period, the results showed a significant change in fucoidan content, and support the claim that sporophyll synthesis in *U. pinnatifida* may affect fucoidan content in the alga. In addition, results showed an increase in uronic acid content of blade-derived fucoidan and an increase in sulphate content of sporophyll-derived fucoidan. Changes in the fucose content of blade-derived fucoidan were evident but not for sporophyll-derived fucoidan. These changes suggest that chemical components in fucoidan were likely linked with the maturation of *U. pinnatifida* (Honya et al., 1999; Skriptsova et al., 2009).

Unlike other fucoidan studies, the fucoidan in this study was extracted from individual alga and not from a homogeneous batch. Some individual variations in fucoidan content of alga harvested from the same farm and month existed. Variations between farms and individual alga may be influenced by exogenous factors such as water temperature, amount of sunlight present, amount of freshwater input, and concentration of nutrients in the water. Endogenous factors that may also play a role in the variations seen in our results include growth rate of each alga being different. This in turn could lead to different morphological changes and reproductive rates. Controlling such factors was practically impossible in this study. In general, our work showed that each alga, even though harvested at the same time and from the same farm, may contain varying amounts of fucoidan and chemical components, as each alga may be at different reproductive stages from one another.

5 Antioxidant activity of fucoidan

5.1 Introduction

Fucoidans have a wide variety of bioactivities that are closely related to their molecular weight, sulphate content, uronic acid content, and sugar composition that include the sulphate/fucose ratio (Li, Rui et al., 2008; Skriptsova et al., 2009). Fucoidan is an excellent natural antioxidant and has been claimed to have great potential for preventing diseases that are mediated by free radicals (Li, Lu et al., 2008). In order to obtain fucoidan with the best bioactivity, researchers fractionate fucoidan using ion-exchange chromatography (Bilan et al., 2002; Li et al., 2006; Ly et al., 2005; Wang et al., 2007). Once purified, fucoidan fractions are then analyzed separately as each fraction has its own unique composition and bioactivity. Consequently, the fraction with the highest bioactivity can be further characterized in relation to its composition.

5.2 Materials and methods

5.2.1 Sample collection

As discussed in Chapter 4, the fucoidan content was the highest in the September harvest. Hereafter, the fucoidan extracted from the *U. pinnatifida's* sporophyll collected in September was combined from three plants to provide enough material for further analysis in this chapter.

5.2.2 Fucoidan extraction

The $CaCl_2$ extraction method from Bilan et al. (2002) was used in this chapter for the same reasons described in Chapter 3.

5.2.3 Fractionation of fucoidan

5.2.3.1 Swelling the gel

Crude fucoidan was fractionated using anion-exchange chromatography through a diethylaminoethyl (DEAE) Sephadex A-25 (Pharmacia) column. Buffer was prepared by dissolving 6.057 g of Tris base (US Biochemical Corp) into 500 mL deionized water. The addition of 1 M HCl (RCI Labscan Ltd) was added dropwise until pH 7.4 was achieved. This buffer solution was then made up to 1 L to give 0.05 M Tris-HCl.

DEAE Sephadex A-25 (3 g) was suspended in excess buffer (100 mL) and heated to 85^{o}C in a water bath for 5 hours. The heat swelled the gel a lot quicker and also degassed the gel by removing trapped air pockets. After heating, the gel was allowed to settle overnight at room temperature and excess buffer and fine gel particles which had not settled to the gel bed were decanted off. Fresh buffer (50 mL) was then added to make a slurry.

5.2.3.2 Packing and washing the column

A glass column (25 cm x 4 cm) with a mesh sieve was mounted vertically on a laboratory stand and rinsed with buffer. Buffer was poured into the column to a level just above the mesh sieve and the outlet tap was closed. The gel suspension was then poured into the column using a glass rod as a guide with its end touching the inner wall until approximately 80% of the column was filled.

The outlet tap was opened to allow flow of the buffer by gravity, settling the gel in the column. Further gel suspension was added. The column was then washed with 50 mL of buffer in order to further pack the column bed and also equilibrate the gel with buffer. The column was placed on top of the fraction collector as shown in Figure 14, and the outlet tap was closed until required.

Figure 14 Ion-exchange chromatography apparatus.

5.2.3.3 Fractionation of fucoidan for analysis

Crude fucoidan (2000 mg) from the September harvest was dissolved in 20 mL Tris-HCl buffer and mixed. This solution was filtered through filter paper before applying it to the column. The first fraction was eluted with deionized water at a flow rate of 50 drops per tube. This was followed by sodium chloride (NaCl) (Ajax Finechem Ltd) elution at increasing concentrations (1 M and 2 M NaCl each time) until the absence of a positive reaction of the phenol-sulphuric acid assay in the test tubes containing the eluted sample.

The original phenol-sulphuric acid assay used was according to Dubois et al. (1956) to check for the presence of sugars in the tubes. Test tubes containing the eluted samples were transferred (1 mL) into more robust glass test tubes (5 mL). Then 0.05 mL 80% phenol (BDH Limited) and 2.5 mL concentrated H_2SO_4 (J.T. Baker Limited) were added to each test tube and mixed thoroughly. Test tubes were placed on a rack and heated in a 35^{o}C water bath for 20 minutes. The absorbance was measured at 480 nm (Ultrospec 2100) for any indication of sugars and uronic acids (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

When no sugar was found to be present in the tube, the absorbance was zero or close to zero (< 0.01). This was an indication that no more fucoidan was eluted off the column with the specific eluent, before the next eluent was applied. The carbohydrate-positive fractions with the same eluent were pooled together and dialyzed over 3 days at room temperature against deionized water, with a buffer change daily, in order to remove NaCl from the fractions. Dialysis tubes with a cut-off membrane of 12-14,000 Da were used (Medicell International Ltd) and fractions were then lyophilized using a Christ LOC-1M freeze dryer for 48 hours.

5.2.4 Fucoidan fraction composition

Fucoidan fractions were analyzed for fucose, sulphate, protein, and uronic acid content as described in sections 3.2.4.1 to 3.2.4.4.

5.2.5 Antioxidant activity

5.2.5.1 DPPH free-radical scavenging activity

Fucoidan and its fractions were analyzed for their antioxidant activity based on their scavenging activity of the 1, 1-diphenyl2-picryl hydrazyl (DPPH) free radical, using the method of Mensor et al. (2001). DPPH is a stable free radical and acts as a scavenger for other radicals. Rate reduction of a chemical reaction using DPPH is a useful indicator of the radical state of a reaction.

Fucoidan samples (2.5 mL) were prepared in triplicates at different concentrations (100-4000 µg/mL) and transferred into 1 mL 0.3 mM methanolic DPPH solution (Sigma Aldrich). Samples were left to stand for 30 minutes in the light and the absorbance was measured at 517 nm, zeroing the spectrophotometer with a methanol blank. The DPPH radical had a dark violet colour in solution, and once neutralized, became pale yellow allowing visual monitoring of the radical reaction (Mensor et al., 2001). Ascorbic acid (BDH laboratories) was used as a positive control and commercial fucoidan from Sigma was also used as a comparison. The % inhibition was calculated using the following equation:

$$\% \, inhibition = 1 - \frac{(Absorbance \, of \, sample - absorbance \, of \, blank)}{(Absorbance \, of \, control)} \, x \, 100$$

5.2.5.2 CUPRAC Assay

The CUPRAC (cupric reducing antioxidant capacity) method was applied to fucoidan and its fractions. Copper chloride ($CuCl_2$) solution (0.01 M) was prepared by dissolving 0.426 g $CuCl_2$ (Ajax Chemicals) in water and diluting the solution to 250 mL. Ammonium acetate (NH_4Ac) buffer (pH 7, 1.0 M) was made by dissolving 19.27 g of NH_4Ac (Ajax Chemicals) in water, and diluting this solution to 250 mL. Neocuproine (Nc) solution (0.075 M) was prepared fresh by dissolving 0.039 g Nc (Sigma Aldrich) in 96% ethanol and diluting to 25 mL with ethanol.

Fucoidan samples (1 mL) were prepared in triplicates at different concentrations (100-2500 µg/mL) and added into a solution containing 1 mL $CuCl_2$, 1 mL NH_4Ac, 1 mL neocuproine, and 0.1 mL water. Test samples were incubated for 10 minutes at room temperature, and the final absorbance was measured at 450 nm, zeroing the spectrophotometer with a water blank (Apak, Guclu, Ozyurek, & Celik, 2007).

5.2.6 Molecular weight determination

To determine the molecular weight of fucoidan and its three fractions, gel permeation chromatography (GPC) was applied using Sephadex G-200 (Pharmacia). By applying molecules through the gel, small molecules become trapped in the gel beads and those with a larger molecular weight will flow through the gel. Thus, this technique allowed larger molecules to elute first, followed by smaller molecules which are held longer inside the beads (Garrett & Grisham, 1999). Blue Dextran 2000 (Pharmacia), blue dextran 500 (Pharmacia), bovine serum albumin (BSA) (ICP Bio Limited), cytochrome C (BDH laboratories), and cobalamin (Sigma) with molecular weights of $2x10^6$, $5x10^5$, 67 000, 11 700, and 1355 Da, respectively, were used as standard molecular weight markers. Standards and samples were dissolved in phosphate buffer (pH 7) at a concentration of 10 mg/mL. Sephadex G-200 was swelled and packed in the same manner as described in 5.2.3.1 and 5.2.3.2 using 0.1 M sodium acetate (CH_3COONa) buffer (pH 5) into a 30 cm x 4 cm glass column.

To calibrate the column, the void volume (V_o) was measured, i.e. the amount of mobile phase collected from the time a known standard with a large molecular weight was applied to the column until the first appearance of the standard eluting out. In this case, Dextran 2000 was used as it had the largest molecular weight, therefore providing the quickest elution time (see Figure 15). Each sample will experience V_o, as long as the molecular weight was smaller than the blue dextran 2000. In general, *U. pinnatifida's* fucoidan was known to have a molecular weight that ranged from 30-1200 KDa (Fitton et al., 2007). Standard proteins with known molecular weights were then applied to the column and the volume at which each standard was eluted (V_r) was recorded. This was done by visual inspection of the coloured standards. However, as the BSA standard used was not a coloured compound; 1 mL fractions collected in the test tubes were measured for the presence of aromatic amino acids in the protein at 280 nm. The increase in absorbance at 280 nm indicated when the protein eluted. A calibration curve was plotted with the molecular weight of the protein vs V_r/V_o. Fucoidan samples were collected in 1 mL portions just before the void volume and tested for the presence of sugars using the phenol-sulphuric acid assay described in section 5.2.3.3.

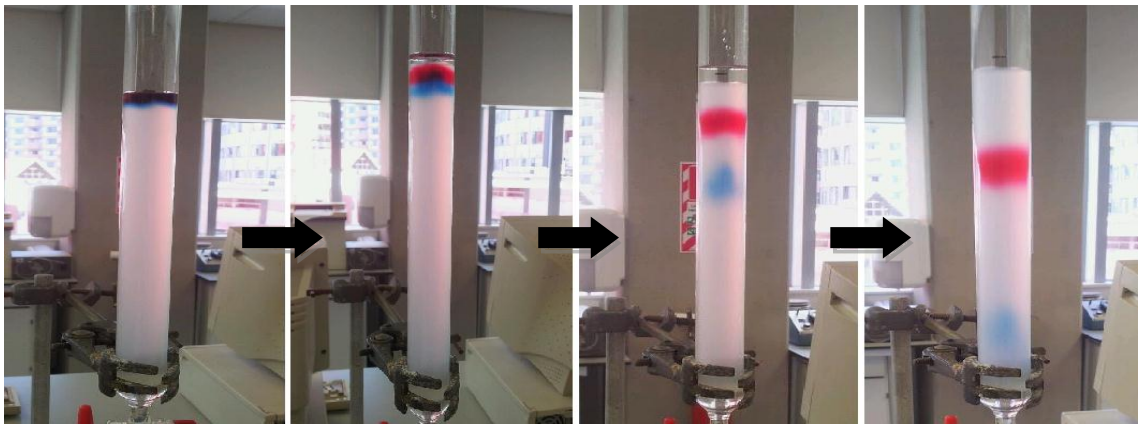

Figure 15 Calibration of gel permeation chromatography. Separation shown in this picture shows a standard mixture of Dextran 2000 (blue) and cobalamin (red).

5.2.7 Statistical analysis

All data are shown as means of triplicates and the degree of significance at $p < 0.05$ was determined using ANOVA, and processed with Minitab® (Version 16) and Excel and

Statistica (2003). Where significant differences occurred, Tukey's HSD was employed to examine where that effect occurred.

5.3 Results

5.3.1 Fractionation and molecular weight determination of fucoidan

The eluting profile from the ion-exchange chromatography of crude fucoidan (F0) is shown in Figure 16, where the absorbance of the individual test tubes after hydrolysis was plotted against the tube number. Three fractions were obtained: F1, F2, and F3 from using water, 1M NaCl and 2M NaCl elution on a DEAE A-25 Sephadex column, respectively. No polysaccharide was detected using 2.5M NaCl. The chromatogram showed that the majority of crude fucoidan applied to the DEAE A-25 Sephadex gel was eluted with water.

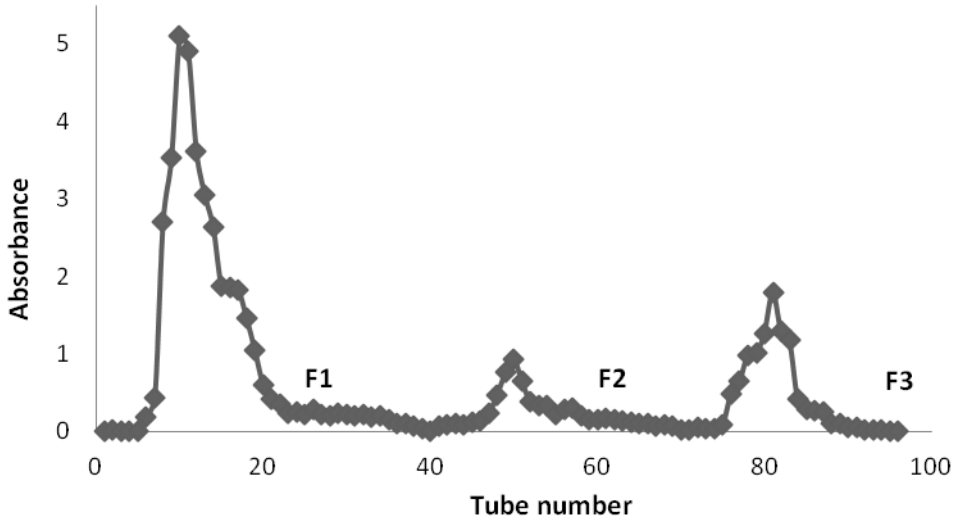

Figure 16 Chromatogram of fucoidan on a DEAE-Sephadex A-25 column. F1 eluted with water; F2 eluted with 1M NaCl; F3 eluted with 2M NaCl.

The GPC calibration curve of the protein standards yielded a correlation factor of R^2 = 0.9943, with a linear formula of y = -2.1542x + 8.4903 (Figure 17). This equation was used to determine the molecular weight of the fucoidan fractions. The average

molecular weight of crude fucoidan was estimated to be about 1.35 x 10^3 KDa. Fractions F1, F2 and F3 had molecular weights of 1067, 870 and 870 KDa, respectively, whereas commercial fucoidan was found to have the lowest molecular mass of 127 KDa.

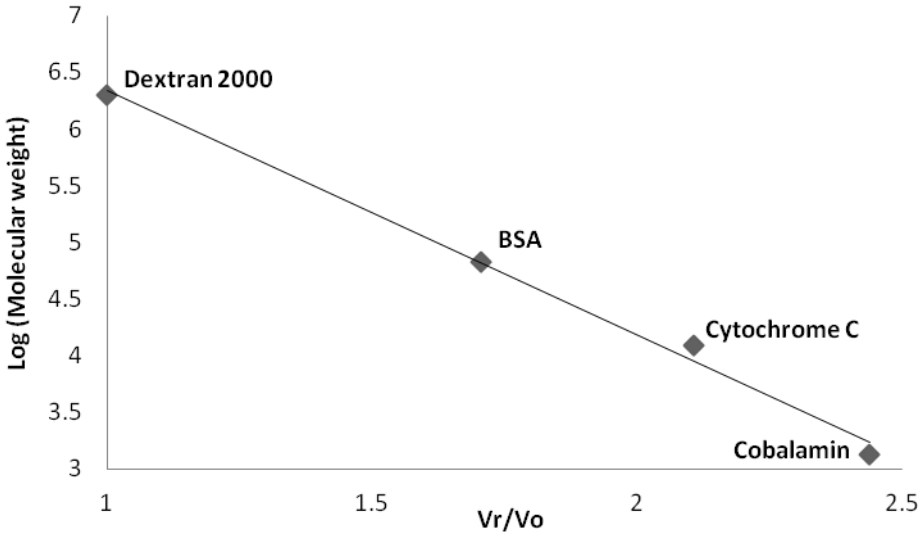

Figure 17 Calibration curve for determination of the molecular weight of fucoidan by Sephadex G-200. Protein standards used in order of increasing molecular weight were Cobalamin (1355 Da), cytochrome C (12,270 Da), BSA (67,000 Da), and Dextran (2,000,000 Da). $R^2 = 0.9943$.

The chemical composition of crude fucoidan, its fractions, and commercial fucoidan is shown in Table 7, in addition to the molecular weight and the sulphate:fucose ratio. The results showed that all fucoidan fractions contained the major sugar component fucose, along with sulphate groups being another major constituent, and uronic acid and protein as minor stituents. However the proportion of these components varied from one another. F1 had the highest uronic acid content of 4.34% ($p = 0.000$), whereas F3 had the highest sulphate content of 25.19% ($p = 0.000$). Fucose content showed no significant differences between F0, F1, F2 and F3. However all four fucoidan samples contained significantly less fucose than commercial Sigma fucoidan. The high fucose concentration in commercial fucoidan therefore had a relatively low sulphate:fucose ratio of 0.41. In addition, the sulphate:fucose ratio increased with an increase in NaCl concentration as an eluting solvent for fractions F1 to F3.

Table 7 Chemical composition (% dry weight) of crude fucoidan (F0), its fractions and commercial fucoidan (Sigma) (n = 3).

Fraction	NaCl	UA	SO_4	Protein	Fucose	MW, KDa	SO_4:Fucose
F0	-	1.24^{c}	15.02^{d}	0.36^{bc}	17.24^{b}	1350	0.87
F1	0M	4.34^{a}	6.96^{e}	0.86^{a}	19.87^{b}	1067	0.35
F2	1M	0.84^{c}	22.78^{b}	0.63^{b}	16.94^{b}	840	1.34
F3	2M	0.67^{c}	25.19^{a}	0.11^{c}	17.45^{b}	840	1.44
Sigma	-	3.14^{b}	17.96^{c}	0.41^{bc}	43.74^{a}	127	0.41

Different superscript letters indicate significant differences at $p < 0.05$ by one-way ANOVA followed by Tukey's test. Same superscript letters indicate no significant differences.

5.3.2 Antioxidant activity

Crude fucoidan, the three fractions, and commercial fucoidan were tested for antioxidant activity via the DPPH and CUPRAC assays. The experimental results are presented in Figures 18 and 19, where fucoidan and all three fucoidan fractions were established to possess antioxidant activity. Vitamin C was used as a positive control for the DPPH assay.

Antioxidant activity was determined by assaying the reduction of DPPH radicals. The inhibition percentage of all tested samples showed a concentration-dependent pattern as shown in Figure 18. The percentages of inhibition of the fucoidans at concentrations ranging from 10 – 4000 µg/mL however, were lower than vitamin C. Vitamin C had over 90% inhibition at a concentration of 100 µg/mL, whereas F0 (crude fucoidan) required a concentration of 4000 µg/mL to reach a similar percentage. The percentage inhibition at 4000 µg/mL for F1, F2, F3, and commercial Sigma fucoidans were 70.34, 72.22, 78.55, and 76.61%, respectively. Among the three fractions, F3 had the strongest scavenging ability (p = 0.000) while F1 had the lowest. Commercial fucoidan also showed strong inhibition properties, with a higher percentage inhibition than F1 and F2 (only at 2000 and 4000 µg/mL), but not F3. F0 had the strongest inhibiting potential compared to all tested samples apart from vitamin C (p = 0.000).

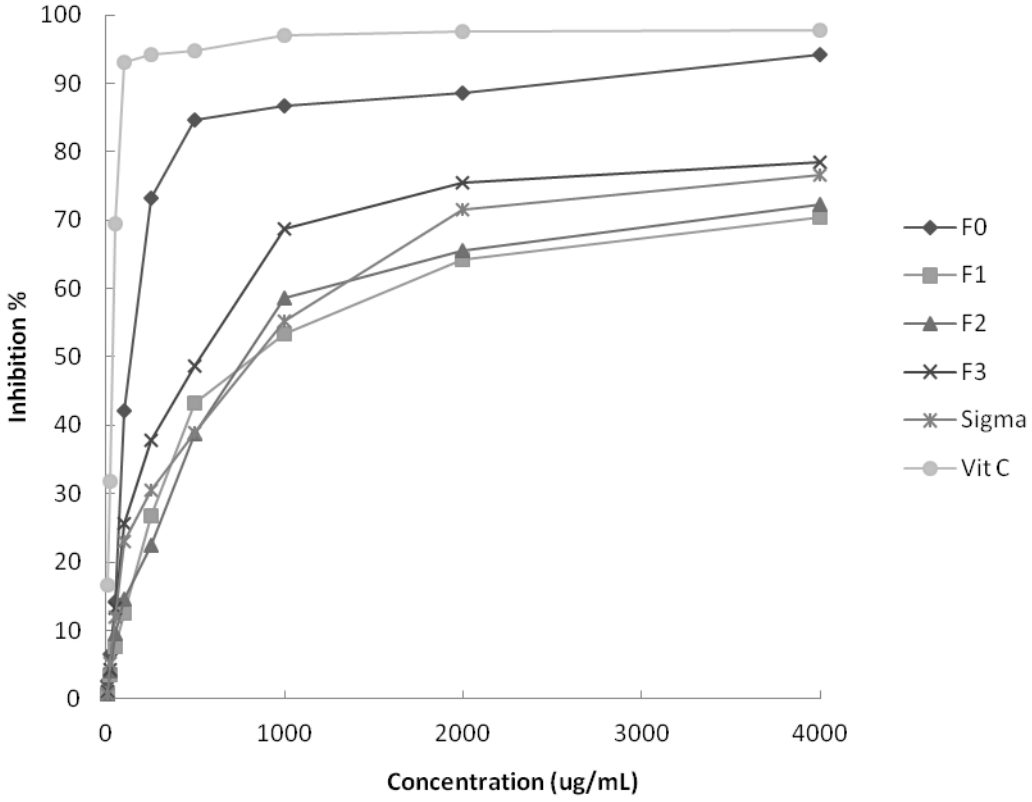

Figure 18 Scavenging effects on DPPH radical by crude fucoidan (F0), different fucoidan fractions (FI, F2 and F3), commercial fucoidan (Sigma) and Vitamin C. Values are means from three independent tests.

Figure 19 shows the reducing power of fucoidan samples on copper ions using the CUPRAC assay. Higher absorbance readings indicated higher reducing ability of the samples. All samples exhibited the ability of reducing coppers ions from Cu(II) to Cu(I) in a concentration-dependent manner. F0, as with the DPPH assay, showed the highest reducing activity when compared to the three fractions and commercial fucoidan (p = 0.008). At a concentration of 2.5 µg/mL, the absorbance of F0, F1, F2, F3 and commercial fucoidan were 1.547, 0.225, 0.262, 0.656 and 0.513, respectively. These results similar to those obtained from the DPPH assay in which F0 showed the highest total antioxidant capacity (TAC), followed by F3, commercial fucoidan, F2, and lastly F1.

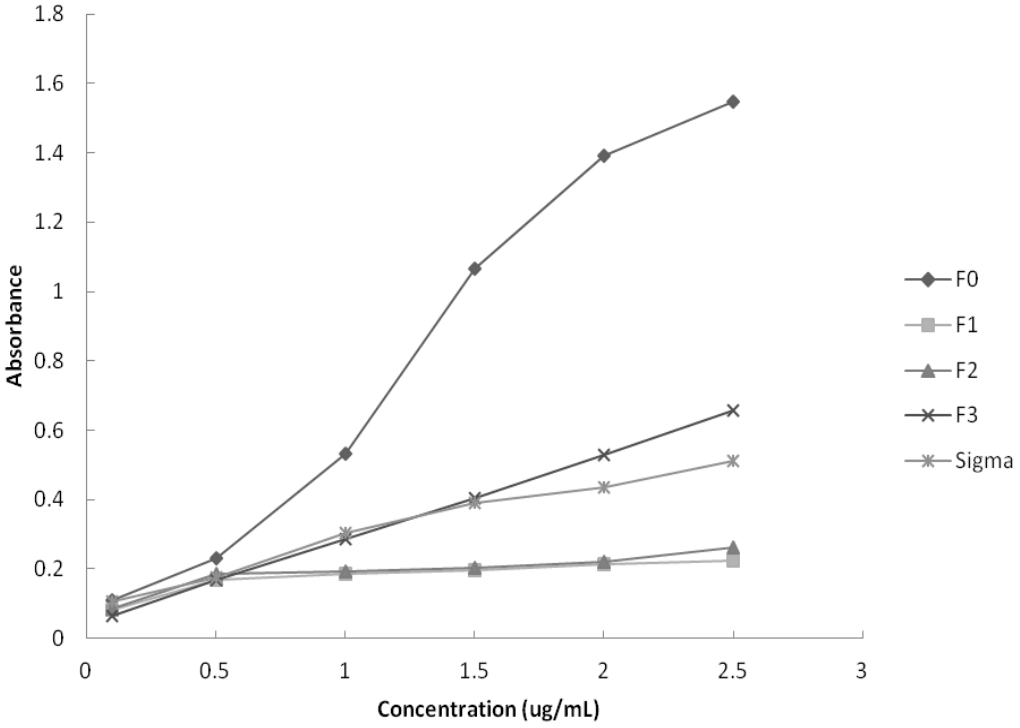

Figure 19 Total antioxidant capacity of crude fucoidan (F0), different fucoidan fractions (FI, F2 and F3), and commercial fucoidan (Sigma) with the CUPRAC reagent. Values are means from three independent tests.

5.4 Discussion

Fractionation of fucoidan (F0) by anion-exchange chromatography yielded three fucoidan fractions: F1, F2 and F3 by chromatography on a DEAE Sephadex A-25 column. The first fraction eluted out with water resulted in a major peak. This was followed by elution using 1M NaCl then 2M NaCl which produced two smaller peaks. Anionic groups such as sulphate are the main contributors to the overall negative charge on fucoidan (Ponce et al., 2003). Once crude fucoidan was introduced into the column, branched sulphate groups would automatically be drawn to the positively charged ion-exchange groups of DEAE. Only the use of strong ionic solvents like NaCl will release the sulphate groups from the gel. This was the case in this study as there was an increase in sulphate content in the fractions from F1 to F3. As the concentration of NaCl increased in the mobile phase, more sulphate groups were released. It was worth noting that a previous study using the same type of gel but a different species of seaweed (*S. swartzii*) required a concentration of 3.5M NaCl solution to wash out all the remaining fucoidan in the gel, whereas this study only required a 2M concentration.

This implied that fucoidans from different species of brown seaweed not only have varying amounts of constituents found in fucoidan, but also have varying levels of ionic strength caused by those contituents (Ly et al., 2005).

Fucoidan fractions were analyzed for their fucose, sulphate, uronic acid and protein content, which gave varying results from one fraction to another. A trend was observed where the fraction eluted with a lower NaCl concentration (1M) had higher uronic acid content and fewer sulphate groups. In contrast, the fraction eluted with higher NaCl concentration (2M) was higher in sulphate groups and lower in uronic acid content. The same trend was previously reported using the alga *S. swartzii* harvested in Vietnam that was also fractionated on a DEAE Sephadex A-25 column (Ly et al., 2005). There was also a significant (p = 0.000) but small reduction in protein content of the fractions as the NaCl concentration of the eluting solvents increased suggesting that the protein groups in fucoidan are not anionic even though both cationic and anionic proteins exist (Lin, Yu, Chang, & Tseng, 2007). On the other hand, the sulphate:fucose ratio increased in the fucoidan fractions as molarity of NaCl eluting solvents increased. The F2 sulphate:fucose ratio of 1.34, was considerably close to the F2 sulphate:fucose ratio of 1.24 in another study using *L. japonica* (Wang et al., 2007). The three fucoidan fractions and crude fucoidan showed no significant changes in fucose content. Similar results have been reported for fucoidans from the same species and different species (*S. swartzii*) (Ly et al., 2005; Skriptsova et al., 2009).

The components of commercial fucoidan was also examined and showed the highest fucose content out of all three purified fractions. However, this result was expected as the commercial fucoidan was obtained from *F. vesiculosus*. This fucoidan only contains fucose as the major monosaccharide (Li, Lu et al., 2008). In contrast, fucoidan derived from *U. pinnatifida* not only contained fucose as the primary component, but also galactose, xylose and mannose as secondary components (Li, Lu et al., 2008) leading to an overall lower fucose percentage. The high fucose content of commercial fucoidan contributed to a low sulphate:fucose ratio, but not quite as low as F1. Uronic acid content in commercial fucoidan was significantly higher than that of F0, F2 and F3 (p = 0.000) except for F1. However the sulphate content of commercial fucoidan was significantly higher than F0 and F1 (p = 0.000) but not F2 and F3 suggesting that sulphate groups within commercial fucoidan were less anionic than F2 and F3.

This study found the molecular weight of crude fucoidan extracted from the sporophyll of *U. pinnatifida* collected at the Marlborough Sounds, New Zealand to be around 1350 KDa. It was reported that the general molecular weight of crude fucoidan extracted from *U. pinnatifida* may range from 30 – 120 KDa (H. Fitton & Dragar, 2006). The large difference in molecular weight may depend on the seaweed's harvesting time, the habitat it was grown in, as well as the difference in extraction techniques (W. Kim, J et al., 2007). Taken collectively, these factors suggest there were more diversity in the polysaccharides molecular structure besides chemical composition. A previous study reported a molecular weight of 2100 KDa from fucoidan isolated from *U. pinnatifida* collected at Wando, Korea (W. Kim, J et al., 2007) that had a relatively large molecular mass similar to the crude fucoidan in this study. Besides *U. pinnatifida*, fucoidan extracted from *C. okamuranus* had a molecular weight of 2000 KDa (Sakai, Ishizuka, & Kato, 2003). Commercial fucoidan had a molecular weight of 127 KDa, which was within the specified molecular mass from the Sigma database (Sigma-Aldrich, 2011) of 20 – 200 KDa, confirming that the GPC technique worked well.

Both F2 and F3 fractions gave a positive sugar reaction at the same volume of eluent during GPC. As a result, both fractions had a similar molecular mass of around 840 KDa. There seem to be a tendency that molecular weight decreases when the fucoidan fractions were eluted out with stronger ionic solvents (Skriptsova et al., 2009). In this study, crude fucoidan had a molecular weight of 1350 KDa, while the molecular weight of its fractions eluted with water, 1M and 2M NaCl were 1067, 840 and 840 KDa, respectively. Similarly crude fucoidan from *U. pinnatifida* collected at Tasmania, Australia, had a molecular weight of 710 KDa that decreased to 290 KDa when purified with 2M NaCl (Hemmingson, Falshaw, Furneaux, & Thompson, 2006). This decrease in molecular mass was most likely due to depolymerisation of fucoidan during the purification process (Skriptsova et al., 2009).

Commercial fucoidan, crude fucoidan and three purified fucoidan fractions were tested for their antioxidant activity using the DPPH and CUPRAC assays. The experimental results showed that all samples demonstrated scavenging activity against DPPH radicals and copper ions. Fraction F3, which had the highest sulphate content and highest sulphate:fucose ratio in the fractions showed the strongest antioxidant activity in both assays proving that sulphate content and ratio of sulphate to fucose played a significant part in the antioxidant activity of fucoidans (Li, Lu et al., 2008; Ly et al., 2005).

Previous studies also reported similar findings (Li, Lu et al., 2008; Ly et al., 2005). The inhibition of vitamin C towards DPPH radicals was the highest compared to all fucoidan fractions and crude fucoidan. Conversely, Maritech® fucoidan was reported as being a stronger antioxidant than vitamin C, but details of extraction method and species of seaweed used were not specified (Fitton et al., 2007). Previous work has shown that fucoidan was only stronger than vitamin C at inhibiting superoxide radicals but not the DPPH radical and copper ions. This was probably due to superoxide radicals being less active, so was scavenged much more easily than DPPH radicals and copper ions (Wang et al., 2009). The scavenging abilities of DPPH radicals from another study using fucoidan from *L. japonica*, cultured in Qingdao, China, and its purified fractions had approximately half the inhibiting power than the fucoidans in this study at concentrations ranging from 1 mg/mL to 4 mg/mL (Wang et al., 2009). The CUPRAC assay values were however not comparable with previous studies due to more common application of the FRAP assay in other studies. This study proved that sporophyll-derived fucoidan possessed more antioxidant activity than commercial fucoidan.

In this study, crude fucoidan which had the highest molecular weight tested also had the strongest inhibiting activity towards DPPH radicals and copper ions even though its sulphate:fucose ratio was lower than F2, F3 and the commercial fucoidan. The molecular weight of fucoidan was likely to be related to the bioactivity potential of fucoidan. An earlier study also reported that crude fucoidan derived from *Padina gymnospora* had the strongest inhibiting activity when using superoxide and hydroxyl radicals compared to its purified fractions due to higher amounts of sulphate and a larger molecular mass (Souza et al., 2007). F3 exhibited more antioxidant activity than F1 despite a smaller molecular weight. However, the sulphate:fucose ratio of F3 was higher than F1 indicating that the ratio of sulphate to fucose content may also be related to the bioactivity of fucoidan besides molecular weight. Ponce, et al. (2003) stated that both sulphate content and high molecular weight of fucoidans are required for their bioactivity. Although commercial fucoidan had a lower sulphate:fucose ratio and a smaller molecular weight than F1, it still possessed a higher antioxidant activity implying that other factors such as the position of sulphate groups, monosaccharide content and the linear backbone of the polysaccharide (Li, Lu et al., 2008; Skriptsova et al., 2009) may all contribute to the bioactivity of fucoidan.

5.5 Conclusion

The three polysaccharide fractions (F1, F2 and F3) successfully fractionated using anion-exchange chromatography varied in their sulphate and uronic acid contents. Fucose content among the fractions was constant. However the sulphate content and sulphate:fucose ratio showed a significant increase from F1 to F3. Conversely, the uronic acid and molecular weight of the fractions significantly decreased from F1 to F3.

The antioxidant activity of fucoidan was determined using two established techniques, the DPPH and CUPRAC assays. Results demonstrated that all purified fucoidan fractions, and crude fucoidan from the sporophylls of *U. pinnatifida* had considerable antioxidant activity. The TAC of each fraction was not as high as vitamin C that had the strongest antioxidant ability in the DPPH test. F0 and F3 purified fucoidan fractions exhibited a stronger antioxidant activity than commercial fucoidan. These results evidently indicate that polysaccharides from brown seaweed may have beneficial effects as natural antioxidants.

6 Additional experiments

6.1 Fucoidan in commercial seaweed

6.1.1 Introduction

The presence of fucoidan in freshly harvested brown seaweed has been well documented and has been further reported in this study. The popularity of seaweed as a food source is continuously growing as more and more people discover the health benefits of fucoidan (Li, Lu et al., 2008). As a result, consumers would want to purchase seaweed fresh, frozen or commercially dried on market shelves. Many studies have reported that fresh seaweed contains substantial amounts of fucoidan (Bilan et al., 2002; Li, Lu et al., 2008; Li et al., 2006). However, no report has been published on the presence of fucoidan in commercial processed seaweed. In this section, the fucoidan content in a commercial *U. pinnatifida* product was determined.

6.1.2 Materials and methods

The commercial seaweed used was the "Chung Jung Won Seaweed" which is a commercially dried *U. pinnatifida* product and requires reconstitution in water before consumption (Figure 20). The fucoidan extraction method was the $CaCl_2$ procedure (Bilan et al., 2002), described previously in Chapter 3.

Figure 20 Commercial *U. pinnatifida* 'Chung Jung Won' Seaweed.

6.1.3 Results and discussion

Results are summarized in Table 8. This experiment was carried out in triplicates with a starting weight of approximately 5 g of commercially dried *U. pinnatifida*. All procedures and chemicals were the same as described in Chapter 3. The yields of fucoidan for the triplicates were considerably lower than to the yield in Chapters 3 and 4 where the freshly harvested *U. pinnatifida* had at least a minimum yield of 2.78% ± 0.17SE fucoidan in the blade using the same technique.

Even though there was a small amount of fucoidan extracted from the commercial sample, there were obvious differences when compared to fucoidan from this study. A simple reason why was because it was not water-soluble. Fucoidan was described as a water-soluble polysaccharide (Li, Lu et al., 2008; Usov et al., 2005; Zvyagintseva et al., 2003). Fucoidan extracted from fresh *U. pinnatifida* in this study and commercial fucoidan easily solubilised in tepid water with mixing. However, fucoidan extracted from the commercial seaweed was not water-soluble and when mixed vigorously, it sank to the bottom of the test tube. This behaviour was not normal for true fucoidan. This material may be a contaminant during the extraction process or some sort of ingredient which was added into the product and not part of the seaweed itself.

Table 8 Fucoidan in commercial seaweed.

Weight of seaweed (g)	Weight of fucoidan (mg)	Yield (%)
4.9170	8.4	0.17
4.9344	9.9	0.20
4.9425	7.8	0.16

The low amount of fucoidan in this product was probably due to the processing procedures of this dried product. The usual processing method of *U. pinnatifida* comprised numerous steps (Yamanaka & Akiyama, 1993). After harvesting, seaweed is often washed with seawater followed by fresh water then blanched. The midrib is then removed and the remaining pieces are dried either in the sun or in an oven (Yamanaka & Akiyama, 1993).

What makes *U. pinnatifida* difficult to process compared to other types of seaweed is that various enzymes in *U. pinnatifida* are still active after the drying process, that may lead to decomposition of the seaweed over a short period of time (Yamanaka & Akiyama, 1993). Commercially, this is overcome by mixing ash derived from wood or straw into the *U. pinnatifida* and spread the mixture onto a flat surface for 2-3 days. The alkalinity of the ash inactivates the enzymes, leading to a much longer shelf life for the final product. The seaweed is re-washed with freshwater to remove the salt and ash and dried again. This final product is referred to as 'haihoshi wakame' (Yamada, Ishizaki, & Tanaka, 1996).

Another way to process *U. pinnatifida* is by blanching, where fresh *U. pinnatifida* is plunged into an 80^{o}C water bath for around 1-2 minutes and then cooled quickly in cold running water. Salt is then added to dehydrate the seaweed and further dried for a few days then stored at 10^{o}C ready for packaging (McHugh, 2003). The majority of *U. pinnatifida* products when blanched turn green, leading to consumer preference for green rather than the brown colour of unprocessed *U. pinnatifida*. The added salt also acts as an inhibitory agent against microorganisms, preserving the product for long periods of time (Yamanaka & Akiyama, 1993).

Due to the high solubility of fucoidan in water, there is no doubt that blanched *U. pinnatifida* would contain, if any, very small amounts of fucoidan as most of the

fucoidan within the cell walls would have leached out into the surroundings during the blanching process. For that very reason, consumers wanting to obtain fucoidan in their diet would need to consume seaweed which has not been boiled in any way, such as the 'haihoshi wakame'. Some seaweed products such as the one used in this section did not state the processing procedures used in creating this product. Hence, based on the fucoidan yield obtained, it was assumed that the processing procedures in developing this product involved some blanching process.

6.2 Re-extracting fucoidan from *U. pinnatifida*

6.2.1 Introduction

The use of $CaCl_2$ to extract fucoidan has been used extensively in previous studies (Bilan et al., 2002; Usov et al., 2005). The method of Bilan et al. (2002) gave the best quality fucoidan in this study (see Chapter 3). The effectiveness of $CaCl_2$ as an extracting solvent was highly recommended as it can solubilise water-soluble polysaccharides such as fucoidan, and remove insoluble components which could affect the purity of the fucoidan (Kawamoto et al., 2006; Usov et al., 2005). In this section, we investigated the effectiveness of $CaCl_2$ as an extracting solvent by re-extracting *U. pinnatifida* previously extracted with $CaCl_2$ to determine if there are traces of fucoidan leftover in the extracted *U. pinnatifida*.

6.2.2 Materials and methods

Extracted *U. pinnatifida* (sporophyll and blade) from the July collection was re-extracted using the method of Bilan et al. (2002). All procedures and chemicals were the same as previously described.

6.2.3 Results and discussion

As shown in Figure 21, *U. pinnatifida* extracted for the first time (referred to as 'new') with $CaCl_2$ had a much darker solution compared to *U. pinnatifida* extracted for the

second time (referred to as ‘old’). The ‘old’ *U. pinnatifida* extract was creamy to colourless solution after extraction with $CaCl_2$ while the ‘new’ *U. pinnatifida* gave a very distinct yellow/brown solution. The colour gives an indication on the amount of material extracted from the alga. After the addition of cetavlon, the chemical which precipitates out the fucoidan, the ‘new’ *U. pinnatifida* precipitated as clearly shown in Figure 21 (left). However, ‘old’ *U. pinnatifida* did not show any signs of precipitation but turned cloudy, even when left to precipitate overnight at 4^oC. No fucoidan was found in the re-extracted *U. pinnatifida*. This experiment was critically important in confirming that all fucoidan was removed from the first extraction. These results confirmed the effectiveness of $CaCl_2$ as the extracting solvent.

Figure 21 Comparison between *U. pinnatifida* which have been extracted using $CaCl_2$ for the first time (new) and *U. pinnatifida* which have been re-extracted for the second time (old). *U. pinnatifida* after $CaCl_2$ extraction (left) and after precipitation with Cetavlon (right).

7 Conclusions

7.1 Summary of main findings

The main purpose of the *Undaria* research program at Auckland University of Technology was to turn an unwanted pest and into a profitable and environmentally useful organism. *U. pinnatifida, H. fusiforme,* and *L. japonica* are farmed in large scale in Asia due to its high popularity as a food source and the increase in awareness of fucoidan (Li, Lu et al., 2008). *U. pinnatifida* is a $400 million industry in Japan, South Korea, and China, yet it is an unwanted byproduct of mussel farming here in New Zealand. Previously, farming *U. pinnatifida* in New Zealand was not considered an option as there were concerns about environmental effects of the alga in the surrounding waters (Parsons, 1994). However eradicating it would also be ineffective (Brown & Lamare, 1994). After two and a half decades of coexisting with this seaweed, there were still signs of *U. pinnatifida* spreading to new marine ecosystems (Bedford, 2011). It is only recently that this so-called 'pest' was proposed to be turned into a worldwide food source. The Marlborough Sounds, Lyttelton, Akoroa and Wellington harbor could become lucrative seaweed farming grounds as these places are already infected with *U. pinnatifida* (Parsons, 1994). In addition, farming seaweed may be beneficial to nearby mussel and fish farms as it can soak up nutrients out of the water that could contain fertilizers and nitrogen as a result of mussel and fish farming (Wright, 2012).

This project was carried out to determine the fucoidan content of *U. pinnatifida* grown in New Zealand. In Chapter 3, three extraction techniques of fucoidan were compared. The study proved that different solvents used to extract fucoidan varied in terms of yield, fucose, sulphate, uronic acid and protein content. Although various studies reported on methods of extracting the best quality fucoidan and stated advantages of the methods over others, it was important to compare the three most common solvents used to solubilise fucoidan in seaweed in order to determine the best method for New Zealand seaweed. Fucoidan content from both the blade and sporophyll parts of the seaweed was also determined.

The sporophyll part of the alga contained nearly 5 times more fucoidan than the blade. The $CaCl_2$ extraction method produced the best quality fucoidan in terms of percentage yield, and the fucose and sulphate content from the sporophyll. However this technique yielded the lowest amount of crude fucoidan from the blade. As the blade of the seaweed is normally processed as food and the sporophyll part usually discarded as a byproduct, the potential to extract fucoidan from the sporophyll for pharmaceutical purposes exists and should be exploited.

It was also important to investigate the best time to harvest *U. pinnatifida* for its fucoidan content. This was carried out in Chapter 4 by extracting fucoidan from *U. pinnatifida* on a monthly basis during the growing season of the alga. A maximum amount of fucoidan (69.98% dry weight) was found in the sporophyll of the seaweed harvested in September 2011. The sulphate content was also maximum at that time. The sulphate content has been reported to be related to the bioactivity of fucoidan (Li, Lu et al., 2008). Chemical changes within the cell walls of the alga are likely caused by the maturation and synthesis of the sporophyll (Skriptsova et al., 2009). The yield and chemical composition of fucoidans derived from two different locations, Port Underwood and Pelorus Sound were also investigated. This determined whether environmental factors affected fucoidan yield and composition. In terms of the percentage yield and chemical composition of fucoidan, *U. pinnatifida* from Pelorus Sound (farm 353) was more similar to the one in Port Underwood (farm 327). However, the yield of fucoidan from *U. pinnatifida* from farms 327 and 106 from Port Underwood were significantly different in both blade and sporophyll-derived fucoidan. Uronic acid content from blade fucoidan and fucose content from sporophyll fucoidan were also significantly different from these two farms. *U. pinnatifida* from Pelorus Sounds were also much smaller than those from Port Underwood. This was probably due to high amounts of fresh water input from land near Richmond Bay that slowed down the seaweed growth and as a result did not reach peak maturity.

It was also critical to examine the antioxidant activity of fucoidan in order to promote its use in the pharmaceutical industry. Fucoidans very existence is due to its bioactivity. Crude fucoidan was fractionated using ion-exchange chromatography. Antioxidant activities of the fractionated fucoidans, crude fucoidan and commercial fucoidan were compared. Crude fucoidan showed the strongest antioxidant property than the purified fractions and commercial fucoidan, but not vitamin C. Fucoidan fractions also varied

significantly in terms of sulphate content and molecular weight, which may influence its antioxidant activity. It is clear that some of these activities have diverse structural specificities. The biological activities could be improved by modifying these attributes (Cho et al., 2011; Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). The present findings in the study provide a basis for future experiments and may be used as natural antioxidants in the food and pharmaceutical industry.

Production of fucoidan from New Zealand *U. pinnatifida* may be a good selling point to the Asian market due to the "clean green" image most foreigners have of New Zealand. Further studies on the structure of fucoidans from New Zealand *U. pinnatifida* can provide theory groundwork for utilizing this unlimited resource that could be potentially farmed.

7.2 Caveats

Although the yield and composition of fucoidans derived from different species of alga were compared with fucoidan extracted from the current study, this does not take into consideration the extraction technique used to extract fucoidan as well as the location and month the alga was harvested. Hence the variability of fucoidan yield and chemical composition between our findings with other studies was to be expected.

The size and lack of seaweed growing in the Pelorus Sounds made it impossible for a monthly evaluation as with Port Underwood. Hence comparison between farms was compared for only one month when the alga was big enough for fucoidan to be extracted.

Usov et al. (2005) showed that the midrib in brown seaweed also contained fucoidan that could be even more than present in the blade. This could have affected the actual yield and composition of fucoidan derived from the blade in this study. Commercially, the midrib is removed from the blade just after harvesting. In this study, the midrib was not removed as it was not convenient to do so on the boat and removal of the midrib after it has been freeze dried was problematic. Hence in this study, the midrib was included as part of the blade in contrast to other studies (Lee et al., 2006; Usov et al., 2005).

The use of a liquid chromatography-mass spectrophotometry (LC-MS) system or a high performance size exclusion chromatography-multiangle laser light scattering (HPSEC-MALLS) system was planned for the determination of the average molecular mass of the fucoidan and its fractions. However, the LC-MS that we planned to use was only capable of detecting molecular masses of up to 2000 Daltons, while the average fucoidan molecule had an average mass of 25,000 – 800,000 Daltons (Li et al., 2006). Hence, the gel permeation chromatography was employed instead which only gave an estimate of the average molecular weight of the fucoidans. Most studies also used gel chromatography to estimate molecular masses of fucoidans (Choosawad, Leggat, Dechsukhum, Phongdara, & Chotigeat, 2005; Li et al., 2006; Patankart, Oehninger, Barnett, Williams, & Clark, 1993; Zvyagintseva et al., 2003).

Numerous months of work was put in on getting the high pressure-liquid chromatography (HPLC) method to work to determine the monosaccharide content in fucoidan but the results were not successful. Monosaccharides were analyzed using a Shimadzu LC-10AD HPLC system together with a Shimadzu CBM-20A as the controlling software. Detection of monosaccharides was carried out using a 385-ELSD detector (Agilent Technologies) with nitrogen as the ELSD nebulizer gas set at 0.90 bar. Sugar standards used were: L-fucose (Sigma), D-mannose (Sigma), D-glucose (Sigma), D-xylose (Sigma), D-galactose (Serva), and L-rhamnose (Sigma). At first, the HPLC system consisted of a column (HILIC Luna 5µ 250 x 4.6 mm, Phenomenex) set at 40^{o}C and the mobile phase used contained a mixture of acetonitrile (Thermofisher) and Millipore water (97:3). Poor separation of sugars was achieved with these conditions even after the mobile phase was adjusted for gradient elution.

A phenylhydrazine derivatization technique was further applied to the sugar standards as it was reported to give better sensitivity during HPLC separation of saccharides (Lattova & Perreault, 2009). The first attempt gave excellent separation, giving rise to two peaks for some sugars that could have been caused by rotational isomerism. Later attempts on producing reproducible results were not achieved; indicating that the tagging of phenylhydrazine onto sugar mixtures to form phenylhydrazones was highly variable. Further attempts were made using a Luna NH_2 column (250 x 4.6 mm, 5 µm, Phenomenex), a Rezex RPM-Monosaccharide column (300 x 7.8 mm, 8 µm, Phenomenex), and a Rezex RSO-Oligosaccharide column (200 x 10 mm, 12 µm,

Phenomenex) with again no reasonably good separations. Hence the fucose assay was used instead.

7.3 Future research directions

It would be of great research interest to further elucidate the structure of fucoidan derived from New Zealand's *U. pinnatifida*. Sulphate content has been linked with fucoidan's bioactivity, hence increasing the degree of sulfation in fucoidan can be a key aspect in enhancing its bioactivity (Teruya, Konishi, Uechi, Tamaki, & Taka, 2007). Previous studies have shown that bioactivity increased with increase in sulphate content (Cho et al., 2011; Teruya et al., 2007). Further experiments in producing an oversulfated fucoidan would be interesting to further enhance the bioactivity of fucoidan from *U. pinnatifida* grown in New Zealand.

Fucoidan has been proven, on many occasions, to inhibit tumour growth in rats (Ko & Joo, 2011; Maruyama, Tamauchi, Lizuka, & Nakano, 2006; Synytsya et al., 2010). Hence testing the antitumor activity of our fucoidan would have been interesting. As fucoidan has a great potential to be used as a chemotherapy drug, it would be useful to test the fucoidan on apoptosis using human cancer cells. Testing apoptosis using HT-29 and HCT116 cells via the Hoechst staining and Annexin V staining have been proven to work well with fucoidan in the past (E. Kim, J, Park, Lee, & Park, 2010).

The application of nuclear magnetic resonance (NMR) spectroscopy would also be very useful in order to obtain more structural information on fucoidan by identifying the residues present and how this polysaccharide is linked together. The side chains can also be determined, and should lead to much better understanding of the various biological properties that fucoidan have.

Molecular weight of fucoidan could be more accurately determined using a LC-MS, HPSEC-MALLS or MALDI-TOF-MS system. The bioactivity of fucoidan is related to its molecular weight as shown in other studies (Choosawad et al., 2005; Patankart et al., 1993; Zvyagintseva et al., 2003) and further supported in this study. This information would be essential in discussing the polysaccharides bioactivity in relation to its molecular mass. Monosaccharide content of the fucoidan should also be determined

using a HPLC or gas-chromatography (GC) method. This is because the fucose and galactose ratio of fucoidan was said to be related to its bioactivity (Li, Lu et al., 2008).

References

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

Apak, R., Guclu, K., Ozyurek, M., & Celik, S. (2007). Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay. *Microchimica Acta, 160*(413-419).

Arunkumar, K., Sivakumar, S., R, & Rengasamy, R. (2010). Review on Bioactive Potential in Seaweeds (Marine Macroalgae): A Special Emphasis on Bioactivity of Seaweeds Against Plant Pathogens *Asian Journal of Plant Science, 9*, 227-240.

Bedford, J. (2011). *Working together in Fiordland to oust Undaria*. Retrieved 24/02/12

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

Bitter, T., & Muir, H., M. (1962). A Modified Uronic Acid Carbazole Reaction. *Analytical Biochemistry, 4*, 330-334.

Bradford, M., M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Brown, M., T, & Lamare, M., D. (1994). The distribution of *Undaria pinnatifida* (Harvey) Suringar within Timaru harbour, New Zealand. *Japanese journal of phycology, 42*, 63-70.

Carter, D. (2010). *Pests and Diseases: Undaria*. Retrieved 21/03/11

Chandia, N., P, & Matsuhiro, B. (2008). Characterization of a fucoidan from Lessonia vadosa (Phaeophyta) and its anticoagulant and elicitor properties. *International Journal of Biological Macromolecules, 42*, 235-240.

Cho, M., L, Lee, B., Y, & You, S., G. (2011). Relationship between Oversulfation and Conformation of Low and High Molecular Weight Fucoidans and Evaluation of Their *in Vitro* Anticancer Activity. *Molecules, 16*, 291-297.

Choi, J., Raghavendram, H., R., B, Sung, N., Y, Kim, J., H, Chun, B., S, Ahn, D., H, et al. (2010). Effect of fucoidan on aspirin-induced stomach ulceration in rats. *Chemico-Biological Interactions, 183*, 249-254.

Choosawad, D., Leggat, U., Dechsukhum, C., Phongdara, A., & Chotigeat, W. (2005). Anti-tumour activities of fucoidan from the aquatic plant *Utricularia aurea* lour. *Journal of Science and Technology, 27*, 799-807.

Chopin, T., & Sawhney, M. (2008). Seaweeds and their Mariculture. *Encyclopedia of Ocean Sciences*, 317-326.

Chotigeat, W., Tongsupa, S., Supamataya, K., & Phongdara, A. (2004). Effect of Fucoidan on Disease Resistance of Black Tiger Shrimp. *Aquaculture, 233*, 23-30.

Chung, H., Jeun, J., Houng, S., Jun, H., Kweon, D., & Lee, S. (2010). Toxicological evaluation of fucoidan from *Undaria pinnatifida vitro* and *in vivo*. *Phytotherapy Research, 24*, 1078-1083.

Colliec, S., Fischer, A. M., Tapon-Bretaudiere, J., Boisson, C., Durand, P., & Jozefonvicz, J. (1991). Anticoagulant properties of a fucoidan fraction. *Thrombosis Research, 64*, 143-154.

Dische, Z., & Shettles, L., B. (1948). A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination *Journal of Biological Chemistry, 175*, 595-603.

Dodgson, K., S, & Price, R., G. (1962). A Note on the Determination of the Ester Sulphate Content of Sulphated Polysaccharides. *Biochemistry, 84*, 106-110.

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

Dubois, M., Gilles, K., A, Hamilton, J., K, Rebers, P., A, & Smith, F. (1956). Colorimeter method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350-356.

Eluvakkal, T., Sivakumar, S., R, & Arunkumar, K. (2010). Fucoidan in Some Indian Brown Seaweeds Found along the Coast Gulf of Mannar. *International Journal of Botany, 6*, 176-181.

Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., & Parkin, D. (2008). *Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10.* Retrieved 7th April, 2011

Fitton, Irhimeh, M., & Falk, N. (2007). Macroalgal Fucoidan Extracts: A New Opportunity for Marine Cosmetics. *Cosmetics and Toiletries, 122*(8), 55-64.

Fitton, H., & Dragar, C. (2006). U. S. Patent.

Garrett, R., H, & Grisham, C., M. (1999). *Experimental Biochemistry*. Charlottlesville: Brooks Cole.

Guclu, K., Ozyurek, M., Bektasoglu, B., & Apak, R. (2010). *Summary of CUPRAC antioxidant assay methodology and current applications by other CUPRAC users.* Istanbul University, Istanbul.

Hagiwara, H. (2010). La Jolla, CA, US Patent No. Patentdocs: H. Foundation.

Hay, C., H, & Luckens, P., A. (1987). The Asian kelp Undaria pinnatifida (Phaeophyta, Laminariales) found in a New Zealand harbour. *New Zealand Journal of Botany, 25*, 329-332.

Hayakawa, K., & Nagamine, T. (2009). Effect of Fucoidan on the Biotinidase Kinetics in Human Hepatocellular Carcinoma. *Anticancer Research, 29*, 1211-1217.

Hayashi, K., Nakano, T., Hashimoto, M., Kanekiyo, K., & Hayashi, T. (2008). Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *International Immunopharmcology, 8*, 109-116.

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*, 185-193.

Honya, M., Mori, H., Anzai, M., Araki, Y., & Nishizawa, K. (1999). Monthly changes in the content of fucans, their constituent sugars and sulphate in cultured *Laminaria japonica*. *Hydrobiologia, 398*, 411-416.

Huang, Y., & Lam, U. (2011). Chitosan/Fucoidan pH Sensitive Nanoparticles for Oral Delivery System. *Journal of the Chinese Chemical Society, 58*, 779-785.

Hunt, L., Chadderton, L., Stuart, M., D, Cooper, S., & Carruthers, M. (2009). *Results of an attempt to control and eradicate Undaria pinnatifida in Southland, New Zealand, April 1997 - November 2004*. Invercargill: Department of Conservation.

Irhimeh, M., R. (2005). A Quantitative Method to Detect Fucoidan in Human Plasma Using a Novel Antibody. *Methods and Findings in Experimental and Clinical Pharmacology, 27*, 705-710.

Kawamoto, H., Miki, Y., Kimura, T., Tanaka, K., Nakagawa, T., Kawamukai, M., et al. (2006). Effects of Fucoidan from Mozuku on Human Cell Lines. *Food Science Technology Resources, 12*, 218-222.

Kim, E., J, Park, S., Y, Lee, J., Y, & Park, J., H. (2010). Fucoidan present in brown algae induces apoptosis of human colon cancer cells. *BMC Gastroenterology, 10*, 96.

Kim, W., J, Kim, S., M, Kim, H., G, Oh, H., R, Lee, K., B, Lee, Y., K, et al. (2007). Purification and Anticoagulant Activity of a Fucoidan from Korean *Undaria pinnatifida* Sporophyll. *Algae, 22*, 247-252.

Ko, E., J, & Joo, H., G. (2011). Fucoidan Enhances the Survival and Sustains the Number of Splenic Dendritic Cells in Mouse Endotoxemia. *Korean Journal of Physiological Pharmacology, 15*, 89-94.

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

Kylin, K. (1913). Biochemie der Meeresalgen. *HoppeSeyler's Z Physiol Chem, 83*, 171-197.

Lattova, E., & Perreault, H. (2009). Method for investigation of oligosaccharides using phenylhydrazine derivatization. *Methods in Molecular Biology, 534*, 65-77.

Laurence, J. (2008). *Global rise in breast cancer due to 'Western lifestyles'*. Retrieved 7th April, 2011

Lebeau, J., Furman, C., Bernier, J., L, Duriez, P., Teissier, E., & Cotelle, N. (2000). Antioxidant properties of di-tert-butylhydroxylated flavonoids. *Free Radical Biology & Medicine, 29*, 900-912.

Lee, Y., K, Lim, D., Lee, Y., & Park, Y. (2006). Variation in Fucoidan Contents and Monosacharide Compositions of Korean *Undaria pinnatifida* (Harvey) Suringar (Phaeophyta). *Algae, 21*, 157-160.

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

Li, B., Rui, X., Z, & Xin, J., W. (2008). Anticoagulant activity of fucoidan from *Hizikia fusiforme*. *Agro Food Industry Hi-tech, 19*, 22-24.

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*. *Carbohydrate Research, 341*, 1135-1146.

Lin, C., Y, Yu, C., J, Chang, H., C, & Tseng, W., L. (2007). Simultaneous separation of anionic and cationic proteins by capillary electrophoresis using high concentration of poly (diallyldimethylammonium chloride) as an additive. *Journal of Chromatography A, 1165*, 219-225.

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. *Asean Journal For Science And Technology Development, 22*, 371-380.

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

MAF. (2010). *The commercial use of Undaria pinnatifida - an exotic Asian seaweed*. Wellington: Ministry of Agriculture and Forestry.

Mamatha, B., S, Namitha, K., K, Senthil, A., Smitha, J., & Ravishankar, G., A. (2006). Studies on use of *Enteromorpha* in snack food. *Food Chemistry, 101*, 1707-1713.

Marais, M., F, & Jacob, M., P. (2001). A fucoidan fraction from *Ascophyllum nodosum*. *Carbohydrate Research, 336*, 155-159.

Maruyama, H., Tamauchi, H., Lizuka, M., & Nakano, T. (2006). The role of NK cells in antitumor activity of dietary fucoidan from *Undaria pinnatifida* sporophylls (Mekabu). *Planta medica, 72*, 1415-1417.

McHugh, D. (2003). *A guide to the seaweed industry*: Food and Agriculture Organization of the United Nations.

McNally, A. (2007). *Fucoidan extract process protected*. Retrieved 6 May, 2011

Mensor, L., Menezes, F., Leitao, G., Reis, A., Santos, T., Coube, C., et al. (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research, 15*, 127-130.

MOF. (2001). *Action plan for unwanted species - Undaria (Undaria pinnatifida)*. Wellington: Ministry of Fisheries.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology, 26*, 211-219.

Moore, B. (2011). *Pest potential prize export*. Retrieved 17/01/12

Morelissen, B. (2012). *Ecological effects of Undaria pinnatifida (Harvey) Suringar and nutrient-enrichment on intertidal assemblages in Wthe Wellington region of New Zealand.* Victoria University of Wellington, Wellington.

Nimura, K., & Mizuta, H. (2001). Differences in photosynthesis and nucliec acid content between sterile and fertile parts of the sporophyte of *Laminaria japonica* (Phaaeophyceae). *Algae, 16*, 151-155.

Nishino, T., Yokohama, G., & Dobahi, K. (1989). Isolation, purification and characterization of fucose-containing sulfated polysaccharides from the brown

seaweed *Ecklonia kurome* and their blood-anticoagulant activities. *Carbohydrate Research, 186*, 119-129.

Ohigashi, H., Y, Yamaguchi, S., K, Umezaki, I., & Koshimizu, K. (1992). Possible anti-tumor promoting properties of marine algae and in vivo activity of Wakame seaweed extract. *Bioscience, biotechnology, and biochemistry, 56*, 994-995.

Parsons, M., J. (1994). *Status of the introduced brown seaweed Undaria in New Zealand*. Wellington: Department of Conservation.

Patankart, 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. *The Journal of Biological Chemistry, 268*, 21770-21776.

Ponce, N., M., A, Pujol, C., A, & Damonte, E., B. (2003). Fucoidans from the brown seaweed *Adenocystis utricularis*: extraction methods, antiviral activity and structural studies. *Carbohydrate Research, 338*, 153-165.

Prior, R., L, Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural Food Chemistry, 53*, 4290-4302.

Raffo, M., Eyras, M., & Iribarne, O. (2009). The invasion of *Undaria pinnatifida* to a Macrocystis pyrifera kelp in Patagonia (Argentina, south-west Atlantic). *Journal of the Marine Biological Association of the United Kingdon, 1*, 1-10.

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

Ruperez, P., Ahrazem, O., & Leal, J. (2002). Potential antioxidant capacity of sulphated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *Journal of Agricultural Food Chemistry, 50*, 840-845.

Sakai, T., Ishizuka, K., & Kato, I. (2003). Isolation and characterization of a fucoidan-degrading marine bacterium. *Journal of Marine Biotechnology, 5*, 70-78.

Schwarz, K., Bertelsen, G., Nissen, L., R, Gardner, P., T, Heinonen, M., I, Hopia, A., et al. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research and Technology, 212*, 319-328.

Schweiger, R., G. (1962). Methanolysis of Fucoidan. II. The Presence of Sugars Other Than L-Fucose. *Journal of Organic Chemistry, 27*, 4270-4272.

Shibata, H., Takagi, K., & Nagaoka, M. (2000). Properties of fucoidan from Cladosiphon okamuranus Tokida in gastric mucosal protection *Biofactors, 11*, 235-245.

Shimizu, J., Wada-Funada, U., Mano, H., Matahira, Y., Kawaguchi, M., & Wada, M. (2005). Proportion of Murine Cytotoxic T Cells in Increased by High Molecular-Weight Fucoidan Extracted from *Okinawa mozuku (Cladosiphon okamuranus)*. *Journal of Health Science, 51*, 394-397.

Sigma-Aldrich. (2011). *Fucoidan from Fucus vesiculosus*. Retrieved 02/03/12

Silva, P., C, Woodfield, R., A, Cohen, A., N, Harris, L., H, & Goddard, J. (2002). First report of the Asian kelp Undaria pinnatifida in the northeastern Pacific Ocean. *Biological Invasions, 4*, 333-338.

Sinner, J., Forrest, B., Taylor, M., Dodgshun, T., Brown, S., & Gibbs, W. (2000). *Options for a National Pest Management Strategy for the Asian Kelp, Undaria: A Discussion Paper*: Cawthron Institute

Skriptsova, A., V, Shevchenko, N., M, Zvyagintseva, T., N, & Imbs, T., I. (2009). Monthly changes in the content and monosaccharide composition of fucoidan from Undaria pinnatifida (Laminariales, Phaeophyta). *Journal of Applied Phycology, 22*, 79-86.

Souza, M., C., R, Marques, C., T, Dore, C., M., G, Silva, R., F, Rocha, H., A., O, & Leite, E., L. (2007). Antioxidant activities of sulphated polysaccharides from brown and red seaweeds. *Journal of Applied Phycology, 19*, 153-160.

Stuart, M., D. (2004). *Review of research on Undaria pinnatifida in New Zealand and its potential impacts on the eastern coast of the South Island*. Wellington.

Synytsya, A., Kim, W., J, Kim, S., M, Pohl, R., Synytsya, A., Kvasnicka, F., et al. (2010). Structure and antitumor activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida Carbohydrate Polymers, 81*, 41-48.

Szabo, M., R, Iditoiu, C., Chambre, D., & Lupea, A., X. (2007). Improved DPPH Determination for Antioxidant Activity Spectrophotometric Assay. *Chemical Papers Chemicke Zvesti, 61*, 214-216.

Tachikawa, D. (2003). *Amazing Power of Fucoidan: You Can Beat Cancer Too!!* Retrieved 7th April, 2011

Tachikawa, D. (2010). *Immune System Booster with Fucoidan, what is this really?* Retrieved 29/07/11

Teas, J., Zhang, Q., & Muga, S. (2006). *Seaweed as chemoprevention: From breast cancer epidemiology to breast cancer cell culture*. Columbia: University of South Carolina.

Teruya, T., Konishi, T., Uechi, S., Tamaki, H., & Taka, M. (2007). Anti-proliferative activity of oversulfated fucoidan from commercially cultured *Cladosiphon okamuranus* TOKIDA in U937 cells. *International Journal of Biological Macromolecules, 41*, 221-226.

Tiroba, G. (2007). *Seaweed Quality Manual: a practical guide for seaweed farmers, seaweed extension officers, buying agents, fisheries officers and exporters*. Noumea: Secretariat of the Pacific Community.

Tseng, C., K. (1981). Marine phyoculture in China. *Proceedings of the International Seaweed Symposium, 10*, 124-152.

Umeda, Y., Kihara, H., Ikai, K., & Kato, I. (2003). Japan Patent No.

Usov, A., I, Smirnova, G., P, & Klochkova, N., G. (2005). Polysaccharides of Algae 58. The polysaccharide composition of the Pacific brown alga *Alaria fistulosa* P. *et* R. (Alariaceae, Laminariales). *Russian Chemical Bulletin, 54*, 1282-1286.

Uwai, S., Nelson, W., Neill, K., Wang, W., Rosas, A., Boo, S., et al. (2006). Genetic diversity of *Undaria pinnatifida* (Laminariales, Phaeophyceae) deduced from

mitochondria genes - origins and succession of introduced populations. *Phycologia, 45*, 687-695.

Verlaque, M. (2007). *Undaria pinnatifida (aquatic plant, alga)*. Marseille: Invasive Species Specialist Group.

Wang, J., Zhang, Q., Zhang, Z., & Li, Z. (2007). Antioxidant activity of sulphated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules, 42*, 127-132.

Wang, J., Zhang, Q., Zhang, Z., Zhang, J., & Li, P. (2009). Synthesized phosphorylated and aminated derivatives of fucoidan and their potential antioxidant activity in vitro. *International Journal of Biological Macromolecules, 44*, 170-174.

Watanabe, H. (2006). *Miso and its biological effects*. Hiroshima: Hiroshima University.

Werner, A., Clarke, D., & Kraan, S. (2006). *Strategic Review of the Feasibility of Seaweed Aquaculture in Ireland*. Parkmore Galway: Marine Institute.

Wright, M. (2012). *Seaweed pest may become green gold*. Retrieved 24/02/12

Yamada, Y., Ishizaki, S., & Tanaka, M. (1996). Degradation of Chlorophyll in Dried Wakame (*Undaria pinnatifida*) During Storage. *Asian Fisheries Science, 9*, 153-160.

Yamanaka, R., & Akiyama, K. (1993). Cultivation and utilization of *Undaria pinnatifida* (wakame) as food. *Journal of Applied Phycology, 5*, 249-253.

Yang, C., Chung, D., Shin, S., Lee, H., Y, Kim, J., C, Lee, Y., J, et al. (2008). Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *International Journal of Biological Macromolecules, 43*, 433-437.

Yang, C., Chung, D., & You, S., G. (2008). Determination of physicochemical properties of sulphated fucans from sporophyll Undaria pinnatifida using light scattering technique. *Food Chemistry, 111*, 503-507.

Zvyagintseva, T., N, Shevchenko, N., M, Chizhov, A., O, Krupnova, T., N, Sundukova, E., V, & Isakov, V., V. (2003). Water-soluble polysaccharides of some far-eastern brown seaweeds. Distribution, structure, and their dependence on the development conditions. *Journal of Experimental Marine Biology and Ecology, 294*, 1-13.

Appendices

9.1 L-fucose standard curve and linear regression

An assay for L-fucose content was conducted for the extracted fucoidan from the blade and the sporophyll. The absorbance values were read at both 396 nm and 427 nm and values of (A396 nm – A427 nm) was used for the standard curve which directly correlates to L-fucose concentration. Assays for the standard curve were performed together with the samples on that particular day. A graph of the absorbance versus concentration of the known standards was plotted. For example, a linear regression was achieved, yielding a correlation factor of R^2 = 0.9859, with a line formula of y = 0.0136x as shown in Figure 22. This equation derived from the standard curve performed on that day was used to calculate the L-fucose content in the fucoidan samples analyzed on that same day. All samples and standards were done in triplicates.

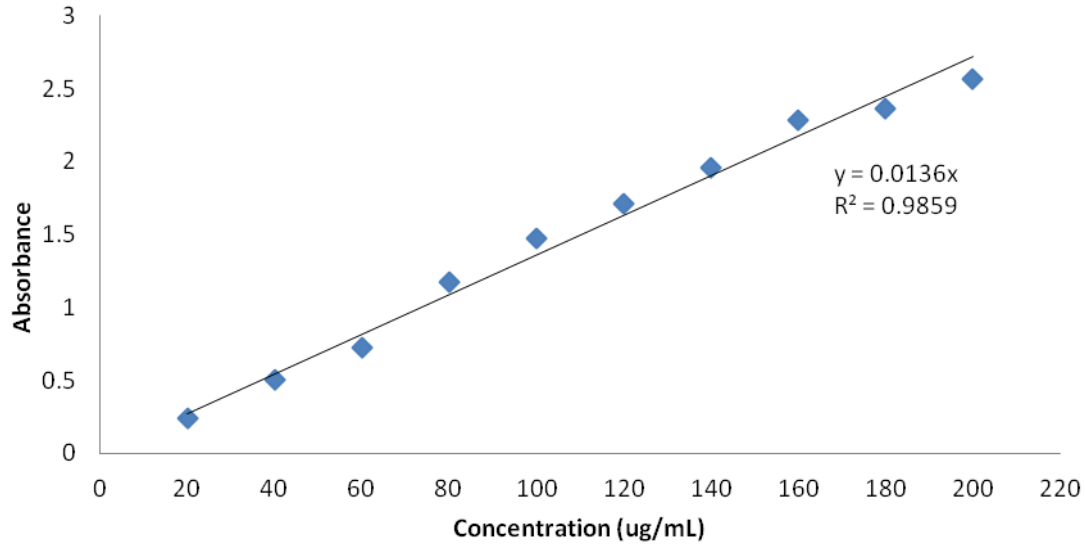

Figure 22 Standard curve for the cysteine-sulphuric acid assay for the determination of L-fucose concentration (µg/mL). Points are means of three assays.

9.2 Sulphate standard curve and linear regression

An assay for sulphate content was conducted for the fucoidan. A standard sulphate solution was prepared by weighing 0.18 g K_2SO_4 and diluting it to one litre, giving 100 mg/L of sulphate. A ten-fold dilution was made with this solution giving a final concentration of 10 mg/L. This stock solution was then used to prepare a series of standard solutions with concentrations of between 200-1000 µg. The absorbance values were measured at 420 nm, and the results were recorded. A standard curve was plotted with absorbance values versus the known standard concentrations. Standard curves were performed together with the samples on each occasion. For instance, a trend line was formed with the absorbance values, yielding a correlation factor of $R^2 = 0.9565$ and $y = 0.0018x$ line formula (Figure 23). This equation was used to calculate the sulphate content in fucoidan. All samples and standards were done in triplicates.

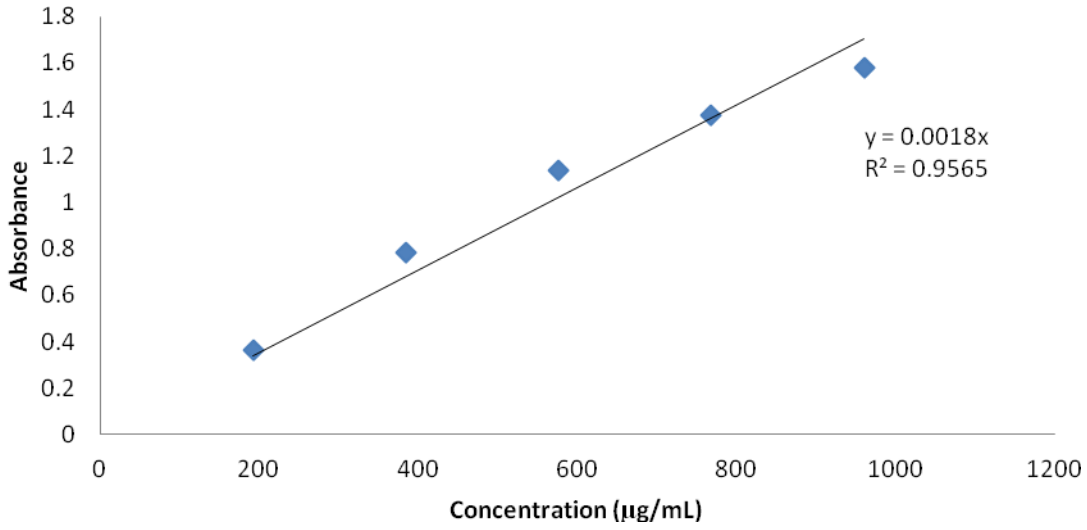

Figure 23 Standard curve for the $BaCl_2$-gelatin assay for the determination of sulphate concentration expressed in K_2SO_4 equivalents (µg/mL). Points are means of three assays.

9.3 Uronic acid standard curve and linear regression

Fucoidan was analyzed for its uronic acid content. Glucuronic acid, the uronic acid derived from glucose, was used to make standards with concentrations of 10-50 µg/mL using a 200 µg/mL stock solution. All standards, samples and blanks were prepared with deionized water saturated with benzoic acid. The absorbance values were read at 530 nm, and the results were recorded. Standard curves were performed together with the samples on each occasion. A graph was plotted with the absorbance values versus the known standard concentrations of glucuronic acid. A trend line was generated using these values. E.g. a linear regression was performed, giving a correlation factor of R^2 = 0.9895, and y = 0.0139x line formula (Figure 24). All standard and samples were done in triplicates.

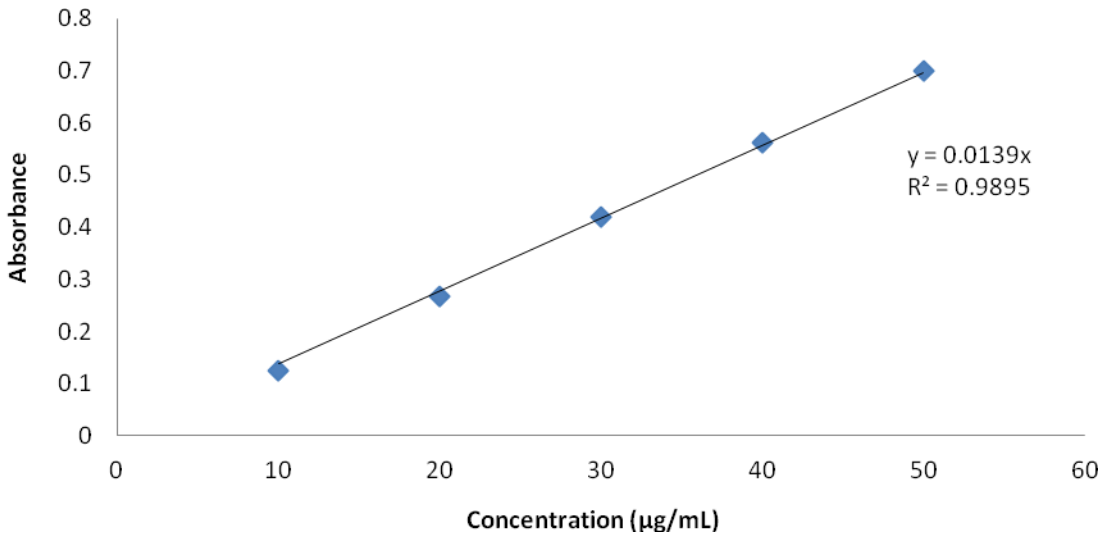

Figure 24 Standard curve for the carbazole assay for the determination of uronic acid concentration expressed in glucuronic acid equivalents (µg/mL). Points are means of three assays.

9.4 Protein standard curve and linear regression

Fucoidan was analyzed for its protein content. The absorbance values were read at 595 nm and the results were recorded. Standard curves were performed together with the samples on each occasion. A linear standard curve was plotted with the absorbance values versus the known standard protein concentrations. This assay only performs linearly up to a limited concentration. In this circumstance, the standard curve was linear up to an absorbance of approximately 0.8. Samples with absorbance higher than 0.8 was diluted accordingly and the protein content was converted back to its original concentration. Protein standards ranging from 10-100 µg/mL were prepared using a stock solution of 100 µg/mL bovine serum albumin. A trend line, for example, was created with these values, yielding a correlation factor of $R^2 = 0.9663$ and $y = 0.0077x$ line formula (Figure 25). This equation derived from the standard curve made on that particular day was used to calculate the protein content in the fucoidan samples analyzed on that same day. All samples and standards were done in triplicates.

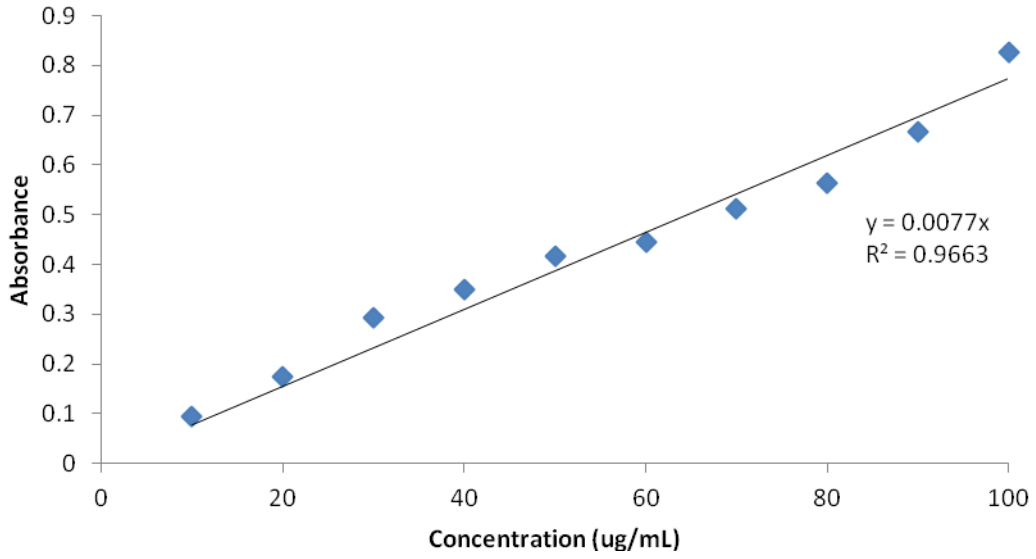

Figure 25 Standard curve for the Bradford assay for the determination of protein concentration expressed in bovine albumin equivalents (µg/mL). Points are means of three assays.

9.5 Monthly variations in protein content of fucoidan

Protein content of the fucoidan throughout the four months of harvest is shown in Figure 26. No significant differences were observed in blade fucoidan from farm 106 (p = 0.072) but not for sporophyll fucoidan from the same farm (p = 0.023). As for farm 327, protein content in blade fucoidan increased significantly from July to August but decreased back down in October (p = 0.000) while sporophyll fucoidan remained constant (p = 0.122). Farm 353from Pelorus Sound produced fucoidan with the highest protein content in both tissues during September.

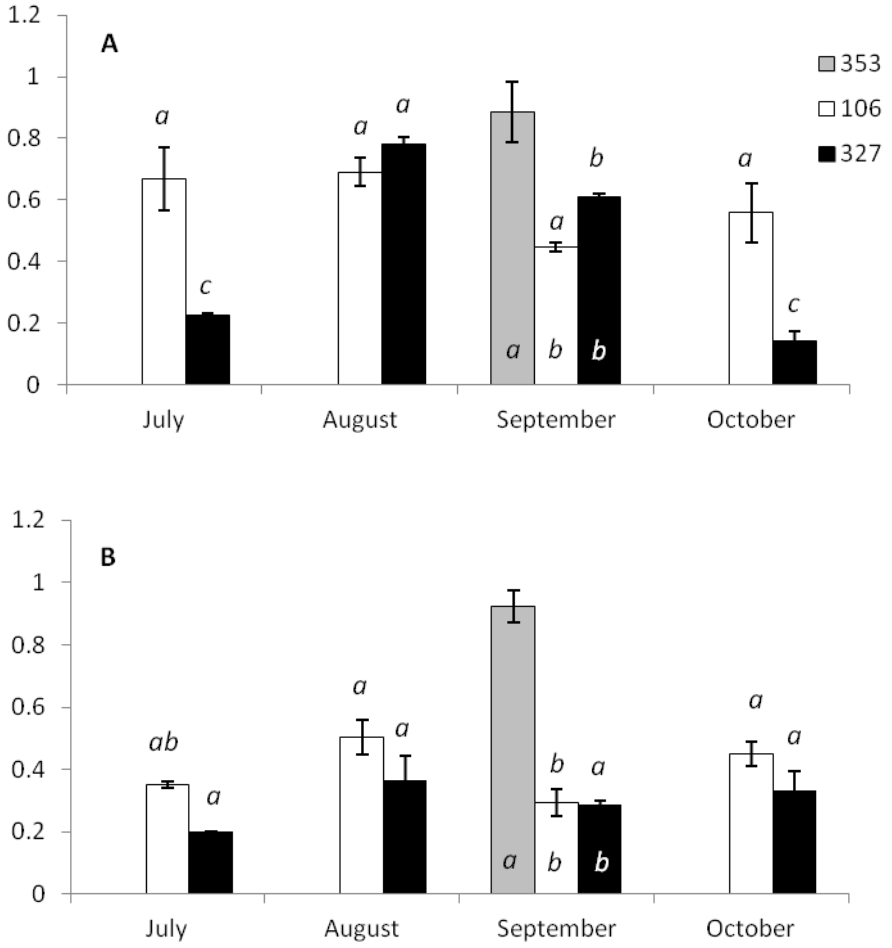

Figure 26 Monthly variations in the protein content in fucoidan from the blade (A) and sporophyll (B) (106/327/353 = farm number). Mean ± SE (n = 3). Farms were examined separately. Different superscript letters indicate significant differences at p < 0.05 by Tukey's test. Subscript letters examine differences between farms.