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Fucoidan from New Zealand *Undaria pinnatifida*: Monthly variations and determination of antioxidant activities

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ABSTRACT

The content and composition of fucoidans extracted from *Undaria pinnatifida* from mussel farms at the Marlborough Sounds, New Zealand were investigated using $CaCl_2$ extraction. Crude fucoidan (F0) was subsequently extracted on a monthly basis from *U. pinnatifida* harvested from July to October 2011 from mussel farms in the Marlborough Sounds, New Zealand. Fucoidan yield varied between the frond and sporophyll parts of the algae, with the sporophyll consistently the highest content. The yield from the sporophyll increased significantly from July (25.4–26.3%) to September (57.3–69.9%). Sulphate content in the extracted fucoidan increased more than twice within the same period, while fucose content remained constant. F0 was further purified by ion-exchange chromatography to yield three fractions, F1, F2 and F3. All three fucoidan fractions contained fucose as the primary sugar component followed by galactose, with xylose, glucose and mannose as minor constituents. All fractions exhibited strong antioxidant activities using the DPPH scavenging and CUPRAC assays. This study showed that sporophyll maturation of *U. pinnatifida* in New Zealand influenced fucoidan content and composition. Sporophyll fucoidan could potentially be a good resource for natural antioxidants.

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1. Introduction

Seaweed utilization is a multi-million dollar industry, mostly based on the production of phycocolloids such as agar and carrageenan, or farming for human consumption (Fung, Hamid, & Lu, 2013; Ly, Buu, Nhut, Thinh, & Van, 2005). However, other compounds extracted from seaweeds have raised great interest from pharmaceutical companies due to their potential as pharmaceuticals and/or nutraceuticals (Ly et al., 2005). Fucoidan is one of these compounds, which are heterogeneous sulphated polysaccharides commonly found in marine invertebrates and brown seaweed (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2009). Their composition varies between species of brown seaweed, but usually consists of α-1,3-linked and α-1,4-linked L-fucose residues, and sulphate as major constituents along with small amounts of uronic acid and other sugars such as galactose, mannose, xylose, glucose, etc. (Bilan et al., 2002; You, Yang, Lee, & Lee, 2010). For the past decade, fucoidans have been extensively studied due to their diverse biological activities including antioxidant, anticoagulant, antiviral, antitumor, anti-inflammatory and antithrombotic properties (Hemmingson, Falshaw, Furneaux, & Thompson, 2006; Li, Lu, Wei, & Zhao, 2008; Ngo, Wijesekara, Vo, Ta, & Kim, 2011; Park et al., 2011; You et al., 2010). The differences in biological activity between different fucoidans are probably linked to their molecular weight, degree of sulphation and monosaccharide composition (Skriptsova et al., 2009). In addition, anticancer activity of fucoidans can be significantly enhanced by lowering the molecular weight after depolymerization under mild conditions (Yang et al., 2008).

The structural characteristics of fucoidan are likely to be dependent on the extraction technique (Ponce, Pujol, & Damonte, 2003), species of seaweed, season of harvest (Honya, Mori, Anzai, Araki, & Nishizawa, 1999), geographic location (Rioux, Turgeon, & Beaulieu, 2007), and algal maturity (Zvyagintseva et al., 2003). Different parts of the seaweed have also been shown to contain varying amounts of fucoidan. In particular the sporophyll, which is the reproductive part of the alga, contains several times more fucoidan than the blade (Usov, Smirnova, & Klochkova, 2005).

Studies on the bioactivity of fucoidan from brown seaweed have been focused on their antioxidant activities (Ngo et al., 2011; Ruperez, Ahrazem, & Leal, 2002; Souza et al., 2007; Wang, Zhang, Zhang, & Li, 2007; Wang, Zhang, Zhang, Zhang, & Li, 2009). Antioxidants are substances that prevent or delay cellular damage caused

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by free radicals by scavenging and inhibiting the production of reactive oxygen species (ROS) (Wang et al., 2007). The most commonly used antioxidants are butylated hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and tertiary-butyl hydroquinone (TBHQ), which are all manufactured synthetically (Choong & Lin, 2000). However, there are increased safety concerns on the use of synthetic antioxidants in food supplements (Choong & Lin, 2000; Igbal et al., 2006), and only recently there have been concerns raised about the use of vitamins A, C and E supplementation and their potential to cause harm from overdosing (Howes, 2007; Trichopoulou & Critselis, 2004). Because of these safety concerns, safer sources of antioxidants from natural sources may be the primary focus of antioxidant research in the near future.

Undaria pinnatifida is a brown seaweed native to Japan, China and Korea (Stuart, 2004) where it is commercially cultivated for food production (Lee, Lim, Lee, & Park, 2006). It has now spread to over twelve countries including France, Spain, Italy, Argentina, North and South America, Australia and New Zealand. The ability to grow in a diverse range of habitats made *U. pinnatifida* an unwanted organism in New Zealand (MAF, 2010). As a result, there is little information about the structure and seasonality of fucoidan from New Zealand *U. pinnatifida*. However, in 2010, NZ Biosecurity changed the policy for *U. pinnatifida*, and while it is still classed as an unwanted organism, they will issue permits for the harvest of this alga from artificial structure (such as mussel farms) and limited farming activities in highly infested areas (MAF, 2010).

In the present study, monthly variations in the content and composition of fucoidans were investigated. Fucoidans extracted from *U. pinnatifida* harvested from two locations were also compared. Crude fucoidan (F0) was then purified using anion-exchange chromatography to yield three fractions and their antioxidant activities were investigated. The relationship between fucoidan antioxidant activity and chemical composition was also examined.

2. Materials and methods

2.1. Materials

U. pinnatifida was harvested from a mussel farm at Great Barrier Island, New Zealand (36° 11.355′S, 175° 18.922′E) during December 2010. Harvests were also made in Port Underwood (sites 106 and 327) and Pelorus Sound (site 353), Marlborough Sound, New Zealand from June 2011 to October 2011 (Fig. 1). Once collected, the algae were washed in seawater to remove epibionts. Then the sporophyll and blade were separated into labelled bags and lyophilized within 48 h of frozen storage. The dried sample was milled using a coffee grinder (Breville CG2B), sieved (<0.6 mm) and then stored in PET containers at room temperature. All chemicals and reagents were of analytical grade.

2.2. Extraction of fucoidan

The extraction of fucoidan was carried out using a previously described technique (Bilan et al., 2002). To study the monthly variations of fucoidan composition and yield, fucoidan was extracted from individual *U. pinnatifida* plants using homogenous batches of both blade and sporophyll tissues from July 2011 to October 2011 (Fig. 1). Site 353 was harvested on one occasion in September as adult sized kelps were necessary for extractions from one single plant.

Calcium chloride ($CaCl_2$) extraction was implemented using the method of Bilan et al. (2002). Four replicate samples of dried ground algae (15 g) were treated at room temperature for 24 h with a MeOH-$CHCl_3$-water mixture (4:2:1) to remove lipids, protein and coloured pigments. This was filtered through Whatman's filter paper (90 mm GF/D), washed with acetone and dried overnight. The resulting powder (10 g) was mechanically stirred with 2% aq $CaCl_2$ (100 mL) at 85 °C for 5 h, centrifuged at 18,500 × *g*, and the supernatant collected. Hexadecyltrimethylammonium bromide (Cetavlon) solution (10%, 50 mL) was added to the extract and left to precipitate at 4 °C overnight. The precipitate formed was centrifuged at 18,500 × *g* (Eppendorf Centrifuge 5810R V3.1), washed with water and mechanically stirred with 60 mL ethanolic NaI solution (20%) for 72 h. The precipitate was removed by centrifugation, washed with ethanol and dissolved in deionized water (30 mL). The solution was dialyzed for 24 h (MWCO 12–14,000) and then lyophilized (Christ LOC 1-M Alpha 2-4, Martin Christ, Osterode am Harz, Germany) to give crude fucoidan.

2.3. Determination of chemical composition

Fucose content of fucoidan was determined by the cysteine-sulphuric acid method using L-fucose (Sigma) as the standard (Dische & Shettles, 1948). The sulphate content was quantified using the $BaCl_2$-gelatin method using K_2SO_4 (BDH Limited) as the standard after hydrolysing fucoidan (15 mg) in 4 M HCl for 2 h at 100 °C (Dodgson & Price, 1962). Uronic acid and protein content of fucoidan were determined using the carbazole-sulphuric acid-borate reaction using D-glucuronic acid (Sigma) as the standard (Bitter & Muir, 1962), and the Bradford assay using bovine serum albumin (ICP Bio Limited) as the standard (Bradford, 1976), respectively. All yields were calculated from the dried weight of fucoidan and converted to a percentage. Absorbance measurements were recorded in triplicate using an Ultrospec 2100 UV/visible spectrophotometer.

2.4. Fractionation of fucoidan

Crude fucoidan was further purified by fractionation using anion-exchange chromatography. Crude fucoidan (2 g) was dissolved in 20 mL of Tris-HCl buffer (0.05 M, pH 7.4) and applied to a column (25 cm × 4 cm) of DEAE-Sephadex A-25 (Pharmacia Ltd) equilibrated with Tris-HCl buffer connected to a Bio-Rad 2110 fraction collector. The first fraction was eluted with deionized water at a flow rate of 40 drops per tube followed by NaCl elution at increasing concentrations (1 and 2 M). Each elution change was detected until the absence of a positive reaction when using the phenol-sulphuric acid method according to Dubois, Gilles, Hamilton, Rebers, and Smith, 1956 to check for the presence of sugars in the test 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 and 2.5 mL concentrated H_2SO_4 were added to each test tube and mixed thoroughly. Tubes heated in a 35 °C water bath for 20 min. The absorbance was measured at 480 nm (Ultrospec 2100) for any indication of sugars and uronic acids (Dubois et al., 1956). Each carbohydrate-positive fraction was pooled together, dialyzed for 48 h (MWCO 12–14,000) in deionized water and lyophilized.

2.5. Determination of monosaccharide composition

Monosaccharides were analyzed by gas chromatography of their alditol acetate derivatives (Melton & Smith, 2001). Fucoidan (10 mg) was hydrolyzed in 0.5 mL of 2 M trifluoroacetic acid at 121 °C in sealed glass tubes flushed with nitrogen. After cooling, 25 μL of 20 mg/mL allose was added as an internal reference. The hydrolysate was filtered through a 0.2-μm Phenex-RC syringe filter into clean glass tubes and evaporated to dryness with a gentle stream of filtered air. Milli-Q water (100 μL) was added into dried hydrolysates, and incubated at 40 °C for 90 min after the addition of 20 μL of 15 M ammonia and 1 mL of 0.5 M sodium borohydride in dimethyl sulphoxide. After cooling to room temperature,

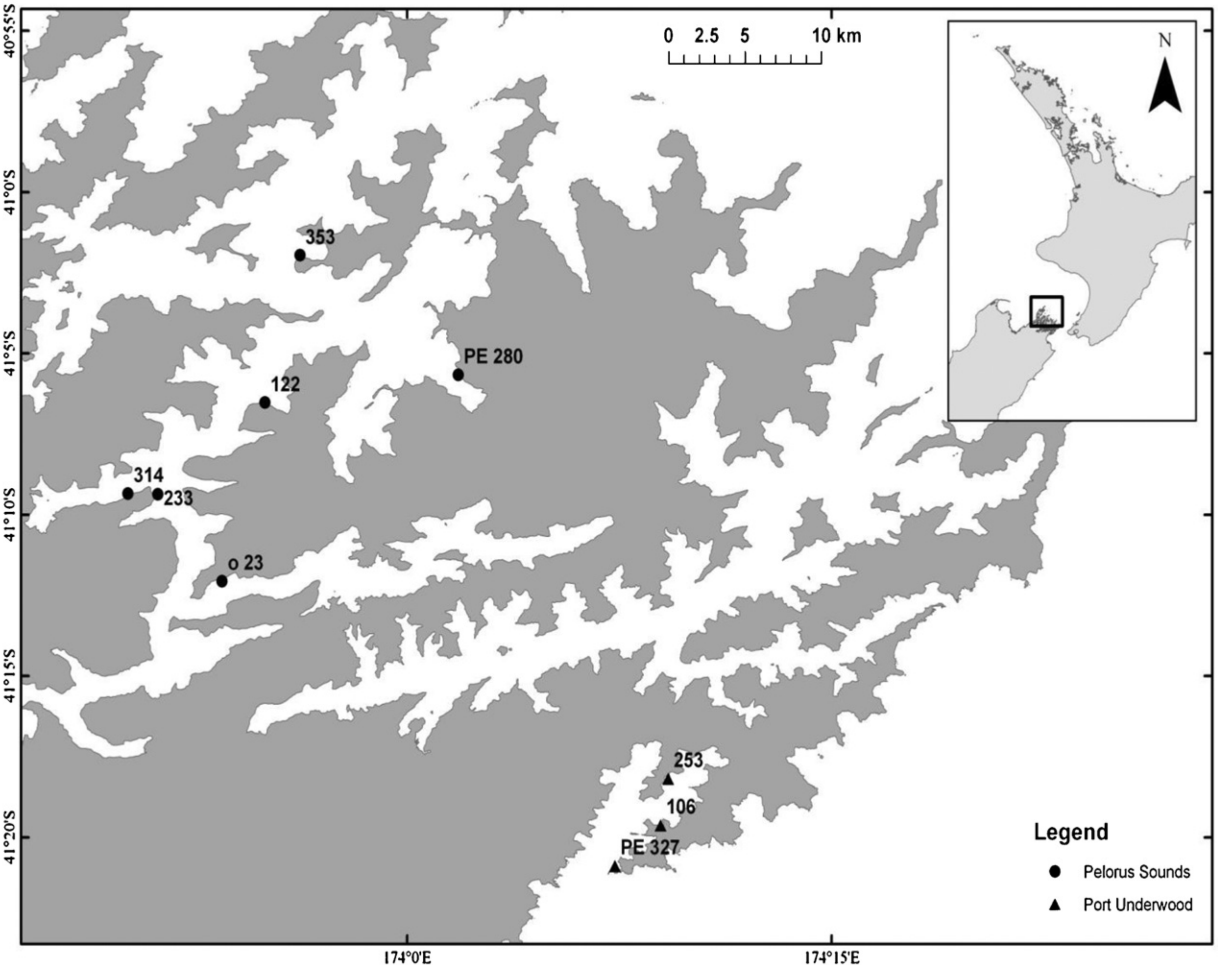

Fig. 1. Mussel farming sites in the Marlborough Sounds, New Zealand.

1-methylimidazole (200 μL) and acetic anhydride (2 mL) were added to the mixture and incubated for a further 10 min. Milli-Q water (5 mL) was added into each tube to neutralize the excess acetic anhydride and allowed to cool. Alditol acetates were extracted by the addition of 1 mL dichloromethane (DCM), and the lower DCM phase was transferred using a Pasteur pipette into a clean glass tube. This extraction was repeated once more. The combined DCM extracts were washed with 4 mL Milli-Q water and the upper water phase was discarded. The wash was repeated twice more. Alditol acetates were analyzed by GC (GC-2010, Shimadzu) using a ZB-5 capillary column (30 m × 0.25 mm) coupled to a flame ionization detector. The detector temperature was held at 280 °C while the injector temperature was set at 250 °C. Nitrogen was used as the carrier gas at a flow rate of 1.5 mL/min and a split ratio of 10. The oven temperature was set at 38 °C for 1 min, increased to 200 °C at 50 °C/min, further increased to 210 °C at 2 °C/min, and then held for 5 min at the final temperature. Sugar standards used were L(−)-fucose, D(+)-galactose, D(+)-xylose, D(+)-mannose, α-D(+)-glucose and β-D-allose.

2.6. Determination of average molecular weight

Average molecular weight (Mw) of the polysaccharides from the sporophyll was determined by gel permeation chromatography (GPC) using a column (25 cm × 4 cm) with Sephadex G-100 equilibrated with phosphate buffer (10 mM, pH 7). Crude fucoidan and its fractions (10 mg) were dissolved in 1 mL phosphate buffer, applied to the column and collected at a flow rate of 40 drops per tube. The presence of sugars was detected using the phenol-sulphuric acid method (Dubois et al., 1956). Dextrans (10, 20, 40, 80 KDa) purchased from Sigma were used as standard molecular weight markers. Blue dextran 2000 (Pharmacia) was used as a void volume marker.

2.7. Determination of antioxidant activity

2.7.1. DPPH scavenging activity

The scavenging activity of DPPH free radicals was assessed as previously described by Mensor et al. (2001). 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. Samples were mixed vigorously and left to stand for 30 min at room temperature, and the absorbance was measured at 517 nm after zeroing the spectrophotometer with a methanol blank. Ascorbic acid was used as a positive control and commercial fucoidan from Sigma was used for comparison as well. Lower absorbance values indicated higher scavenging activity of DPPH radicals. The % inhibition was calculated using the following equation:

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

2.7.2. Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was applied to fucoidan and its fractions according to the method of Apak, Guclu, Ozyurek, & Celik, (2007).

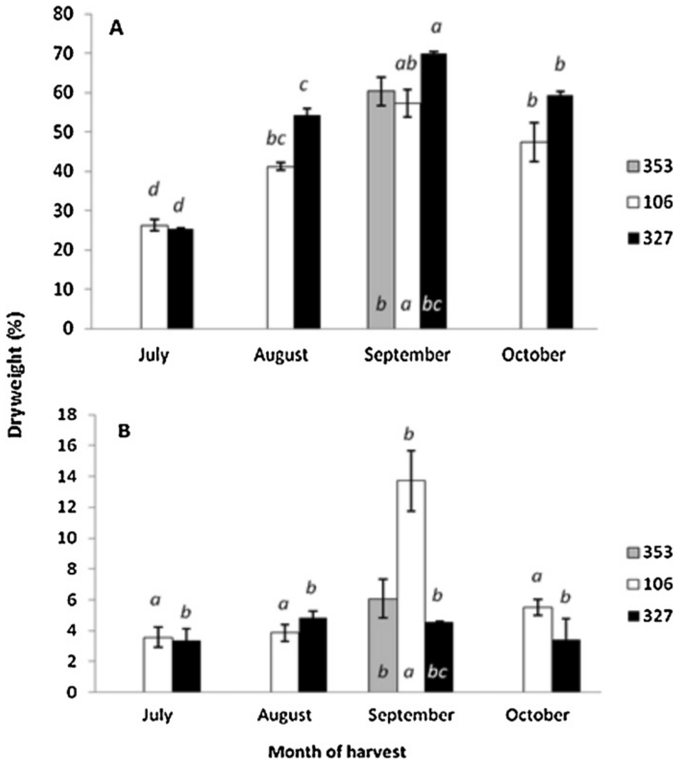

Fig. 2. Monthly variations in the percentage yield of crude fucoidan from *U. pinnatifida* sporophyll (A) and blade (B) (106/327 = farm number). Mean ± SE ($n = 3$). Both farms were examined separately. Different superscript letters indicate significant differences at $p < 0.05$ by Tukey's test. Subscript letters examine differences between farms in September. Same superscript letters indicate no significant differences. Note differences in scale on *y* axis.

Fucoidan samples (1 mL) were prepared in triplicates at different concentrations (100–2500 μg/mL), and added into a solution containing 1 mL $CuCl_2$ (0.01 M), 1 mL NH_4Ac (1 M, pH 7), 1 mL ethanolic neocuproine (0.075 M) and 0.1 mL deionized water to yield a total volume of 4.1 mL. Test samples were incubated for 10 min at room temperature, and the final absorbance was measured at 450 nm after zeroing the spectrophotometer with a water blank. Higher absorbance readings indicated higher reducing power of copper ions.

2.8. Statistical analysis

Analysis of variance (ANOVA) was carried out using Minitab® (Version 15) to test for differences between fucoidan composition from different months of harvest. Where significant differences occurred, Tukey's Honest Significance Difference (HSD) test was employed to examine where that effect occurred.

3. Results and discussion

3.1. Monthly changes in crude fucoidan content and composition

The yields of crude fucoidan extracted from *U. pinnatifida* sporophylls and blades collected from July to October 2011 are shown in Fig. 2. Yield of blade-derived fucoidan from farm 106 increased significantly from July to September by nearly four-fold from 3.56 to 13.71% dry weight ($P < 0.001$) and then decreased significantly ($P = 0.007$) to 5.98% in October. Blade-derived fucoidan from farm 327 started with a similar yield of 3.35% dry weight in July that remained constant up to October ($P = 0.183$). Fucoidan from sporophyll in farm 106 increased more than two-fold between July and 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 a significant increase in yield between July and September from 25.38 to 69.98% dry weight ($P < 0.001$) that then decreased significantly ($P = 0.007$) to 59.30% in October. These changes in fucoidan content were more than those reported from the same species grown in Russia (Skriptsova et al., 2009), *Laminaria digitata* (Haug & Jensen, 1956) and *Laminaria japonica* (Honya et al., 1999). The results also indicated that the maturity of *U. pinnatifida* may have influenced the monthly changes in fucoidan content in terms of yield. *U. pinnatifida* in the Marlborough Sounds grew largest during the start of spring in September when the water temperature begins to increase. In this study, over the months of June and July 2011, the algae started to mature due to the large size of the sporophylls that was encountered. This change in the life cycle of *U. pinnatifida* may result in physiological and chemical changes, including the degradation of blade and photosynthetic pigments (Honya et al., 1999; Nimura & Mizuta, 2001), increase in nucleic acids (Nimura & Mizuta, 2001), decrease in alginic acid (Skriptsova et al., 2009), and maturation of sporophyll (Honya et al., 1999).

Constituents in fucoidan were significantly affected by the month of harvest (Fig. 3). 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 and farm 327 had a significant increase in sulphate content of more than two-fold from July to September ($P < 0.001$). Blade derived fucoidan however 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 between July and September, and then significantly decreased in October ($P < 0.001$). These results suggest that the maturation of *U. pinnatifida* may directly affect the amount of sulphate groups in the polysaccharide, especially in the sporophyll. These results were in accordance with results reported by Honya et al. (1999), which showed a similar trend in of fucoidan sulphate content changes for *L. japonica*, where the molar ratio of sulphate increased as the alga matured, while sulphate content in blade fucoidan remained stable over its growing season.

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. An increase in uronic acid content from July to September was observed for farms 106 ($P < 0.001$) and 327 ($P < 0.001$). A maximum amount of uronic acid was found in blade derived fucoidan obtained in September from farm 327 that constituted 3.66% dry weight. Conversely for sporophyll fucoidan, uronic acid content from farm 106 decreased significantly from 0.89% dry weight in July to 0.62% dry weight in October ($P < 0.001$). 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 of *U. pinnatifida*, which may be linked to algal maturity. 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 maturation of *U. pinnatifida*, and the increase in uronic acid from blade-derived fucoidan may be associated with blade degradation (Honya et al., 1999; Skriptsova, Shevchenko, Tarbeeva, & Zvyagintseva, 2012). The uronic acids found in crude fucoidan are not structural components attached to fucoidan itself, but rather parts of ballast alginates supporting fucoidan preparations (Ale, Mikkelsen, & Meyer, 2011).

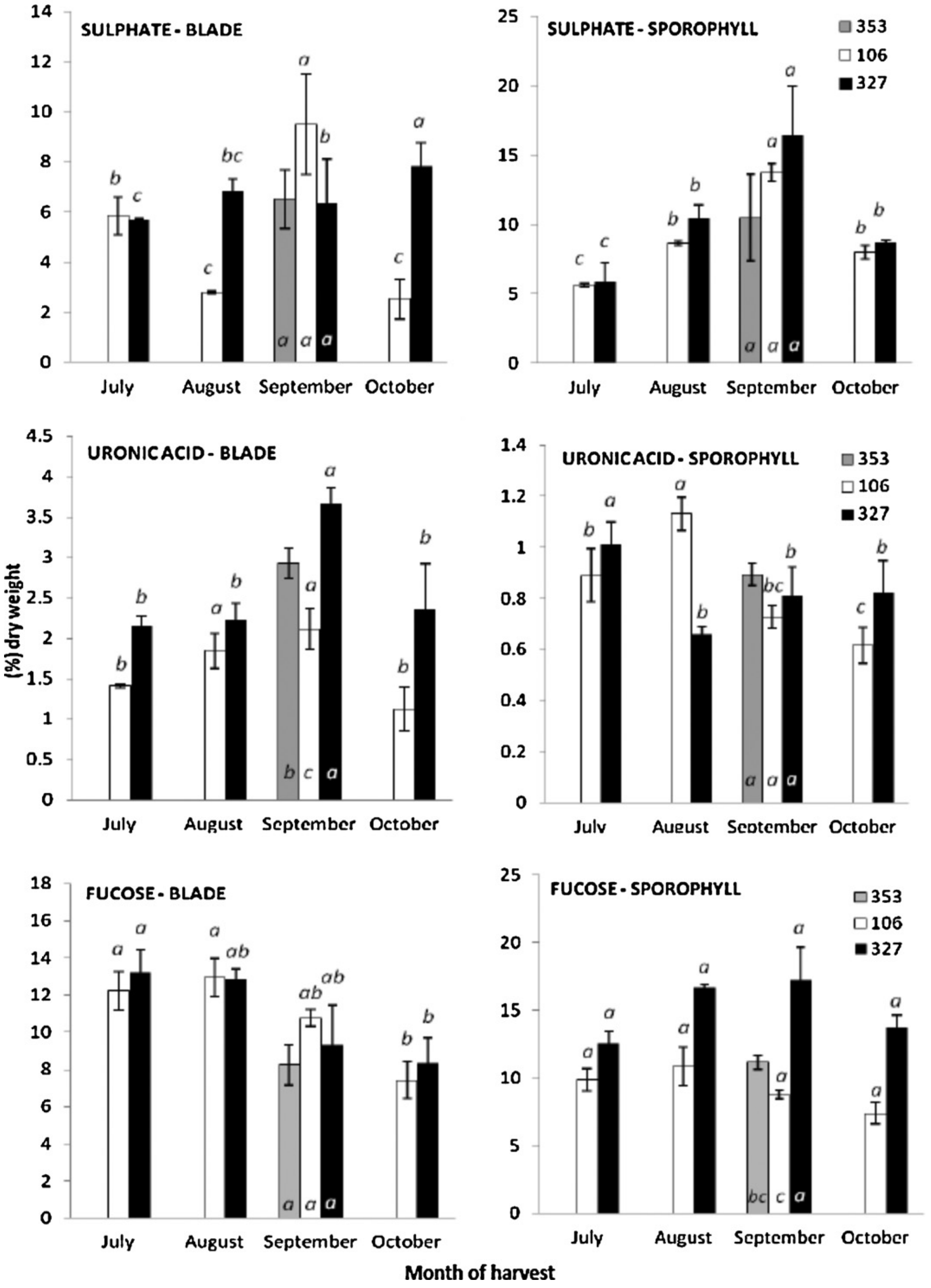

Fig. 3. Monthly variations in the sulphate, uronic acid and fucose content in fucoidan (106/327/353 = farm number). Mean ± SE ($n = 3$). Farms were examined separately. Different superscript letters indicate significant differences at $p < 0.05$ by Tukey's test. Subscript letters examine differences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

Protein content of the fucoidan throughout the four months of harvest however was below 1% dry weight (data not shown). This indicated little protein contamination occurred. Fucoidan has an 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 and our study, suggested that the fucoidan was crude (Li, Wei, Sun, & Xu, 2006; Mabeau, Kloareg, & Joseleau, 1990; Ponce et al., 2003; Wang et al., 2007; Yang et al., 2008).

Fucose composition of fucoidan was significantly affected by the month of harvest. Investigation of the fucose content of fucoidan revealed that blade-derived fucoidan from both farms 327 and 106 had significantly decreased between July and October ($P < 0.05$). 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 ($P = 0.176$). Much like the increase in uronic

Table 1
Yields and chemical compositions of the fucoidans extracted from *U. pinnatifida* by different methods ($n = 3$, ±SE).

Method	Yield (%)	Fucose (%)	SO_4^{2-} (%)	Uronic acid (%)	Protein (%)
SW	10.7 ± 0.13	12.7 ± 0.05	24.5 ± 0.02	2.8 ± 0.04	0.06 ± 0.002
SC	13.3 ± 0.45	15.0 ± 0.11	34.6 ± 0.61	2.4 ± 0.02	0.34 ± 0.005
SA	5.1 ± 0.16	8.6 ± 0.09	14.4 ± 0.16	4.4 ± 0.02	0.23 ± 0.004
BW	4.8 ± 0.04	9.5 ± 0.25	27.9 ± 0.03	2.1 ± 0.12	0.09 ± 0.006
BC	2.8 ± 0.17	16.4 ± 0.29	28.0 ± 0.09	4.9 ± 0.03	0.19 ± 0.001
BA	3.4 ± 0.25	4.3 ± 0.31	22.1 ± 0.68	3.7 ± 0.04	0.16 ± 0.006

S, sporophyll; B, blade; W, water; C, calcium chloride; A, acid.

acid content in the blade was likely due to blade degradation; degradation of the blade may also be responsible for the decline in fucose content in blade-derived fucoidan. Similarly Skriptsova, Shevchenko, Zvyagintseva, & Imbs (2009) 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 required to determine whether a correlation between the chemical composition of fucoidan and sporulation of *U. pinnatifida* existed.

3.2. Variations in crude fucoidan content and composition between two different locations

Extraction yields of fucoidan in the September harvest from farms 106 and 327 in Port Underwood, and farm 353 in Pelorus Sound are shown in Fig. 2. Significant differences were found in the yields of sporophyll-derived fucoidan between farm 353 in Pelorus Sound and farm 106 in Port Underwood ($P = 0.033$). Both farms 106 and 327 in Port Underwood also showed significant differences in fucoidan yield between the sporophyll ($P = 0.003$) and blade ($P = 0.001$). In addition, blade-derived fucoidan from farm 353 was significantly lower than farm 106 ($P = 0.010$) but not 327 ($P = 0.290$).

As shown in Fig. 3, no significant differences were observed between the sulphate content of sporophyll- and blade-derived fucoidan from all three farms. Uronic acid content in blade-derived fucoidan from all three farms showed significant differences ($P < 0.001$), with farm 327 having the highest content. The content of uronic acid in sporophyll-derived fucoidan was similar between farms, with all less than 1% dry weight. Blade-derived fucoidan had similar fucose content between farms ($P = 0.512$). However significant differences in fucose content were found in sporophyll-derived fucoidan between farms ($P = 0.028$). This study suggests that the endogenous changes in the alga during sporogenesis are much more likely to affect the composition of fucoidan than environmental factors. Fucoidan yield, sulphate and fucose content from both farms 353 (Pelorus Sound) and 327 (Port Underwood) were similar even though the farms were approximately 40 km 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).

3.3. Fractionation of crude fucoidan

Crude fucoidan from the September 2011 harvest was fractionated by DEAE-Sephadex A-25 to yield three fractions, F1, F2 and F3. No polysaccharide was detected when eluted with 3 M NaCl. All fractions contained fucose as the major sugar component along with galactose, with small amounts of mannose, xylose and glucose also present. However the sugar composition varied among the three fractions (Table 1). Further purification led to removal of xylose and other minor neutral sugars. As galactose was defined as one of two major components, together with fucose, purified fucoidans in all preparations should be sulphated galactofucans that is in agreement with the literature (Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004; Lee et al., 2006; Synytsya et al., 2010; Vishchuk, Ermakova, & Zvyagintseva, 2011). The fraction eluted with a low NaCl concentration was higher in uronic acid and lower in sulphate content. Conversely, the fraction that was eluted with high NaCl concentration was higher in sulphate content and lower in uronic acid. This was similarly encountered with fucoidan from *Sargassum swartzii* (Ly et al., 2005). The content of fucose increased significantly ($P < 0.001$) from F0 to F3 (Table 1), followed by a decrease in xylose content. No noticeable changes between fractions were observed for galactose, mannose and glucose, and the protein content of fractions was similar. These results showed that crude fucoidan from New Zealand *U. pinnatifida* were heterogeneous in terms of its sulphate content, uronic acid content and monosaccharide composition (Table 2). This is supported in previous studies on fucoidan from *S. swartzii, Pelvetia canaliculata, Fucus vesiculosus, Sargassum muticum and L. digitata* as being heterogeneous (Ly et al., 2005; Mabeau et al., 1990).

3.4. Molecular weight (Mw) estimation by GPC

The average Mw of crude fucoidan from the sporophylls of New Zealand *U. pinnatifida* was estimated to be 171 kDa by GPC, after calibration with known dextran standard molecular weight markers. The mass of crude fucoidan from New Zealand *U. pinnatifida* was higher than crude sporophyll-derived fucoidan from the same species harvested from Kijang, Korea that had a Mw of 38 kDa (Koo, Jo, Do, & Woo, 1995). Conversely, fucoidan extracted from the sporophylls of *U. pinnatifida* grown in Wando, Korea had a higher average Mw of 2100 kDa (Kim et al., 2007). The large differences in Mw from the same species was reportedly due to the variation in different fucoidan extraction techniques that may render fucoidan unstable when heating (Sakai, Ishizuka, & Kato, 2003). Variation in Mw of fucoidans from *U. pinnatifida* was also reported by Fitton and Dragar (2006). In this study, the Mw of fucoidan fractions eluted with water, 1 M and 2 M NaCl were 81, 22 and 27 kDa, respectively (Table 1). The molecular weights of the fucoidan fractions were less than half of that crude fucoidan. A decrease in Mw was observed from F0 to F3 fractions as NaCl concentration increased. This pattern was also observed with fucoidan extracted from *U. pinnatifida* grown in Peter the Great Bay, Russia when using a GPC DEAE-Sephadex A-25 column (Skriptsova et al., 2009). The decrease in Mw of the fractions was assumed to be caused by depolymerization of fucoidan during the fractionation process (Hemmingson et al., 2006). The Mw of commercial fucoidan purchased from Sigma was estimated to be 54 kDa, which was within the specified range obtained from the Sigma database of 20–200 kDa. Purified fucoidan fractions from New Zealand *U. pinnatifida* harvested in the Marlborough Sounds, New Zealand were sulphated galactofucans with high molecular weight.

3.5. Scavenging activity on DPPH radical

The antioxidant activity of fucoidan and its fractions were further investigated by the DPPH scavenging assay. DPPH is a stable free radical, where the reduction of DPPH to DPPH-H

Table 2
Chemical composition of crude fucoidan, its fractions and commercial fucoidan (Sigma) obtained by anion-exchange chromatography from *U. pinnatifida*. Different superscript letters indicate significant differences at $p < 0.05$ by one-way ANOVA followed by the Tukey's post hoc comparison test.

Fraction	Uronic acid	Sulphate	Protein	M_W (kDa)	Monosaccharide composition (%)				
					Fuc	Gal	Xyl	Glc	Man
F0	1.24^c	15.02^d	0.36^{bc}	171	39.24^d	26.48^b	28.85^a	0.95^b	5.04^b
F1	4.34^a	6.96^e	0.86^a	81	48.51^c	37.86^a	3.74^b	2.91^a	6.97^a
F2	0.84^c	22.78^b	0.63^b	22	53.21^{bc}	42.12^a	1.15^b	1.28^b	2.24^c
F3	0.67^c	25.19^a	0.11^c	27	59.71^b	28.74^b	1.58^b	2.77^a	7.19^a
Sigma	3.14^b	17.96^c	0.41^{bc}	54	87.12^a	5.69^c	4.85^b	0.94^b	1.39^c

after encountering a proton-donating substance can be used as an indicator of antioxidant activity. All fucoidan fractions exhibited strong DPPH scavenging ability at concentrations between 10–4000 μg/mL. Fig. 4 showed that the DPPH scavenging activity or inhibition percentage of crude fucoidan, its fractions and commercial fucoidan from Sigma were dose-dependent. At a concentration of 1000 μg/mL, crude fucoidan (F0) showed significantly higher scavenging activity of 86.80% ($P < 0.05$) followed by F3 (68.65%), F2 (58.65%), commercial fucoidan (55.22%) and F1 (53.45%). However, the inhibition percentages were all relatively lower than vitamin C over the same range of concentration. Fucoidan from Maritech® was reported as being a stronger antioxidant than vitamin C, but details of extraction method and species of seaweed used were not specified (Fitton, Irhimeh, & Falk, 2007). Conversely another study reported that fucoidan was only stronger than vitamin C at inhibiting superoxide radicals, but not DPPH radicals (Wang et al., 2009) due to superoxide radicals being less active. The scavenging abilities of DPPH radicals from another study using fucoidan from *L. japonica*, cultured in Qingdao, China, and its purified fractions possessed approximately half the inhibiting power (Wang et al., 2009) compared to the fucoidan in this study. In addition, New Zealand fucoidan showed more or less higher scavenging activity of DPPH radicals than fucoidan extracted from *U. pinnatifida* in Qingdao, China (Hu, Liu, Chen, Wu, & Wang, 2010). Crude fucoidan that had the strongest inhibiting activity towards DPPH radicals also had the highest molecular weight compared to F2 and F3. This indicated that high molecular weight of fucoidan fraction may have higher bioactive potential. This was in line with an earlier study that reported crude fucoidan derived from *Padina gymnospora* as having the strongest inhibiting activity when using superoxide and hydroxyl radicals compared to its purified fractions due to a larger molecular mass (Souza et al., 2007). The strongest antioxidant activity of crude fucoidan F0 could be explained by the presence of polyphenols. Fitton and Dragar (2006) stated that these compounds may enhance fucoidan bioactivity. Purification may have led to the removal of polyphenols that may explain the decrease of antioxidant activity in the fractions. On the other hand, F3 exhibited significantly more antioxidant activity than F1 ($P = 0.105$) at a concentration of 4 mg/mL despite a smaller molecular weight. However, the sulphate content of F3 was also significantly higher than F1 ($P < 0.001$) indicating that sulphate 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 necessary for bioactivity. Although commercial fucoidan had a higher sulphate:fucose ratio, and a slightly smaller molecular weight than F2, 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 et al., 2008; Skriptsova et al., 2009) that may all contribute to the bioactivity of fucoidan.

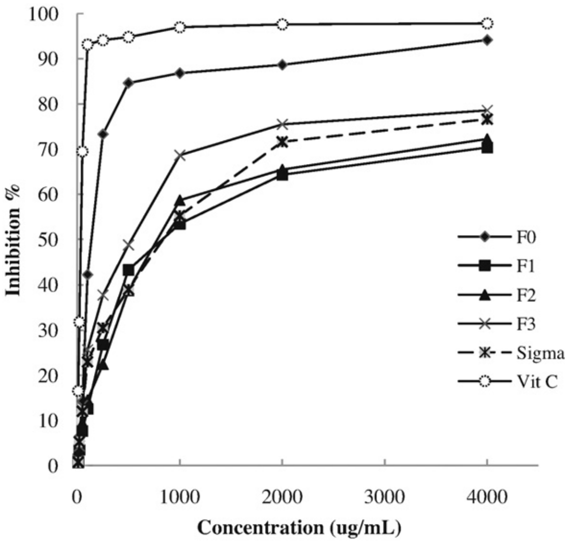

Fig. 4. Scavenging effects on DPPH radical by crude fucoidan (F0), its fractions (FI, F2 and F3), commercial fucoidan (Sigma) and Vitamin C. Values are means from three independent tests.

3.6. CUPRAC assay

The CUPRAC method is based on the reduction of the CUPRAC reagent, Cu(II)-neocuproine after reacting with an antioxidant to form the CUPRAC chromophore, Cu(I)-neocuproine, which has a higher the absorbance and stronger chelating ability (Guclu, Ozyurek, Bektasoglu, & Apak, 2010). The ability to chelate Cu(I) is an important mechanism of antioxidants. The significance of copper ions in biological systems is crucial for cellular regulatory functions. The imbalance of copper in our system can cause severe health conditions such as osteoporosis, hypothyroidism, schizophrenia, premenstrual syndrome, etc. (Yruela, 2005). As shown in Fig. 5, all fucoidan fractions showed dose-dependent antioxidant activity. At a concentration of 2.5 mg/mL, the chelating effect of F0 was significantly stronger ($P < 0.05$) than F1, F2, F3 and commercial fucoidan. Both F0 and F3 fractions exhibited stronger chelating activity than commercial fucoidan. The highest absorbance recorded by the chelating effects of F0, F1, F2, F3 and commercial fucoidan, were 1.547, 0.225, 0.262, 0.656 and 0.513 respectively, at a concentration of 2.5 mg/mL. Although commercial fucoidan contained significantly more sulphate groups than F0 ($P = 0.006$), the chelating activity of commercial fucoidan was significantly lower than F0 ($P < 0.001$) at a concentration of 2.5 mg/mL. This may be due to the much larger molecular mass of F0 compared to commercial fucoidan (Table 1). The results of this study suggest that the ratio of sulphate:fucose content of fucoidan was related to their chelating ability. A higher sulphate:fucose ratio of both F0 and F3 fractions may contribute to the higher chelating activity compared to commercial fucoidan. The CUPRAC scavenging assay values were however not comparable with previous studies due to the more commonly applied method of the ferric reducing antioxidant power (FRAP) assay in

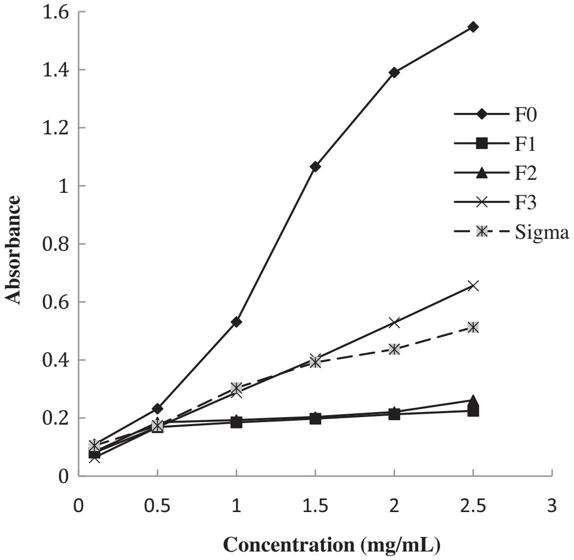

Fig. 5. Reducing power of crude fucoidan (F0), its fractions (F1, F2 and F3) and commercial fucoidan (Sigma) with the CUPRAC reagent. Values are means of three independent tests.

other studies (Ajisaka et al., 2009; Nabanita et al., 2010; Ruperez et al., 2002).

4. Conclusions

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. This study showed that New Zealand *U. pinnatifida* had an increase in crude fucoidan yield from July to September which may be related to the maturation of the algae. Hence *U. pinnatifida* should be collected in September to obtain the highest amount of crude fucoidan. Changes in the fucose content of blade-derived fucoidan were evident but not for sporophyll-derived fucoidan suggesting that chemical components in fucoidan were likely linked with the maturation of *U. pinnatifida*. Exogenous factors such as water temperature, amount of sunlight present, amount of freshwater input, and concentration of nutrients in the water may influence fucoidan content between farms and individual alga. Fractions obtained after gel permeation chromatography varied in terms of monosaccharide and chemical composition. F0 exhibited the strongest antioxidant activity compared to all three fractions and commercial fucoidan. A correlation was observed between both sulphate content and molecular mass with antioxidant activity of fucoidan fractions. The antioxidant activity of sporophyll-derived fucoidan from New Zealand algae makes it an attractive potential natural antioxidant resource. Comparison of bioactive properties between different types of fucoidans remains to be unclear. Therefore, it would be beneficial to derive crude fucoidans from different extraction technologies in order for the active compounds in different fucoidans to be compared.

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