The estimation of the digestibility of fucoidan extracted from New Zealand *Undaria pinnatifida*

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'The estimation of the digestibility of fucoidan extracted from New Zealand *Unndaria pinnatifida*', 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: ……Yi Yi…….

Signed: … …….

Date: ……24/11/2017…….

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Abstract

Undaria pinnatifida is an unwanted seaweed species in New Zealand that heavily infests local mussel farms. Fucoidan, a sulfated polysaccharide extracted from *U. pinnatifida*, is known to have greater number of bioactivities. As a dietary supplement, fucoidan is mainly consumed orally. As fucoidan were regarded as indigestible carbohydrates, the enzymatic digestibility in human body has not been studied. It is also important to investigate the digestibility of fucoidan to help understand how fucoidan exerts its health benefits.

The objective of this study was to investigate the enzyme digestibility of fucoidan extracted from the New Zealand *U. pinnatifida* in human body by using an *in vitro* digestion model.

In order to determine the digestibility, fucoidan was digested in the *in vitro* digestion model with 5-min oral, 2h-gastric and 2h-intestinal phases, respectively. The dissolved undigested sample and the fucoidan digesta were fractionated by filters with different molecular weights (100, 10-100, 3-10 and 3 kDa). After the separation, samples were freeze dried and weighed. The weighed sample was then hydrolyzed with Trifluoroacetic acid (TFA) and derivatized with 1-phenyl-3-methyl-5- pyrazolone (PMP). The monosaccharide contents were detected by LC-MS afterwards.

My results suggest that the fucoidan extracted from New Zealand *U. pinnatifida* can be partly digested in *in vitro*. The weight and the neutral sugar contents (fucose, galactose, mannose, xylose, rhamnose, glucose and glucuronic acid) of > 100 kDa was decreased significantly after the digestion process. While in small fractions (10-100 kDa, 3-10 kDa and < 3kDa), both weight and neutral sugar contents were significantly increased. Approximately 4 % (w/w of dry weight (dw) fucoidan) and 14 % (w/w) of sugar were detected in 10-100 kDa and 3-10 kDa fractions, respectively after the simulated digestion. The results indicated that the > 100 kDa fraction were cleaved by salivary and intestinal amylases and the gastric juice. Fucoidan was partly digested.

Structural analysis, bioactivities and interactions with the gut microbiota of the partly digested fucoidan can be investigated in the further.

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List of Abbreviations

ABEE	p-aminobenzoic ethyl ester
CF	Crude fucoidan
DEN2	Dengue virus type 2
DMSO	Dimethyl sulfoxide
FID	Flame ionization detector
FTIR	Fourier-transform infrared spectroscopy
G1	1 hr gastric phase
G2	2 hr gastric phase
GC	Gas chromatography
GI	Glycemic index
HPSEC	High-performance size exclusion chromatography
I1	1 hr intestinal phase
I2	2 hr intestinal phase
LMWF	Low molecular weight fucoidan
MAF	Ministry of Agriculture and Foresty
MALLS	Multi-angle laser light scattering
MPI	Ministry for Primary Industries
MPI	Ministry for Primary Industries
Mw	Molecular weight
NMR	Nuclear magnetic resonance
NZMF	New Zealand Ministry of Fisheries
O	Oral phase
PMP	*1-phenyl-3-methyl-5- pyrazolone*
SCFA	Short chain fatty acids
SD	Standard Deviation
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid
TFA	Trifluoroacetic acid
U. pinnatifida	*Undaria pinnatifida*

Chapter 1 Introduction

Recently, nutraceuticals derived from marine resources have drawn great interest of consumers (Kim, Vo, and Ngo, 2013). As is known to all, more than 70% of the earth surface is covered by oceans where provide various living environment for marine organisms. In recent decades, numerous novel bioactive compounds were isolated from marine bionts, many of which are utilized in pharmacy industry and healthcare market for their potential biological activities (Blunden, 2001; Blunt, Copp, Munro, Northcote, and Prinsep, 2006; Mayer, Rodríguez, Berlinck, and Hamann, 2009). In 2014, approximately 28.5 million tonnes of seaweeds and other algae were harvested from wild oceans or cultivated in farms (FAO, 2016). Annually, the largest share of seaweed industry value is from human consumption. Beyond that, about one-sixth of the income is based on the extracting seaweed products, like hydrocolloids and fucoidan for their bioactivities, animal feed additives, cosmetics ingredients, fertilizer, etc. (Nayar, S., and Bott, 2014; Phang, 2010). Although these plant-derived foods and nutraceuticals have lower potency compared to the pharmaceutical drugs, since these products are recommended a dietary ingest, they may have a significant physiological effect for long term consumption (Espín, García-Conesa, and Tomás-Barberán, 2007). In addition, they are more acceptable because of their safety, economy, medicinal synergy and less side effects than most drugs (Raskin et al., 2002).

U. pinnatifida (wakame) is a kind of brown seaweed, native in cold temperate seas of Northern hemisphere, such as China, Japan and Korea. It was introduced to New Zealand accidentally in 1987 probably by international shipping or fishing vessels and spread rapidly to numerous sites on North, south and Stewart islands in the next two decades (Silva, Woodfield, Cohen, Harris, and Goddard, 2002). Since it is a highly fertile species and serious invader, the New Zealand Ministry of Fisheries (NZMF) permitted to harvest this extraneous species for both indigenous seaweed protection and economical purpose. Farmers and researchers are encouraged to develop and utilize the New Zealand *U. pinnatifida*. The bioactive compound, fucoidan was extracted and studied. Numerous therapeutic and health benefits have been revealed clinically. It seems to be one of the most commercially viable products of *U. pinnatifida*. In 2015, White (2015) first reported the commercial scale extraction of fucoidan from New Zealand *U. pinnatifida*.

Fucoidan was often regarded as resistant to digest by human digestion enzymes. However, the fucoidan structure is complex, the presence of glycosidic bonds are still debated. Moreover, fucoidan structure is various from species, harvesting season and many habitat conditions, the digestibility of fucoidan extracted from the New Zealand *U. pinnatifida* was unknown. The aim of this project is to investigate the enzyme digestibility of fucoidan extracted from the New Zealand *U. pinnatifida* in human body through a simulated gastrointestinal (GI) digestion model. It is hypothesized that depending on the stages of digestion, size and sugar content of fucoidan fragments would be different.

This thesis is composed of six chapters. The summaries of previous studies of fucoidan structures, human GI system and *in vitro* digestion models are presented in chapter two. The preliminary studies are explained in chapter three. Chapter four listed the materials, methodology and statistical data analysis used in this study. The results acquired from the project are shown and discussed in chapter five. Conclusion and the outlooks for further studies are included in chapter six.

Chapter 2 Literature review

2.1 Background of research objective

2.1.1 Brown seaweed—— *Undaria pinnatifida*

The classification of seaweed is shown in Figure 1. Brown seaweeds hold the biggest portion of marine algae, which have been classified 13 orders, about 300 genera and calculated around 1836 known species (Okolie, Udenigwe, Aryee, and Mason, 2017). They are tough and normally live in wave-exposed waters near the surface. It is easy to find them in the intertidal rocky shore and sublittoral fringe (Anthoni, 2007). Brown seaweeds are mostly large in size, the giant kelp can grow 2 to 4 meters while red seaweeds can only be farmed to a meter.

U. pinnatifida (Suringar, 1873) is one of the most commercially important species the big seaweed group. According to the statistics reported by Phang (2010), native to Japan sea. New Zealand *U. pinnatifida* was first found in Willington Harbour in 1987, it may be carried by a shipping or fishing vessels from Asia. In the past two decades, it has spread internationally which already occupied some region of New Zealand and Australia's coastlines (Hemmingson, Falshaw, Furneaux, and Thompson, 2006).

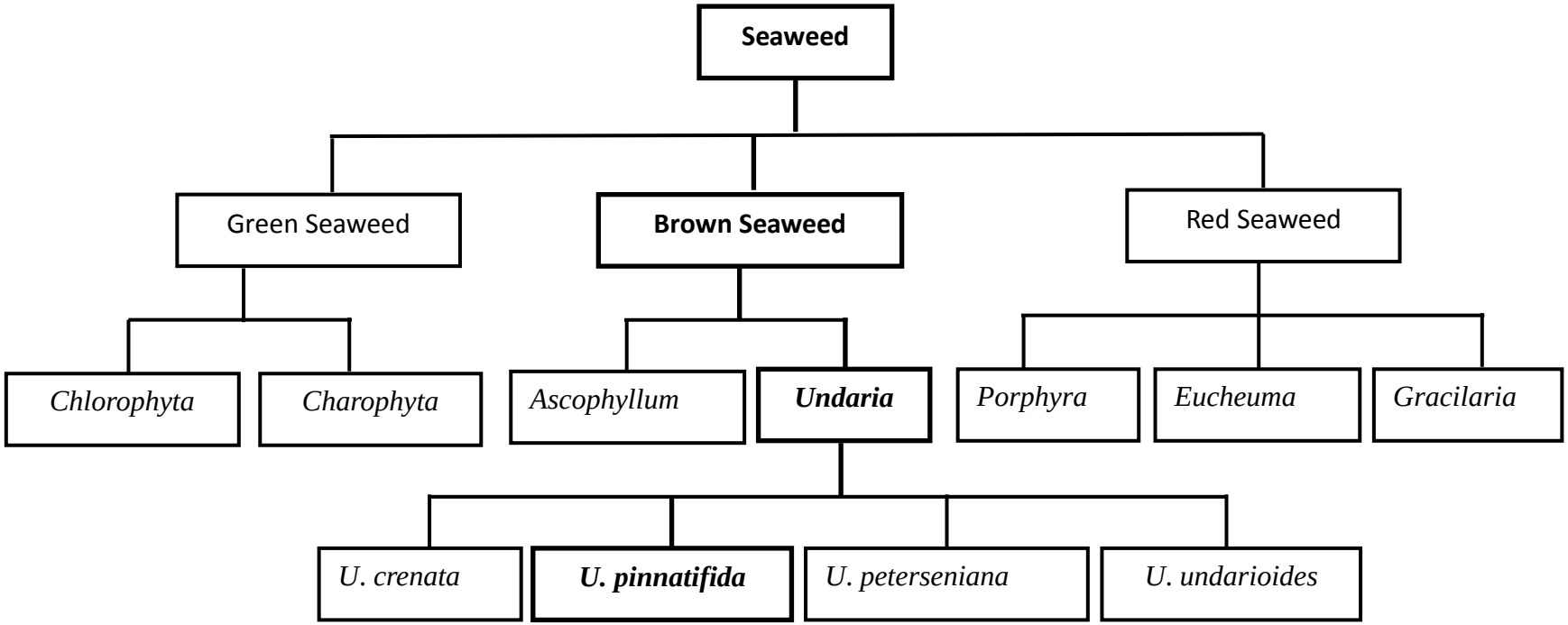


Figure 1 The classification of seaweeds

Previous study (Figure 2) has suggested that the *Undaria* may have positive impacts on improving food availability in areas which have little native seaweed reserve and may enhance the populations of the grazers and filter-feeders (Hay, 1992). However, Curiel, Bellemo, Marzocchi, Scattolin, and Parisi (1998) has reported that the colonization by *Undaria* can cause the decrease in biodiversity and reduce of indigenous seaweeds surface cover (*Rhodymenia ardissonei, Gracillaria verrucosa, Ulva rigida* and *Enteromorpha spp.*). It has been reported that the contribution of the *Undaria* to algal drift could influence the faunal populations as well. *Undaria*, replaced the native algae *Maraka* and *Gracilaria chilensis* was used as a feeding source for a sustained benefit to commercially important marine organism, paua (*Haliotis iris*) (Redfearn, 1994). Paua fed on *Undaria* grew slower than which fed on the indigenous algae. Though, more *Undaria* was consumed at 4-day intervals, it was shown an inferior nutritive value compared with the native seaweeds and less was converted to biomass. Beyond doubt, *Undaria* has been affirmed as a highly invasive and unwanted species by NZ biosecurity (Biosecurity Act 1993 No. 95) (MPI, 1993).

Though Ministry of Agriculture and Foresty (MAF) made attempt to wiped out *U. pinnatifida* from New Zealand in the 1990s, little effects were received. Before it was allowed to be harvested as a by-product in 2004 (Stuart, 2004), 11 studies were published by New Zealand scholars, besides researched on the mechanisms and spread, ecology and ecophysiology extension of the *Undaria*, the studies were also focused on the development of control management of the *Undaria*. Instead of pest the *U. pinnatifida*, the Ministry for Primary Industries (MPI) has allowed to harvest *U. pinnatifida* from 2009 (Ministry of Agriculture and Forestry, 2010). In view of the commercial beneficial and establishing a stable marine environment Farm owners and investors have been encouraged to commercialize this exotic Asian species in the selected heavily infested area and farm in a limited area and artificial surfaces to protect native marine organism (Ministry of Agriculture and Forestry, 2010). Since then, more studies were popped out. The scientific interest can be *U. pinnatifida*, as an edible seaweed used as an ingredient in pasta (Prabhasankar et al., 2009), or used for pharmaceutical and medicinal purpose because of its high content of bioactive components (Misurcova, 2011; MacArtain, Gill, Brooks, Campbell, Rowland, 2007).

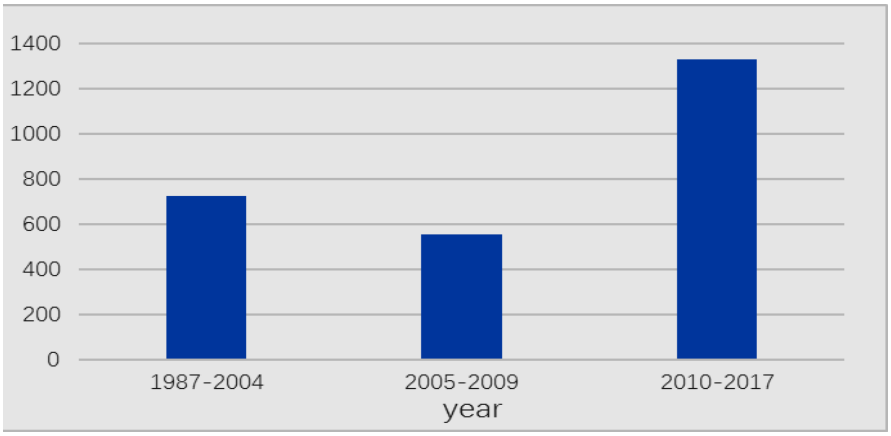


Figure 2 Number of publications with “Undaria pinnatifida” and “New Zealand” in the title or keywords (Source: google scholar)

The chemical compositions of seaweeds are various from the type of spices and harvesting season, geographic habitat and many habitat conditions like light intensity, water temperature and nutrition concentration in water (Indergaard, M. and Minsaas, 1991; Marsham, Scott, and Tobin, 2007; Misurcova, 2011; Watts, Goldstien, and Hopkins, 2016). The general nutrition facts of *U. pinnatifida* was listed in table 1 (USDA, 2016). Higher ash contents (33.02 % and 26.79 % in the marine and freshwater *U. pinnatifida*, respectively) were reported by Vacek (2010). Similar vitamin, macro- and trace elements content was reported by Kolb, Vallorani, Milanović and Stocchi, (2004), Awczynski, Chäfer, Eiterer and Ahreis (2007), Santoso, Gunji, Yoshie-Stark and Suzuki (2006) and Mišurcová, Stratilová, and Kráčmar (2009). In 100 g of *U. pinnatifida* dry matter, 21.3 % of protein (Marsham et al., 2007), 1 to 5 % of lipids and 35.3 % to 71.4 % of total carbohydrates (Misurcova, 2011; Yang, Chung, and You, 2008) have been observed.

Table 1 General nutrition facts of U. pinnatifida (Source: United States Department of Agriculture Food Composition Databases)

Amount Per 100 grams	
Calories 45	
Total Fat 0.6 g	
Saturated fat 0.1 g	
Polyunsaturated fat 0.2 g	
Monounsaturated fat 0.1 g	
Total Carbohydrate 9.14 g	
Dietary fiber 0.5 g	
Sugar 0.65 g	
Protein 3.03 g	
Water content 79.99 g	
Minerals	
Calcium 150 mg	Sodium 872 mg
Iron 2.18 mg	Potassium 50 mg
Magnesium 107 mg	Zinc 0.38 mg
Phosphorus 80 mg	
Vitamins	
Thiamine (B_1) 0.06 mg	Folate 196 μg
Riboflavin (B_2) 0.23 mg	Vitamin C 3 mg
Niacin (B_3) 1.6 mg	Vitamin E 1 mg
Pantothenic acid (B_5) 0.697mg	Vitamin K 5.3 μg

2.1.2 Polysaccharides in brown seaweeds

Seaweed polysaccharides were divided into insoluble fibers, including cellulose and lignin, and another big group of biological functional soluble carbohydrates, including laminarin, alginate and fucoidan are the three major polysaccharides of brown seaweeds (Okolie et al., 2017). Mabeau and Fleurence (1993) reported the soluble and insoluble fiber content of *U. pinnatifida* were 30.0 % and 5.3 % of the dried matter, while Rupérez and Saura-Calixto (2001) has shown the content as 17.31 % and 16.26 %, respectively. The difference in fiber contents may be caused by different growing environment.

Laminarin

Laminarin is the main storage polysaccharide which is located in cell vacuoles with confirmed antibacterial, antioxidative and anticoagulant properties (Lee, J. Y., Kim, Y. J., Kim, H. J., Kim, Y. S., and Park, 2012; H. Zhang and Row, 2015). The chemical structure of the laminarin is formed by (1,3)-β-D-glucan with β-(1,6) branching. Damas, Forget, Dandrifosse, and Devill (2004) has revealed *laminarin* extracted from *Laminaria Saccharina* was indigestible by human digestive enzymes, however, can consumed as a good source of dietary fiber with a modulatory impact during the metabolism in the intestines.

Alginate

Alginates are extracted mostly from the cell wall of brown seaweeds with a well-established gelling feature. Alginates are widely used as stabilizers, thickeners, gel formers in food industries (Draget and Taylor, 2011). It has conclusively been shown that over 80 % of alginate oligosaccharides remained intact after enzymatic hydrolysis (dissolved in phosphate buffer and incubated with 5 U amylases or proteases for 72 hours). Alginate oligosaccharides were proved to resisted to digest in the upper intestinal tract (Wang, Han, Hu, Li, and Yu, 2006).

Fucoidan

Fucoidans are compound of a series of complex sulfated fucose-rich polysaccharides which are in abundance in the cell walls of brown seaweeds (Kim et al., 2013; Ponce, Pujol, Damonte, Flores, and Stortz, 2003), soluble in water and sparingly soluble in dimethyl sulfoxide (DMSO). According to other studies, marine invertebrates can be the source of fucoidan as well, like sea cucumbers (Mourão and Bastos, 1987) and the egg jelly coat of sea urchin (Vilela-Silva, Alves, Valente, Vacquier, and Mourão, 1999).

Fucoidan was first isolated a hundred years ago and was named as ”fucoidin” (Killing, 1913). The fucoidan content in *U. pinnatifida* varies from location (Mak, Hamid, Liu, Lu, and White, 2013) and seasons (Mak et al., 2013; Skriptsova, Shevchenko, Zvyagintseva, and Imbs, 2010). the fucoidan extracted from *U. pinnatifida* originated from Korea by Yang, Chung and You (2008) had a 8.8 % yield, which was slightly lower (9.4 %) than the same species originated from Japan (Nishide, Anzai, Uchida, 1987). White, (2015) reported the lowest yield (0.52 %) from New Zealand *U. pinnatifida*.

Commonly used extraction method of fucoidan was acid/base extraction, ion-exchange chromatography or gel filtration were the two mainstream method of fucoidan purification (Bilan et al., 2002; Yang, Chung, Shin, et al., 2008). It was reported that the extraction with acidic solvent like hydrochloric acid can result a higher yield of crude fucoidan. However, more undesirable products like alginic acid and metals may also obtained from the acidic extraction and fucose chain degradation may happened with the presence of acid (Kawamoto et al., 2006). Recently, hot water extraction was utilized more frequently as it can maintains the stability of the large polysaccharide structures and safer for oral consumption (B. Li, Lu, Wei, and Zhao, 2008; White, 2015; Yang, Chung, and You, 2008).

2.1.3 The structure of fucoidan

During the last decades, a great deal of interest has been developed by the research towards the content of fucoidan extracted from different brown seaweeds. For example: *Adenocystis utricularis* (Ponce et al., 2003), *Ascophyllum nodosum* (Chevolot, Mulloy, Ratiskol, Foucault, and Colliec, 2001; Clément et al., 2010; Daniel et al., 2001; Marais and Joseleau, 2001), *Chorda filu* (Chizhov et al., 1999), *Cladosiphon okamuranus* (Nagaoka et al., 1999), *U. pinnatifida* (Synytsya et al., 2010), *Fucus distichus* (Bilan et al., 2004), *Fucus evanescens* (Bilan et al., 2002), *Fucus vesiculosus* (Mabeau, Kloareg, and Joseleau, 1990), *Fucus serratus* (Bilan, Grachev, Shashkov, Nifantiev, and Usov, 2006), *Sargassum stenophyllum* (Duarte, Cardoso, Noseda, and Cerezo, 2001), *Turbinaria conoides* (Marudhupandi, Kumar, Lakshmanasenthil, Suja, and Vinothkumar, 2015), *Pelvetia canaliculata* (Mabeau et al., 1990), *Laminaria digitate* (Mabeau et al., 1990), *Sargassum muticum* (Mabeau et al., 1990).

The molecular weight of fucoidan can be 100 to 1,600 kDa, when measured by gel-permeation FPLC (Skriptsova et al., 2010) , or using a high-performance steric exclusion chromatography (HPSEC) equipped with different column (Nardella et al., 1996) or coupled with multi-angle laser light scattering (MALLS) (Ramnani et al., 2012; Yang, Chung, Shin, et al., 2008; Hemmingson et al., 2006). Another fraction method of fucoidan was using a Millipore Ultra filtration System with different molecular weight cut-off membranes (You, Yang, Lee, and Lee, 2010).

The composition and proportion of the monosaccharides in fucoidan have been studied comprehensively (Table 2). Fucose and galactose are the two major monosaccharides to make up the fucoidan (White, 2015). Glucose, mannose and rhamnose are specialized in fucoidan of some seaweed species (Kim et al., 2013; Misurcova, 2011). The monosaccharides content of fucoidan was analyzed by the acid hydrolysis. Fucoidan was hydrolyzed to monosaccharides with 2M TFA at 120 °C for 90 min or at 100 °C for longer incubation time (Ponce et al., 2003; Skriptsova et al., 2010). After derivatized to aldononitrile acetates, the monosaccharides content was analyzed by Gas chromatography (GC) equipped with a flame ionization detector (FID) (Ponce et al., 2003). High performance liquid chromatography was also used for the quantitative analysis for monosaccharides very frequently after monosaccharides were derivatized by p-aminobenzoic ethyl ester (ABEE) or 1-phenyl-3-methyl-5- pyrazolone (PMP) (Blanco, Muro, and Mangas, 2001; Gucek and Pihlar, 2000; Skriptsova et al., 2010; Yang,

Chung, and You, 2008; Zhang, Zhang, Wang, Shi, and Zhang, 2009).

Though the composition and proportion of the monosaccharides in fucoidan can be easily determined, the technology for today can't scan the intact fucoidan with complex "sugar-sugar" and "sugar-sulfate" bonding and branching. The core structures of fucoidan are described as (1,2) and/or (1,3)-linked fucose and sulfates are linked at the C2 and/or C4 position of L-fucopyanosyl residues seldom on C3 various from different source. The structure analysis was normally speculated by a combination of different method, such as Nuclear Magnetic Resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), FT-Raman, methylation analysis and so on.

Table 2 Monosaccharides composition of fucoidan extracted from different brown seaweed species

Brown seaweed species	Composition	Reference
Adenocystis utricularis	Fucose, galactose and sulfate ester	Ponce et al. (2003)
Ascophyllum nodosum	Fucose and sulfate	Marais and Joseleau (2001)
	Fucose, xylose, mannose, galactose, glucuronic acid and sulfate	Nardella et al. (1996)
Chorda filu	Fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate	Chizhov et al. (1999)
Cladosiphon okamuranus	Fucose, glucuronic acid and sulfate	Nagaoka et at. (1999) Sakai, Ishizuka, Shimanaka, Ilai and Kato (2003)
	Fucose, xylose, glucuronic acid, acetic acid and sulfate	Sakai et al. (2003)
Undaria pinnatifida	Fucose, galactose and sulfate 43.26: 11.63: 41.5	Yang et al. (2008)
	Fucose: xylose: galactose: mannose 72.3: 1.5: 14.6: 10.9 Neutral sugar: uronic acid: sulfate esters 52.34: 26.2: 7.4	Kim et al.,(2007)
	The content of elements C: H: N: S 23.03: 3.97: 0: 9.18 Fucose: galactose: xylose: mannose 50.9: 44.6: 4.2: 0.3	Synytsya et al.,(2010)
	Fucose: galactose 1.0: 1.1	Lee, Hayashi, Hashimoto, Nakano, and Hayashi, (2004)
	Fucose: galactose: xylose: mannose 72.3: 14.6: 1.5: 10.9	Kim et al., (2007)

The stracture of fucoidan extracted from *A. nodosum* was investigated by Marais and Joseleau (2001), the core region is dominantly α-(1→3)-linked fucose with a few α-(1→4) linkages, single and multi-units fucosyl residues were branched at C-2 of the →3-linked internal residues. The sulfate groups were carried at position 2 and 4 of both main chain and terminal chain. The same result was motioned by Bilan et al. (2002) and Pomin, Valente, Pereira and Mourão (2005). A 3-sulfate linked fucopyranans were found in *A. nodosum* fucoidan by Nardella et al. (1996) and Daniel et al. (2001). Chevolot et al. (2001) reported the backbone fragment of low molecular weight fucoidan (LMWF) which was established as a repeating unit:

$$[\rightarrow 3)\text{-}\alpha\text{-L-Fuc}(2SO3^{-})\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc}(2,3diSO3^{-})\text{-}(1]_n.$$

The structure of fucoidan isolated from *C. filu* was studied by Chizhov et al. (1999). Most fractions were constructed by (1→3)-linked poly-α-L-fucopyranoside backbone branched with a high degree α-(1→2)-linked single fucopyranose residues which sulfated mainly at the position of O-4 and sometimes O-4 position.

The everage structure of fucoidan in *C. okamuranus* was described as a linear backbone of (1→3)-linked α-fucopyranosyl unit. 1 mol sulfate group occupied the O-4 position for every 2 mol of fucose and 1 mol glucuronic acid residue branched as a side chain at position 2 for every 6 mol of fucose. The structure was shown as follow: $-[(\rightarrow 3\text{Fuc-4}(\pm OSO3^{-})\alpha 1\text{-})5\rightarrow 3[\text{GlcAa}1\rightarrow 2]\text{Fuc}\alpha 1\text{-}]_n-$ (Nagaoka et al., 1999). Sakai, Ishizuka, Shimanaka, Ilai and Kato (2003) represented the main structure as: (-3L-Fucα1-3L-Fuc(4-O-sulfate)α1→3L-Fuc(4-O- sulfate)α1→3[GlcAa1→2]Fucα1-), while the ratio of fucose, glucuronic acid and sulfate group was 4:1:2 which reported less glucronic acid contant than the previous study.

Ponce et al. (2003) reported the stracture of fucoidan fractions are primarily constituted by 3-linked α-L-fucopyranosyl backbone, 2-, with mostly sulfated groups at C-4 and sulfated fucopyranosyl units branched at C-2, and non-sulfated fucofuranosyl and fucopyranosyl units branched at C-2. It is also reported that different fucoidan structures were obtained by increasing the extraction tempertature from 25 to 70 °C. Fucose conbined with mainly monnose and uronic acid and low propotions of glucose, xylose, rhamnose, galactose and sulfate ester.

α-1,3 and/or 1,4 linked sulfated L-fucose unit built up the backbone of the fucoidan, various 1-3-linked fucose, 1,3-, 1,4- and 1,6- linked galactose and other monosaccharides residues are brunched to the fucose backbone (Figure 3). The sulphation may occur at position mostly C2 and/or lesser C4 of the fucose and/or C3 and C6 of the galactose residues (Holtkamp, Kelly, Ulber, and Lang, 2009; Skriptsova et al., 2010; Vishchuk, Ermakova, and Zvyagintseva, 2011), while common fucoidans are mainly cosist of only sulfated fucose (Vishchuk et al., 2011). Synytsya et al., (2010), Vishchuk, Ermakova and Zvyagintseva (2011) and Ho et al., (2015) reported a significant amount of acetylation of sugar monomers in *U. pinnatifida* which is differ from the fucoidan extracted from *F. vesiculosus*. The sulphated component of *U. pinnatifida* varies from 4.5 -6.5 % (Fitton, Dragar, 2006), 7.4 % (Kim et al., 2007), 9.18 % (Synytsya et al., 2010), 41.5 % (Yang et al., 2008), 14.4 % - 34.6 % with different extraction methods. The content of sulfate, uronic acid and fucose shown significant difference due to monthly variations and harvest location (Mak et al., 2013). The change of mannose and galactose concentration were also observed in *U. pinnatifida* fucoidan via harvest at different months (Skriptsova et al., 2010). Every fucoidans is unique, no two isolated fucoidan are exactly the same in structure or composition or biological activities (He and Jeon, 2012) .

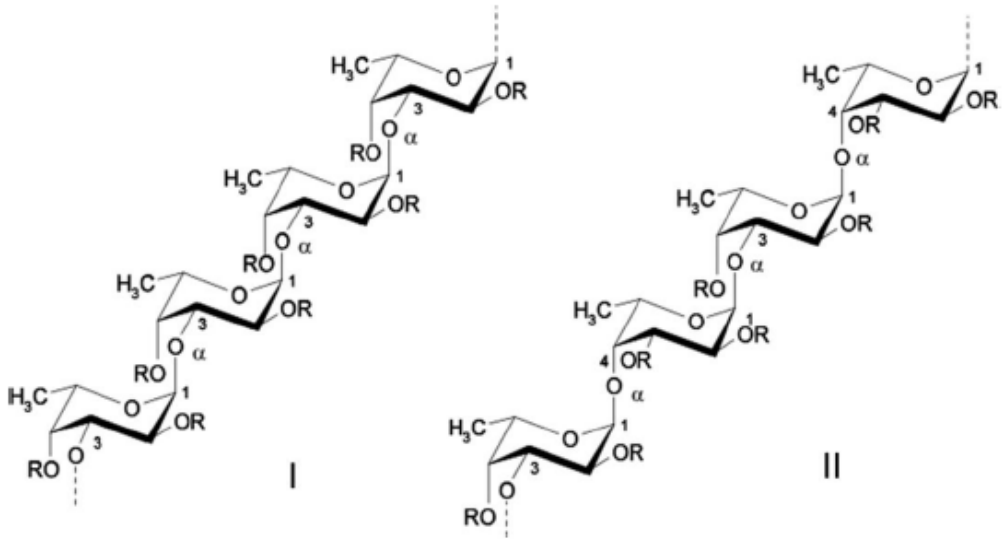


Figure 3 Schematic chemical structure of U. pinnatifida

Note: Ⅰ α-1,3 linked sulfated L-fucose backbone Ⅱ α-1,3 and α-1,4 linked sulfated L-fucose backbone, R = α-L-fucopyranoside, galactosyl residue, sulfuric base, acetyl base (from White, 2015)

2.1.4 The benefits of fucoidan

Numerous studies have been published to verify the bioactivities of fucoidan (Table 3). The degree of sulfation (Chevolot et al., 1999; Mak and White Nazimah, 2012; Preeprame, Hayashi, Lee, Sankawa, and Hayashi, 2001; Rupearez, Ahrazem, and Leal, 2002), composition of monosaccharides, the structure and the molecular weight (Ho et al., 2003; Nardella et al., 1996; Ramnani et al., 2012) were all correlated to the bioactivities. For example, it was reported that the oversulfation of fucoidan enhances the antitumor, anti-angiogenic activities and anticoagulant activities (Bouhedja, Ellouali, Sinquin, and Vidal, 2000; Koyanagi, Tanigawa, Nakagawa, Soeda, and Shimeno, 2003; Pielesz, Biniaś, and Paluch, 2011; Teruya, Konishi, Uechi, Tamaki, and Tako, 2007), while fucoidan with low molecular weight, ranged from 10 to 300 kDa displayed a better anticoagulant effects (Lin et al., 2004; Nardella et al., 1996; Nishino, Ura, and Nagumo, 1995). Since most of these bioactivities were tested on the rats (Ho et al., 2003; Katai et al., 2015; Min, Kwon, Lee, Park, and Kim, 2012; Roberfroid, 2007) or on the cells (Adhikari et al., 2006; Khil et al., 2011; Lin et al., 2004; Vishchuk et al., 2011) the study on how human digest fucoidan is necessary to help understanding how fucoidan was utilized and interacted in human body.

Table 3 Previous studies on fucoidan bioactivities

Bioactivity	Sources of fucoidan	References
Antioxidant	*F. vesiculosus*	Rupearez et al. (2002)
	U. pinnatifida	Mak and White Nazimah (2012)
	Turbinaria conoides	Marudhupandi et al. (2015)
	Ecklonia radiata	Charoensiddhi, Franco, Su, and Zhang (2015)
Anticoagulant	*Ascophyllum nodosum*	Chevolot et al. (1999) Nardella et al. (1996)
	Lessonia vadosa	Chandía and Matsuhiro (2008)
	U. pinnatifida	Kim et al. (2007)
	Sargassum stenophyllum	Duarte et al. (2001)
Antithrombotic	*U. pinnatifida*	Min et al. (2012)
	F. vesiculosus	Min et al. (2012) Soeda, Ohmagari, Shimeno, and Nagamatsu (1993)
Anti-inflammatory	*Fucus evanescens*	Cumashi et al. (2007)
	Laminaria saccharina	
Anti-tumour/ anti-cancer/ anti-metastatic	*U. pinnatifida*	Sheng (2012)
Anti-virus	*Cystoseira indica*	Min et al. (2007)
	Cladosiphon okamuranus	Hidari et al. (2008)
	U. pinnatifida	Hayashi, Nakano, Hashimoto, Kanekiyo, and Hayashi (2008)
	S. tenophyllum	Sinha, Astani, Ghosh, Schnitzler, and Ray (2010)
Anti-obesity	Commercial fucoidan	Kim, Jeon, and Lee (2014)
Immunostimulatory effects	*F. vesiculosus*	Kim and Joo (2008)
	F. evanescens	Khil et al. (2011)
Inhibitory effect on parasites	*U. pinnatifida*	Chen, Lim, Sohn, Choi, and Han (2009)
Anti-depression effects	*L. saccharina,* *L. digitata,* *Fucus distichus* *Cladosiphon okamuranus*	Lee, Shim, Lee, and Hahm (2013)
Cholesterol modulation effects	*Sargassum henslowianum*	Cuong, Thuy, Huong, Ly, and Van (2015)
Downregulation of allergic reaction	Commercial fucoidan	Vo, Ngo, Kang, Jung, and Kim (2015)
Anti-fatigue effects	*Laminaria japonica*	Chen et al.(2015)
Anti-ulcer effects	*C. okamuranus* *F. vesiculosus*	Chen et al. (2010)
Hyperphosphatemia (renal failure) modulation effects	*U. pinnatifida*	Katai et al. (2015)
Protective effects on gastric and liver injuries	*C. okamuranus*	Kim et al., 2014; Nagaoka et al. (1999)
Protective effects on the nervous system	Commercial fucoidan	Hu, Zhang, and Zhao, 2014; Resident and Holders (2011)
Therapeutic and healing effect in surgery and brain injury	Commercial fucoidan	Hu, Zhang, and Zhao, 2014; Resident and Holder (2012)

2.2 Study of human carbohydrates digestion

2.2.1 Human digestion process

Human digestion is a complex process for breaking down food to nutrients to fuel body and maintain metabolism (Vander, Sherman and Luciano, 2001). Take carbohydrate-based food as an example, the digestion of food starts in the mouth. Food is first reduced food particles size by teeth cut and increased the surface area for better enzymatic reactions. The salivary glands in mouth secrete saliva which helps to moisten food. Salivary amylase is also secreted by salivary glands simultaneously, which is the first enzyme to have an impact on carbohydrates digestion (Etienne and Denis, 2012). After a relatively short time of breaking down food particles and polysaccharides in the carbohydrate food, the bolus of food is easily pass through the esophagus to stomach by peristaltic waves (Dona, Pages, Gilbert, and Kuchel, 2010).

Food is further dissolved in stomach, the secretion of HCl by stomach parietal cells leads to a regular decrease in pH from 6 (oral phase) to ~1.5 (gastric juice). The presence of HCl retards the activity of salivary amylase and kill most of the bacteria ingest along with food. Since no carbohydrates-related enzymes are secreted in stomach, instead of enzymatic hydrolysis, acid hydrolysis is the major reaction to the dietary carbohydrates (Vander et al., 2001). Polysaccharides along with droplets of lipid, molecular fragments of proteins and other small particles in food formed a solution called chyme. The gastric ends at the pylorus, which works as a filter and a pump, selectively emptying chyme of 1 to 2 mm size to the duodenum. The large particles remained in the gastric phase and have been further degraded. Hardly any molecules in chyme, except water can cross the epithelium of gastric wall, hence very little organic nutrients are being absorbed in the stomach (Vander et al., 2001). The rate of food utilized in stomach is closely related to the consumption and absorbance of the nutrients and calories which is regulated by the gastric emptying time (Reviews and Science, 2008). The gastric empty time is affected by food biological properties, food physical properties like the type of macronutrients in meal, the meal volume, food particle size viscosity and pH and food physical properties or structures (Rayner, Samsom, Jones, and Horowitz, 2001).

The acidic chyme is then delivered to the next section, the small intestine, which is about

3.8 cm in diameter and 2.7 m in length. It is divided into three compartments: a short section, the duodenum, and followed with two longer section jejunum and ileum. Normally, most of the chyme is digested and absorbed in duodenum and jejunum. Pancreas and liver secrete digestive fluid through ducts into duodenum. Digestive enzymes and fluid rich in bicarbonate ions are secreted by the exocrine portion of pancreas to the stomach. The sodium bicarbonate neutralized the acidic chyme to give an appropriate pH (7) for pancreatic enzymes activities. A complex mixture of amylases, lipases and proteases made up the pancreatic enzymes. Pancreatic amylase catalyses the polysaccharide to break down into disaccharides. Other digestive enzymes such as lactase, sucrose and maltase produced by the inner wall of small intestine and ileal microbiota involved in the breaking down of disaccharides to monosaccharides (Etienne-mesmin and Denis, 2012). The Bile is produced by liver which plays an important role for emulsifying ingested fats into small droplets in lipid digest. Mechanical digestion such as peristalsis activity, propels the chyme move through the small intestine and segmentation movements, allows chyme to mix thoroughly with digestive enzymes. Since the inner lining of the small intestine gives a huge epithelial surface, villus enterocytes absorb nutrients and water by active transport, simple diffusion or facilitated diffusion.

Small volume of water, salts and non-absorbed material, are slowly advanced toward the large intestine. Some remained dietary fibers can be metabolized by colonic microbiota and bile get reabsorbed in colon. Other material is temporary stored in the colon and concentrated by absorbing water and electrolytes. The feces are finally eliminated by the contraction of the rectum and relaxation of associated sphincter muscles (Etienne-mesmin and Denis, 2012; Vander et al., 2001).

Around 800 g of solid food and 1200 ml are consumed by an adult in average per day, whereas, additional 7000 ml of fluid is secreted from salivary glands (1500 ml), gastric glands (2000 ml), intestinal glands (1500 ml), pancreas (1500 ml) and liver (500 ml) into the gastrointestinal tract to help digestion and absorption every day. Among 8L of fluid enter the lumen, 99 % can be absorbed, only about 100 ml of water is expelled with the feces. Almost all the salts in the digestion juice are reabsorbed into blood. The digestive enzymes are self-digested to amino acids and absorbed into blood.

2.2.2 Digestion enzyme of carbohydrates

α-amylase is present in the mouth and intestine, mainly responsible for carbohydrates-based food products, roughly separated into two categories by its distinct modes of catalysis, endoamylase and exoamylase (Zhang, Han, and Xiao, 2017). The main function of endoamylase is to hydrolyze the internal α-1,4 glycosidic linkages to glucose, maltose and dextrin while not changing the α-anomeric configuration (Gupta, Gigras, Mohapatra, and Goswami, 2003; Zhang et al., 2017) (Figure 5). The catalyze activity causes the formation of linear and branched oligosaccharides of various chain lengths (Gupta et al., 2003). They affect nutrient digestion and absorption, glucose metabolism and prevention against risk of cardiovascular disease in the small bowel (Scheppach, Luehrs, and Menzel, 2017). The molecular weight of α-amylase varies from 10 kDa (*Bacillus caldolyticus*) (Grootegoed, Lauwers, and Heinen, 1973) to 210 kDa (*Chloroflexus aurantiacus*) (Eriksen, Jensen, and Olsen, 1998), while human salivary amylase has an apparent molecular weight of less than 22 kDa (Wilding, 1963)

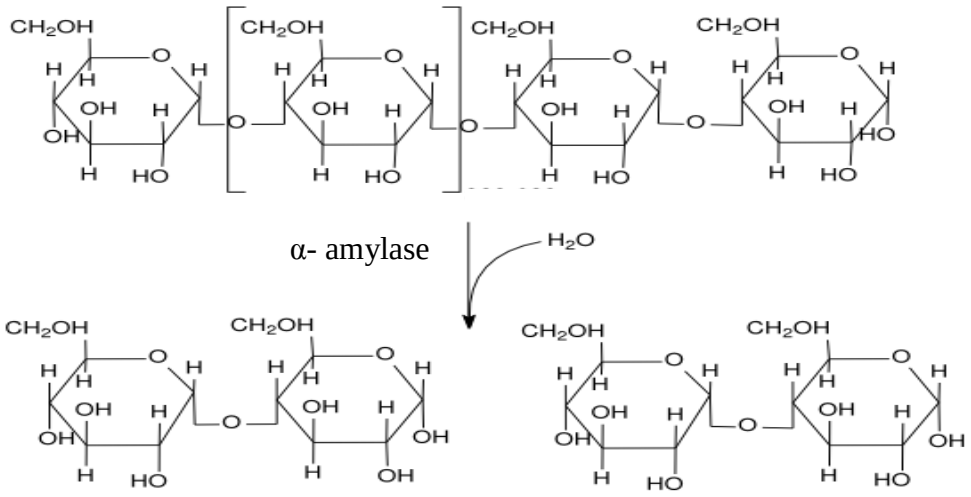


Figure 4 The mechanism of α-amylase activity

2.2.3 Indigestible carbohydrates

The classification of carbohydrates is presented in Figure 4. The indigestible carbohydrates are complex dietary fibers which are enzyme-resistant saccharides (Hipsley, 1953). They are also called non-glycemic carbohydrates which are generally accepted the definition based on their physiological characteristic, cannot absorbed or utilized directly in human small intestine (Mudgil and Barak, 2013). It can be categorized to two types based on their solubility, insoluble and soluble fiber, or classified according to physiological relevance by plant cell-wall substances, non-starch polysaccharides, resistant starch, and non-digestible oligosaccharides (resistant short-chain carbohydrates) (Englyst and Englyst, 2017). Types of dietary fiber can also be classified based on their source and fermentability (Mudgil and Barak, 2013)

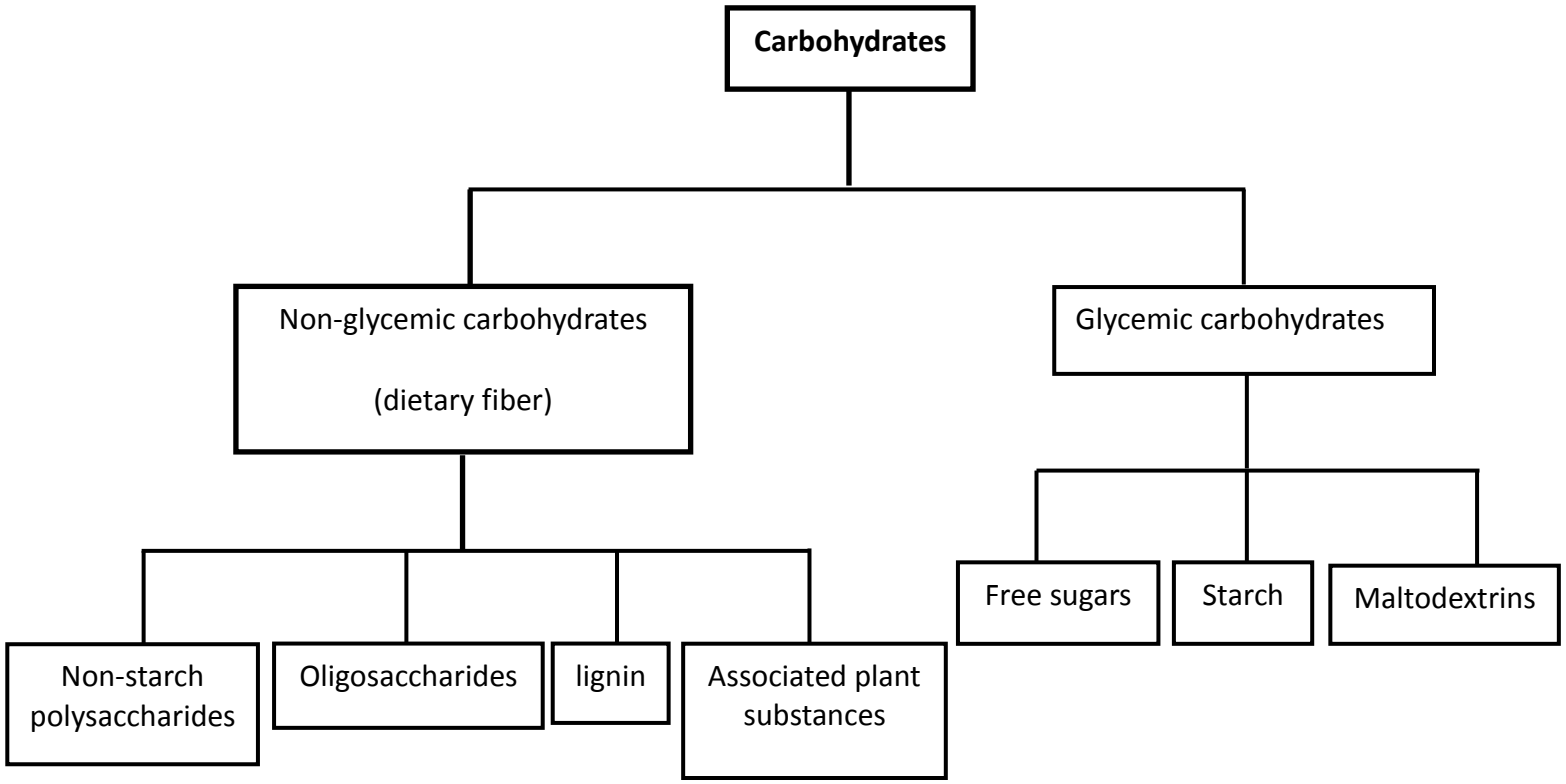


Figure 5 Classification of carbohydrates

The non-starch polysaccharides are marker of the natural fiber-rich diet recommended as beneficial to health. Their diverse physical and chemical properties made them showing different biological functions and utilization in the gastrointestinal tract and gut (Englyst and Englyst, 2017). Some of the indigestible fibers pass through the intestinal tract and excrete out of the body directly, rest stimulate the growth of friendly colonic microflora and mainly degraded by anaerobic bacteria in the colon which is known as prebiotic activity. Inulin, an example of the indigestible fiber which found mostly in yams, is reported to stimulate the growth of friendly intestinal bacterial (Nyman, 2017).

The consume of resistant starch helps people stay full for longer time since they are not easily to digest. It also enhances the fecal nitrogen excretion (Birkett, Muir, Phillips, Jones, 1996). The fecal nitrogen is used to prevent or treat hepatic encephalopathy clinically and implicated as a protective factor resist colon cancer (Munster, Tangerman, and Nagengast, 1994) . On the contrary, an excess consuming of plant fibers in the form of grains, fruits and vegetables may lead to intestinal gas, abdominal pain, bloating and constipation. Both Young et al. (1996) and Burn, Katheuser, Fodde, Coaker (1996) reported resistant starch significantly enhanced the cancer formation in rat model.

It is not appropriate to say all the indigested carbohydrates are beneficial to health, detailed biological function of various types and amounts of non-glycemic carbohydrates should be investigated before consuming (Englyst and Englyst, 2017; Wasan, 1996).

Insoluble fibers

Insoluble fibers which absorb or attract water instead of dissolve in water. The fibers normally have strong bulking ability (Mudgil and Barak, 2013). Fibers “bulk up” within the large intestine, as the result, the indigestible fiber accelerates intestinal motility and bowel movements and helps cleaning the large intestine (Groff, 2009). Spiller (1993) was using wheat bran as an example for these less fermentable fibers. Scheppach et al. (2017) further reported these unfermented carbohydrates as a treatment of chronic functional constipation, the irritable bowel syndrome and diverticulosis (Elleuch et al., 2011).

Soluble fibers

According to Werbach (1999), the soluble fibers pass through intestinal tract and

correlated with the reducing of blood cholesterol levels. The fibers dissolve in water and made the digest fluid becomes sticky and gel-like. Consequently, they are bound with the bile molecule and removed from the body. Bile carries significant amounts of fatty acids and cholesterol, which will be cleaned out of the body contemporary (Elleuch et al., 2011). The water holding capacity of different particle size of soluble fibers in *L. digitate*, a brown seaweed, was determined by Fleury and Lahaye (1991), it was shown that small size particles (125-250 μm) tend to have a higher water binding capacity.

Soluble fibers undergo gut bacteria fermentation to form short chain fatty acids (SCFA), like butyrate, acetate and propionate and absorbable gases, like hydrogen and methane (Pylkas, Juneja, 2005). These fermentable fibers were proved to reduce the risk of diseases and have many other potential effects on mammalian cell function via altering the composition and populations of the colonic microflora (prebiotic effect). The fermentation of some soluble fibers was reported to stimulate human liver to produce less low-density lipoprotein and/or LDL cholesterol which both are considered harmful. Ten healthy man was asked to consume 90 g carbohydrate portion of either barley or brown rice for dinner with white rice as control. Thorburn, Muir and Proietto (1993) reported serum fatty acid and hepatic glucose production were suppressed significantly in the next morning, especially barley consumption with more fermentable carbohydrates, caused more reduction. The fermentation product, butyrate, has been attested to have effects on protein synthesis, mammalian cell gene transcription and cellular proliferation and apoptosis (Erik et al., 2010). Other SCFA from fermentation were evaluated as new therapy in acute colitis and considered in a primary prevention of colorectal cancer (Scheppach et al., 2017).

A positive relation between colonic bacterial fermentation and improved glucose tolerance was reported by Erik et al., (2010). Eight cereal based meals with different content and glycemic index (GI) of indigestible carbohydrates was fed to seventeen healthy participants. The postprandial blood sugar response was inversely influenced by plasma butyrate ($p < 0.01$) and acetate ($p < 0.05$) concentration. Compared with white wheat bread, meal with high-amylose barley kernels and high-β-glucan barley kernels resulted a higher plasma butyrate concentration. The result supported the view that consuming products rich in indigestible carbohydrates may be protectively against type 2 diabetes and cardiovascular diseases through a mechanism coming down to intestinal

bacterial fermentation and generation SCFA, in particular butyric acid, improving the glucose tolerance. SCFAs also inhibit harmful non-acid tolerant bacteria by decreasing colonic pH, or inhibit negative flora by supporting the growth of beneficial bacteria. Moreover, they participate in cleaning bile salts and serum cholesterol (Pylkas, Juneja, 2005).

Fucoidan was mostly defined as indigestible fibers (Charoensiddhi et al., 2015; Misurcova, 2011; Okolie et al., 2017), the molecular structure remains to be established, as the presence of glycosidic bond, the digestibility is still debated (Daniel, Berteau, Jozefonvicz, and Goasdoue, 1999)

2.3 Digestion models

2.3.1 *In vivo* digestion

In vivo digestions are achieved by feeding animals or humans food directly. Erik et al., (2010) have tested the digestibility of cereal fructans in an animal model. Cereal fructans were extracted and intubated into the rats' stomachs. The content of fructans in the small intestine and colon were quantified and shown approximately the same amount indicating no or very low absorption of fructans in the small intestine. Mannose, was used as control to show higher absorption through passive diffusion than fructans.

The digestibility can also be assessed mediately by measuring the blood sugar and insulin levels at clinical trial stage. Alternatively, the undigested molecules can be detected from the faces and distal ileum of human volunteers to directly quantify the digestive degree (Roberfroid, 2007).

The *in vivo* digestions usually offer more accurate and vivid results. However, these methods are normally time consuming and costly. Englyst and Cummings (1985) assumed to be difficult to measure the extent of digestion of polysaccharides in small bowel accurately in human body. For this reason, *in vitro* digestion models were alternatively applied to simulate the human and animal digest and give a relatively accurate result in a short time (Boisen and Eggum, 1991; Jin, Ou, Decker, and Mcclements, 2011).

2.3.2 *In vitro* digestion model

Among the previous studies related to *in vitro* digestion, food structural changes, digestibility, bioaccessibility and release of food components were mostly studied under a simulated gastrointestinal condition. Sometimes, the *in vitro* digestion models show a different result with a *in vivo* model because it is difficult to simulate the highly complex physicochemical and physiological condition of the individual and animal digestive tracts accurately (Jin et al., 2011). The scholars are still trying to develop and improve the digestion models. When choosing the *in vitro* digestion models protocol, consideration should be given on the method being simple, rapid, reproducible and the compatibility with the *in vivo* results for the same food materials (Boisen and Eggum, 1991).

To date, food samples tested with the *in vitro* digestion models were plants, meats, dairy, fish and emulsion-based foods. The biological molecules included in the digestion models were digestive enzymes (α-amylase, pepsin, pancreatin, peptidase, lipase, trypsin, chymotrypsin), bile salts and mucin. The utilized enzymes can be collected from human subjects or extracted from animal or plant sources. In essence, it has been reported that the porcine pancreatic lipase and co-lipase is acceptable to replace human pancreatic lipase and co-lipase, however, there was no study showing the advantages and disadvantages or the difference between using different sources of the enzymes (Zangenberg, Müllertz, Kristensen, and Hovgaard, 2001). There was no doubt that different enzymes have various optimum temperature, the digestion temperature was mostly set at 37 °C to simulate the human body condition (Jin et al., 2011).

The *in vitro* digestion models are different from one another in their operations. The most important factor of *in vitro* digestion system is the characteristics of the enzymes, concentration, temperature, pH, incubation time, stability, activators and inhibitors all effect the enzyme activities (Jin et al., 2011). Some enzymes require additional components within the simulated digestive fluids to active more efficiency. For example, bile salts promotes pancreatic lipase activities (Boisen and Eggum, 1991). Calcium not only helps free fatty acids to precipitate, but also stabilizes the architecture of the catalytic cleft of α-amylase and help with thermostability (Minekus et al., 2014; Zhang et al., 2017) . Other factors affected the digestion system, including the phases involved, like mouth, stomach, small intestine, large intestine, the time in each phase, the composition

and concentration of the digestive fluids, like salts, buffers, biological polymers and surface-active components, or the mechanical stresses and fluid flows utilized in dynamic models (Jin et al., 2011). However, the transit and digestion time must be optimized according to the food physical characteristics (Jin et al., 2011).

2.3.3 Application of *in vitro* digestion

The *in vitro* model was more often associated with bioaccessibility of food (Brandon et al., 2006), food structural changes, digestibility and release of components.

In vitro digestion model can be also used to hamper the risk assessment by testing the bioaccessibility of contaminants released from the food in human and animals. The bioaccessibility of elements, like K, Mn, Zn, Fe and Na from wheatgrass, wheatgrass tablets and wheat seeds were measured by the *in vitro* gastro-intestinal model. The total elements bioaccessibility were 37-57%, 17-43% and 9-38%, respectively after 3hr gastric digestion. The bioaccessibility increased to 39-60%, 34-55% and 15-23% after another 4hr intestine digestion (Kulkarni, Acharya, Rajurkar, and Reddy, 2007). Yeung, Glahn and Miller (2003) reported the iron bioacailability of common raisin-containing food with the *in vitro* digestion model (pepsin digestion for 1hr and pancreatin-bile digestion 2hr).

The digestibility of food can by examined by simply test the enzyme digestibility or using *in vitro* digestion models. The simplified enzyme digestibility was mostly accomplished by incubated the mixture of enzymes and samples for 24 hours for an optimized temperature, usually 37 °C for simulating body temperature. This method is useful for predicting the digestibility of single nutrients (Boisen and Eggum, 1991). The °Brix of amylopectin, total sugar and the ratio of hydrolyzed starch versus total starch have been compared before and after steamed and incubated under an enzyme condition (α-amylase 60 U. mL^{-1} and amyloglucosidase 24 U. mL^{-1}) at 15 °C for 24 hours to assess the enzyme digestibility of five endosperm starch mutants (Okuda, Aramaki, Koseki, Satoh, and Hashizume, 2005). Using a single purified enzyme was considered consistent laboratory-to-laboratory comparisons. Though it was reported advantageous since it promotes the standardization of the *in vitro* digestion system, it is often more realistic to use complex mixture enzymes when digesting more than one nutrient (Boisen and Eggum, 1991). It is defined material indigestible or hard to digest when the recovered amount of this material was not altered significantly after pass though the *in vitro* digestion model with the addition of digestive enzymes. (Baba et al., 1988; Green, Murphy, Schulz, Watkins, and Ferruzzi, 2007)

The gastrointestinal fate of carbohydrates determines whether the dietary carbohydrates

are glycemic (be absorbed in the small intestine and release energy for body metabolism), or non-glycemic (fermented by colon bacteria or excreted as faeces) (Englyst and Englyst, 2017; Jin et al., 2011). The conditions of carbohydrates digestion were listed in the following table:

Table 4 Studies of *in vitro* digestion systems of dietary fibers

Study materials	Conditions	Findings	References
Soya bean, cowpea and maize	α-amylase 30 min lipase pepsin 1 hr pancreatin 30 min	40.2-45.5 %, 27.5-42.1% and 25.5.4-67.1 % defatted sample have been digested	Kiers, Nout, and Rombouts (2000)
Barley β-glucan in wheat bread	Pepsin 30min α-amylase 5hr	The digestibility of starch was reduced by both low and high molecular weight barley β-glucan	Cleary, Andersson, and Brennan (2007)
Alginate oligomer	α-amylases 24hr	80 % of alginate oligomer remained intact	Wang et al. (2006)
Seaweeds and mushrooms prebiotic extractions	α-amylases 30 min pepsin 2hr panreatin 3hr	The intermediate steps of faecal batch-culture fermentation	Rodrigues et al. (2016)
Isomalto-oligosaccharides		Partly digestible	Roberfroid (2007)
Laminarin extracted from *Laminaria saccharina*	Human saliva 5 hr	No hydrolysis of laminarin has been detected	Damas et al. (2004)
Cereal fructans (fructo-oligosaccharides)	Simulated gastric juice 2 hr	The intermediate step of feces fermentation	Nilsson, Oste, and Jägerstad (1988)
Sulfated polysaccharides extracted from *Enteromorpha prolifera* and *Laminaria japonica*	Human salivary α-amylase 15 min Pepsin 30 min Pancreatin 1.5 hr	The intermediate steps of faecal batch-culture fermentation	Kong, Dong, Gao, and Jiang (2016)

Chapter 3 Preliminary studies

In this section, the optimization of *in vitro* digestion, sugar hydrolysis and monosaccharides derivatization conditions are presented base on the other studies and the preliminary experiments done during the earlier stage of this study. It helped to confirm the applied method for the main studies investigating the digestibility of fucoidan extracted from New Zealand *U. Pinnatifida*.

3.1 Optimization of the conditions the *in vitro* digestion protocol

The conditions of the static *in vitro* digestion protocol was optimized based on the standardized method reported by Minekus et al. (2014) and modified refer to other *in vitro* digestion studies (Kedia, Vazquez, and Pandiella, 2008; Kong et al., 2016; Moon et al., 2015; Oomen et al., 2003; Versantvoort, Oomen, Kamp, Rompelberg, and Sips, 2005).

3.1.1 Sample size for digestion

Fucoidan extracts, as health supplements, are predominantly made in the form of tablets, capsules, powder and drinks. The purity, as a selling point, varies from different products. As a compromise for commercial suggested daily dose and for the ease of calculation, 250 mg of pure fucoidan was used as the starting sample size for the *in vitro* digestion study.

3.1.2 Oral enzyme activity and pH

As a carbohydrate-based food with a solid form, oral phase is speculated to have effect during the fucoidan digestion. The human salivary amylase activities were compared among different gender and age groups (Sahu, Upadhyay, and Panna, 2014). It was reported that the 18- 25 age group has a relatively higher baseline amylase activity (91.86 - 249.61 U. mL^{-1} for male subjects and 85, 75 - 189.25 U. mL^{-1} for female subjects) compared with 40 - 60 age group (76.23 to 159.14 U. mL^{-1} for male subjects and 75.75 and 178.62 U. mL^{-1} for female subjects). Considering that the major consumers of health care products are middle-aged people, the simulated saliva amylase was made to achieve 75 U. mL^{-1} in the mixture of food and saliva fluid in this study. Salivary α-amylase has a pH optimum at 6.8, after 20 s to 30 s oral digestion *in vivo*, α-amylase is inactivated by the gastric acid secretion and proteolytic activity. Even so, recent studies has confirmed that 25 to 50 % of the starch in pasta and bread boluses was hydrolyzed in oral cavity during the short retention time (Bornhorst, Hivert, and Singh, 2014; Hoebler et al., 1998). Since, it is hard to ensure an accurate and reproducible digestion in a lab situation with short digestion time, a 2-min simulated oral phase was suggested by Minekus et al. (2014) to allow food to have the contact time with enzyme. While Versantvoort, Oomen, Kamp, Rompelberg and Sips (2005) and Moon et al. (2015) recommended to increase the oral digestion time to 5 min to guarantee proper simulation of mastication movements. In summary, 5 min oral digestion was designed in this study. The digesta was sampled and analyzed after the oral digestion.

3.1.3 Simulated salivary fluid

99.5 % of water and 0.3 % of proteins along with various electrolytes like sodium, calcium, potassium, magnesium phosphate and bicarbonate constitute human saliva. simulated salivary fluid (SSF) was added to simulate the wetting and lubrication of solid food masses by salivation (Minekus et al., 2014; Moon et al., 2015). The constituents and concentrations of the SSF was made differently in previous studies (Table 5). Most studies were using a 1: 1 (g: mL) ratio as the amount of SSF. However, Kedia, Vazquez, and Pandiella (2008) and Oomen et al. (2003) reported a relatively high ratio (1: 20 and 1: 98 (g: mL)) to form a paste-like consistency. The solid-to-fluid ratios from 1: 5 to 1: 5000 (g: mL) were observed not likely to limit the bioaccessibility (Buckley, Lioy and Hamel, 1998; Oomen et al., 2003). Considering that the current fucoidan sample dissolves very poorly in water (up to 0.8 g. L^{-1}), 100 mL of SSF was used to wet and lubricate the fucoidan in oral phase. The SSF was first prepared according to Moon et al. (2015). Due to the large mass of various electrolytes (approximately 351 mg in 100 mL SSF), the change of fucoidan mass (250 mg) from before and after digestion was hard to weigh accurately (data not shown). A better method to adopt for this investigation was to use water only, instead of making electrolytes solution, with optimized pH and enzymes.

3.1.4 Gastric digestion time

In gastric phase, the gastric emptying time of a solid meal (steak, potato, bread and egg) is usually required 3 hr to 4 hr (Dressman et al., 1990; Jerry, Ciociola and Robinson, 2002). The content in stomach was observed an initial lag phase (the first 30 min) followed by a linear decrease with time (Lin et al., 2005). The length of gastric digesting homogenized meal, completed one-hour less than ordinary meal (Malagelada, Go, and Summerskill, 1979). By contrast, liquid meal requires a relatively rapid emptying process. Lin et al. (2005) reported emptying 300 mL of water need 1 hr. To make a compromise, a two-hour gastric digestion was applied for this model. The digestibility in gastric phase was described with 1-hr and 2-hr value.

3.1.5 Simulated gastric fluid and pH

A liquid meal usually requires half to one volume of gastric juice for digestion (Malagelada, Go, and Summerskill, 1979; Wisén and Johansson, 1992). In comparison, solid meal needs two volumes of gastric secretion (Burton et al., 2005). The simulated gastric fluid (SGF) used by others is shown in Table 5. HCl is secreted to lower the pH to construct an acidic condition for the optimal enzyme activities *in vivo*. One volume of simulated gastric juice for one volume of oral content was finally decided, in other word, the total gastric fluid was added up to 200 mL. Concerning the low content of fucoidan in gastric suspension solution (250 mg in 200 mL) and the large mass of electrolytes in the synthetic gastric juices (approximately 696 mg in 200 mL SGF), the fucoidan mass change from before and after gastric digestion was hard to weigh accurately. It was decided to use HCl solution to simulate the acidic condition (pH = 2), instead of making electrolytes solution. Since protein and lipid digestibility was not focused in this study, pepsin and gastric lipase were omitted in the digestion experiment. As the result, no enzymes were added in the gastric phase to guarantee weighing the fucoidan mass change accurately.

3.1.6 Intestine phase

In the small intestine digestion, the previous gastric fluid was further diluted with 50:50 v/v simulated intestinal fluid (SIF), the total intestine fluid was aggregated to 400 mL.

Fucoidan was completely dissolved in this phase with a concentration of 625 μg. mL^{-1}. The constituents and concentrations of SIF of previous studies are shown in Table 5. The pH in this phase was increased to 7 by the addition of NaOH. The amount of pancreatin added to the simulated fluid was based on the pancreatic amylase (200 $U.mL^{-1}$). 2 hr digestion simulating the intestinal digestion was used. Again, due to the large mass of various electrolytes (approximately 1259 mg in 400mL intestine digesta), the fucoidan mass (250 mg) change from before and after intestinal digestion was hard to weigh accurately. Only water was decided to be use, instead of making electrolytes solution, with optimized pH and enzyme.

To sum up, the optimized *in vitro* digestion procedure is shown in the methods and materials section (chapter 4).

Table 5 SSF constituents and concentrations in different studies

Study materials	Constituents of simulate fluid	SSF		SGF		SIF		Sources
		Concentration (mmol. L^{-1})	Food: SSF (g: mL)	Concentration (mmol. L^{-1})	SGF: oral fluid (mL: mL)	Concentration (mmol. L^{-1})	SIF: gastric fluid (mL: mL)	
Linear arabino-oligosaccharides and Debranched arabinan from sugar beet	KCl	15.1	1:1	6.9	1:1	6.8	1:1	Moon et al. (2015)
	KH_2PO_4	3.7		0.9		0.8		
	$NaHCO_3$	13.6		25		85		
	$MgCl_2$	0.15		0.1		0.33		
	NaCl	-		47.2		38.4		
	$(NH_4)_2CO_3$	0.06		0.5		-		
	$CaCl_2(H_2O)_2$	1.5		0.15		0.6		
	$CaCl_2$	0.75		0.075		0.3		
Food mix of peanut slurry, buckwheat	KCl	24.1	1:1	22.1	1:1	15.2	5:6	Versantvoort et al. (2005)
	KSCN	4.1		-		-		
	NaH_2PO_4	14.8		4.4		-		
	Na_2SO_4	8.0				-		
	NaCl	10.2		94		239.7		
	$NaHCO_3$	40.3				80.7		
	Urea	6.7		2.8		3.4		
	Uric acid	0.18		-		-		
	Mucin	50 mg. L^{-1}		6 $g.L^{-1}$		-		
	$CaCl_2.2H_2O$	-		5.4		2.7		
	NH_4Cl	-		11.4		-		
	Glucose	-		7.2		-		
	Glucuronic acid	-		0.2		-		
	Bull serum albumin	-		2 $g.L^{-1}$		2 $g.L^{-1}$		
	KH_2PO_4			-		1.17		
	$MgCl_2$			-		1.1		
Soil contaminants	Same as above	Same as above	1:98	Same as above	1:1	Same as above	1:1	Oomen et al. (2003)
sulfated polysaccharides	Phosphate buffer	20	Food suspended in buffer	water	1:4.2	Water	1:1.3	Kong et al. (2016)
Oats	Na_2SO_4	20	1:20	-	1:134	20	1:67	(Kedia, Vazquez, and Pandiella, 2008)
	NaCl	10		1.5		10		

3.2 Optimization of sugar hydrolysis

Analysis of monosaccharides composition is fundamentally important for polysaccharides structure and characteristic analysis. This experiment was designed to quantitatively determine the monosaccharides in fucoidan samples with a rapid, repeatable and accurate method.

Numerous method have been used for sugar hydrolysis with different acids like TFA (Albersheim, Nevins, English, and Karr, 1967; Amelung, Cheshire, and Guggenberger, 1996) , sulfuric acid (Christine Hoebler, Barry, David, and Delort-laval, 1989; Roberto, Mussatto, and Rodrigues, 2003; Willför et al., 2009), HF (Yu, Manam, Hepler, and Hennessey, 1992) and HCl (Meinita, Hong, and Jeong, 2012; Pomin, Pereira, et al., 2005) with different concentrations, incubation times and temperatures. TFA was selected to hydrolyze the fucoidan in this study. Compared with HCl and sulfuric acid, TFA is less oxidative, therefore using TFA to hydrolyze can minimize the damage to sugar cyclic structure or the carbonization reaction (Ruiter, Schols, Voragen, and Rombouts, 1992; Li, Zong, Chen, Zeng, and Zhang, 2013). The strong electronegativity of $-CF_3$ groups can attract more proton to catalyze the hydrolysis. As it is volatile, the acid could be simply removed by evaporation without neutralizing the solution with alkali, which makes the procedure more efficient (Li et al., 2013). Fucoidan hydrolyzed with 1, 2 and 4 M TFA at 100 and 121 °C and 1, 2, 3, 6, 9, 12 hr were compared. The total amount of carbohydrates liberated (w/w %) from the dry fucoidan sample under different conditions are listed in Table 6 The total sugar yield (69.7 % ±1.39) from fucoidan was the highest after the hydrolysis under the condition of 2M TFA, incubated in 121°C for 2 hr. The low yield of fucoidan may due to the incompletely hydrolyzed with short time or low acid concentration, or the sugar carbonization with high concentration of TFA (4M) or prolonged incubation time (>3hr). Hence, hydrolysis with 2M TFA at 121 °C for 2 hr was chosen for the rest of the samples.

Table 6 The yield of TFA hydrolysis fucoidan sample under different conditions (w/w %)

2hr	**100 °C**	**121 °C**
2M TFA	53.59 ± 3.21	69.71 ± 1.39

2 hr	**1M TFA**	**2M TFA**	**4M TFA**
121 °C	30.47 ± 0.25	69.70 ± 1.39	36.51 ± 3.23

2M TFA	**1 hr**	**2 hr**	**3 hr**	**6 hr**	**9 hr**
121 °C	45.67 ± 1.89	69.70 ± 1.39	62.22 ± 2.76	34.87 ± 1.09	32.12 ± 0.71

It has been observed that, brown precipitates were formed if the hydrolysis tubes were not sealed well (Figure 6). The hydrolyzed simple sugars were oxidized under high temperature with the presence of oxygen. This thermal degradation is commonly named as caramelization (BeMiller and Whistler, 1996). Woo, Kim, Hwang, Lee, and Jeong (2015) reported a 5 % of decrease of glucose contents after heating at 120 °C for 2 hr. This could be an indication to see if the hydrolysis is successful or not.

Together, the optimized hydrolysis condition is shown in the methods and materials section (chapter 4).

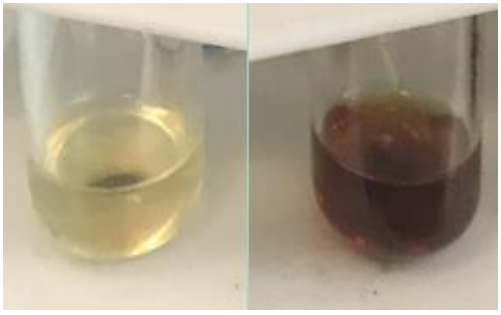


Figure 6 Fucoidan solution after 2 hr TFA hydrolysis a) well sealed b) not well sealed

3.3 Optimization of monosaccharides derivatization

Numerous methods have been used for pre-column derivatization of monosaccharides. For example, reacting with reductive amination reagents (eg: 4-aminobenzonitrile (ABN) (Schwaiger, Oefnel, Huber, Grill, and Bonn, 1994), ρ-Aminobenzoic acid ethyl ester (ABEE) (Gomis, Tamayo, and Alonso, 2001; Shen and Perreault, 1998; Yasuno, Murata, Kokubo, Yamaguchi, and Kamel, 2014) and 2-aminobenzamide (2AB) (Campbell, Royle, Radcliffe, Dwek, and Rudd, 2008), amine coupling with glycamine (eg: phenylisothiocyanate (PITC) (Spiro and Spiro, 1992)), forming hydrazine with dansylhydrazine (DNS-hydrazine) (Avigad, 1977) and fluorenylmethoxyl carbonylhydrazone (FMOC-hydrazine) (Zhang, Cao, and Hearn, 1991) and condensing with the active methylene group, including PMP (Lv et al., 2009; Sun et al., 2010) and 1-(4-methoxy)phenyl-3-methyl-5-pyrazoline (PMPMP) (Zhang, Wang, Xie, Nie, and Huang, 2010). After a careful consideration of the above mentioned methods, and based on the available facility, cost and safety, the monosaccharides were derivatized with three different labeling reagents: ABEE, procaine and PMP. Although all three labeling reagents were effective for derivatizing the monosaccharides, PMP was most favorable among these three reagents, since PMP was the only one found to derivatize glucuronic acid. Glucuronic acid is one of the constituent parts of fucoidan extracted from *C. filu* (Chizhov et al., 1999), *C. okamuranus* (Cumashi et al., 2007), *A. nodosum* (Chevolot et al., 1999) and *S. stenophyllum* (Adhikari et al., 2006). Glucuronic acid is also an uronic acid of interest in this study. In most of the previous studies, the 0.5 M methanolic solution of PMP was used to derivatize the monosaccharides (Gucek and Pihlar, 2000; Lattová, Snovida, Perreault, and Krokhin, 2005; Zhang et al., 2010). In this study, the effectiveness of PMP with dimethyl sulfoxide DMSO and ethanol was compared (saturated solutions). It was found that PMP with DMSO presented a greater peak in the chromatogram of the precursor scan for the monosaccharide standards. From a safety standpoint, DMSO is less flammable and less harmful to human body. By comparison, DMSO was used to dissolve the PMP reagent in this study.

In addition to derivatization, hydrochloric acid was used to neutralize the solution after the incubation, formic acid was selected as an alternative in this study. It was considered that formic acid was happened to be the acid used to adjust the pH of the mobile phase in LC-MS system, it was a better choice without changing the effectiveness of

neutralization.

The removal of the excess reagent was an important step to establish an efficient procedure. Unlike in most other methods for PMP derivatization using chloroform, ethyl acetate was used in this study. By contrast, ethyl acetate could easily remove the excess PMP from the reaction solution and minimize the loss of the derivatives due to its slightly higher hydrophobicity compared with chloroform (Honda, Suzuki, and Taga, 2003).

Overall, the above conditions were optimized for the PMP derivatization.

Chapter 4 Methods and materials

4.1 Materials

Fucoidan was provided by Pharmalink Extraction Ltd, New Zealand, and extracted from New Zealand *U. pinnatifida* in Auckland University of Technology. Fresh seaweed was extracted in MilliQ water (Select edi 60, Purite Ltd, Bandet Way, UK) for 4 hours at 80 °C with 1:1 ratio of seaweed to water and stirring occasionally. After the extraction, the seaweed residue was filtered with a sheet of muslin. Calcium chloride (ACS reagent, ≥ 96 %, Sigma-Aldrich®) (2 % (w/v) solution) was added to precipitate the alginic acid. It was removed from the residue-free extract by filtrating with the muslin. 99.6 % ethanol (HPLC grade, Sigma-Aldrich®) was added to the supernatant to form a 70 % ethanol solution. Fucoidan precipitation was developed after stored the mixture overnight at 4 °C in the fridge. By centrifuging the mixture at 18 °C for 20 mins at 400 rpm (Eppendorf Centrifuge 5810R V3.1), fucoidan was collected and dried for 24hours in a freeze-dryer (Alpha 1-2LD plus Entry Freeze Dryer, Martin Christ). The dried fucoidan was ground and filtered with a sieve of mesh aperture (size 106 μm, certified reference material, 140 mesh, Sigma-Aldrich®). Fucoidan under 106 μm was collected and sealed in the foil ZipLock bag (Size: 4" x 6", 900 Series StandPAK®, Thomas Scientific, U.S.A.) and stored in a desiccator (Corning® PYREX® 5.8 L Small Knob Top Desiccator) in the fridge (- 4 °C) for further studies.

4.2 Static *in vitro* digestion of fucoidan

The static *in vitro* digestion modal was modified according to Minekus et al. (2014) and Moon et al. (2015). Three simulated consecutive stages include: 5 min for oral phase, 120 min for gastric phase and 120 min for small intestinal phases. The simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were simplified by using MilliQ water. The condition was optimized in the preliminary studies.

Enzyme solutions needed to be prepared freshly every time. Enzymes were dissolved in the MilliQ water and pre-warmed in a 37 °C oven (SANYO Electric Biomedical Co. Ltd, Japan) for half an hour before the digestion process. To meet the final enzymatic activities in digest solutions (75 U. mL^{-1}), 0.5 g α-amylase (from *porcine pancreas* α-amylase, (≥ 150 units.mg^{-1} protein, A9857, Sigma-Aldrich®, contained 10.58 % of potato dextrin) was weighed accurately and dissolved in 50 mL MilliQ water to achieve 1500 U. mL^{-1}. 1.07 g pancreatin (from *porcine pancreas*, 3X, U.S.P., MP Biomedicals, LLC, contained 58.72 % of potato dextrin) was dissolved in 50 mL of MilliQ water to acquire 1600 U. mL^{-1} solution based on pancreatic amylase's activity, since only carbohydrates but no protein or fats was involved in the enzymatic digestion.

1M and 6 M of HCl (prepared from ACS reagent, 37 %, Sigma-Aldrich®) and NaOH (prepared from ACS reagent, ≥ 97.0 %, pellets, Sigma-Aldrich®) were used to adjust the pH of different phases (7, 2 and 7 for three consecutive phases). pH and sample temperature were monitored by a pH meter (HI 4221, HANA Instrument) through the whole digestion process. A shaking water bath (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific Co., INC, USA) was pre-warmed to 37 °C and shaking at 5 rpm to simulate the mechanical movement happened in the body.

250 mg of fucoidan was set as the sample size, since 250 mg is one dose for commercial fucoidan products (purity 35 ~ 95 %). Five individual digestion tests were performed to monitor the consecutive digestion process with different time points (Table 7 and Figure 7): 5 min oral phase (O), 1 hr gastric phase (G1), 2 hr gastric phase (G2), 1 hr intestinal phase (I1) and 2 hr intestinal phase (I2). Each phase was duplicated along with a blank (250 μL MilliQ water instead of 250 mg fucoidan) to control any contamination from the condition and compared with the samples before digestion (CF). After reached the

incubation time, the fluid was divided to 50 mL falcon tubes (Corning ®, polypropylene, conical bottom w/ CentriStar cap, Sigma-Aldrich®) and snap-frozen in liquid nitrogen to inactivate enzymes activities and stored in a freezer for further analysis.

Table 7 The procedure of digestion model

Sample	Oral phase			Gastric phase		Intestinal phase		
	Enzyme	pH	Time	pH	Time	Enzyme	pH	Time
CF	-	-	-	-	-	-	-	-
O	Amylase 75 $U.mL^{-1}$	7	5 min	-	-	-	-	-
G1				2	1 hr	-	-	-
G2					2 hr	-	-	-
I1						Pancreatin 200 $U.mL^{-1}$	7	1 hr
I2								2 hr

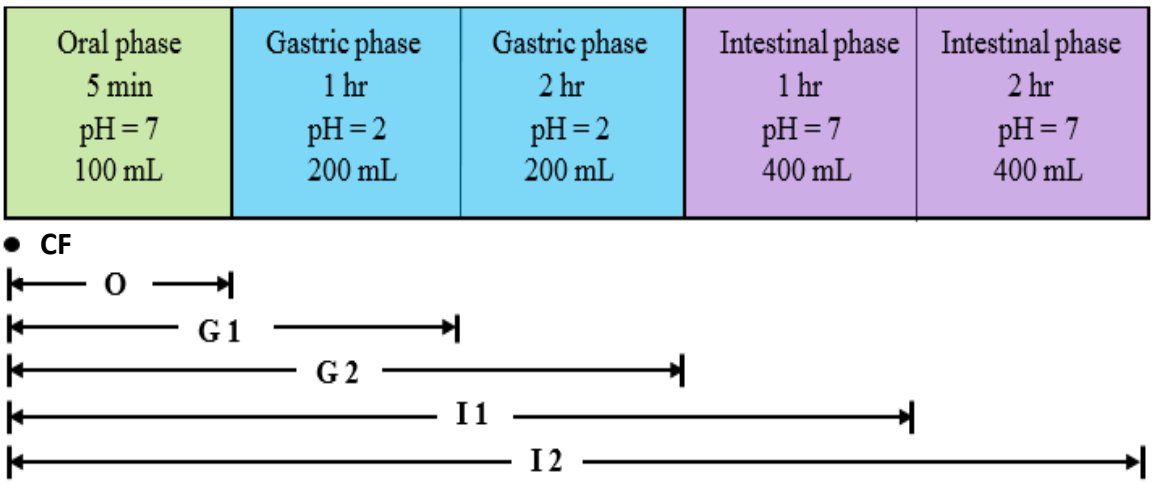


Figure 7 The procedure of digestion model

4.2.1 Oral phase

250 mg (one dose) of crude fucoidan was weighed into a 400 mL Schott bottle and mixed with 95 mL MilliQ water. The mixture was shaken thoroughly with a vortex machine (Vortex-Genie 2, Scientific Industries, Inc.) for 1 min. Considered to individuals eating habits that we do not always melt the pills in the mouth, there was no need to dissolve the fucoidan completely, as no caked particles would do. The pH of the suspension was adjusted to 7.0 with NaOH according to the pH in human oral cavity (Minekus et al., 2014). After the mixture was heated to 37 °C in the water bath, 5 mL of α-amylase stock solution was added to the bottle to achieve 75 U. mL^{-1} total enzyme activity of the mixture and 100 mL of total volume. The bottle was quickly capped tightly and incubated in the shaking water bath for 5 min at 37 °C. Sample O was collected into two 50 mL falcon tubes. It was snap-frozen in the liquid nitrogen as soon as possible to inactivate the amylase activity and stored in the freezer (-20 °C) for the separation step. Sample G1, G2, I1 and I2 were treated with the following gastric digestion.

4.2.2 Gastric phase

1 mL of 1 M HCl was added immediately to the Schott bottle to inactivate the amylase after 5-min-oral-digestion, 99 mL of MilliQ water was added to filled the final SGF up to 200 mL to achieve the final ratio of 100 mL oral and gastric digestion fluid = 50: 50 (v/v). After the pH was adjusted to 2.0 to acquire the acid digest condition in gastric phase, the fluid was mixed well and put back into the shaking water bath for a further incubation at 37 °C for 2 hr. G1 and G2 were taken out from the water bath, divided and stored in four 50 mL falcon tubes after the first one hour and at the end of the gastric digestion, respectively. Samples were frozen immediately with liquid nitrogen and stored in the freezer (-20 °C) for the separation step. I1 and I2 continued digesting for the next step of intestinal digest.

4.2.3 Intestinal phase

1 mL 1 M NaOH was added to the digest solution, followed with 149 mL MilliQ water to reach the final ratio of gastric and intestinal digestion fluid = 50: 50 (v/v). After pH was regulated to 7.0 to achieve the human pancreatic condition, 50 mL of pancreatin stock solution was added to the Schott bottle to acquire 100 U. mL^{-1} of pancreatic amylase in

final mixture. The mixture was incubated in the shaking water bath at 37 °C for 2 hr. The Schott bottles of I1 and I2 were taken out from the water bath after the first one hour and at the end of the intestinal digestion, respectively. 400 mL of simulated digestion fluid were transferred to eight 50 mL falcon tubes and snap-frozen using liquid nitrogen to inactivate pancreatic. The samples were kept in the freezer (-20 °C) for further treatments.

4.3 Separation of different molecular weight cut-offs

50 mL falcon tubes were labeled (Table 8) and weighed before storing separated solutions. To avoid repetitive freeze and thaw cycles, appropriate-volume samples (CF, O, G1, G2, I1 and I2) were taken out and defrosted at room temperature before the following treatment.

Table 8 Sample labels of 24 digested samples

	> 100 kDa	10- 100 kDa	3- 10 kDa	< 3 kDa
Before digestion	>100 CF	10- 100 CF	3- 10 CF	< 3 CF
Oral phase 5 min	>100 O	10- 100 O	3- 10 O	< 3 O
Gastric phase 1hr	>100 G1	10- 100 G1	3- 10 G1	< 3 G1
Gastric phase 2hr	>100 G2	10- 100 G2	3- 10 G2	< 3 G2
Intestinal phase 1hr	>100 I1	10- 100 I1	3- 10 I1	< 3 I1
Intestinal phase 2hr	>100 I2	10- 100 I2	3- 10 I2	< 3 I2

Amicon Ultra-15 centrifugal Filter Devices was used to separate the digested samples. The separation device was constituted with three parts (Figure 8): cap, filter device and centrifuge tubes. The solution was poured into the filter device, after centrifuging, small molecules were “slipped away” through the filter to the centrifuge tube, while big molecules were trapped and remained in the filter. Three different molecular weight cut-offs filter specification were used: 3 kDa, 10 kDa and 100 kDa which separated over/under 3 kDa particles, over/under 10 kDa particles and over/under 100 kDa particles. In other words, every sample were divided in to four portions: < 3 kDa, 3- 10 kDa, 10- 100 kDa and > 100 kDa.

The procedure of separation in shown in Figure 9. Digested sample (labeled with yellow in Figure 9) was first pipetted into the 15 mL 100 kDa centrifugal filter device. The cap was screwed tightly and the filter device was centrifuged at 4000 rpm in a swinging bucket rotor (Centrifuge 1680R, Gyrozen) for 20 min. Particles with smaller molecular weight (under 100 kDa (labeled with pink in Figure 9) were “washed out” into the centrifuge tube along with the fluid, while particles over 100 kDa molecular weight were trapped in the filter device. The residue (pink) was then poured into the 10kDa filter.

Particles with 10- 100 kDa molecular size were remained in the filter device after 20 min of centrifuge. Molecules under 10 kDa (label with green in Figure 9) was collected and transferred to the 3 kDa filter. Over/under 3 kDa particles were separated by the 3 kDa centrifugal filter with centrifuge for 20 min. The upon steps were repeated for another 20-min centrifuging once there was liquid remained in the membrane trap. The solution with under 3 kDa fraction portions (labeled with blue in Figure 9) was collected from the centrifuge tube and transferred to the weighed 50 mL falcon tubes. After all the solutions were separated or liquid stopped been filtered out from the filter device (the filter was clogged up with big molecules), the blocked particles were dissolved by adding 3 ml of MilliQ water. The 3-mL solution was then transferred to a 50-mL falcon tube carefully with a 200 μL pipette. The membrane trap was washed for another 2 times to make sure all the concentrated solution was shifted. The upon steps were repeated until all digest fluid was separated.

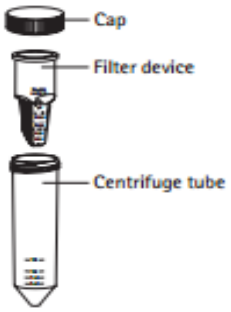


Figure 8 The structure of Amicon Ultra-15 centrifugal Filter Devices

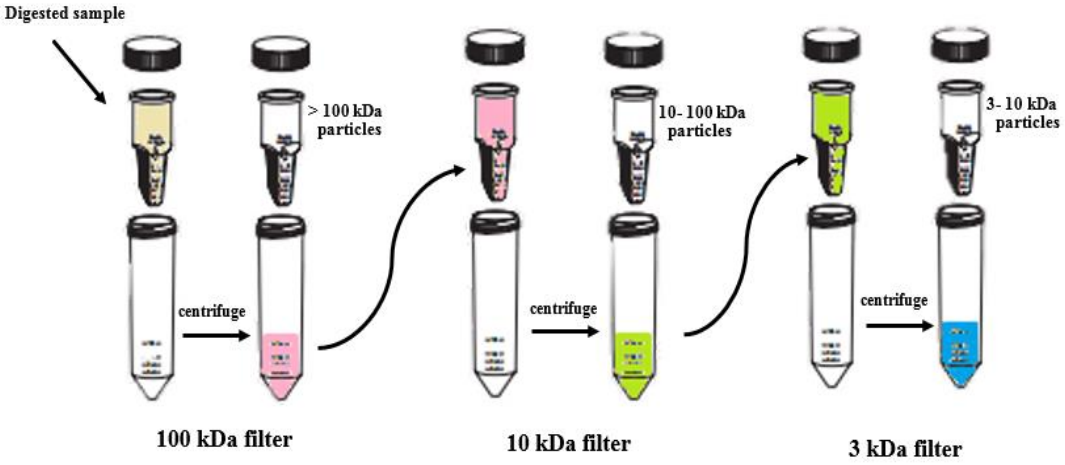


Figure 9 Different molecular weight cut-offs separation procedure (different color was used to distinguish different solution, not the color of the solution)

The separated samples were then frozen in liquid nitrogen. The caps of the falcon tubes were loosened and the samples were dried in a freeze dryer (Alpha 1-2LDplus Entry Freeze Dryer, Martin Christ) for 3 days at -80 °C, 0 atm. The tubes were weighted after drying. The proportion of different molecular weight cutoff was calculated by subtracting the empty tube weight from the final dried tube weight. Samples were stored in a -20 °C freezer for further analysis.

4.4 Monosaccharides composition analysis with LC-MS

Crude fucoidan and 24 dried samples were hydrolyzed with (TFA (ReagentPlus®, 99%, Sigma-Aldrich®) to figure out the monosaccharides composition change during the digestion process. Samples were derivatized with PMP and quantitively analyzed by LC-MS.

4.4.1 Fucoidan hydrolysis with trifluoracetic acid (TFA)

The hydrolysis process was modified from Dai and the others (2010), Melton and Smith (2005), and the optimization of the hydrolysis is shown in the preliminary studies.

5 mg crude fucoidan/dried sample was completely dissolved in 500 μL MilliQ water in an Eppendorf tube (1.5 mL natural sterile, polyprocpylene conical type LabServ®, Thermofisher Scientific New Zealand Ltd). 20 μL of 2 g. L^{-1} D-ribose (Solgar Vitamin and Herb, Leonia, N.J. 07605 U.S.A) was used as internal standard and added into the dissolved solution with 500 μL of 4 M TFA to achieve a 2 M TFA in the final hydrolysis mixture. Fucoidan was hydrolyzed under 121 °C for 2 hr in a nitrogen atmosphere. The concentration of TFA (1 M, 2 M and 4 M), incubation time (1hr, 2hr, 6hr, 9hr and 12hr) and temperature (70, 100 and 121 °C) was optimized. After 2 hr hydrolysis, the solution was dried with a consecutive airflow for overnight (Figure 10). The hydrolysate was then dissolved in 1 mL 20 % MeCN (for HPLC, gradient grade, ≥ 99.9 %, Sigma-Aldrich®) for further treatment.

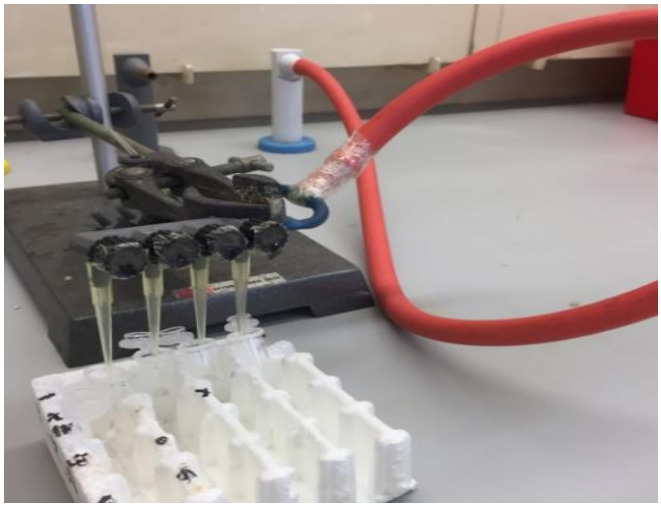


Figure 10 The device of air-dry hydrolysate

4.4.2 Preparation of monosaccharides standard curve

The method of making monosaccharide standard curve was modified from (Zhang, Zhang, Wang, Shi, and Zhang (2009). Ribose, D-(+)-xylose (≥ 99 %, Sigma-Aldrich®) , L-(-)-fucose (≥ 99 %, Sigma-Aldrich®), L-rhamnose (≥ 99 %, Sigma-Aldrich®), D-(+)-mannose (BioUltra, ≥ 99.5 % (sum of enantiomers, HPLC), Sigma-Aldrich®), α-D-(+)-glucose (European Pharmacopoeia (EP) Reference Standard, Sigma-Aldrich®), D-(+)-galactose (≥ 99 %, Sigma-Aldrich®) and glucuronic acid (≥ 98 % (GC), Sigma-Aldrich®) were dissolved in 20 % MeCN in MilliQ water to make 10 $g.L^{-1}$ stock solutions for each monosaccharide in the Eppendorf tubes. Standard "A" was made by mixing 100 μL of arabinose, xylose, fucose, rhamnose, mannose, glucose, galactose and glucuronic acid stock solution and 200 μL of 20 % (w/w) MeCN to achieve 1000 mg. L^{-1} for all eight sugars. 500 μL of standard "A" was mixed with 500 μL of 20 % MeCN to make "B" (500 mg L^{-1}). in the same way, standard "C", "D"," E"," F" (250, 125, 62.5, 31.25 mg. L^{-1}) was prepared by serial dilution. 20 μL 2 $g.L^{-1}$ ribose was mixed to all six standard solutions as internal standard. The serial standard solutions, hydrolyzed samples and blank were derivatized by the following procedure.

4.4.3 Sugar derivatization with PMP

The process of sugar derivatization was adapted from Dai et al. (2010) and Lv et al. (2009) with optimized condition shown in the preliminary studies section.

4 g PMP (purum, ≥ 98.0 % (NT), Sigma-Aldrich®) was dissolved in 20 mL of dimethyl sulfoxide (DMSO) (for HPLC, ≥ 99.7 %, Sigma-Aldrich®). This was the first study to use a 20 $g.L^{-1}$ PMP solution. The high concentrated PMP solution ensured the derivatization process to be completely reacted. 100 µL of PMP reagent was used to derivatize 20 µL of the hydrolyzed samples, standards or crude fucoidan. The solution was alkalized by adding 13 µL of 10 M NaOH. The reaction was taken in a 70 °C oven for 100 min. After cooled down to room temperature, 100 µL of MilliQ water and 5 µL of formic acid (puriss., meets analytical specifications of DAC, FCC, 98.0-100 %, Sigma-Aldrich®) were added to neutralize the solution. The excess PMP reagent was extracted with 500 µL ethyl acetate (for HPLC, ≥ 99.8 %, Sigma-Aldrich®) for 3 times. The final derivatized monosaccharides samples were diluted for 50 folds in a 2-mL screw-top vial (amber, Aglient, Thermofisher Scientific New Zealand Ltd) for LC-MS analysis.

4.4.4 LC-MS condition

Separation and quantification of monosaccharides were carried out on a Kintex column (1.7 μm EVO C18 100 Å) equipped on the Agilent 6420 Triple Quad LC/MS. The LC conditions were as follows: the flow rate was kept at a constant speed of 0.3 $mL.min^{-1}$. The solvent A was composed of 0.6 % formic acid in MilliQ water and Solvent B was composed of 0.1 % formic acid in Acetonitrile. After injected 1 μL of sample, 77 % of solvent A and 23 % of solvent B was gradually changed into 74 % of solvent A and 26 % of solvent B in the first 10 min. 10 % of solvent A and 90 % of solvent B was applied from 10 min to 11 min. The rate changed back to 77 % of solvent A and 23 % in the next 1.2 min. The MS condition was set as: the electron ion spray was used as the ionization source, the parameters used for MS was from 60 to 430. The scan time was 4.16 milliseconds, fragmentation voltage of 180 V, cell acceleration voltage was 7 V. Positive mode was applied to the derivatized sample. Source parameters consisted of a gas temperature maintained at 300 °C with a flow of 6 $L.min^{-1}$, the constant pressure of the nebulizer was 60 psi, concluding with the capillary voltage at 2200 V.

The retention time of each monosaccharide was identified by comparing with individual monosaccharide. The data and chromatogram were shown and analyzed by MassHunter Qualitative Analysis software (B.06.00) qualitatively and by MassHunter QQQ Quantitative Acquisition software (B.07.00) quantitatively. The concentrations of monosaccharides were corrected by the relative correction factor according to the value of internal standard and subtracted the blank reading. The results are expressed as mg/250 mg of undigested fucoidan or in %.

4.5 Sulfate analysis

0.2mg solid sample was weighed accurately and placed on the Nicolet 6700 spectrophotometer (Thermo Scientific, USA). The FT-IR condition was set as follow: spectral region 4000–400 cm^{-1}, resolution 2cm^{-1}. Omnic 6.0 software was used for data analysis.

4.6 Data analysis

Data were analyzed statistically by Rstudio (version 1.0.153, RStudio, Inc.) with different monosaccharides and molecular weight cut-offs as the main effects. Analysis of variance (ANOVA) was carried out by using Statistica (TIBCO® Statistica™) to test for weight differences and sugar content differences between different time points and control (CF). Least squares means (LSM) were used for comparing multiple group means. Significant differences were found when $p < 0.05$. A further Tukey test was employed to investigate where the difference occurred. Data are presented as the mean ± SD in the tables.

Chapter 5 Results and discussion

5.1 Validation of the monosaccharides analysis method (LC-MS)

The mass spectrum identification studies on individual monosaccharide sample was carried out with precursor ion scan. Table 9 shows retention time and characteristic fragment ions for each PMP-derivatized monosaccharide. The common product ion of [PMP- H+] was detected at 175.2 m/z, so it was used as the target fragment for sugar derivatives. The characteristic fragment ions [M- H+] of pentose, deoxyhexose, aldohexose and uronic acid were detected at 481.2, 495.1, 511.3 and 525.3 m/z, respectively. These characteristic fragment ions were set for further product scan. Collison energy was also optimized, all pentose, aldohexose and uronic acid showed biggest peak at 25 au collision energy while deohexose presented best at 21 au collision energy for ESI condition for further studies.

The standard curve of monosaccharides is shown in Figure 11. The concentration range of the individual sugar was from 0 to 1 g. L^{-1}, the peak area of PMP- arabinose, xylose, rhamnose, fucose, mannose and glucuronic acid have shown good linear relationships with their concentrations ($r^2 > 0.99$), while the r^2 of PMP-glucose and PMP-galactose were over 0.98. As shown in Figure 12, PMP-glucose and PMP-galactose were co-eluted at 9.0 to 9.5 min, the two peaks could not be separated distinctly, which gave difficulties to integrate the exact peak area for both sugar. It was because glucose and galactose are very similar in structure (Figure 13). Duarte, Cardoso, Noseda, and Cerezo (2001) reported that glucose and galactose was presented in α and β form respectively. However, same sugar with different configurations were shown in the same peak in the chromatogram. The only difference between D-glucose and D-galactose is the orientation of the hydroxyl group (OH) at C4 which caused the co-elution. The pH of mobile phase, gradient elution and column back pressure conditions were all adjusted up and down in the limited condition of the instrument, PMP- glucose and PMP-galactose could not be separated better.

The repeated standard deviations (RSD) (n=5) of the individual PMP derivatized sugar standard are shown in Table 10. The threshold of < 2 % was required for the RSD value. In other words, the reproducibility was good for all PMP-sugars except for

PMP-glucose. The co-elution peaks of PMP- glucose and galactose could be responsible for the slightly higher RSD value than the standard.

Table 9 Sugar sample with their molar mass, retention time, fragment piece and collision energy optimized by precursor ion scan and set for further quantitative analysis

Type	Sugar	Molar mass (Da)	Retention (min)	Precursor ion	Product Ion	Collision Energy (au)
Pentose	Ribose	150.13	8.218	481.2	175.2	25
	Arabinose	150.13	10.493	481.2	175.2	25
	Xylose	150.13	11.404	481.2	175.2	25
Deoxyhexose	Rhamnose	164.16	9.017	495.1	175.2	21
	Fucose	164.16	12.461	495.1	175.2	21
Aldohexose	Mannose	180.16	6.722	511.3	175.2	25
	Galactose	180.16	9.574	511.3	175.2	25
	Glucose	180.16	9.932	511.3	175.2	25
Uronic acid	Glucuronic acid	194.14	9.733	525.2	175.2	25

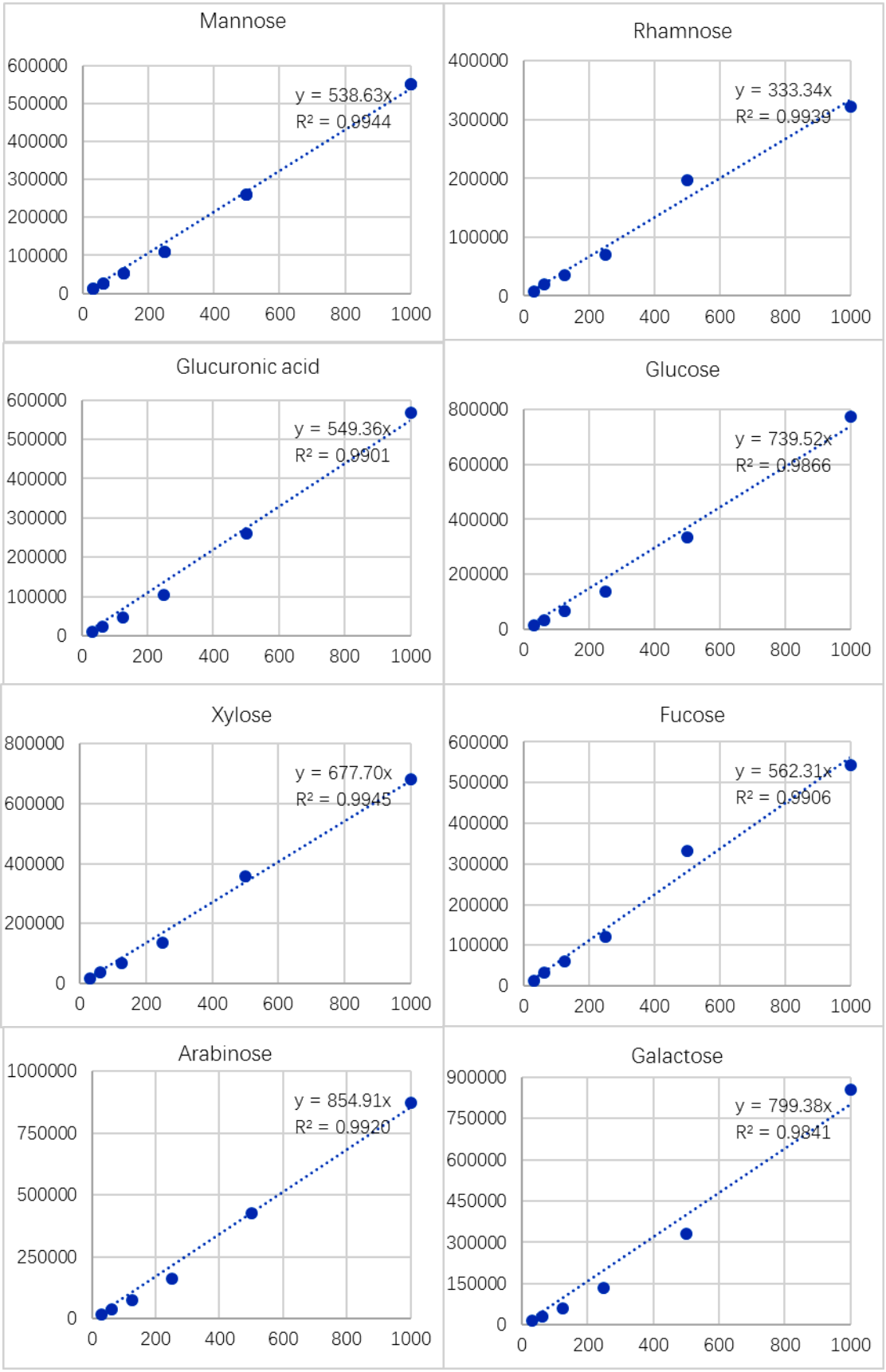


Figure 11 The standard curve of eight monosaccharides (ribose, as the internal standard, is not shown)

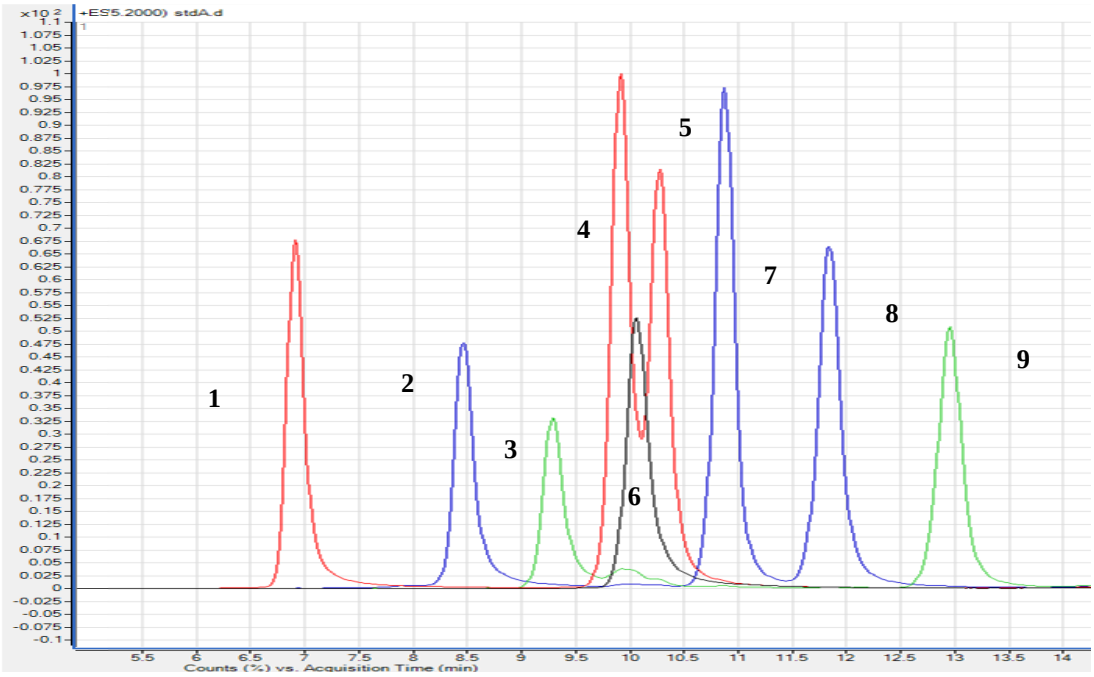


Figure 12 LC-MS separated PMP-monosaccharides standard

Note: numbers represent different monosaccharides: 1. mannose, 2. ribose (internal standard), 3. rhamnose, 4. galactose, 5. glucose, 6. glucuronic acid, 7. arabinose, 8. xylose 9. fucose. Different color is showing different characteristic fragment ions at: Blue. 481.2 m/z, Green. 495.1 m/z, Red. 511.3 m/z, Black. 525.2 m/z

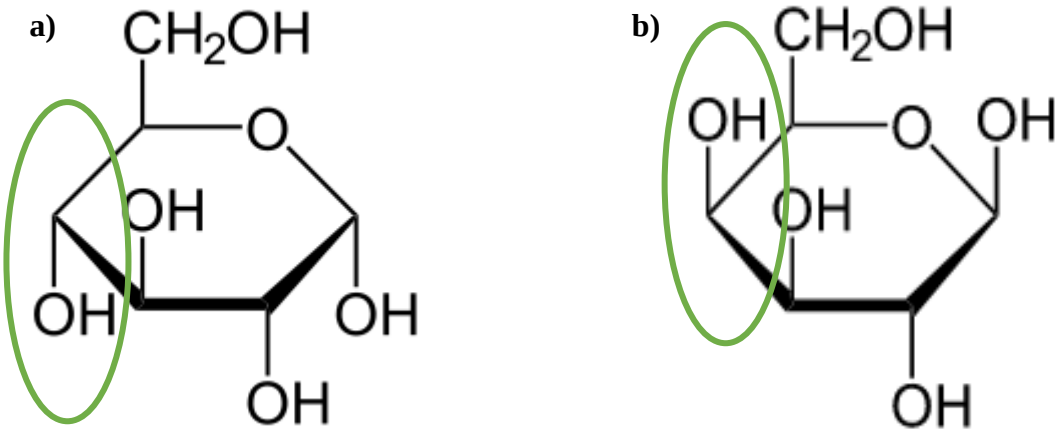


Figure 13 Structure of a) α-D-glucose and b) β-D-galactose

Table 10 Reproducibility of PMP-derived monosaccharides (Relative standard deviations) (n = 5)

PMP-sugar	RSD (%)
Mannose	1.56
Ribose	1.11
Rhamnose	1.85
Galactose	1.67
Glucuronic acid	1.34
Glucose	2.01
Arabinose	1.50
Xylose	1.45
Fucose	1.78

The limitation of the column lead to the bad separation of PMP-glucose and PMP-galactose, while the high reproducibility of the sugars represents no errors were made manually or instrumentally. A slightly overlap peak of PMP derivatized xylose and arabinose was observed by Dai et al. (2010) with a ZORBAX Eclipse XDB-C18 column (Agilent, USA). Blanco et al. (2001) reported the ABEE-Glucose and galactose, arabinose and xylose derivatives were co-eluting by using a Kromasil C8 column (200 mm × 2.1mm i.d., 3.5 μm), while C18 and C16 columns were not able to separate the aldoses and uronic acids derivatives completely. The PMP derivatized glucose and galactose was showing a better separation with YMC-Pack ODS-AQ column (250 × 4.6 mm, 5 μm) (Zhang et al., 2009) and CHO C-18 column, 220 × 2.1 mm (Perkin Elmer, Applied Biosystems Division) (Fu and Neill, 1995). If the funding was allowed, a more suitable column would be used in the study to achieve a better separation of PMP-glucose and PMP- galactose.

5.2 Constitutions of monosaccharides in crude fucoidan

Using the method outlined above, the constitution of each individual monosaccharide to crude fucoidan was determined after acid hydrolysis (Figure 14). From the LC-MS analysis, the monosaccharides composition of the crude fucoidan was shown to be fucose, galactose, glucose, mannose, rhamnose, glucuronic acid and xylose with a molar

ratio of 1, 0.92, 0.06, 0.04, 0.02, 0.01, and 0.01, respectively. Fucose (47.97 % ± 1.27 in mol percentage) and galactose (45.24 % ± 1.42) were present as two major monosaccharides, minor components were mannose (1.88 % ± 0.16), rhamnose (0.68 % ± 0.12), glucose (3.20 % ± 0.19), xylose (0.57 % ± 0.17) and glucuronic acid (0.46 % ± 0.03) which to made up a total carbohydrate of 69.75 % (w/w) to dry-weight crude fucoidan (Figure 15). The proportion of each monosaccharides was obtained by setting the sum of total neutral sugars at 100 %. The recovery rate of the internal standard, ribose, was 75.4%, which was lower than expected, for more than 80%. The low recovery rate of ribose may possibly be due to the poor stability of the TFA hydrolysis. Due to the low ribose recovery, the calibrated monosaccharides mass may be a little higher than the actual value in this section. Based on the assumption that all of the monosaccharides would be equally affected, the ratio or the proportion of each monosaccharides was not changed. Arabinose was not found in the crude fucoidan. Hence, no arabinose is compared in the further experiments.

As a contrast, Yang, Chung, and You (2008) reported a higher proportion of fucose being present in fucoidan extracted from *U. Pinnatifida* harvested from Korea (78.8 %) accompanied with a lower proportion of galactose (21.2 %). No other monosaccharides were found to constitute the fucoidan. Another study focused on Korea *U. Pinnatifida* gave the similar result on fucose content with 72.3 % of total natural sugars (Kim et al., 2007). Other monosaccharides compositions were shown to be fucose: galactose: xylose and mannose with a ratio of 1: 0.2: 0.02: 0.15. A much higher uronic acid (26.2 % w/w) was discovered from the fucoidan isolated from Korea *U. Pinnatifida* (Kim et al., 2007) compared with which detected in this study. Skriptsova, Shevchenko, Zvyagintseva, and Imbs (2010) reported a monthly changeable molar ratio of fucose and galactose, which varied from 1: 0.34 to 1: 0.69. Fucose and mannose varied from 1: 0.6 to 1: 0.07 for *U. Pinnatifida* fucoidan from Japan. New Zealand *U. Pinnatifida* fucoidan measured by Mak, Hamid, Liu, Lu, and White (2013) presented a lower composition in fucose (39.24 %), galactose (26.48 %) and glucose (0.95 %) and significant higher proportion of xylose (28.85 %) and mannose (5.04 %) than my data. It is now commonly accepted that the sugar proportion of fucoidans vary depending on the brown seaweed species, their regions of cultivation and harvesting time (Kim et al., 2007; Mak et al., 2013; Mori, 1982; Sakai et al., 2003).

The total monosaccharides occupied 69.75 % (w/w) of the original fucoidan sample. The value is greater than Vishchuk, Ermakova, and Zvyagintseva (2011) (40 %), Yang et al. (2008) (54.9 %) and Ho et al. (2015) (55.0 %) but lower than the results from Kim et al. (2007) (78.54 %) and (Hemmingson et al., 2006) (85%). It should be noticed that Kim and Yang's fucoidan were extracted from Korean *U. Pinnatifida*, while Ho and Hemmingson's fucoidan samples were extracted from *U. Pinnatifida* harvested from Australia coastline and Vishchuk's sample was from Japan *U. Pinnatifida*. The result indicates that fucoidan from same species could have similar characteristics or more likely to have different features, it may attribute to the harvesting regions. Mak et al., (2013) investigated the chemical compositions in fucoidan isolated for New Zealand *U. Pinnatifida* with different harvesting times and extraction methods. It was reported that fucoidan extracted with CaCl2 revealed largest percentage of fucose (14.96 % ± 0.11 w/w) and lowest uronic acid (2.38 % ± 0.02 w/w) compared to water (12.69 % ± 0.05 w/w and 2.77 % ± 0.04 w/w) and acid (8.57 % ± 0.09 w/w and 4.42 % ± 0.02 w/w) extraction. Moreover, a decrease of uronic acid content from 0.89 % w/w in July to 0.62 % w/w in October was reported (Mak et al., 2013).

Taken collectively, the constitution of monosaccharides in New Zealand *U. Pinnatifida* reported aboved was used as nontreatment control for further studies. Moreover, it's comparable to other studies focused on *U. Pinnatifida* fucoidans and researches base on fucoidans extracted from various brown seaweed sources.

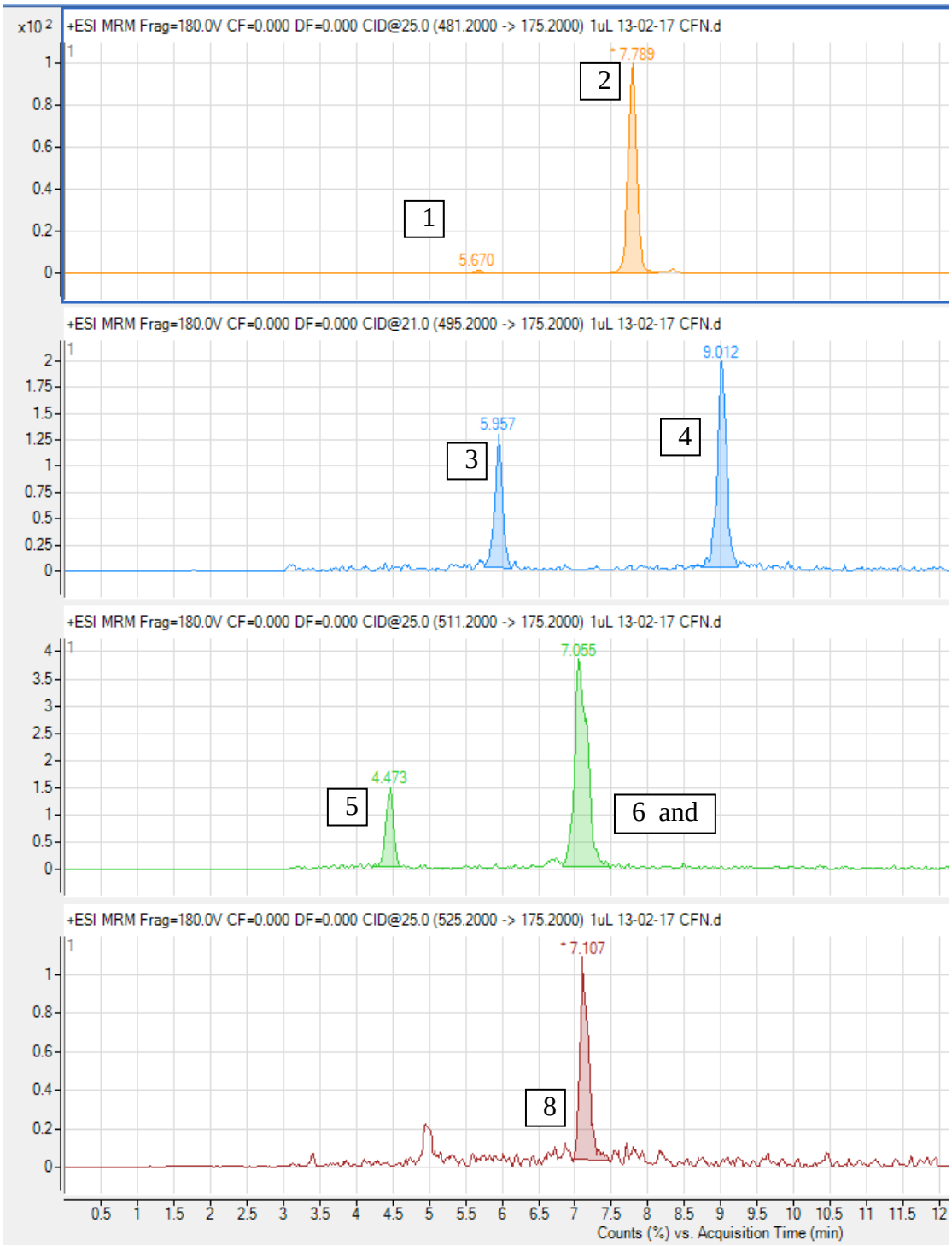


Figure 14 Chromatogram of acid hydrolysates of crude fucoidan,

Note. 1. xylose, 2. ribose (internal standard) 3. rhamnose 4. fucose, 5. mannose, 6. galactose, 7. glucose 8. glucuronic acid. Orange: pentose, Blue: deoxhexose, Green: aldohexose, Red: uronic acid

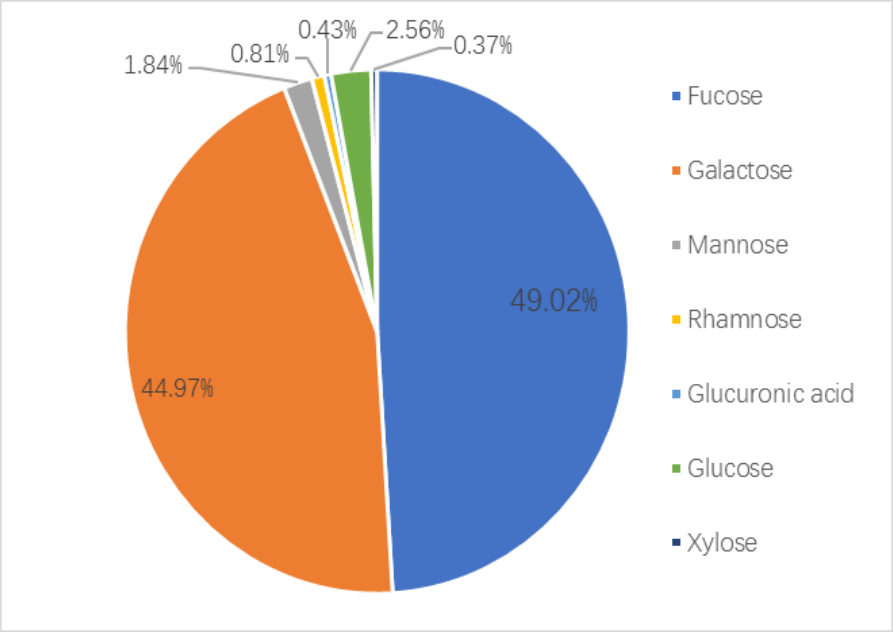


Figure 15 Monosaccharides contribution for acid hydrolyzed crude fucoidan (values were obtained by setting the sum of total carbohydrates at 100 %)

5.3 Fractionation of crude fucoidan

Crude fucoidan was fractionated to four fractions with three different molecular weight cut-offs filters. 3, 10 and 100 kDa were chosen for the analysis for the availability of the manufactural product of the filters. Fucoidan can be subdivided to more fractions if more sizes are available for the filters. The percentage of different fractions were measured as percentage the dry-weight crude fucoidan (Table 11). The most abundant fraction was > 100 kDa, making up to 71.39 % ± 0.21, the second biggest fraction was under 3 kDa with 26.63 % ± 0.19 of dry-weight crude fucoidan. The fractionate yield of this method (n = 5) was 97.96 % ± 5.55 and the repeated determinations is presented with RSD value (5.66 %). The results were unlike previous studies from the same laboratory (Wang, White, Lu, Talor, and White, 2014; White, 2015) with same fractionate method. The > 10 kDa fraction only yield 30-50 %, while < 3 kDa fraction yield about 70 % in fucoidan extracted from New Zealand *U. Pinnatifida* in past September, which confirmed that seaweed harvested in different season has different fucoidan content. Thus more > 100 kDa fractions was found in my study.

The Mw distributions were as expected showing that the larger molecules (> 100 kDa) was made up the majority of the crude fucoidan comparing with other studies applied with different measurements. Hemmingson et al. (2006) reported the average Mw of fucoidan polymers was 710 kDa by using high performance size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALLS). Rupearez, Ahrazem, and Leal (2002) found that the average molecular weight was 1,600 kDa which made 92 % of dry weight fucoidan, while the average molecular weight of minor fraction was 43 kDa, by using the molecular exclusion HPLC. Ho et al. (2015) reported more detailed distributions: > 1600 kDa (10.2 %), 1100-1600 kDa (4.3 %), 200-1100 kDa (25.9 %), 60-200 kDa (25.6 %), 20-60 kDa (16.1 %), 5-20 kDa (5.2 %) and <5 kDa (11.7 %) Methods used for quantifying the molecular weight fractions were not mentioned in their study.

Unlike the fractionation method applied in this study, the mostly used fractionation method of fucoidan study was using the ion exchange column which fractionates fucoidan with different solvent gradients. The fucoidan was fractionated to two fractions with the average molecular weight of 150, 290 kDa, respectively (Hemmingson et al.,

2006). Same fractionation method was used by (Skriptsova et al., 2010) to acquire fractions with 30-80 kDa in molecular weights. However, the Mw of the fractions were normally less than half of the untreated crude fucoidan. Hemmingson et al. (2006) pointed out that the reduction of the average Mw of the fractions was caused by the fucoidan depolymerization during the fractionation with the acid eluent. Though the depolymerization will not change the sugar compositions, the structural profile would change undesirably.

Although the elution can be influenced by the molecular adsorption, formation of aggregate and ion exclusion (Zhang and Row, 2015), HPLC coupling with the size-exclusion column was commonly used for determining molecular weights recently, for its simple procedure and efficiency. As the limitation of the funding, Amicon centrifugal filter is a good alternative way to measure the molecular weight range of the samples. The separation process was easy to operate and had high recovery rate without changing the structures. However, this method was very time consuming limited by the filter volume (15 mL solution took 20 min to separate), and the separation range was largely limited with the size of the filters (only 3, 10 and 100 kDa were provided).

Monosaccharide proportions of each molecular fraction was analysis by LC-MS after acid hydrolysis. The monosaccharides composition of different fucoidan fractions are shown in mol % in Table 11 and the distribution of different monosaccharides is shown in Figure 16. The neutral sugar (%) indicates the mass percentage of total neutral sugar in different dry fucoidan fractions (Table 11).

As shown in Table 11, 96.93 % (mass) of the > 100 kDa fucoidan fraction was constituted with neutral sugar, while in the second abundant fraction, < 3 kDa, only 0.48 % of total mass was sugar. Correspondingly, comparing seven monosaccharides across different molecular fractions, no significant difference was found between >100 kDa fucoidan fraction and crude fucoidan, while the percentage of monosaccharides compositions in 10-100 kDa, 3-10 kDa and < 3 kDa differed significantly across other fractions. Mannose, rhamnose, galactose, glucuronic acid and fucose were mostly presented in > 100 kDa fractions, while glucose and xylose were more abundant in under 100 kDa fractions. Since the two basic monosaccharides, fucose and galactose, were not found in < 3 kDa fraction, the < 3 kDa fraction was considered not “true”

fucoidan. Same conclusion was obtained by White (2015). Since only monosaccharides changes were interested in this study, the remained compositions were not analyzed. According to other studies on fucoidan (Kim et al., 2007; Mak et al., 2013; Ponce et al., 2003), the remained compositions could be the mix of sulfate, acetyl groups, protein and other uronic acids in the big molecular portions (> 100 kDa). In small molecular portions (< 100 kDa) of given fucoidan sample, sugars were most likely to be the depolymerized fucoidan branches, other polysaccharides or free sugars, while non-sugar compounds were considered to be depolymerized sulfate groups, acetyl groups and other contaminates from the seaweed extraction. To ensure the sample integrity, the unseparated crude fucoidan sample was used for the *in vitro* digestion process, the weight of different fractions and monosaccharides content were compared and the above data were used as undigested control.

Table 11 Mass distribution and monosaccharides compositions of four fractions of New Zealand U. Pinnatifida (n = 4)

	Mass distribution (%)[1]	**Neutral sugar (%)[2]**	**Proportion of monosaccharides (mol %)**						
			Fucose	**Galactose**	**Glucose**	**Mannose**	**Rhamnose**	**Xylose**	**Glucuronic acid**
Crude fucoidan[3]	100	69.75 ± 0.41	47.97 ± 1.27^{a}	45.24 ± 1.42^{a}	3.20 ± 0.19^{c}	1.88 ± 0.16^{d}	0.68 ± 0.12^{c}	0.57 ± 0.17^{c}	0.46 ± 0.03^{d}
> 100 kDa	71.39 ± 0.21	96.93 ± 1.67	49.02 ± 0.67^{a}	44.97 ± 0.21^{a}	2.56 ± 0.07^{d}	1.84 ± 0.01^{d}	0.81 ± 0.02^{c}	0.37 ± 0.0^{c}	0.43 ± 0.00^{d}
10-100 kDa	1.19 ± 0.04	11.11 ± 0.10	27.43 ± 0.17^{b}	24.21 ± 0.08^{b}	2.04 ± 0.00^{e}	29.86 ± 0.29^{a}	2.15 ± 0.00^{a}	12.66 ± 0.43^{a}	1.64 ± 0.03^{b}
3-10 kDa	0.79 ± 0.02	35.47 ± 0.05	8.80 ± 0.01^{c}	3.83 ± 0.09^{c}	70.54 ± 0.11^{a}	11.02 ± 0.51^{b}	2.12 ± 0.13^{b}	2.38 ± 0.00^{b}	1.33 ± 0.15^{c}
< 3 kDa[4]	26.63 ± 0.19	0.48 ± 0.00	ND.	ND.	67.10 ±0.30^{b}	7.27 ± 0.29^{c}	11.14 ± 0.01^{a}	12.12 ± 0.35^{a}	2.37± 0.05^{a}

Note:
1) Mass proportion (%): The mass percentage of fractions in dry weight fucoidan
2) Neutral sugar (%): The mass percentage of neutral sugar in corresponding dry fucoidan fraction
3) Crude fucoidan is shown as nontreatment control
4) Fucose and galactose were not detected in under 3 kDa fraction

Results are presented as Mean ± Standard Deviation. Values with different superscripts ($^{a, b, c, d, e}$) in the same column for the same monosaccharides differ significantly across the fractions

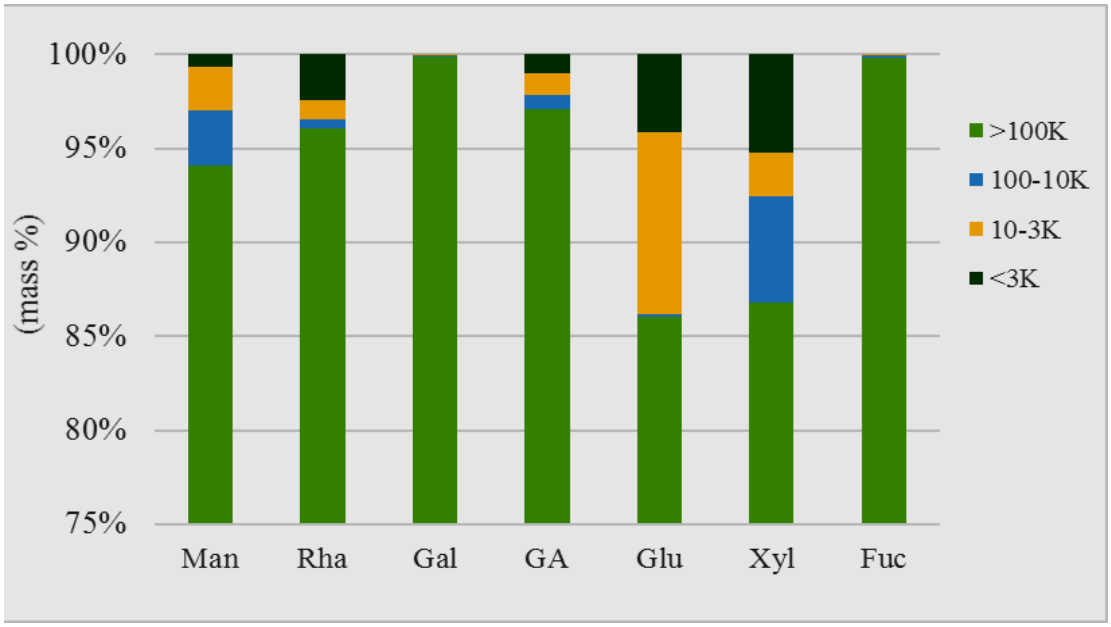


Figure 16 Distribution of monosaccharides in weight % for different molecular weight fractions.

Note: Abbreviation indicate: Man = mannose, Rha = rhamnose, Gal = galactose, GA = guluronic acid, Glu = glucose, Xyl = xylose Fuc = fucose).

5.4 Digestibility of fucoidan —— weight change

The digestibility of fucoidan was determined based on changes in the weight and monosaccharides of different fractions during the simulated *in vitro* gastrointestinal digestion. In this study, different molecular weight fractions were weighed, after the separation by Amicon filters. The weights of different molecular weight fractions before and after each phase of *in vitro* digestion are shown in Table 12. The results are given as mean (mg/ 250 mg dw fucoidan) ± standard deviation.

As is shown in Table 12, the amount of > 100 kDa fractions were showing a continues decrease along with the increase of digestion time from 180.04 mg to 139.77 mg, except in the first hour in intestine phase with an unexpected increase. The significant decrease of weight was first present in oral phase, no statistically significant different was found in oral versus G1 and G1 versus G2. After the short leveling off in gastric phase, the weight of over 100 kDa fraction continued to decrease significantly in the two hours intestine digestion. For 10-100 kDa fraction, the mass was slightly decreased after the oral digestion from 3.01 mg to 1.75 mg, followed by a sharp increase in the first hour gastric digestion. The value reached the bottom after the second hour gastric digestion and climbed gradually in the next two-hour-intestine digestion. Similarly, the weight of 3-10 kDa fraction showed a slight reduction from 2.00 mg to 0.86 mg after oral digestion. The proportion increased significantly during the two-hour-gastric digestion to 6.53 mg. The value dropped significantly to 2.04 mg in the next 2 hours in intestine phase. The second abundant proportion, < 3 kDa, showed a continuous increase through the whole process. Except for G2 and I1 showing significantly difference, no significant differences were found between two continues phases.

Table 12 Weight of different molecular weight fractions at different time cut-offs during the *in vitro* digestion (mg/ 250 mg dw crude fucoidan)

	> 100 kDa	10-100 kDa	3-10 kDa	< 3 kDa	Total mass
CF	180.04 ± 0.50^{a}	3.01 ± 0.10^{bc}	2.00 ± 0.05^{bc}	67.15 ± 0.50^{d}	252.20 ± 0.05^{b}
O	154.13 ± 0.90^{bc}	1.75 ± 0.24^{c}	0.86 ± 0.03^{c}	69.38 ± 3.22^{d}	226.11 ± 3.86^{d}
G1	150.05 ± 0.78^{b}	17.21 ± 2.22^{a}	1.12 ± 0.04^{c}	73.23 ± 0.12^{cd}	241.52 ± 1.60^{bc}
G2	146.73 ± 2.42^{c}	0.48 ± 0.10^{c}	6.53 ± 0.53^{a}	82.90 ± 0.83^{bc}	236.64 ± 2.22^{cd}
I1	161.27 ± 4.32^{b}	9.22 ± 1.11^{abc}	3.81 ± 0.28^{b}	94.57 ± 3.26^{a}	268.87 ± 6.18^{a}
I2	139.77 ± 4.89^{d}	11.21 ± 6.10^{ab}	2.04 ± 1.45^{bc}	90.94 ± 5.42^{ab}	243.95 ± 5.65^{bc}
P value	***	*	**	**	**

Results are presented as Mean ± Standard Deviation (SD).

Note: CF= non-digested crude fucoidan, O= oral, G1= gastric 1hr, G2= gastric 2hr, I1= intestine 1hr, I2= intestine 2hr. Values with different superscripts ($^{a, b, c, d, e}$) in the same column differ significantly across the digestion time cut-offs. $P < 0.05$ presented as $*$ for level of significance. $P < 0.01$ presented as $**$ for level of significance. $P \leq 0.001$ presented as $***$ for level of significance.

Table 12 exhibits the statistically significant difference in the total mass before and after different stages of *in vitro* digestion (O: oral, G1: gastric 1hr, G2= gastric 2hr, I1= intestine 1hr, I2= intestine 2hr). The total mass of I1 (268.87mg) was significantly higher than other phases, while the total mass of Oral phase (226mg) was showing significantly lower than CF, G1, I1 and I2. There was no significant difference among other phases. As same amount, 250 mg crude fucoidan, was used as the starting material of all 5 stages, the final total mass of four fractions was theoretically to be equal or lower than 250mg caused by the unavoidable loss through the experiment which explained the low total mass in Oral phase.

The unexpected increase in I1 phase may have resulted from the presence of potato dextrin and galactose-construct-sugar polymers in the commercial *Aspergillus oryzae* amylase and *porcine pancreas* pancreatin used in this study. The result exhibited that 10.5 % and 58 % of dry weight enzyme products were composed of glucose and galactose. Both have shown a positive result in Benedict's test and iodine test (Bhandary, Kumari,

Bhat, and Prasad Bekal, 2012) which indicates the presence of reducing carbohydrates and polysaccharides. Sigma-Aldrich® technical support team confirmed that about 10 % potato dextrin was used as the diluent in the amylase used for this study which agrees with my results. Unfortunately, *porcine pancreas* pancreatin has been discontinued and no related test or ingredients information were given by the manufacturer (MP Biomedicals). It was undeniable that, sugar polymers existed in the enzymes products. Potato dextrins, as mixtures of polymers of D-glucose linked by α-(1-4) and/or α-(1-6) glycosidic bonds (Haas and Hill, 1913), and other unknown galactose-construct-sugar polymers, were assumed to be involved in the enzymatic hydrolysis. Meanwhile, previous studies indicated that fucoidan extracted from 11 different brown seaweeds (*U. Pinnatifida* haven't been tested) exhibited the inhibition against the α-amylase activity (7-100 %) after incubated at 25 °C for 10 min (Kim, Rioux, and Turgeon, 2014; Shan et al., 2016). There was reason to believe New Zealand *U. Pinnatifida* could inhibit the enzyme activities as well. Although a parallel experiment (no fucoidan was added to the *in vitro* digestion model) was set as control to eliminate the enzymatic hydrolysis of other sugar polymers brought from the enzyme products, the inhibitory effect could cause the sugar polymers in experiment less digested compared with the control. In this case, more big sugar polymers (> 100 kDa) were undigested and remained in big fraction, while most big polymers were partly or completely hydrolyzed to small fractions in control. After subtracted the control, excessive mass was obtained in > 100 kDa fraction in I1, which explained the significant difference in the amount of >100 kDa in I1. Further experiments on compensation analysis of enzyme products needed to be done to confirm the explanation. Carbohydrates-free enzymes were suggested for further studies examining the digestibility of carbohydrate-based food with the *in vitro* model.

Figure 17 exhibits the weight percentage changes (mass % dw fucoidan) and the statistical relation among different *in vitro* digestion phases. The observed weight changes in all five time cut-offs indicates a structural degradation during the digestion process.

In oral phase, salivary amylases were responsible for the hydrolysis of the fucoidan. It was reported that 25 % of pasta starch and 50 % of bread starch was hydrolyzed and transformed into molecules of smaller molecular mass in 20-30 s *in vivo* oral digestion (Hoebler et al., 1998). However, a less percentage (approximately 10 % dw fucoidan)

was hydrolyzed and transformed into small fractions in oral *in vitro digestion* (Figure 17). This result was expected since fucoidan has more complexed structures compared with starch. In previous studies, fucoidan was reported to be dominated with 1-3, 1-4 and/or 1-6 fucan backbones along with various oligosaccharides branches (Cuong, Thuy, Huong, Ly, and Van, 2015; Mak and Hamid, 2012). α-amylases randomly cleave α-1,4 bonds (Bornhorst et al., 2014), thus increase in < 3 kDa fragments can be explained as the result of cleavage of α-1,4 glycosidic bonds.

There is no significantly difference in the first hour between the weight % of >100kDa, 10 – 3 kDa, and <3 kDa fractions of gastric digestion, while the fractions with 100 - 10 kDa had significantly changed. The result showed one significant changes in 100-10 kDa fractions, it can be discovered the gastric juice (HCl and water) may influence gastric digestion without enzyme. Bornhorst et al. (2014) has mentioned HCl will predominantly hydrolyze the α-1,4 bonds compared with α-1,6 bonds, while acid may also be responsible for the cleavage of non-glycosidic bonds. In the second hour gastric digestion, almost none fragments greater than 100 kDa fragment was hydrolyzed. Nevertheless, fragment with 10-100 kDa was further broken down to smaller molecules of 3-10 kDa and < 3 kDa (both showed significant difference). Compared to the oral phase with G2, significant difference was found among in > 100, 3-10 and < 3 kDa fractions which also verified the participation of gastric acid in fucoidan digestion.

In the intestinal phase, intestine α-amylase present in pancreatin further digested on fucoidan. G2 and I2 was showing significant difference in 3-10 kDa and 10-100 kDa fractions. In the other two fractions, the percentage of weight change was showing significantly different with I2, while no significantly different with I2. This result could have largely effected by the unexpected increase of mass in I1 (explained above). Buy the cover of the excessive mass, real changes of fucoidan in intestine digestion could not be seen by weight analysis clearly. Further monosaccharides analysis would be necessary.

From the result, approximate 14 % of > 100 kDa molecules were degraded to smaller fractions. However, it cannot be said that only 14 % of fucoidan was involved the digestion. Due to the limitation of the weight average molecular weight analysis, the change of over 100 kDa could not be analyzed in more details. For example, if the crude fucoidan fraction was 300 kDa to begin with, and after digestion, it was cleaved to one of 100 kDa fraction and one of 200 kDa. With, the largest molecular weight cut-off being

100 kDa, both fractions would be counted as > 100 kDa fraction, which allows no observational change. For further study, a more advanced instrument, high performance size exclusion chromatography, should be used for fractionating and determining the average molecular weight.

100 kDa		O	G1	G2	I1	I2
	CF	√	√	√	√	√
	O		-	-	-	√
	G1			-	√	√
	G2				√	-
	I1					√

10-100 kDa		O	G1	G2	I1	I2
	CF	-	√	-	-	-
	O		√	-	-	√
	G1			√	-	-
	G2				-	√
	I1					

3-10 kDa		O	G1	G2	I1	I2
	CF	-	-	√	-	-
	O		-	√	√	-
	G1			√	√	√
	G2				√	√
	I1					-

<3 kDa		O	G1	G2	I1	I2
	CF	-	-	√	√	√
	O		-	√	√	√
	G1			-	√	√
	G2				√	-
	I1					-

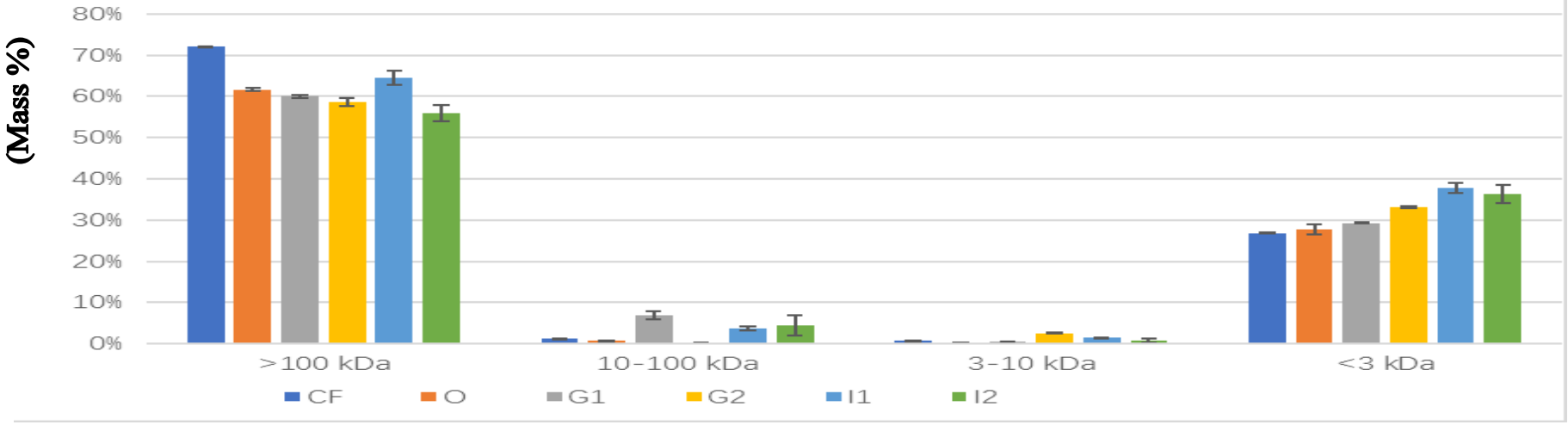


Figure 17 Weight % of four Mw fractions at the end of each *in vitro* digestion stages are compared with undigested crude fucoidan control (mass % of dw fucoidan).

Note: CF= non-digested crude fucoidan O= oral, G1= gastric 1hr, G2= gastric 2hr, I1= intestine 1hr, I2= intestine 2hr. Mean values are plotted with error bars representing standard deviations. √ with green cell presents significant difference among two phases, - with red cell presents no significant difference among two phases.

5.5 Digestibility of fucoidan----monosaccharides analysis

Monosaccharides of each fraction were analyzed by LC-MS after acid hydrolysis to give more details on the composition of monosaccharides that were digested from the crude fucoidan. Table 13 shows the amounts of monosaccharides present in > 100kDa, 10-100 kDa, 3-10 kDa and < 3 kDa fractions, sampled at different *in vitro* digestion periods. The results are given as mean (μg/ 250 mg dw fucoidan) ± standard deviation. Figures 18 and 19 present the monosaccharides distributions in >100 kDa and < 3 kDa fractions across the different *in vitro* digestion sampling time along with the statistical relation among different phase, respectively. The results are presented as mass % in dw crude fucoidan.

5.5.1 > 100 kDa

In Table 13, the total mass of monosaccharides in > 100 kDa fraction dropped significantly with the increasing digestion time, except for I2, which has shown an unexpected increase compared to that of the G1. Galactose was statistically higher than the previous phase. As mentioned in Section 5.3, sugar polymers comprised of glucose and galactose were detected in the pancreatin enzyme used (MP Biomedicals). The excessive galactose in I1 and I2 was brought from the undigested *porcine pancreas* pancreatin sugar polymers which confirmed the previous speculation. The total mass of galactose amount in I1 and G2, had about 10 mg difference, which met the 10-mg difference in the total mass observed in Section 5.3. After the 2-hour intestinal digestion, the galactose was decreased to the same level compared with G2 (no significant difference) which indicated that the pancreatic amylase was partly inhibited by the fucoidan in this study.

It was noticed that, there was a statistically significant difference in all monosaccharides in > 100 kDa fraction during the *in vitro* digestion except for glucuronic acid, which has shown no significant difference before and after digestion. The glucuronic acid residue was proved to be linked to the C2-positions of the fucose residues in fucoidan isolated from the brown seaweed *C. okamuranus* (Nagaoka et al., 1999) and observed to be linked to C-3 and/or C-4 of fucose in fucoidan extracted from *S. stenophyllum* (Duarte et al., 2001). Since only (1→4)- α- D-glucuronic acid can be targeted by α-amylase, which may explain the no significant difference in glucuronic acid content in > 100 kDa fraction statistically. However, it was showing a continuously decrease along with the digestion time in over 100 kDa, and increase in small fractions in value. Hidari et al., (2008) noted that, the glucuronic acid of fucoidan accounts for the inhibition of dengue virus type 2 (DEN2) infection, while a carboxy-reduced fucoidan derivative in which glucuronic acid substituted with glucose did not inhibit the viral infection. From anti-virus perspective, it was desirable to see glucuronic acid not separated from the fucoidan polymers.

Comparing the monosaccharide contents across the digestion time cut-offs in > 100 kDa fraction (Table 13), mannose, glucose, galactose, xylose and glucose and fucose decreased significantly after oral digestion, except for glucuronic acid. Rhamnose and fucose decreased significantly in the first hour of gastric digestion, while mannose,

rhamnose, galactose and glucose were cleaved markedly in the second hour gastric digestion, followed with significantly decrease in mannose, glucose contents along with significant increase of galactose content in the first-hour intestine digestion. In the second hour of intestinal digestion, monosaccharides have not changed significantly. It could possibly indicate that no more α-1, 4 glycosidic bonds to be cleaved. Or the introduction of sugar polymers slowed down the fucoidan digestion. If so, an extra hour of intestinal digestion may have resulted in a significant difference.

Figure 18 indicated that after full gastrointestinal digestion, more than 25.80 % w/w of fucose were reserved undegraded in form of > 100 kDa fragments. While, around 9 % w/w of fucose participated in the degradation through the *in vitro* digestion. In general, approximately 12 %, 0.7 %, 0. 27 %, 0.08 %, 1.08 %, 0.13 % w/w of galactose, mannose, rhamnose, glucuronic acid, glucose and xylose were degraded to smaller fragments (< 100 kDa). Correspondingly, 19.93 %, 0.59 %, 0.29 %, 0.22 %, 0.71 % and 0.13% w/w were resisted to digest through the simulated *in vitro* digestion. In particularly, monosaccharides in >100 kDa reacted with salivary α-amylase, gastric juice and pancreatic amylase were shown in Figure 18. Fucose content changed significantly in oral phase and the first hour of gastric digestion, no significant changes were happened in G2 and intestinal digestion which revealed α-amylase presented in pancreatin and the second hour gastric juice had no significant influence on fucose in big fraction. Similarly, rhamnose changed significantly in oral and gastric phase, while xylose only changed remarkably in oral phase. Mannose was showing not significantly differ among oral and G1, oral and G2 and I1 and I2. Galactose and glucose were changed significantly among different stages. However, the galactose and glucose changes obtained were not only from fucoidan, but also the sugar polymers brought from the commercial pancreatin Section 5.3.

5.5.2 10 – 100 and 3 - 10 kDa

Comparing the monosaccharides contents sampled in different digestion stages in 10-100 kDa fraction (Table 13), all 7 monosaccharides and the total mass presented statistically significant difference obtained from different digestion time cut-offs. Mannose found in 10-100 kDa was increasing gradually in the first two phases from 99.86 μg/ 250 mg crude fucoidan to 541.72 μg/ 250 mg crude fucoidan. This was because of the degradation of the large fraction. A continuous decrease was shown in the next 3 hours of digestion which resulted in yielding nil of the 10-100 kDa fraction. This indicates the complete degradation of mannose fragments to smaller fractions (< 10 kDa). Fucose in 10-100 kDa increased steadily in the oral and in the first hour of the gastric phase from 91.73 to 6370.31 μg/ 250 mg crude fucoidan. A sharp decreasing of fucose content in 10- 100 kDa was seen in the second hour of gastric digestion, approximately 6000 μg of fucose/ 250 mg crude fucoidan. This indicates further degradation of large fragments to small fragments. During the 2-hour intestinal digestion, 7012.62 μg of fucose in 250 mg was crude fucoidan were degraded and remained in the 10-100 kDa fractions. Similar trend was presented by galactose, the third most abundant monosaccharides in the undigested 10-100 kDa fraction. Total of 3642.63 μg of galactose was detected at the end of gastrointestinal digestion in 10-100 kDa fraction from 250 mg of starting materials.

Comparing the monosaccharides contents sampled in different digestion stages in 3-10 kDa fraction (Table 13), all 7 monosaccharides and the total mass were statistically significant different for different digestion time cut-offs. The total amount of monosaccharides was not significantly differed in oral phase and G1 compared with the undigested fucoidan (CF). There was a significant increase in the total neutral obtained in G2 and I1 and a significant decrease in I2 phase. Glucose and fucose amount were mostly increased ate the end of the simulated digestion in this fraction.

Monosaccharides were fluctuated dramatically in 10-100 and 3-10 kDa fractions. The amount of monosaccharides changed up and down continuously in oral, gastric and intestine phases which indicates that salivary α-amylase, gastric juice and intestine α-amylase effectively cleaved the fucoidan into smaller fragments.

5.5.3 < 3 kDa

Table 13 illustrates the monosaccharides amount change in < 3 kDa fraction. The resistance of acid hydrolysis in < 3 kDa fractions was observed in the first hour of gastric digestion (no significant difference). The total amount of monosaccharides was not showing significant difference between oral digestion and the first hour gastric phase digestion, same result was observed in 3-10 kDa fraction. However, weight difference was shown between these two phases (Section 5.3). A possible reason was that gastric juice was preferentially breaking down non-carbohydrates linkage during the first-hour gastric digestion, like sulfated group at position 2 or 3 or 4 (Cuong et al., 2015). In general, the amount of all 7 monosaccharides increased gradually with the increasing digestion time. The slight decrease of rhamnose in the second hour of intestinal digestion and the decrease of glucuronic acid in the first hour of gastric phase may due to an error. With small peak area shown in LC-MS chromatogram, precise integration the amount was hard.

In Figure 19, all the monosaccharides were more abundant in this fraction for all of the digestion process. Mannose raised from 0.7 % of dw fucoidan to approximately 0.42 %, while rhamnose from 0.01 % to 0.08 % of dw fucoidan and glucuronic acid from 0.005 % to 0.03 % of dw fucoidan. Galactose and fucose were not detected in the starting material (under 1 μg in 250 mg dw fucoidan), equivalently 9.56 % and 1.59 % of dw fucoidan of galactose and fucose were degraded to under 3 kDa fragments. 1.22 % dw fucoidan of glucose and 0.46 % dw fucoidan xylose were occurred in the < 3 kDa fraction. 20 % w/w of sugar was presented in < 3kDa molecules, they could be either free monosaccharides or short chain fucoidan fragments. In particularly, monosaccharides in < 3 kDa reacted with salivary α-amylase, gastric juice and pancreatic amylase, as shown in Figure 19 (statistical relation). Fucose, mannose, rhamnose and xylose were shown almost opposite phenomenon compared to >100 kDa with no significant difference observed in oral phase but significantly differ in the gastric and intestine phases. Both galactose and glucose were not changed significantly in the second hour of the intestinal phase, and this could possibly be explained by complete cleavage of α-(1, 4) linked glycosidic bonds.

The result illustrates that fucoidan, commonly known as indigestible polysaccharides, can be partly digested with human native enzymes and gastric acid. Around 22 % of dw

fucoidan with large molecular weight polysaccharides (greater than 100 kDa) were involved in the *in vitro* digestion, these were cleaved to smaller fractions (approximately 4 % w/w in 10-100 kDa and 14 % w/w in < 3 kDa fractions).

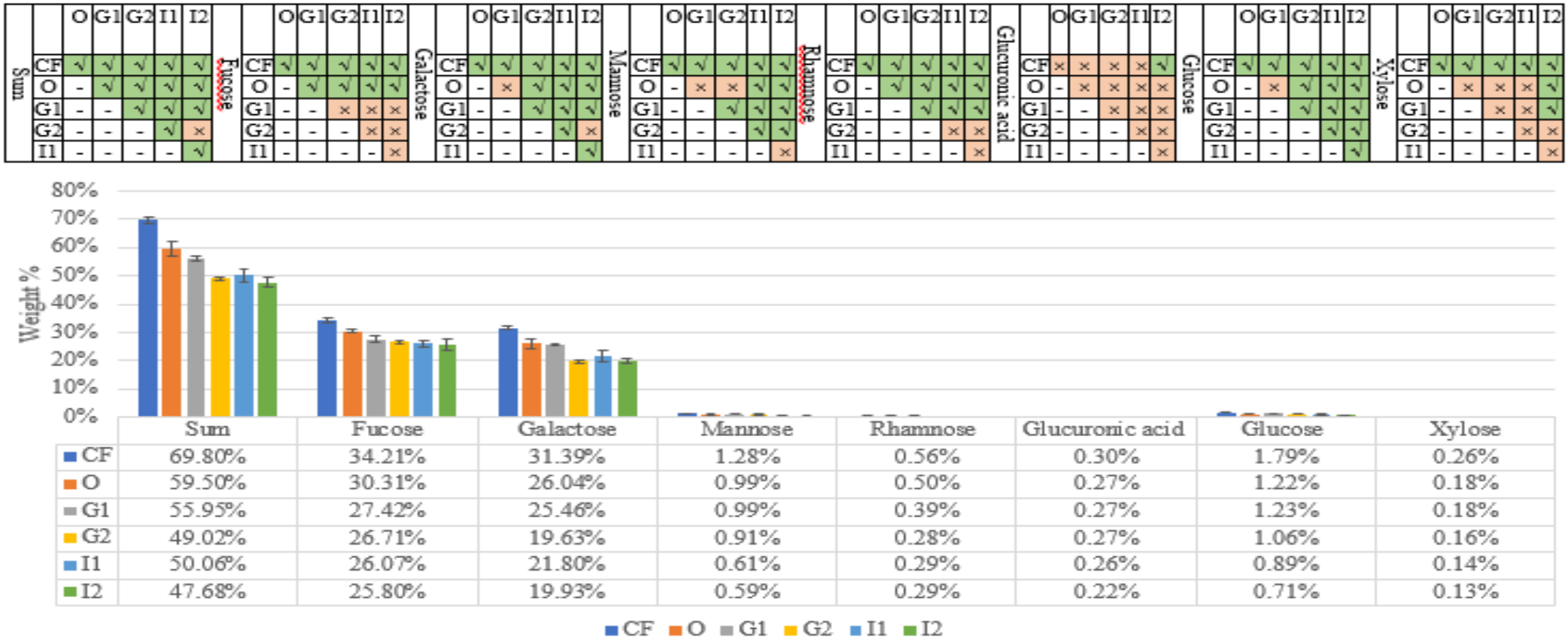
As reported in the previous studies, low molecular weight fucoidan (LMWF) (< 100 kDa) tend to have higher bioactivities. For example, fucoidan fractions with high anticoagulant activity had a Mw of around 21-23 kDa and contained fucose-galactose-sulfate at a ratio of 9:1:9 (Kitamura, Matsuo, and Tsuneo, 2014). Matsubara, Xue, Takashi, Zhao, and Sugawara (2005) reported that fucoidan fraction with Mw ranges between 15 - 20 kDa enhanced human umbilical vein endothelial cells migration, and had proangiogenic effect on angiogenesis. Hence, several attempts have been done to develop a good method for producing LMWF. The mostly used method in previous studies were acid hydrolysis (Yang, Chung, and You, 2008; Zhu et al., 2010), free radical (Colliec-Jouault, Millet, Helley, Sinquin, and Fischer, 2003; Durand et al., 2008) and enzymatic hydrolysis (Daniel et al., 1999; Kim, Lee, Nguyen, Yoon, and Kim, 2010; Kim et al., 2010).

The enzyme used in enzymatic hydrolysis (Yang, Chung, and You, 2008; Zhu et al., 2010) were fucoidanases (EC 3.2.1.44) and α-fucosidases (EC 3.2.1.51) which are mostly found in marine bacteria such as *Vibrio* sp. (Furukawa, Fujikawa, Koga, 1992), *Alteromonadaceae* (A. Sakai, Kawai, and Kato, 2004) and *Pseudoalteromonas* sp. (Ivanova et al., 2002) and in marine invertebrates such as *Patinopecten yessoensis* (Kitamura et al., 2014) and Hepatopancreas, *Littorina kurila* (Kusaykin et al., 2006). An α-fucosidase only cleaves the α-L-fucosyl linkages at the non-reducing termini of the fucoidan, while one type of fucoidanase cleaves somewhere in the middle of the polysaccharide and the other type cleaves off oligosaccharides from the end of the polysaccharide chain thus leading to lower the molecular weights of fucoidan (Holtkamp et al., 2009). Fucoidanase extracted from *Pseudoalteromonas* produced 26 % (w/w of the starting fucoidan) of 2-5 kDa fucoidan fractions, 8 % (w/w) of 2-3 kDa fucoidan fractions. While, fucoidanase from Hepatopancreas, *Littorina kurila* yielded 30 % (w/w) of 3-10 kDa, 8 % (w/w) of 2–7 kDa and 17 % of 3-10 kDa fractions (Kusaykin et al., 2006). Compared to the simulated human hydrolysis (α-amylase and gastric juice) investigated in this study, fucoidanase were cleaving more efficiently. Moreover, a much higher content (> 90 % of total carbohydrates) of fucose was obtained in the fucoidanase-cleaved fucoidan fractions (Kusaykin et al., 2006)

compared with my result. As shown in Figure 19, the < 3 kDa fraction was composed of fucose (1.59 %w/w), galactose, mannose, rhamnose, glucuronic acid, glucose and xylose in the molar ratio of 1.00 : 6.01 : 0.27 : 0.03 ： 0.02 ： 0.77： 0.29 after 4 hour simulated gastrointestinal digestion. Since previous studies suggested that sugar content may play an important role for anticoagulant activity (Dobashi, Nishino, Fujihara, and Nagumo, 1989; Nishino, Yokoyama, Dobashi, Fujihara, and Nagumo, 1989), the fucoidan fractions obtained by human digestion may not have high bioactivities due to the low yield.

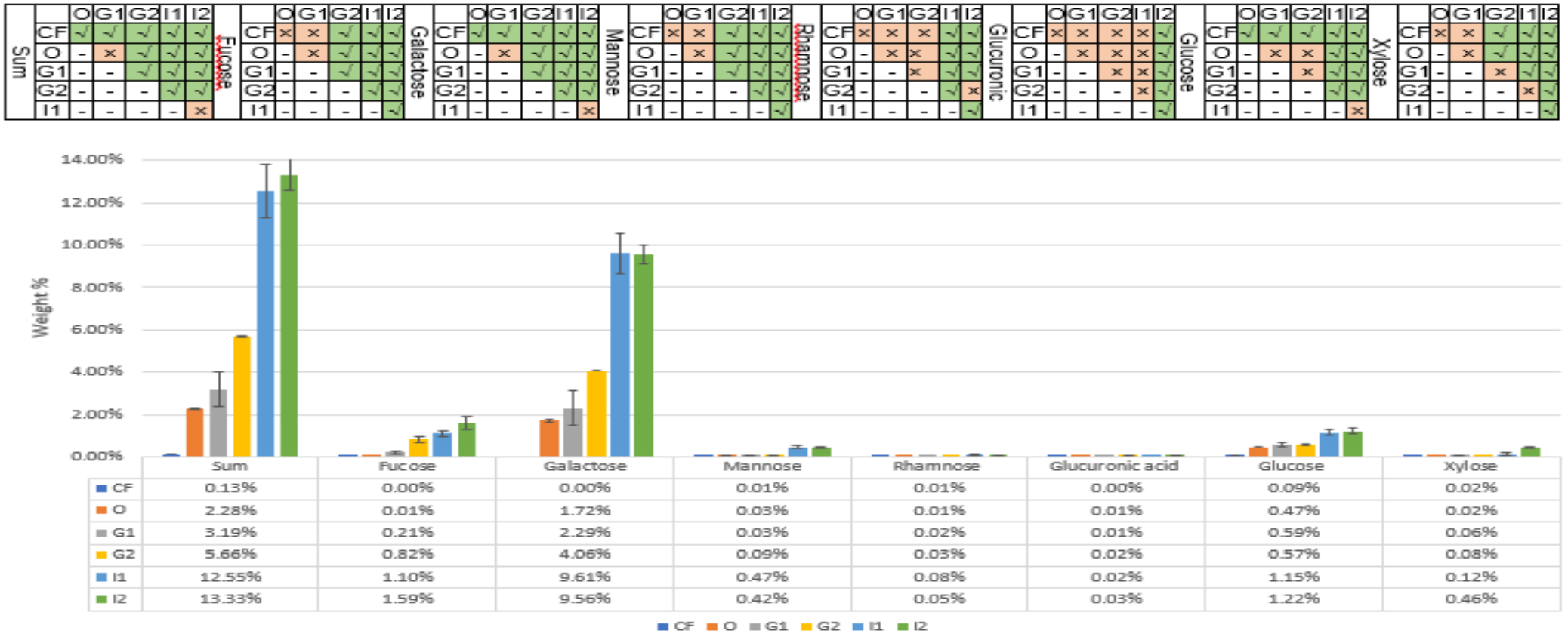
Table 13 Amount of monosaccharides in four Mw fractions at different *in vitro* digestion stages (μg/ 250mg dw fucoidan)

	Phase	Mannose	Rhamnose	Galactose	Glucuronic acid	Glucose	Xylose	Fucose	Sum
>100K	CF	3202.44±10.13^{a}	1412.44±38.58^{a}	78480.05±1430.80^{a}	755.68±0.97^{a}	4472.82±156.62^{a}	646.48±64.57^{a}	85537.17±1557.47^{a}	174507.08±3001.84^{a}
	O	2467.36±172.85^{bc}	1247.34±72.87^{b}	65098.42±4506.23^{b}	670.63±18.49^{ab}	3037.68±225.10^{b}	453.86±22.37^{b}	75764.69±1383.44^{b}	148739.97±5998.34^{b}
	G1	2476.83±106.01^{b}	972.17±87.74^{c}	63656.33±934.75^{b}	682.28±130.81^{ab}	3077.17±342.86^{b}	444.90±114.07^{b}	68554.50±2763.28^{cd}	139864.18±1809.08^{c}
	G2	2279.02±186.08^{c}	704.81±86.64^{d}	49074.17±799.08^{d}	675.53±85.86^{ab}	2650.99±84.80^{c}	388.32±86.90^{bc}	66766.47±1614.37^{d}	122539.31±1644.70^{d}
	I1	1516.38±40.29^{d}	722.04±25.01^{d}	59501.77±4983.58^{c}	650.01±100.64^{ab}	2235.02±180.91^{d}	348.57±5.23^{bc}	65686.52±2944.93^{d}	130660.31±5734.58^{e}
	I2	1470.55±23.47^{d}	733.77±15.04^{d}	49828.40±2037.77^{cd}	553.76±73.11^{b}	1786.40±216.47^{e}	319.00±34.99^{c}	64512.15±5311.11^{d}	119204.03±3994.30^{d}
P value		***	***	***	ns	***	**	***	***
100-10K	CF	99.86±1.42^{c}	7.20±0.21^{d}	80.98±0.39^{d}	5.48±0.07^{c}	6.81±0.01^{d}	42.35±1.37^{c}	91.73±0.55^{e}	334.41±3.03^{d}
	O	255.56±71.00^{b}	26.99±6.09^{c}	366.81±70.28^{d}	30.75±14.24^{b}	86.58±18.51^{c}	68.52±9.18^{b}	541.84±97.84^{d}	1377.05±277.87^{c}
	G1	541.72±40.62^{a}	108.19±8.70^{a}	4545.23±338.32^{a}	50.17±1.97^{a}	343.26±20.05^{b}	125.45±3.25^{a}	6370.31±503.19^{c}	12084.33±888.27^{a}
	G2	23.82±2.94^{d}	3.11±1.21^{de}	227.64±35.57^{d}	2.89±0.64^{c}	15.33±4.25^{cd}	4.36±0.98^{d}	398.86±22.51^{de}	676.02±39.65^{cd}
	I1	22.34±5.01^{d}	44.71±1.20^{b}	2599.46±501.47^{c}	59.73±1.28^{a}	ND^{d}	28.05±17.52^{c}	8258.92±201.44^{a}	11013.22±563.68^{b}
	I2	ND^{d}	ND^{e}	3642.63±264.29^{b}	62.24±12.28^{a}	469.25±95.16^{a}	ND^{d}	7012.62±377.99^{b}	11186.75±593.88^{ab}
P value		***	***	***	***	***	***	***	***
10-3K	CF	78.15±1.09^{d}	15.02±0.21^{bc}	27.15±0.66^{b}	9.41±0.32^{b}	500.33±0.18^{b}	16.86±0.02^{e}	62.38±0.74^{de}	709.31±0.93^{c}
	O	139.28±12.07^{c}	10.61±0.88^{c}	117.98±9.21^{b}	7.28±2.43^{bc}	336.80±16.59^{c}	149.39±3.84^{b}	39.57±2.64^{e}	800.90±21.85^{c}
	G1	77.37±12.07^{d}	4.64±0.18^{c}	169.44±1.75^{b}	1.92±0.16^{cd}	319.50±1.56^{c}	62.60±0.26^{d}	181.80±0.27^{c}	817.27±2.27^{c}
	G2	465.71±0.71^{a}	44.79±2.11^{ab}	668.63±6.98^{a}	13.79±2.58^{ab}	791.24±35.17^{a}	349.41±6.64^{a}	819.11±7.44^{a}	3152.68±7.76^{a}
	I1	368.91±1.65^{b}	75.30±30.73^{a}	602.78±330.62^{a}	16.94±5.64^{a}	797.21±53.71^{a}	119.41±23.41^{c}	312.77±98.67^{b}	2293.32±124.10^{b}
	I2	144.59±17.30^{c}	ND^{c}	ND^{b}	1.45±1.55^{d}	ND^{d}	27.22±8.99^{e}	127.29±16.66^{cd}	300.55±11.38^{d}
P value		***	**	**	**	***	***	***	***
<3K	CF	23.21±1.21^{d}	36.04±0.11^{c}	ND^{d}	7.74±0.30^{b}	215.98±2.19^{c}	39.55±1.54^{d}	ND^{d}	322.53±1.49^{d}
	O	77.81±5.91^{d}	34.87±9.52^{c}	4300.61±166.84^{c}	30.52±15.41^{b}	1176.11±73.36^{b}	52.36±15.65^{d}	26.56±14.77^{d}	5698.84±122.22^{c}
	G1	77.36±24.91^{d}	47.64±23.64^{c}	5719.53±2041.86^{c}	12.66±3.94^{b}	1468.31±250.35^{b}	138.08±15.99^{cd}	513.31±159.76^{d}	7976.88±2024.82^{c}
	G2	217.81±8.48^{c}	68.38±14.85^{bc}	10139.95±366.60^{b}	38.52±8.94^{b}	1435.09±96.69^{b}	195.69±52.42^{bc}	2048.84±78.55^{c}	14144.27±462.07^{b}
	I1	1170.26±112.44^{a}	211.42±42.44^{a}	24035.72±2402.89^{a}	37.79±29.31^{b}	2878.64±350.85^{a}	302.58±136.59^{b}	2748.88±318.92^{b}	31385.29±3111.01^{a}
	I2	1060.18±59.42^{b}	125.14±78.14^{b}	23894.73±1043.73^{a}	82.99±39.12^{a}	3046.45±335.47^{a}	1151.88±56.93^{a}	3974.90±800.14^{a}	33336.27±1851.12^{a}
P value		***	**	***	**	***	***	***	***



	Sum	Fucose	Galactose	Mannose	Rhamnose	Glucuronic acid	Glucose	Xylose
CF	69.80%	34.21%	31.39%	1.28%	0.56%	0.30%	1.79%	0.26%
O	59.50%	30.31%	26.04%	0.99%	0.50%	0.27%	1.22%	0.18%
G1	55.95%	27.42%	25.46%	0.99%	0.39%	0.27%	1.23%	0.18%
G2	49.02%	26.71%	19.63%	0.91%	0.28%	0.27%	1.06%	0.16%
I1	50.06%	26.07%	21.80%	0.61%	0.29%	0.26%	0.89%	0.14%
I2	47.68%	25.80%	19.93%	0.59%	0.29%	0.22%	0.71%	0.13%

Figure 18 Monosaccharides distributions in > 100 kDa before digestion and after digestion (mass % in dw crude fucoidan).
Note: CF= non-treatment crude fucoidan, O= oral, G1= gastric 1hr, G2= gastric 2hr, I1= intestine 1hr, I2= intestine 2hr. Mean values are plotted with error bars representing standard deviations. √ with green cell presents significant difference among two phases, × with red cell presents no significant difference among two phases.



	Sum	Fucose	Galactose	Mannose	Rhamnose	Glucuronic acid	Glucose	Xylose
CF	0.13%	0.00%	0.00%	0.01%	0.01%	0.00%	0.09%	0.02%
O	2.28%	0.01%	1.72%	0.03%	0.01%	0.01%	0.47%	0.02%
G1	3.19%	0.21%	2.29%	0.03%	0.02%	0.01%	0.59%	0.06%
G2	5.66%	0.82%	4.06%	0.09%	0.03%	0.02%	0.57%	0.08%
I1	12.55%	1.10%	9.61%	0.47%	0.08%	0.02%	1.15%	0.12%
I2	13.33%	1.59%	9.56%	0.42%	0.05%	0.03%	1.22%	0.46%

Figure 19 Monosaccharides distributions in < 3 kDa before digestion and after digestion (mass % in dw fucoidan).

Note: CF= non-treatment crude fucoidan, O= oral, G1= gastric 1hr, G2= gastric 2hr, I1= intestine 1hr, I2= intestine 2hr. Mean values are plotted with error bars representing standard deviations. √ with green cell presents significant difference among two phases, × with red cell presents no significant difference among two phases.

5.6 Sulfate analysis

Compared with sugar compositions, more studies pointed out that the bioactivity of fucoidan is highly dependent on the sulfate group, while the enzymatic hydrolysis prevents the loss of sulfate group than the acid hydrolysis (Morya et al., 2012). Unfortunately, the sulfate group analysis trial was not very successful. Figures 20 and 21 present the FTIR spectrum of sulfate content in > 100 kDa fraction (1223.39 cm^{-1}) between undigested fucoidan (orange) and sample after whole process of *in vitro* digestion and in < 3 kDa fraction (1238.61 cm^{-1}) between undigested fucoidan (orange) and sample after whole process of *in vitro* digestion, respectively. It can be noted that the sulfate peak in >100kDa decreased slightly after digestion while peak in < 3 kDa was relatively increased. The result indicated the cleavage of sulfate group among *in vitro* digestion. However, unlike crude fucoidan, enzymes were mixed in the digested samples, the concentration of digested sample was lowered by the presence of enzymes. Quantitively analyze the sulfate group needed a purified sample, or, an alternative method need to be developed for the further study. Moreover, the bioactivities of human digested fucoidan can be tested in the further.

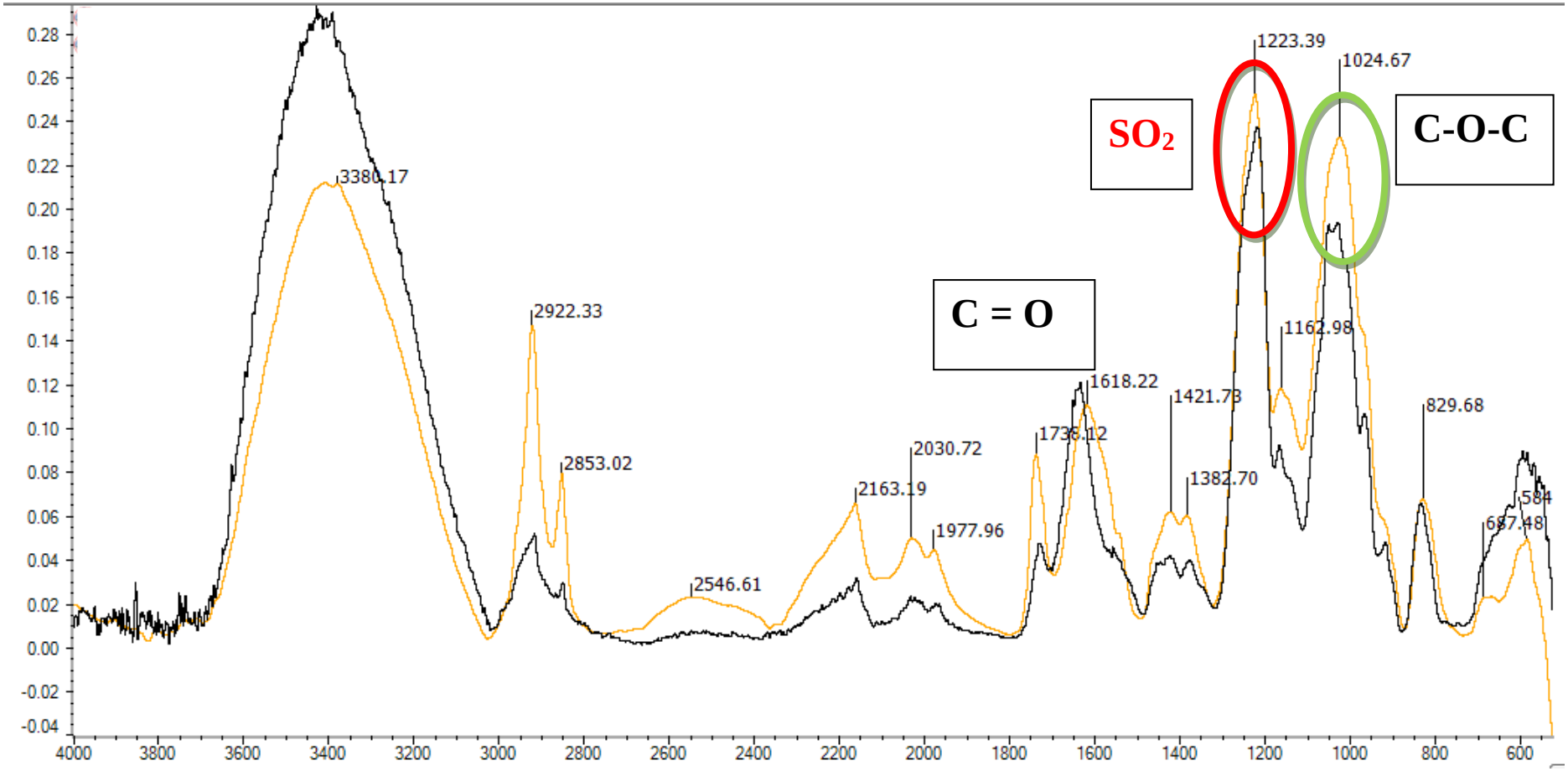


Figure 20 FTIR spectrum showing sulfate content in >100 kDa fractrion (1223.39 cm-1) between undigested fucoidan (orange) and sample after whole process of in vitro digestion

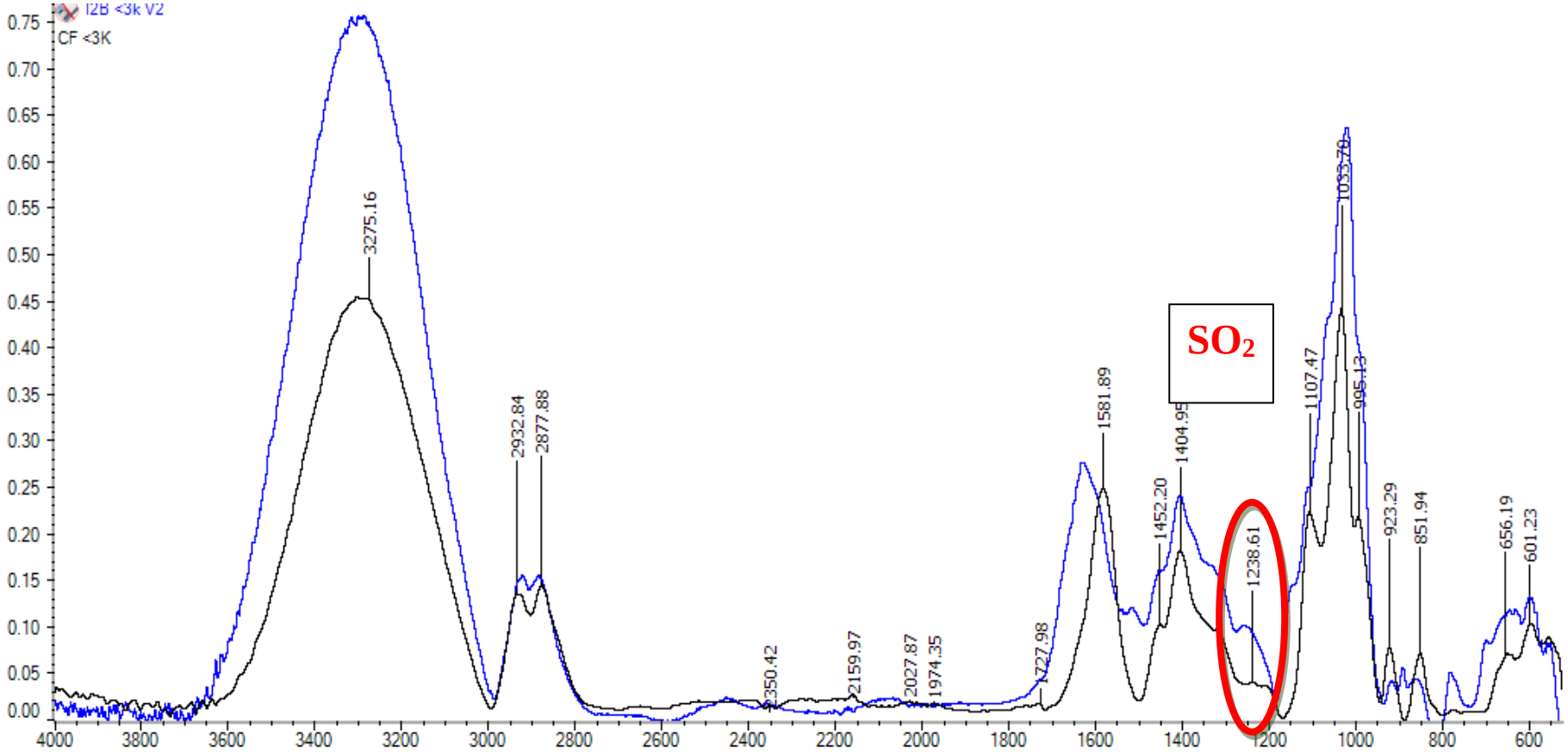


Figure 21 FTIR spectrum showing sulfate content in <3 kDa (1238.61 cm-1) between the undigested fucoidan (blue) and sample after whole process of in vitro digestion

Conclusion

In this study, fucoidan extracted from New Zealand *U. pinnatifida* was studied for its digestibility by using an *in vitro* digestion model. The digestibility was determined by the weight and composition of monosaccharides (fucose, glucose, galactose, mannose, rhamnose, xylose and glucuronic acid) changes in four different molecular weight fractions (<3, 3-10, 10-100, > 100 kDa).

As a result, both weight and the composition were changed significantly after *in vitro* digestion. The weight of > 100 kDa fraction decreased significantly across the digestion time cut-offs ($p < 0.001$), while other three fractions have shown increase. This indicated that the polysaccharide structure of fucoidan polymer has been cleaved to form smaller fragments after the simulated digestion process. Approximately 4 % and 14 % (w/w) of neutral sugar was increased in the 10-100 kDa and < 3 kDa fucoidan fractions which were cleaved from the large Mw fucoidan fraction. The result illustrates that fucoidan extracted from New Zealand *U. Pinnatifida*, commonly known as indigestible polysaccharides, can be partly digested with *porcine* amylase, and gastric acid.

A further structural analysis should be done in the future once a better fractionate instrument is equipped. HPLC with size exclusion column is suggested for better fractionate. Also, the bioactivities of the digested fucoidan fractions can be tested and compared. The introduction of enzymes extracted from bacteria and marine invertebrates to help human to digest fucoidan could also be an interesting task. Moreover, considering that the partly digested fucoidan will be transferred to the large intestine for further digestion, interaction with the gut microbiota can be of interest for further investigation.

There was no doubt that fucoidan, as a functional supplement has various bioactivities. As low molecular weight with high sulfate group fucoidan tend to have better bioactivities. This is the first study investigating the human enzymatic digestibility of fucoidan. The data from this study verified the partly digestibility of fucoidan extracted from Zealand U. *Pinnatifida* will be useful to the New Zealand Seafood industry to increase the value of fucoidan.

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