Metals influence structure and solubility of fucoidan and strategy of metal removal in fucoidan

Xuegeng Luo

A thesis submitted to Auckland University of Technology in partial fulfilment of the requirements for the degree of Master of Science (MSc) School of Science 2020

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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, 'Metals influence structure and solubility of fucoidan and strategy of metal removal in fucoidan', 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 an university or other institution of higher learning.

Name: Xuegeng Luo

Signed:

Date: 15/12/2020

Acknowledgement

I would like to thank my primary supervisor Professor Jun Lu for giving me this great opportunity to work on this subject. Throughout this year of study, I have learnt quite a lot about biomedical science, especially regarding polysaccharides. 2020 has been an especially difficult year, there has a lot of experimental issues, but I feel so lucky that I have had such respectable and knowledgeable supervisors to support me.

Many thanks to all staff members in the laboratories of the Faculty of Health and Environmental Sciences at Auckland University of Technology. I would like to thank Adrian Owens for helping me resolve any mechanical issues and I thank Tony Chen for guiding me in the use of the analytical machines.

Abstract

Undaria Pinnatifida is a widely distributed species of brown seaweed in New Zealand. Fucoidan is a sulfated polysaccharide which is extracted from *U. pinnatifida*. Its numerous bioactivities have been studied for several decades. Fucoidan is heterogenous polysaccharide and its structure is not constant. With degradation of the molecular weight of fucoidan across the same species, the structure and components are also not constant.

However, in *Undaria Pinnatifida* there are few reports that show a molecular weight of fucoidan below 10kDa. Fucoidan should be highly soluble, however, it was found that the fucoidan did not completely dissolve and unknown sediments were found. Therefore, this research is aimed to find out the sugar component of fucoidan fraction among 10kDa to 1kDa, and to find out a good agent to solubilize the solubility of fucoidan.

Here the interaction of polysaccharide and metals might cause a metal-sugar complex gel to insolubilize fucoidan. EDTA-Na_2 was chosen as metal removal agent which detected the amount of sediment present and was used to verify the efficacy of removing metals from fucoidan. The fraction of fucoidan among 10kDa to 1kDa was divided into >10kDa, >3kDa, <3kDa, >1kDa and <1kDa, all of which were seperately treated with EDTA-Na_2 and Milli-Q water. Microwave plasma – atomic emission spectroscopy (MP-AES) was used to determine the metal contents in fucoidan from *U. pinnatifida*, and LC/MS was used to detect the chemical composition of fucoidan. Infrared Spectroscopy was used to measure the total sugar content within the 10kDa and 3kDa fraction.

To conclude, EDTA-Na_2 showed good efficacy in the removal of divalent and transition metal ions. Fucoidan with different treatments had different sugar content, which would indicate that the structure correlated with metal content.

Chapter 1 Introduction

1.1 History of Fucoidan

Fucoidan is a complex group of notable percentages of L-fucose and sulfate ester groups, which were firstly isolated by Kylin (1915) from brown algae in 1913 (Kylin, 1915; Li et al., 2008). Since then, fucoidan has been successfully extracted from several different brown seaweed species. Furthermore, scientists have found that fucoidan from different seaweed species have varied structures and properties. During the past decades, fucoidan has shown its numerous bioactivities such as antioxidant capacity, anti-complementary, anti-cancer, anticoagulant, anti-thrombotic activities and immunomodulation. Nutraceutical, functional food, and cosmetic properties have also been identified. Fucoidan has aroused much research interest because of its confirmed and potential pharmaceutical and food value (Koh, Lu & Zhou, 2019). These special bioactivities demonstrate that fucoidan may be applied to health products and functional foods for disease prevention and health enhancement.

1.2 The Structure and Molecular weight of Fucoidan from *U. pinnatifida*

In 2006, researchers identified that the constituents of *U. Pinnatifida* are mainly fucose, xylose, galactose, glucose, rhamnose, and mannose as well as small amounts of uronic acid, and sulfate (Waffenschmidt & Jaenicke, 1987). Using analytical methods,

including HPLC, gel electrophoresis, elemental analysis, infrared, Raman, and mass spectrometry, it was found that the connections between fucoidan monosaccharides are 1-3, 1-4, 1-6 glycosidic bonds. The various saccharide bonds include 1-3 linked fucose, and 1-3, 1-4, and 1-6 linked galactose. The sulfate substitution sites are mainly at 2- or 4-positions of fucose residues, 3- or 6-positions of galactose residues (Waffenschmidt & Jaenicke, 1987). There is still controversy about the composition and connection of fucose. One claim is that fucose and galactose in galactofucan may form separate blocks and intersperse in one polymer backbone. The other claim is that there are two separate polymers: fucan and galactofucan. However, ESI-FTICR mass spectrometry analysis showed that galactose and fucose residues are successively linked into one polysaccharide molecule (Yang et al., 2018).

The molecular weight (Mw) also acts important part for structural function. The average molecular weight of crude fucoidan from sporophylls of New Zealand (*U. Pinnatifida*) was estimated to be 171kDa by Gel Permeation Chromatography (GPC) (Mak et al., 2013). This was higher than the same species harvested from Kijang, Korea which had a molecular weight of 38kDa (Koo et al., 1995). Furthermore, several other reports showed different environments and sources caused variations in molecular weight of fucoidan extracts. *U. pinnatifida* grown in Wando, Korea had a higher average Mw of 2100 kDa (Kim et al., 2007). Moreover, different fucoidan extraction techniques may make molecular weight of fucoidan indeterminable, such as heating (Sakai, Ishizuka & Kato, 2003). The use of solvents can also impact Mw such as increasing concentration of NaCl solvent (Mak et al., 2013).

1.3 Present research on fucoidan

There is a significant amount of emerging fucoidan research that is growing every day. Over 1600 independent, peer-reviewed scientific studies are available on the bioactive properties and effects of fucoidan. These research papers have focused on

key health topics including immunity support, heart and digestive health, anti-inflammation, and anti-cancer activity. Currently, the majority of fucoidan research has been conducted in, *in vitro* studies and animal studies (Chu, Phang, 2016; Shang et al., 2016), and more recently, human clinical trials (Luthuli et al., 2019) ;

1.4 Interference on Fucoidan

Fucoidan, in terms of pure particles is easily water-soluble. Due to the good solubility of fucoidan, it is efficiently combined with other particles to perform good bioactivities. For example, in Alek et al., (2019), microspheres (sizes ranging from 1 um to 1000 um) was prepared with fucoidan to create so-called “fucospheres”. In their review (Alek et al., 2019), increasing fucoidan content in the fucospheres, synergistically increased bovine serum albumin and zeta potential decreased. Meanwhile, the high solubility of fucoidan allowed microparticles to release drugs in a shorter time and reduced fucospheres-loaded drug’s (rifabutin) toxicity. However, the present extraction methods cannot achieve the 100% solubility. For instance, the cationic surfactant cetyltrimethylammonium bromide (CTAB) was used to purify fucoidan and resulted in precipitate, which had strong interaction between detergent and polysaccharide (Hahn et al., 2012), thereby indirectly lowing the biological efficiency of fucoidan.

Moreover, Fucoidan is heterogeneous polymer, any different factors would vary the structure and component of fucoidan, including extraction method, growth environment and seasons etc (Lu et al., 2018). Due to this characteristic, fucoidan exerts not exactly consistent biological activities, hence it has quite a lot controversy of its efficacy and structural recognition (Yang et al., 2018). This is the main interference of fucoidan for most research.

1.5 Goal of this research

Fucoidan research has been ongoing for several decades, people have discovered a lot of useful information and good utilization in many fields. While dissolving the *U. pinnatifida* species fucoidan, it was found that fucoidan was not completely soluble (Hahn et al., 2012), demonstrated by some rice color precipitate. Due to the existence of metal in fucoidan, metal ions (such as copper, barium, calcium, lead, etc) and polysaccharide may form the precipitate of coordination compounds. In addition, there may also exist a potential issue that can make fucoidan insolvable or decrease its solubility, termed as "conification". Conification is rarely mentioned in articles, conification is a phenomenon may be caused by freeze drying. When fucoidan is freeze dried the metal ions may be squeezed and deeply combined into the polysaccharide, forming cone shape crystals, which become hard to redissolve. Fucoidan, as a branched sulfated L-fucosyl polymer, may exist numerous anionic charge density and 3D structure. It would be able to interact with heavy metal cations whereas the mechanism on fucoidan is not yet concluded. Therefore, investigating the solubility and metal interference in fucoidan extracts for future research would be useful.

EDTA was first synthesized in 1935 and Ethylenediaminetetraacetic disodium acid (Na_2EDTA) was the first clinically introduced chelator (Hargreaves & Cohen, 2011) . Na_2EDTA is a polyaminopolycarboxylic acid which is a white powder and a water-soluble compound. Based on its complex stability with wide variety of metals and commercial availability, it has been widely utilized to chelate metals from soil and water. EDTA-Na2 previously showed excellent effect on metal-removal from soil (Hill-Cottingham, 1957; Ghestem, 1998). Its chemical characteristics highlight its potential as a chelation agent to remove metals from fucoidan. This may solubilize fucoidan and increase certain component of fucoidan, however, it is unknown whether EDTA-Na2 could remove or qualify metals from fucoidan. Therefore, this needs to be checked using a variety of experiments.

This study was to figure out that did metal ion interfere with the solubility or

structure of fucoidan. The aim of this research is to find out whether EDTA-Na_2 is able to remove metals in fucoidan and what would happen to fucoidan when EDTA-Na_2 is used. Meanwhile, this research orientation is meaningful to give more possible explanations about fucoidan structure. The results would also be stocked up for further research.

Chapter 2 Literature review

2.1 Background of brown seaweed

Scientists have divided seaweed species into red (*Rhodophyta)*, green (*Chlorophyta*) and brown seaweed (*Phaeophyceae*). These seaweeds have been introduced to habitats beyond their original range by human's activities like shipping, aquaculture and shellfish farming, and have successfully survived in these new locations (Ribera & Boudouresque, 1995). Over 200 seaweed species are made into phycocolloid (algins, agars, and carrageenans) and food products for international economical trade, which represents a market value of over 6.2 billion U.S. dollars (Zemke-White & Ohno 1999). Seaweed production now has more than doubled over the past two decades (Bixler et al., 2011).

Among these species, brown seaweed is the biggest portion of marine algae, which is sorted into 13 orders, ~300 genera and include ~1836 known species (Okolie et al., 2017). Brown seaweeds are commonly bigger in size, the giant kelp can grow between 2-4m, red seaweeds can only grow to 1m.

2.2 Polysaccharide in brown seaweedss

Laminaran, fucoidan and alginate are the three major polysaccharides in brown

seaweed. Their contents normally differ from 40% to 80% of dry weight of defatted algal biomass. The constituent of water-soluble polysaccharide is different by species, growing environment and so on. (Zvyagintseva et al., 2003).

Laminaran

Laminaran is 5kD glucan with a degree of polymerization among 20 to 25 (Nelson & Lewis, 1974). Laminaran is extracted from cell vacuoles and is composed of D-glucose with β-(1,3) linkages, β-(1,6) intrachain branching (Laurie et al., 2010). Laminaran has exhibited various bioactivities including antibacterial, antioxidative and anticoagulant properties (Zhang & Row, 2015). It exists in two different forms, soluble or insoluble. One form of laminaran can be completely dissolved in cold water with chains that terminate with D-mannitol residues (M-series). The other form is only soluble in hot water which has chains terminal D-glucose residues (G-series) (Nelson & Lewis, 1974).

Alginate

Commercial alginate can be generally extracted from Brown seawead (cell wall) including *Laminaria hyperborea, Laminaria digitata, Laminaria japonica*, *Ascophyllum nodosum* (Fucales), and *Macrocystis pyrifera* (Smidsrod & Skjak-Braek, 1990). Alginate is known to be whole family of linear copolymers, which consist of linear β-D-mannuronic acid (M block) and α-Lguluronic acid (G block) that are linked in position 1,4. If guluronates are connected, they form a so-called GG block and if mannuronates are connected, they form an MM block (Usov & Zelinsky, 2013) .

Fucoidan

Fucoidans are compounds of complex sulfated fucose-rich polysaccharides, which are found in abundance in the cell walls of brown seaweeds (Liu et al., 2017). Fucoidans

are also soluble in water and sparingly soluble in dimethyl sulfoxide (DMSO). According to other studies, fucoidan can also be extracted as source from marine invertebrates, such us sea cucumbers and the egg jelly coat of sea urchin (Vilela-Silva et al., 1999).

2.3 Historic view of fucoidan's extraction procedures

Brown seaweed (class Phaeophyceae) include three orders: the Ectocarpales, Laminariales (Undaria) and Fucales (Ascophyllum). Kylin (1913) first extracted fucoidan from various species of *Laminaria* and *Fucus* using dilute acetic acid, followed by purification. Kylin (1915) also reported that components of this extraction method were predominantly fucose, with smaller amounts of mannitol, alginate and laminaran. It was caused by co-extraction of latter contaminants with fucoidan. In 1995, Kylin isolated fucoidan from *Laminaria digitata,* which contained L-fucose and some other pentoses (Kylin, 1995). The early fucose-containing sulfated polysaccharide extraction procedures began with hydrolyzation of the non-fucose-containing sulfated polysaccharide using diluted acid treatment. Since Kylin's seminal report, the extraction and purification methodologies have been modified to isolate fucoidan from brown seaweed biomass. For composition analysis, H_2SO_4 was used for hydrolysis. However, H_2SO_4 itself may have contributed to a lot of the sulfate amount which biased the analysis result (Kylin, 1995). Bird and Haas (1931) proposed another acid as substitute, but the bias still existed and uronic acid was present.

Hoagland and Lieb (1915) isolated a water-soluble polysaccharide from *Macrocystis pyrifera* which was closely related but not identical to fucoidan, this compound also contained L-fucose and high level of sulfate. Extraction of *M*. pyrifera fucoidan was repeated by Nelson and Cretcher (1931). Nelson and Cretcher (1931) extended the extraction time (48h) with diluted HCl, followed by ethanol precipitation which resulted in sulfate precipitates as form of ester groups. however, fucose was identified as the only sugar to be recognized as an unhydrolyzed residue. Later, in 1937

Lunde, Heen & Oy processed the fucoidan precipitation and proposed a structural unit formula. The hypothesized structure for fucoidan (R-R'-O-SO2-OM)n, suggested that R was fucose or another pentose sugar residue. R' was unknown, and M was either Na+, K+, (½)Ca2+ or (½)Mg2+ (Lunde, Heen and Oy, 1937).

In 1950, for deeper research, Percival and Ross extracted fucoidan from *F. vesiculosus, F. spirales, Himanthalia lorea and Laminaria clustoni.* Lead acetate and barium hydroxide was used to remove alginates and protein from fucoidan to make fucoidan into a crude lead hydroxide complex (Percival and Ross, 1950) . Dilutes H_2SO_4 was used to treat the lead hydroxide complex followed by filtration. They got the purest fucoidan specimen from *H. lorea.* This fucoidan extract comprised of substantial quantities of fucose and sulfate, small amount of uronic acid, galactose, xylose and calcium sulfate. During this period, it was thought that fucoidan's backbone was fucosyl units in the fucoidan were 1→2 linked, however, this was incorrect (Conchie et al., 1950). After more advanced analysis, the Nuclear Magnetic Resonance (NMR) and heteronuclear ^{13}C–^{1}H spectrum (HMQC) was used to recognize that the backone of *F. vesiculosus* consisted of alternating α(1→3) and α(1→4) linkages (Chevolot et al., 1999).

In 1952, Black, Dewar & Woodward launched a laboratory-scale extraction of fucoidan to pursuit extensive quantities in future. Their extracted product was considered as a "polyfucose ethereal sulphate". To prevent the contamination of the target compound, prior treatment before extraction with formaldehyde was used . The fucoidan quantities they got from four different algal species with optimal extraction percentage for *Pelvetia canaliculata*, *F. vesiculosus*, *Ascophyllum nodusum*, and *L. cloustoni* were 76%, 62%, 54% and 20% respectively (Black, Dewar and Woodward, 1952). The authors (Black et al) also predicted that yield output might positively relate to water/seaweed ratio, extraction time and extraction frequency.

Formaldehyde is used as a polymerizing agent that links and fixes phenols, rendering them insoluble (Mian & Percival, 1973). The aldehyde is typically applied in

an ethanolic solution. High ethanol/water ratio can reduce the preliminary simultaneous extraction of fucoidans. The aqueous ethanolic solution can advantageously remove mannitol, a major carbohydrate reserve, and chlorophyll instantaneously. These procedures can be performed without structurally altering the target compound (Mian & Percival, 1973). However, in recent studies it was found that formaldehyde may induce covalent bonding between proteins and polyphenols or nucleic acids, generating high-molecular weight complexes (Rioux, Turgeon & Beaulieu, 2007). Hahn et al., (2012) was concerned that these high-molecular weight complexes may interact with the sulfated polysaccharide resulting in the precipitation of the complex and decreased fucoidan yield.

Meanwhile, Whyte (1970) developed a mixture of methanol/chloroform/water (w/w/w), 4:2:1, to isolate lipid from fish. This ternary system could remove lipids, terpens and phenols. It was found that phenols or flavins have high affinity for fucoidans and alginates and can be absorbed by these polysaccharides tightly during extraction (Whyte and Southcott, 1970). Therefore, this mixture is commonly used in pretreatment. Pretreatment of algae is advantageous to prevent coextraction of other algal compounds during isolation.

Overall, the classical extraction and purification procedures can be described as the following: Raw algae is treated with hot aqueous or acidic solution at temperature ranging among 70 - 100°C for hours. pH is adjusted to increase the amount of extracted fucoidan. Releasing proton or hydroxide ions into solution also increases the yield because protons and hydroxide ions interfere with the hydrogen bonds of polysaccharide (Ale et al., 2011). Next, cycles of acidic or alkaline treatments were repeated, followed by neutralization of the products to limit polysaccharide degradation. Hot acidic solution would be a better choice as this solution can simultaneously precipitate alginate as alginc acid . The mannuronates and guluronates are two forms of alginate. They are protonated below their pKa values which means that the electrostatic repulsion decreases and the polymers can associate via interchain hydrogen bonds

(Draget, Moe,Skjak-Braek & Smidsrod, 2006). However, the application of diluted acid at ambient temperatures causes the partial division of sulfate esters (Pomin, Valente, Pereira & Mourao, 2005). Bioactivity is dependent on fucoidan's molecular weight, monosaccharide composition and sulfate content, so a compromise must be made between the amount of extracted polysaccharide and the sulfate ester content (Holtkamp, Kelly, Ulber & Lang, 2009). Ethanol precipitation is then used to remove salts and other small molecules from the polysaccharide. Due to the high dielectric constant of water, oppositely charged groups are shielded and surrounded by hydration shells. Because of the lower dielectric constant of ethanol, the existence of ethanol would precipitate the sulfate ester, and positive ions form ionic bonds (Hahn, 2012).

In addition, the detergent CTAB was normally applied to purify fucoidan, the charged quaternary ammonium part of CTAB would interact with anionic polysaccharide. Based on the strong interaction between the polysaccharide and the detergent, complete solubilization cannot be achieved and requires high salt concentrations (Hahn et al., 2012).

Variations in extraction methods will influence fucoidan yield, short extraction time will result in low total fucoidan yield but with high fucose contents. While longer extraction time and high frequency may increase the yield, it will also disadvantageously affect the fucoidan structure since HCl will break structural polymers and degrade the chain of fucose residues. Different extraction treatment is one of the factors that confuse fucoidan results, highlighting the need for a unified extraction protocol.

2.4 Glucosidic bonds within fucoidan, bioactivity, and application

Structure

Fucales

Fucoidan from *F. Vesiculosus* was wrongly interpreted as α(1→2) linked and it was

revised in 1993 by Patankar et al., Furthermore, Pereira (1999) found that the presence of 2,4-di-O-methylfucose and 2,3-di-O-methylfucose, demonstrated both α(1→3) and α(1→4) linked fucose residues. In addition, *Ascophyllum nodosum* (Fucales) has similar structurally derived oligosaccharides and about 8-14 monosaccharide units (Chevolot, 1999).

More recently, Bilan in 2002 reported that in the order of Fucales (*Ascophyllum nodosum)*, *F. evanescens* and *F. serratus L* were found to be composed of fucose, sulfate and acetate. Fucoidan of *F. evanescens* has a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues: → 3)-α-L-Fucp(2SO3-)-(1→4)-α-L-Fucp(2SO3-)-(1→, with an additional sulfate occupying position 4 in a part of 3-linked fucose residues, while a part of the remaining hydroxyl groups was randomly acetylated. Fucoidan from *F. serratus L.* has a branched structure, its backbone is →3)-α-L-Fucp-(1→4)-α-L-Fucp-(1→, about half of the 3-linked residues are substituted at C-4 by α-L-Fucp-(1→4)-α-L-Fucp-(1→3)-α-L-Fucp-(1→trifucoside units. Sulfate groups occupy mainly C-2 and sometimes C-4, although 3,4-diglycosylated and some terminal fucose residues may also be nonsulfated. Acetate groups occupy C-4 of 3-linked Fucose and C-3 of 4-linked Fucose at a ratio of about 7:3. The fucoidan also contains small amounts of xylose and galactose (Bilan et al., 2006).

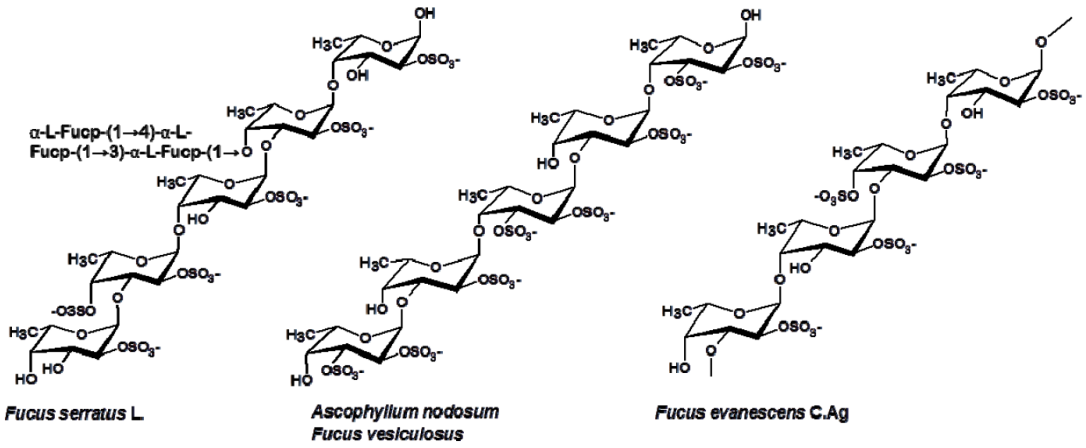


Figure 1. The classic structure of fucoidan extracted from brown seaweed species in the order of A. nodosum (Marcel, Jorn & Anne, 2011). Chevolot et al., (2001)

reported that [→3)- α-L-Fuc(2SO3-)-(1→4)- α-L-Fuc(2,3diSO3-)-(1]n was the predominant repeating structure of fucoidan from Fucales.

Fucoidan extracted from *Sargassum stenophyllum* (Fucales) can be divided into two types (Duarte et al., 2001). Type I contain a relatively high percentage of α-D-glucuronic acid and relatively few sulfate groups, while type II contain relatively small amounts of α-D-glucuronic acid and a high percentage of sulfate (Duarte et al., 2001). Type I polysaccharides are composed of a linear backbone formed mainly by (1→6)-β-D-galactose and/or (1→2)-β-D-mannose with branching chains formed by (1→3) and/or (1→4)-α-L-fucose, (1→4)-α-D-glucuronic acid (Duarte et al., 2001).

Li et al., (2006) isolated fucoidan from *Hizikia fusiforme* which contained a fucose-free core. The sugars (92.7kDa) isolated were mainly fucose, galactose, mannose, xylose and GlcA while 21.8% of the isolate was sulfate. The structural core was composed of →2)-α-D-Man(1→ and →4)-β-D-GlcA(1→, while some →4)-β-D-Gal(1→ was also mixed in . Sulfate groups were at C-6 of →2,3)Man(1→, C-4 and C-6 of →2)Man(1→, C-3 of →6)Gal(1→, C-2, C-3 or C-4 of fucose, while some fucose residues had two sulfate groups (Li et al., 2006)

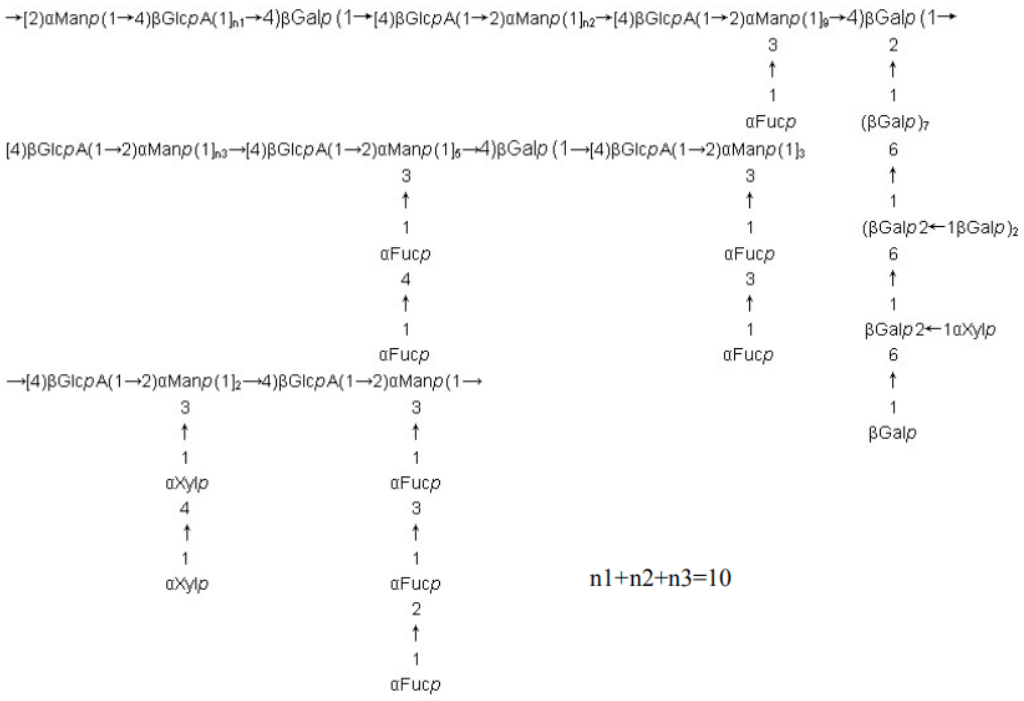


Figure 2. Presumptive fucoidan from *Hizikia fuisforme* (Li, Lu, Wei & Zhao, 2008)

*Undaria (*Laminariales*)*

There are also various structures of *Undaria* have been reported. Schweiger (1967) isolated a polysaccharide from *Macrocytis pyrifera* (Laminariales) with a fucose to galactose raito of 18:1. Schweiger (1967) first reported that fucoidan was not a pure fucan sulfate but the heteropolymer of fucose, galactose and trace xylose. Subsequent testing revealed mannonse, xylose, glucose and glucuronic acid had also been detected as part of fucoidans, further complicating the proposed fucoidan structure.

Sporophyll *Undaria pinnatifida* had a high fucose/galactose ratio, high uronic acid and low sulfate content (Hemmingson et al., 2006). The richest fucopyranosyl units were substituted at the 3-, 2,3-, or 2,3,4-positions and fucose residues were substituted at the 3,4- or 4-positions. The galactopyranosyl units were predominantly substituted at the 3- or at both the 3,4-positions (Hemmingson et al., 2006). Fucoidan isolated from *Chorda filum* (Laminariales) contains a poly-α-(1→3)-fucopyranoside backbone with

a high degree of branching, mainly of α-(1→2)-fucopyranoside single units (Conchie et al., 1950). Some fucopyranose residues are sulfated at O-4 (mainly) and O-2 positions, some α-(1→3)-fucose residues are shown by NMR to be 2-O-acetylated (Chizhov, Dell & Morris, 1999).

It is reported that extracted polysaccharide of *Undaria* had huge amounts of fucose and galactose, while its composition and structure appeared similar to fucoidan from *Fucales* (Hemmingson et al., 2006). However, some recent studies found that *U.pinnatifida* had a big amount of acetylation of sugar monomers which differed from the fucoidan extracted from *F. vesiculosus* (Vishchuk et al., 2011; Ho et al., 2015). The percentage of sulfated groups within the extracts of *U.pinnatifida* also differed across studies based on different extraction methods ranging from 7.4% (Kim et al., 2007) and 9.18% (Synytsya et al., 2010) to 41.5% (Yang et al., 2008). Moreover, the content of uronic acid and fucose varied depending on harvest time and growing environment (Mak et al., 2013). No two isolated Fucoidans are the same, even if they are extracted from the same seaweed species. Each extraction is unique in structure, composition, and bioactivities. What people interpret is normally the most predominant and representative structure.

Chordariales (Ectocarpales), *C. Okamuranus*, *Analipus japonicas* and *Adenocystis utricularis* also exhibit a structure that contains the same fucoidan residues and α(1→3)-backbone. However, C. Okamuranus also contain α(1→3)-linked-L-fucopyranose residues with sulfate substitutions at C-4 and/or with α(1→2)-linked single α-L-fucopyranosyl substitutions. Vicinal glucuronic acid substitutions and some of side chain of fucose residues may be O- acetylated (Nagaoka et al., 1999; Bilan, 2007). Moreover, *Adenocystis utricularis* extracted by different methods has shown different contents. *A. utricularis* at room temperature was mainly consist of galactose, fucose and sulfate ester (as galactofucan); relatively, at 70°C. It mostly contained fucose, uronic acid and low sulfate ester, as well as some other monosaccharide, so-called uronofucoidan (Ponce et al., 2003).

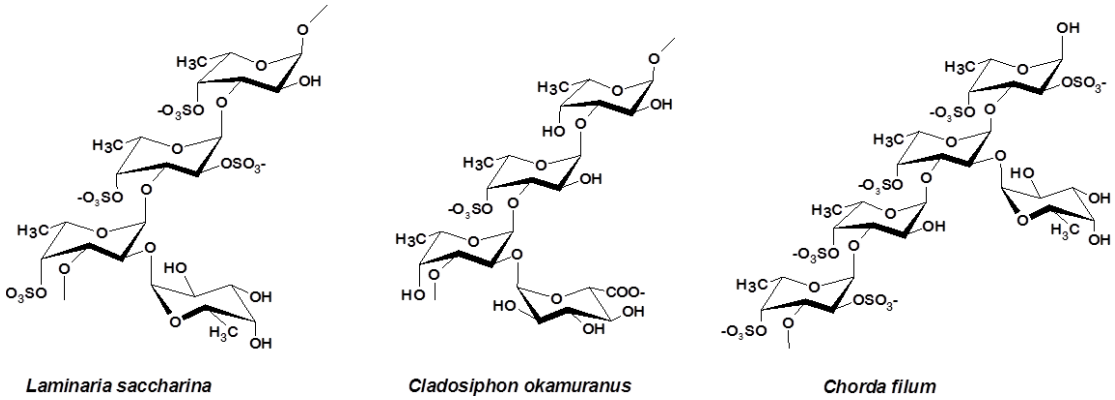


Figure 3. Structure for Undaria and Chordarials species fucoidan

2.5 New Zealand *U. pinnatifida*

Undaria pinnatifida originated in China, Japan, and Korea and has spread to many other places such as the Atlantic, Mediterranean, Australia and New Zealand (Pushkar, 2018). *U. Pinnatifida* was first discovered in New Zealand in 1987 and it spread rapidly to several sites in the North, South, and Steward Island (Silva et al., 2002). *U. pinnatifida* is a highly invasive species and has a high tolerance for light, temperature, and salinity. *U. pinnatifida* also has a high growth rate and large reproductive output, it releases spores all year round (Hui et al., 2019). Since 2009, due to its overpopulation and heavy invasion, the New Zealand Ministry of Fisheries (NZMF) has allowed the commercial harvesting of *U. Pinnatifida*. This abundance of *U. pinnatifida* makes it cheap for both research and industry.

The *U. pinnatifda* fucoidan is normally extracted from sporophyll. The main difference from *U. pinnatifida* among other majority species fucoidans, like *F. vesiculosu*s, is that fucoidan from *U. pinnatifida* are the composition of monosaccharides which form the backbone of polysaccharide chain, mainly about fucose, galactose and sulfate groups (sulfated galacofucan) (Vishchuk et al., 2011). In Vishchuk et al's report, they extracted fresh *U. pinnatifida* with ethanol, acetone, and chloroform; defatted with HCl (1N) for five hours at 60℃ (Vishchuk et al., 2011).

Followed by passing the fucoidan specimen through an anion-exchange chromatograph with 1.4M NaCl. Vishchuk et al found that *U.pinnatifida* contains 51% fucose, 48% galactose and small amount of other monosaccharides. They highlighted the main chain is built up of (1→3)- and/or (1→4)-a-L-fucopyranose residues. The sulfate groups mainly bind to the C2 position and to a lesser degree, the C4 positions of fucose and/or galactose residues (Vishchuk et al., 2011). In a recent study, this structure was confirmed by using the HPLC-RID system equipped with a Sugar-Pak I Column (Koh, Lu and Zhou, 2019).

2.6 Determinant of fucoidan's research field and bioactivity

For several decades research, scientists have found that fucoidan has exhibited bioactivities such as antioxidant capacity, anti-complementary, anti-cancer, anticoagulant, anti-thrombotic activities, and immunomodulation. Nutraceutical, functional food, and cosmetic properties have also been identified. There are still a lot experiments needed to assess its potential biological properties. This section mainly focuses on the most significant properties of fucoidans and the relationship among its structural features and bioactivities.

Antitumor

Antitumor activities of fucoidan has been frequently reported in recent decades. Previous studies have shown that different species of fucoidan can induce apoptosis in various tumors. *S. thunbergia* was found to exert growth inhibitory activity in Ehrlich ascites carcinoma in mice and antitumor effects within Ehrlich carcinoma (Zhuang et al., 1989; Zhuang et al., 1995). *A. nodosum* fucoidan was reported to exhibit an inhibitory effect against NSCLC-N6, non-small-cell human bronchopulmonary carcinoma (Riou et al., 1996). *C. okamuranus* fucoidan showed growth inhibitory

activity on the stomach cancer cell line MKN45 (Kawamoto et al., 2006). *U. pinnatifida* showed strong *in vivo* and *in vitro* antitumor activities against breast cancer cells (Funahashi et al., 2001). Apoptosis is one of the best characterized pathways in which fucoidan inhibits the overall growth of cancer.

Yamasaki-Miyamoto et al., (2009) demonstrated that fucoidan from *C. Okamuranus* inhibited the growth of peripheral blood mononuclear cells (PBMCs) from adult T-cell leukemia patients and human T-cell leukemia virus type 1-infected T-cell lines. In 2015, Han et al., used *F. vesiculous* fucoidan from Sigma to treat on colon cancer HT29 cell lines. Fucoidan also has ability to stimulate G1-phase cell cycle arrest to inhibit proliferation (Yamasaki-Miyamoto et al., 2009).

Furthermore, according to Zhuang et al., 1989, extracts from *Sargassum thunbergia* from Japan were used to treat Ehrlich ascites carcinoma cells which proved to be effective against sarcomas. Zhuang et al., 1989 also found that the only effectual fucoidan fractions were both sulfated polysaccharides composed mainly of L-fucose. One of the fractions had a Mw of 19kDa with 28.4% of SO_4 per fucosyl residue, while the other one of the fractions was had a Mw of 13.5k with 34.2% of SO_4 per fucosyl residue (Zhuang et al., 1989). Ferial et al., (2000) hypothesized that, for *A. nodosum* low molecular weight fucoidan (LMWF) fractions to exhibit antiproliferative activities, sulfate groups were necessary. Fucoidan has shown a relationship between their inhibitory effect and their sulfate groups content. Ferial et al also showed that the negative charges and the molecular size of fucan derivatives rather than a specific carbohydrate structure were more important in the determination of biological activities (Ferial et al.,2000).

Molecular weight is another important parameter that impacts bioactivities. High performance gel permeation chromatography (HPGPC) indicated that all fucoidans consist of both high low Mw fractions (Lu, 2018). There are several studies showing that some of the crude fucoidan or high molecular weight (HMWF) had antitumor activities. These included growth inhibition of implanted Sacroma-180 cells

(Yamamato et al.,1984) and stimulation of apoptosis in HCT-15 colon carcinoma cells (Hyun et al., 2009). In a recent study Alek et al., (2011) tested crude fucoidan from *Sargassum sp* on treating lung and skin cancer *in vitro*. Crude fucoidan induced apoptosis of melanoma B16 cells and exerted antitumor activity through inhibition of the growth of Lewis lung carcinoma and melanoma B16 cells. The researchers concluded that the anti-tumor activity mechanism of crude fucoidan is related to the sulfate content and the ability of fucoidan, which is based on the enhancement of NK cells and can induce tumor cell death. (Alek et al., 2011).

Although high molecular weight fucoidan species exert different inhibitory effects, lower molecular weight exhibits better anticancer activity when compared with native fucoidan, HMWF & crude fucoidan. It was figured that *U. pinnatifida* exhibited stronger anticancer activity with 5-30 kDa fractions than those with Mw>30kDa in human stomach cancer cell line AGS (Cho et al., 2010). Moreover, as the fucoidan from *F. vesiculosus*, low molecular weight fucoidan (7-16 kDa) enhanced in human stomach cancer cell line AGS, human breast cancer cell line MCF-7 and human hepatocellular carcinoma cell line HepG2 better than 217kDa one did. (Choi & Kim, 2013).

Sulfate groups contribute to the negative charge found in fucoidan and help to recognize biological markers, such as plasmaticproteins, adhesion proteins, and growth factors in complex *in vivo* environments (Ferial et al., 2000). Sulfated galactofucan with Mw of 123 kDa was extracted from *S. gurjanovae* and compared to a lower molecular weight extract of 71 kDa from the same sample (Shevchenko et al., 2015). Desulfination at position 2 and removal of galactose residues, did not impact the inhibitory effects on the human colon cancer cell line (DLD-1) in an MTS assay *in vitro* (Shevchenko et al., 2015). Even now, the exact interaction mechanism of action between sulfate groups and molecular weight is yet to be eluded. LMWF fractions are well-characterized which may give more helpful information to explain their interactions with targets and activities. Many reports indicate that the sulfate group is the key factor in fucoidan's antitumor activity.

Anticoagulant and antithrombotic activity

The potent anticoagulant activity of fucoidan has been widely studied. The earliest published report about anticoagulant activity of fucoidan was in 1957 by Springer et al. Springer et al found that extracts from *F. vesiculosus* had strong anticoagulant activity hence they regarded fucoidan into the group of heparinoids while Heparin consists of highly sulfated glucosaminoglycan. Anticoagulant mechanisms of fucoidan are reported to be related to both antithrombin and heparin cofactor II-mediated activity (Grauffel, 1989). Kitamura in 1992 reported that fucoidan from *L. angustata* had antithrombotic activity at 200 U/mg, equal dose of 140 U/mg heparin. However, due to the variations in fucoidan's structure and constituents, the mechanism of action is still undecided. Low molecular weight fucoidan from *L. angustata*, around 21-23 kDa, which had a ratio of 9:1:9 fucose-galactose-sulfate with the sulfate substitutions at C-4 of the fucose residues exerted remarkable antithrombin activity (Kitamura et al., 1992).

Cumashi et al., (2007) compared anticoagulant properties from 9 species of fucoidan. The only sample that did no show anticoagulant activity was the extraction from *C. okamuranus* which had a low sulfate content, linear (1→3)-linked poly-α-fucopyranoside chain and contained a large amount of 2-O-α-D-glucuronopyranosyl branches.

Many studies pointed out that anticoagulant activity of fucoidan were related to sulfate content, sulfate position, molecular weight, and sugar composition. It was shown that if sulfate groups in LMWF made up over 20% of the fraction that fucoidan would exhibit anticoagulant activity (Cho, 2010). While a long polysaccharide chain and a suitable configuration which binds thrombin more effectively, resulting in a larger molecular weight is also advantageous to achieve anticoagulant activity. An experiment by Chandía et al., (2008) showed that fucoidan from *Lessonia vadose* with a 320kDa Mw, compared with the same weight of radical depolymerized fraction, showed stronger anticoagulant activity. The impact of the amount of sugar composition has also

been studied. Nishino et al., (1989) speculated that oligo- or polysaccharides groups might act as an essential role for anticoagulant activity. However, Pereira et al (2002) concluded that 2-sulfated, 3-linked α-L-galactan, instead of α-L-fucan, was the predominant thrombin inhibitor mediated by antithrombin of heparin cofactor II.

2.7 Metal binding of algae

Brown algae can effectively remove toxic metal ions such as lead, mercury and cadmium (Sheng et al, 2004).. The maximum bioabsorption capability of brown algae for heavy metals are very high ranging from 0.39 to 1.66mmol/g. Algae uptaking sequence of metal is Pb> Cu>=Ni > Cd > Zn. Among the three kinds of algae, brown algae have better biosorption capacities than green and red algae (Sheng et al, 2004).

The carboxylic groups are normally the predominant acidic functional groups in brown algae. They occupy the highest proportion of detectable sites (>70%) in dried brown algae biomass (Davis et al., 2003). The adsorption capability of algae is primarily determined by these carboxylic group sites on the alginate polymers, and alginate comprises up to 40% of the dry weight (Percival & McDowell, 1967). The secondary acidic functional groups of fucoidan are the suldonic acids. Suldonic acid groups play an important role in binding metals at pK_a~5.0. Hydroxyl groups also exists in all polysaccharide which can also bind metal at low pH (Percival & McDowell, 1967).

Biosorption is the main way that algae uptake metals from seawater, this mechanism is related to ion-exchange. The ion-exchange takes place between heavy metals and light metals (i.e., Ca^{2+} and Mg^{2+}, but monovalent Na^{+} and K^{+} do not create strong cross-linkages (Chen & Yang, 2005). However, ion-exchange has not yet been clearly identified. Ion-exchange relates to physical (i.e., London–van der Waals forces) and chemical bonding (ionic and covalent). In the term of biosorption of metals by algal biomass, theoretically, intracellular sorption initiates bioaccumulation of alive

organism (Chen & Yang, 2005).

Lots of studies have reported the observations of heavy metals bonded by alginate. G-block structure of alginate polymers exhibit strong selectivity for divalent metals (Mackie et al., 1983; Steginsky et al., 1992). However, only a few papers have reported the metal binding capability of fucoidan. Romera et al (2007) reported that the affinity of metal ions to both alginate and fucoidan was dependent on stereochemical influence. Larger ions could be easier to link two distant functional groups, the order of affinity between fucoidan and metals from strongest to weakest is as follows: $Pb^{2+}>Cu^{2+}>Cd^{2+}>Zn^{2+}>Ni^{2+}>Ca^{2+}>Mg^{2+}$

Coordination is a term to describe the combination of cations with molecules or anionic groups which contain free electron pairs, the coordinated compound could be electrostatic or covalent (Stumm & Morgan, 1996). The heavy metal cation could coordinate with the anions or molecules, called ligands, where cation sit on the central position and anionic groups around it. Several atoms comprised ligand, which the atom has the basic or nucleophilic nature characteristic is called a ligand atom. Over one ligand atom will form multidentate complex and have more position to coordinate. Chelation is defined as complex formation with multidentate ligand. Most metals could involve in the central part to coordination of 2, 4, 6, 8 (number is the quantity of central ligand atom). However, due to steric influence, polymers have lower numbers. (Yano et al., 1996)

Anionic sugar residues anchor to cell membranes, where they might functionate the cation regulation availability for import biological action, for example, biosynthesis and transmission of nervous impulses (Yano et al., 1996). The interactions of sugar and metal are responsible to determine the surface charge, improve various recognition and binding phenomena. Each divalent cation could coordinate three sugar anions and three water molecules in a nine-coordinate trigonal arrangement. The carboxylate and sugar rings could coordinate with cations with their sugar oxygen atoms (Yano et al., 1996).

It has been reported that heavy metal concentrations in the marine environment

are increasing annually (Nubi et al., 2011). Algae is widely spread in the ocean and has strong biosorption of heavy metals, there is always more or less heavy metal content in algae, including transition metals. Zn(II) and Cd(II) can six-coordinate with sugars and result in a coordination sphere around mercury ion with four ligating atoms. The effect of coordination, the backbone of polymers could be bent or transformed. Moreover, in some specific conditions, transition metals such as cobalt can change the conformation of sugar (Yano et al., 1996).

Chapter 3 Methodologies

3.1 Materials

Crude Fucoidan (10k Mw) was extracted from New Zealand *U. Pinnatifida*, provided by Prof. Lu (Auckland university of technology). EDTA-Na_2 is from Ajax Finechem, Amico Ultra centrifugal filters (MWCO 3kDa membrane) and Pur-A-Lyzer^{TM} Mega Dialysis Kit (MWCO 1kDa membrane) were from Sigma-Aldrich. EDTA-Na_2 was dissolved by Milli-Q water at 0.1g/mL (w/w) as solvent for fucoidan. Fucoidan (10k Mw) was separately dissolved into Milli-Q water and 10% (w/w) EDTA-Na_2 solution, made up 500mL (1mg/mL) fucoidan solution for each.

3.2 Fucoidan Pretreatment

Firstly, the 541 paper was labelled and weighed. Next, the 10kDa fucoidan solutions, EDTA-Na_2 solved fucoidan (EF) and Milli-Q solved fucoidan (MF) were passed through a buchner funnel with 541 type filter paper, the precipitate was collected separately. The filtered 10kDa fucoidan solutions were centrifuged (4000PPM) with

3kDa filters for 40mins. This step was repeated several times until no more solution passed though the filters. Over 3kDa Mw precipitate and below 3kDa Mw solutions were collected. Then, part of 3kDa solutions of EF and MF were dialyzed by Milli-Q water via Pur-A-Lyzer^{TM} Mega Dialysis Kit (1kDa MWCO membrane) for 2 days. Once every 2hours dialysate was changed, inner (>1kDa Mw EF and MF) and outer solutions (<1kDa Mw EF and MF) were collected. <1kDA EF and MF were evaporated to the origin volume before dialysis. Finally, these specimens were detected using analytical instruments (HPLC, MP-AES, LCMS, and spectrophotometer).

3.3 Detection of EDTA with HPLC

Molecular weights of EF >3kDa and >1kDa were detected using HPLC (Agilent 1200). The mobile phase were made by 70:30 (v/v) ratio of acetonitrile to ammonium acetate (both purchased from Sigma-Aldrich); flow rate was set to 0.5 mL/min; pressure was set to 205 psi; injection was 10uL; column from Merck KGaA was ZIC- HILIC 150x2.1nm, 5um, 200A; levels of standard curve were made into 25ppm, 50ppm, 100ppm, 200ppm, 800ppm EDTA-Na_2.

3.4 Solubility test

Solubility of crude fucoidan (EF and MF)

The 541 paper with 10kDa fucoidan precipitate were freeze dried then weighed, the 541 paper's weight was subtracted from the total weight in order to calculate the final fucoidan weigh and the solubility of the 10kDa crude fucoidan extract.

Solubility of over 3kDa Mw EF and MF

The precipitates collected over 3kDa were freeze dried for 2 days to obtain completely dried samples. Several 10mg samples were prepared and dissolved by 5 numbers of decreasing volumes of Milli-Q till sample stopped dissolving.

3.5 Metal detection with Microwave plasma – atomic emission spectroscopy (MP-AES)

10mg of each sample of processed EDTA, including 10kDa EF and MF; >3kDa EF and MF, <3kDa EF and MF, >1kDa EF and MF, <1kDa EF and MF, were digested with 68% nitric acid (from Fisher Scientific) via microwave digestion for 40mins, set at 200°C. A stock solution of 100ppm metal ion solution (purchased from Sigma-Aldrich), $HgCl_2$ powder and 1000 ppm arsenic solution (both purchased from Sigma-Aldrich) was made. The complex metal ion solution contained Ca^{2+}, Pb^{2+}, Si^{2+}, Cd^{2+}, Cr^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Zn^{2+}, Al^{3+}. Standard solutions were made to 0.05ppm, 0.1ppm, 0.5ppm, 1ppm, 5ppm, 10ppm. All samples were diluted to 25mL with 2% nitric acid. The MP-AES machine (Agilent 4200) was used to detect the above-mentioned metal content from 5 different fraction classes (10kDa, >3kDa, <3kDa, >1kDa, <1kDa) 2 different solvent fucoidan (MF and EF).

3.6 Analysis of single sugar content with LCMS

First, 10mg of each sample of processed EDTA, including 10kDa EF and MF; >3kDa EF and MF, <3kDa EF and MF, >1kDa EF and MF, <1kDa EF and MF, were hydrolyzed by 10mL 4mol/L TFA, covered with nitrogen and heated at 110°C for 2hrs. Next samples were neutralized by 10mL 4mol/L NaOH, pH was adjusted to pH 7 and volumized to 25mL. Then, 1mL of the sample was combined with 1mL of 0.3 mol/L

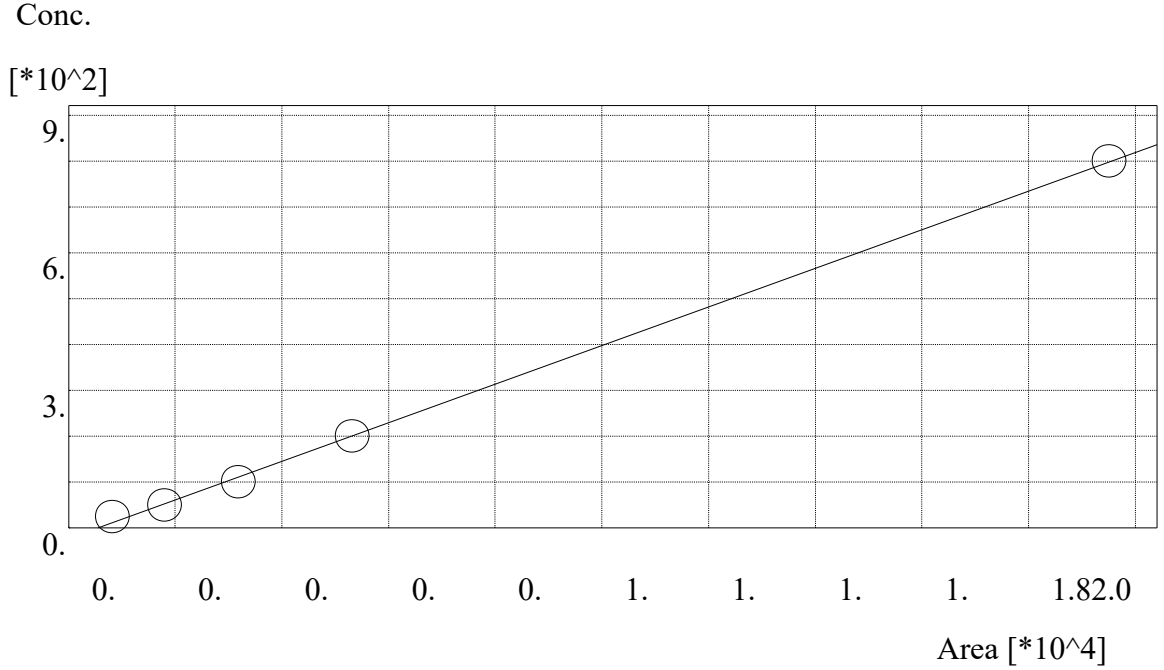
NaOH and vortexed. 1mL of 0.5 mol/L PMP methanol solution was then added and the mixture was incubated in a water bath at 70°C for 70mins. After cooling down, 1mL 0.3mol/L acetate acid was added to neutralize pH, and volumized to 10mL with KH_2PO_4. 1mL of the hydrolyzed samples were processed three times in chloroform. Meanwhile, the standard curve was created by diluting galactose, xylose, mannose, glucose, glucuronic acid, fucose, arabinose and rhamnose to 1ug/mL, 5ug/mL, 10ug/mL, 50 ug/mL, 100ug/mL and 200ug/mL. The standard solutions were derivatized by the same steps as fucoidan samples. Finally, the samples were detected by LCMS (Agilent 6420). The column used was the ZIC- HILIC 150x2.1nm, 5µm, 200A. All chemicals were purchased from Sigma-Aldrich.

3.7 Determination of total sugar content with Spectrophotometer

Standard solutions were made using pure fucose. The standard curve concentrations used were 0ug/mL, 10ug/mL, 20ug/mL, 50ug/mL, 100ug/mL and 200ug/mL. 1mL of 50g/L phenol was added into 10mg of 10kDa EF and MF and >3kDa EF and MF standard solutions. Immediately following this concentrated sulfuric acid was added, vortexed and left to stand for 20mins. The samples absorbance was detected using the spectrophotometer at a 490nm wavelength.

Chapter 4 Results

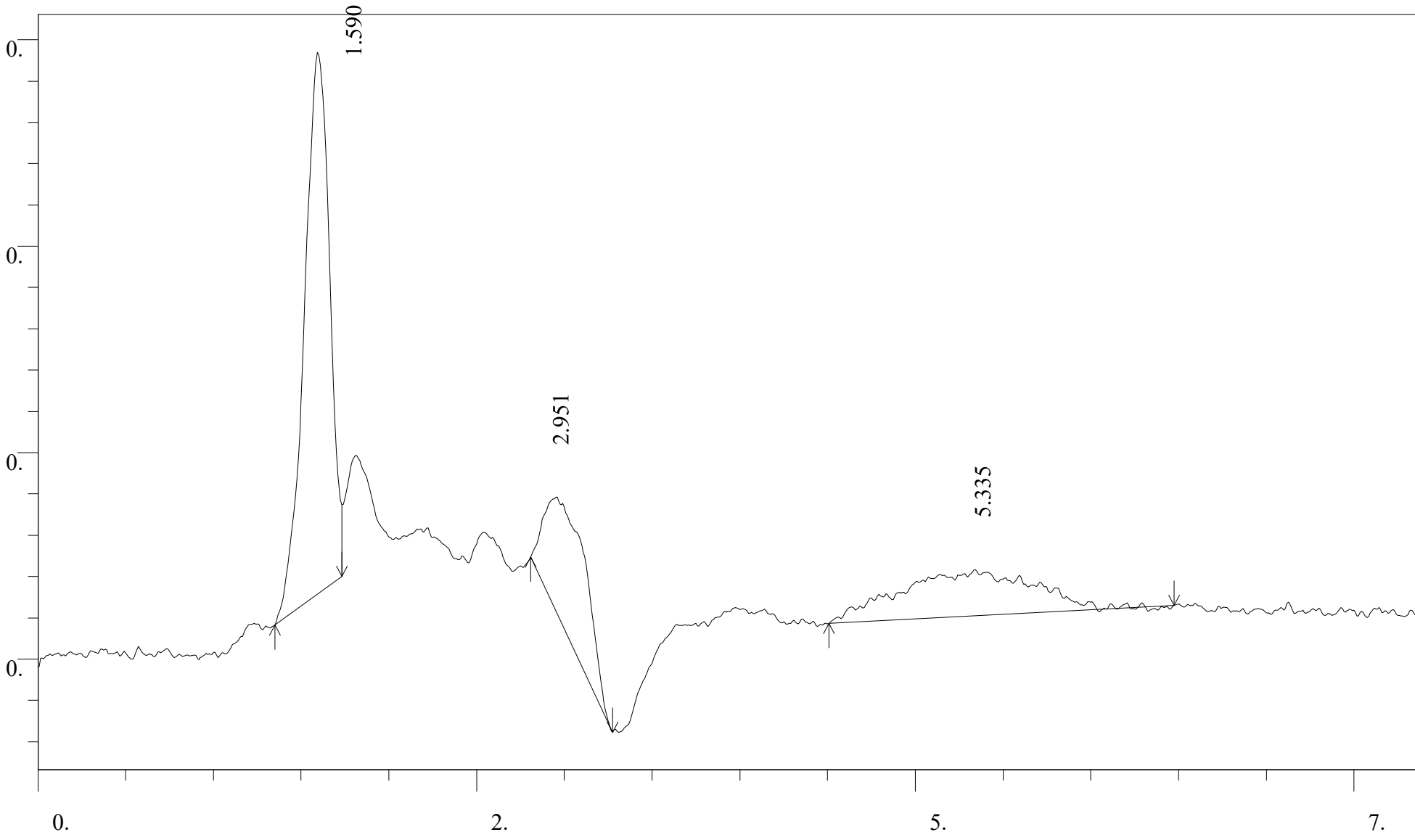
4.1 EDTA detection of HPLC



Rr1=0.9996361 Rr2=0.9992723

Table 1. Standard curve of EDTA-Na_2

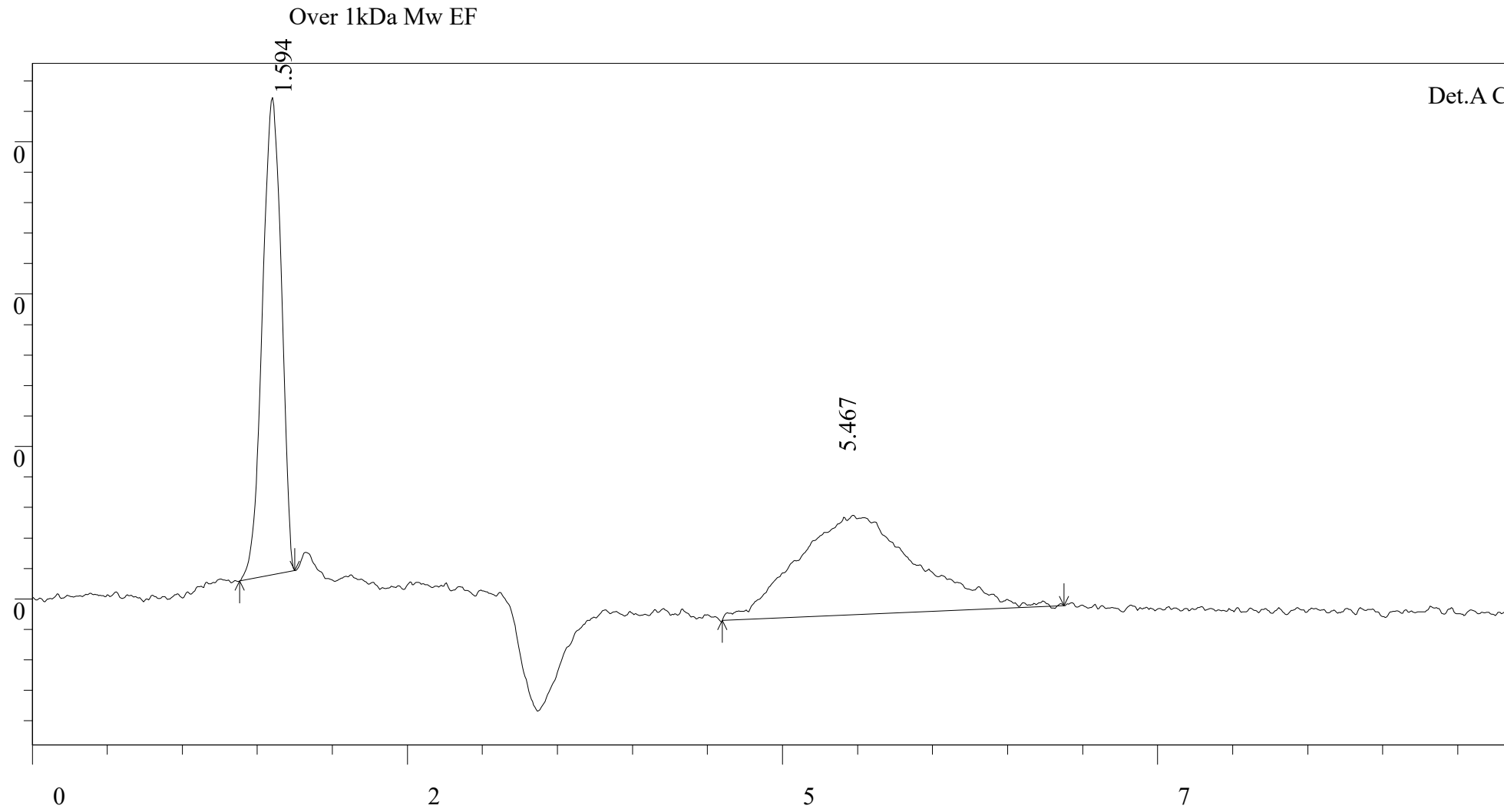
Over 3kDa Mw EF


1.590
2.951
5.335
0.
0.
0.
0.
0.
2.
5.
7.

1 Det.A Ch1/265nm

ID#	Name	Ret. Time	Area	Height	Conc.
1	EDTA Fucoidan	5.335	1168	22	25.944

Table 2. Peak and duration time of over 3kDa Mw EF


Over 1kDa Mw EF
1.594
5.467
Det.A C
0
0
0
0
0
2
5
7

1 Det.A Ch1/265nm

ID#	Name	Ret. Time	Area	Height	Conc.
1	EDTA Fucoidan	5.467	3618	65	129.106

Table 3. Peak and duration time of over 1kDa Mw EF

The standard curve of EDTA-Na_2 is fitted as table 1., the R^2 is over 0.999.

EDTA-Na_2 was used to chelate the metal ion in fucoidan, which molecular weight was below 1kDa Mw, hence, the chelation should have passed over the dialysis membrane easily. Testing EDTA content by HPLC was to make sure there was not too much EDTA left after degradation so that when testing the metal removal efficacy of EDTA, it would not be influenced by EDTA-metal ions.

EDTA-Na_2 signal peak is around 5.4mins, the over 3kDa Mw EF contained 25.944ppm EDTA-Na_2 and over 1kDa Mw EF had 129.106ppm EDTA-Na_2. EDTA can 1:1 ratio combine metal ion, the Mw will not go over 1kDa, hence, the EDTA complex will easily pass over each class filter membrane. The original EDTA, concentration 10% w/w, had been mostly removed by filters, filtering rate is over 99.99%.

The deep freeze drying would remain 1%-5% crystal water compound and that may keep few EDTA-Na_2. The efficacy of filter membrane was acceptable.

4.2 Solubility test

	Fucoidan 10%EDTA base	Fucoidan MilliQ base	Difference
Molecular Weight	10K	10K	EDTA - Non-EDTA
Solubility	98.20%	90.45%	7.75%
Molecular Weight	>3K	>3K	
Solubility	200mg/mL	200mg/mL	Same

Table 4. Solubility of 10kDa and over 3kDa fucoidan

The results showed that while 0.1g fucoidan was dissolved in Milli-Q, it was not completely soluble (Table 4). Fucoidan dissolved by 10% EDTA-Na_2 had less suspended matter. The 541-type paper was weighed, paper for EF was 13.3827g and freeze-dried EF with paper was 13.3845g. Filter paper for MF was 13.0975g and freeze-dried MF with paper was 13.107g. The solubility% was calculated as follows:

$(W_{F\text{-}P}\text{-}W_p)/W_{0.1}$ *100%

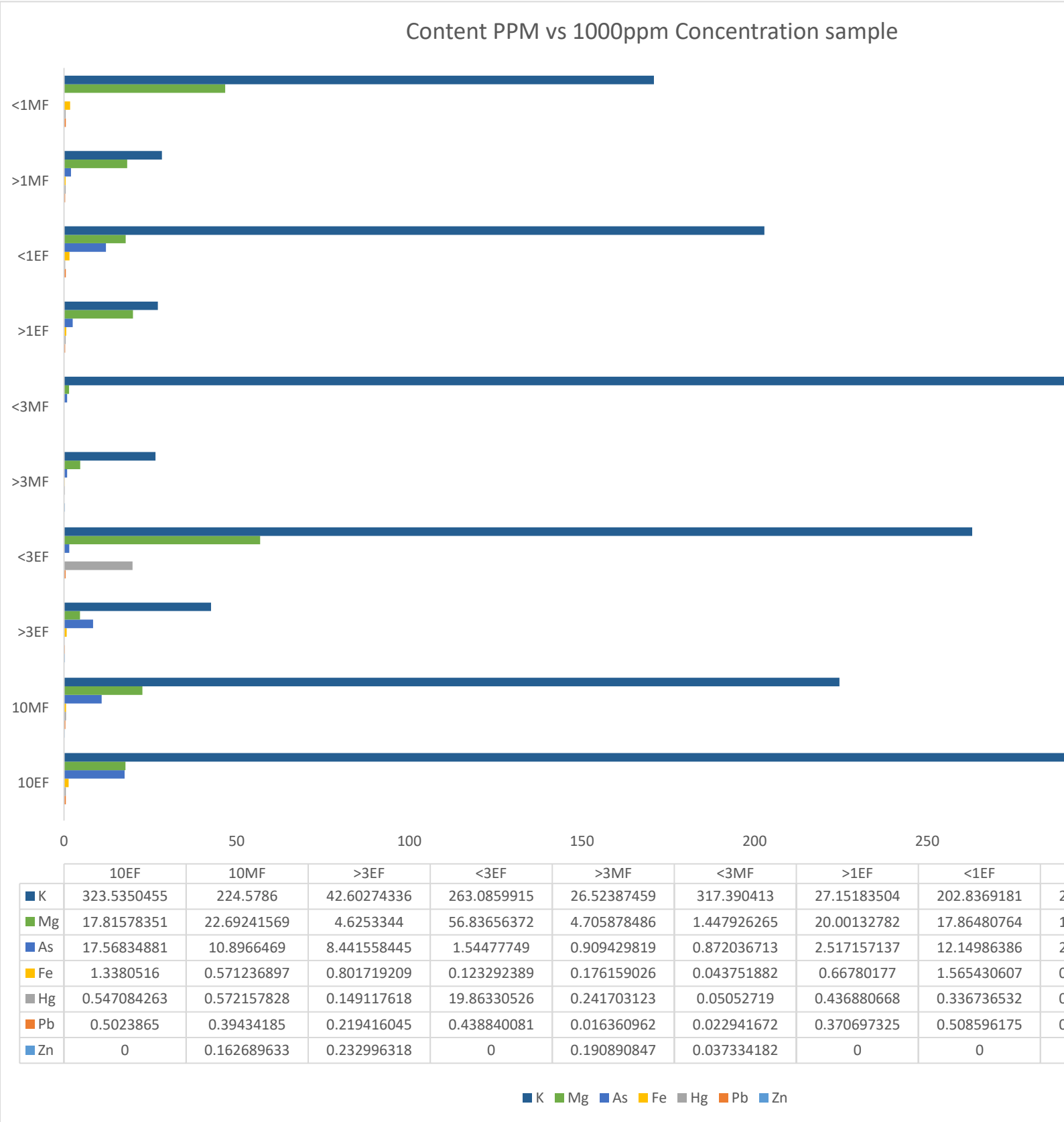
At room temperature 25℃, pH=7, 10kDa Mw Fucoidan processed by 10% EDTA-Na_2 had 98.2% solubility, while pure Milli-Q base Fucoidan had only 90.45%, there was 7.75% difference between 2 bases (Table 4).

At room temperature 25 ℃, pH=7, decreasing volume of Milli-Q were used to dissolve 100mg 3kDa Mw dry EF and MF gradually. The volume sequence was 2000uL, 1500uL, 1000uL, 500uL, 400uL. When the volume was less than 500uL, fucoidan was not completely dissolved. Both fucoidan specimens were tested and solubility were roughly

same.

Polysaccharides consisting of 10-unit monosaccharide, below 3kDa fraction mostly consisted of the monosaccharides, fucose, xylose, mannose, arabinose, rhamnose, galactose, glucuronic acid and glucose. This research mainly focused on fucoidan polymers, hence, the single sugar or oligo-sugar component is meaningless to detect in this research.

4.3 Metal detection class fucoidan

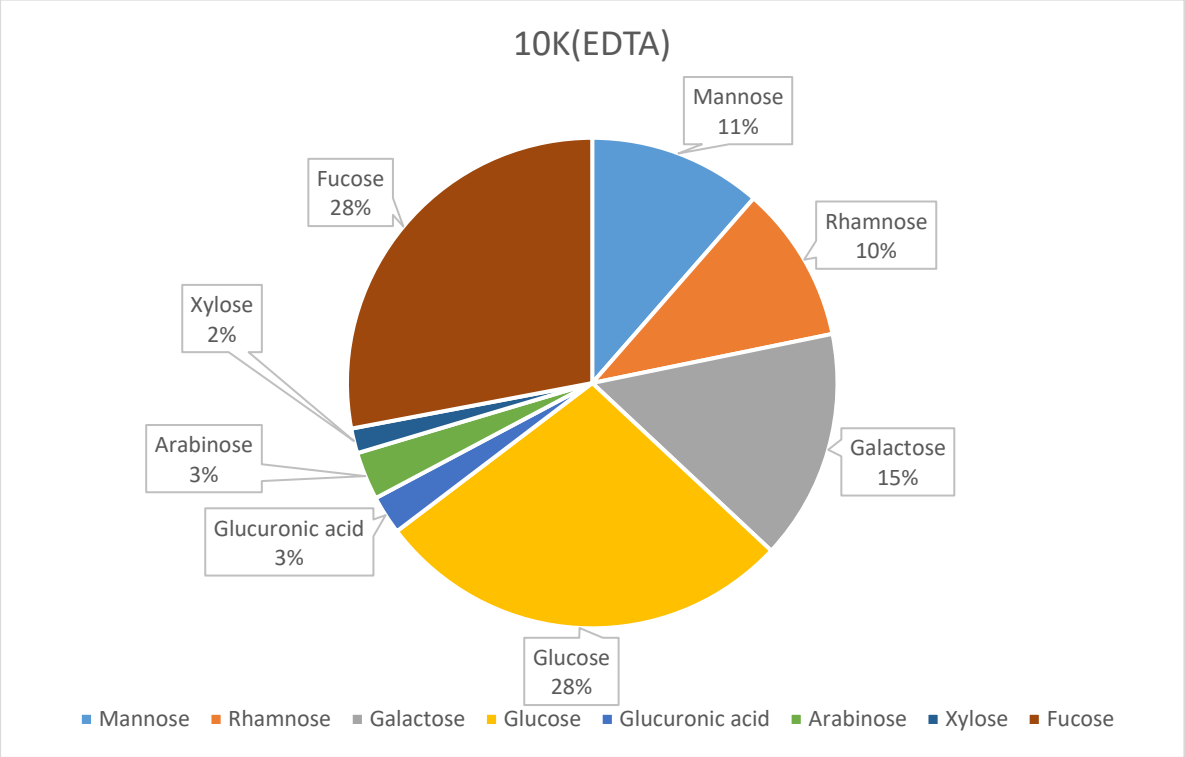


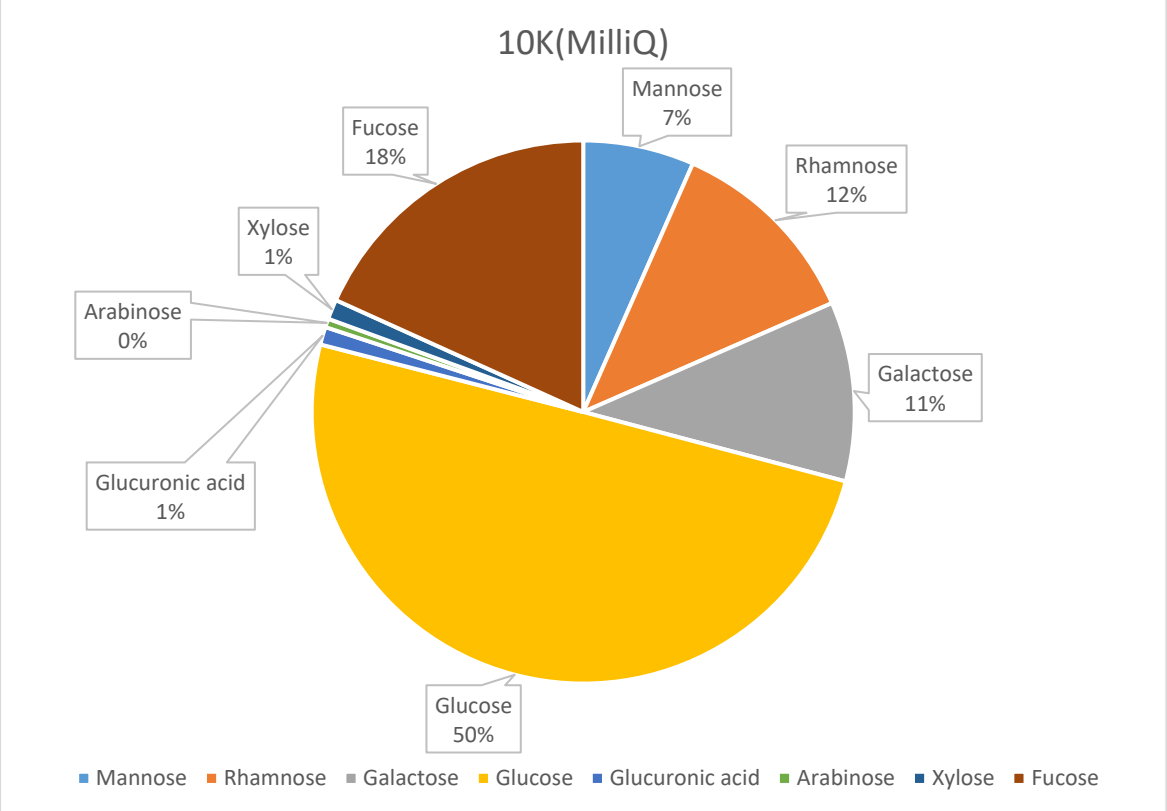
	10EF	10MF	>3EF	<3EF	>3MF	<3MF	>1EF	<1EF
K	323.5350455	224.5786	42.60274336	263.0859915	26.52387459	317.390413	27.15183504	202.8369181
Mg	17.81578351	22.69241569	4.6253344	56.83656372	4.705878486	1.447926265	20.00132782	17.86480764
As	17.56834881	10.8966469	8.441558445	1.54477749	0.909429819	0.872036713	2.517157137	12.14986386
Fe	1.3380516	0.571236897	0.801719209	0.123292389	0.176159026	0.043751882	0.66780177	1.565430607
Hg	0.547084263	0.572157828	0.149117618	19.86330526	0.241703123	0.05052719	0.436880668	0.336736532
Pb	0.5023865	0.39434185	0.219416045	0.438840081	0.016360962	0.022941672	0.370697325	0.508596175
Zn	0	0.162689633	0.232996318	0	0.190890847	0.037334182	0	0

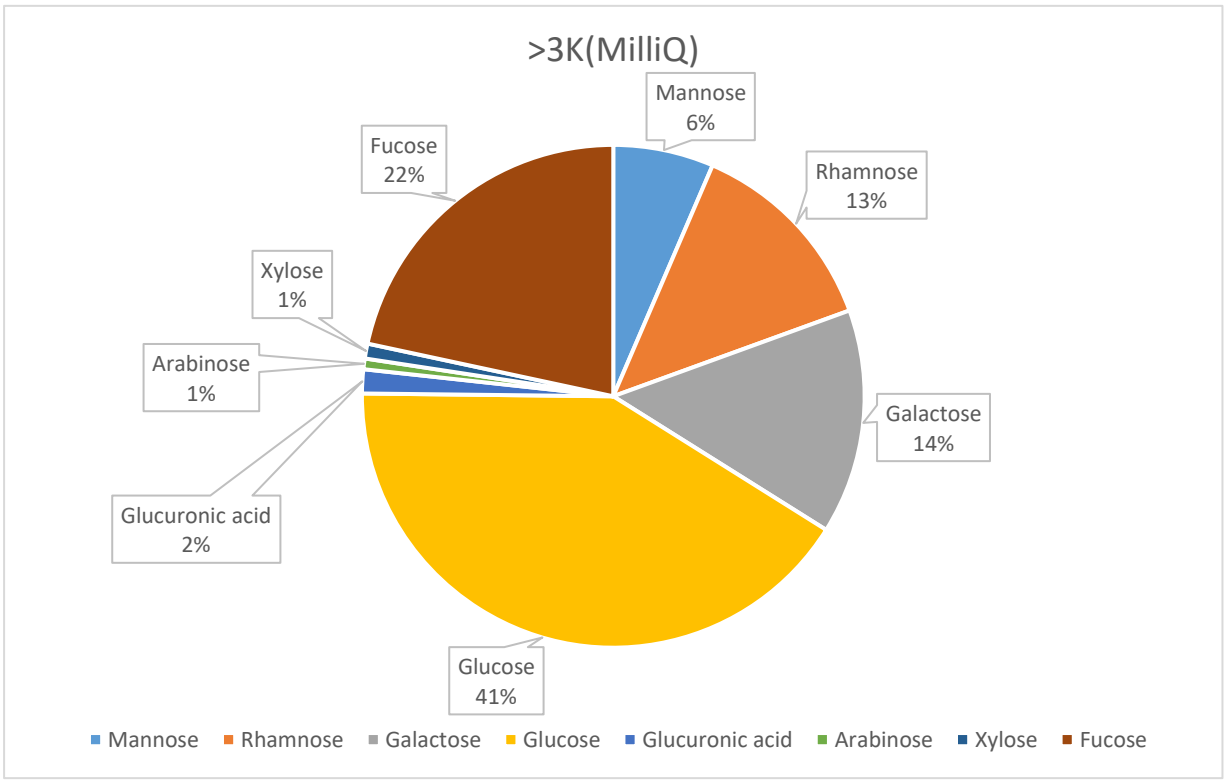
Table 5. Content of metal ion contents of each

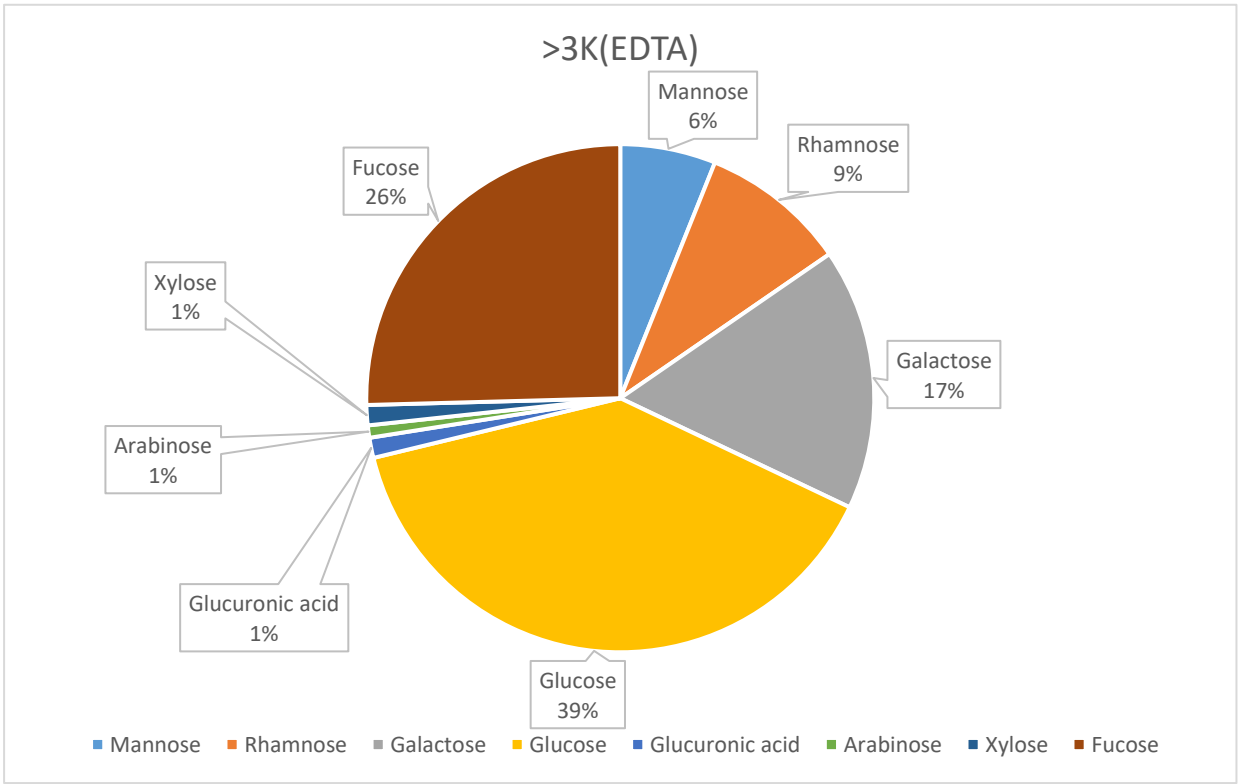
Each class of EF and MF were detected by MP-AES. Fucoidan concentration was uniformly converted to 1000ppm to make observation clearer. All the data was posted on the above list (Table 5). The table only listed the existing metal in my sample. The other metals are not existing or extremely low to be detected. There were 13 elements detected including Cd, Cr, Cu, Ca and Si, these are the most frequent metals in fucoidan reported by other articles. Potassium ion occupied the biggest portion of total ions in its class. In the 10kDa class of EDTA-Na_2 processed fucoidan, potassium ion resulted in 323.5 ppm per 1000ppm solution while MF had only about 224.6 ppm, revealing a 99 ppm difference. The other metals in 10kDa class, EF and MF had similar content of zinc, lead, and mercury ion, which were at a relatively low levels when compared to arsenic and magnesium which were over 10 ppm. Meanwhile, the crude EF contained much more arsenic than MF, 17.57ppm versus 10.90ppm, and with fucoidan degrading, arsenic content of EF was relevantly higher than MF in each class. While below 3kDa class, in both EF and MF, zinc was almost or completely filtered out. Compared among centrifuge solutions, >3kDa and <3kDa; >1kDa and <1kDa, content of K^+ in the upper matters (unpassed the membrane) was significantly lower than underlayer solution (passed the membrane) , most of other metal contents had the same condition as well, except iron in 3kDa fractions and mercury in 1kDa fractions. Moreover, arsenic did not exist in <1kDa MF solution.

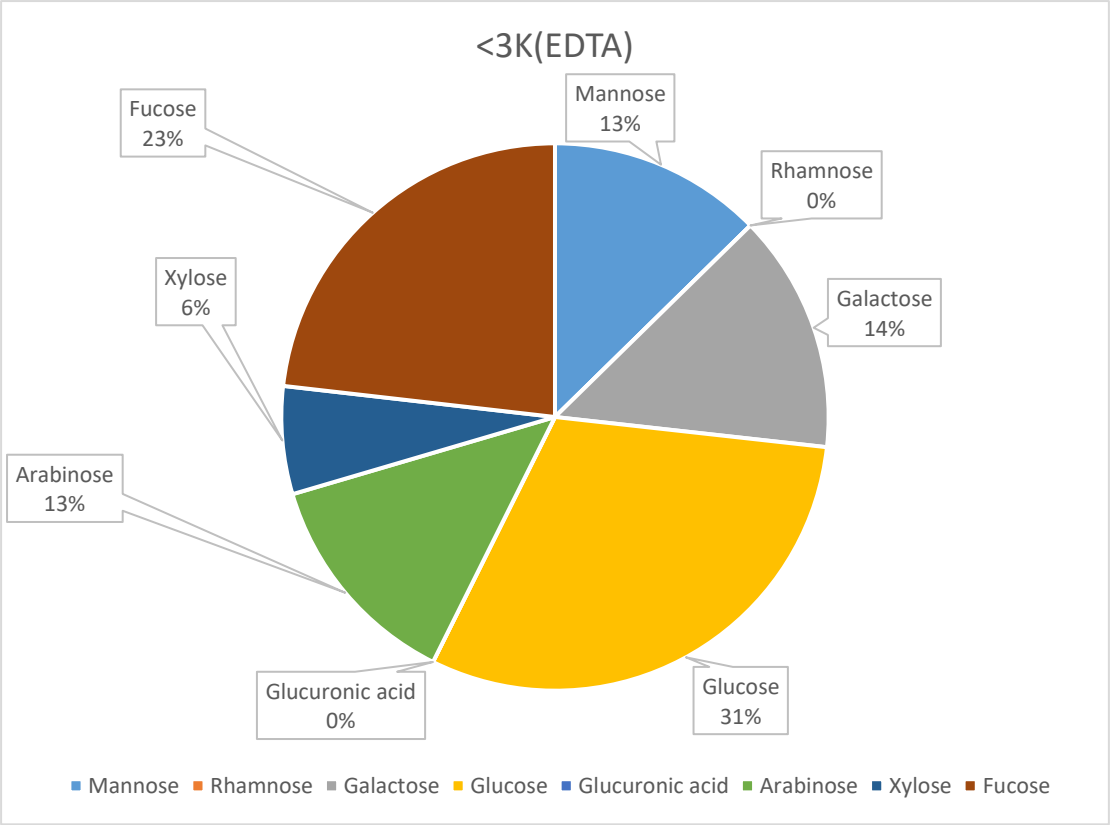
4.4 Single sugar content

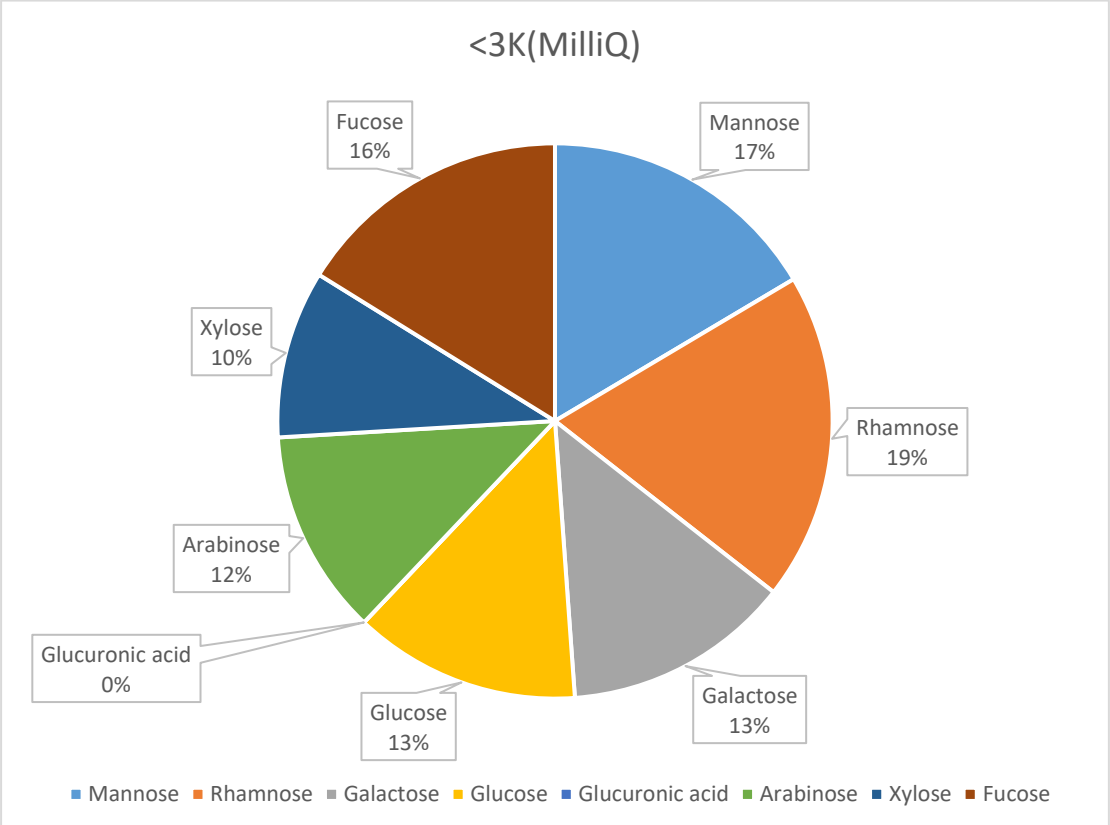


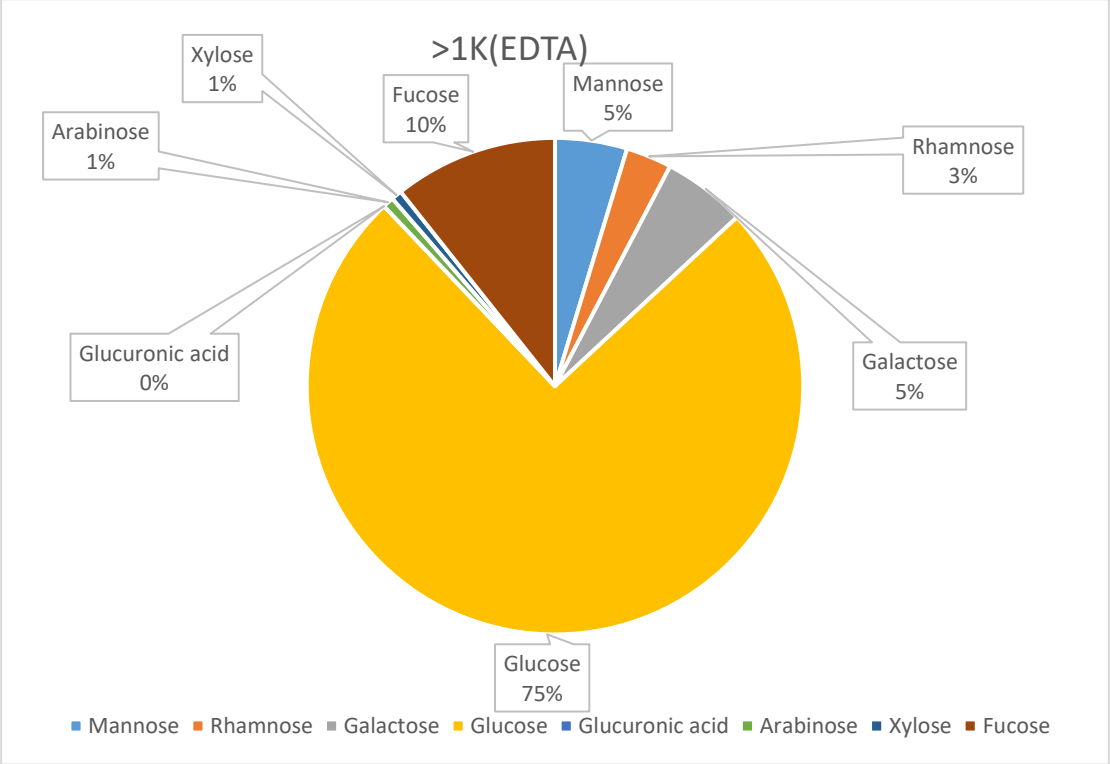


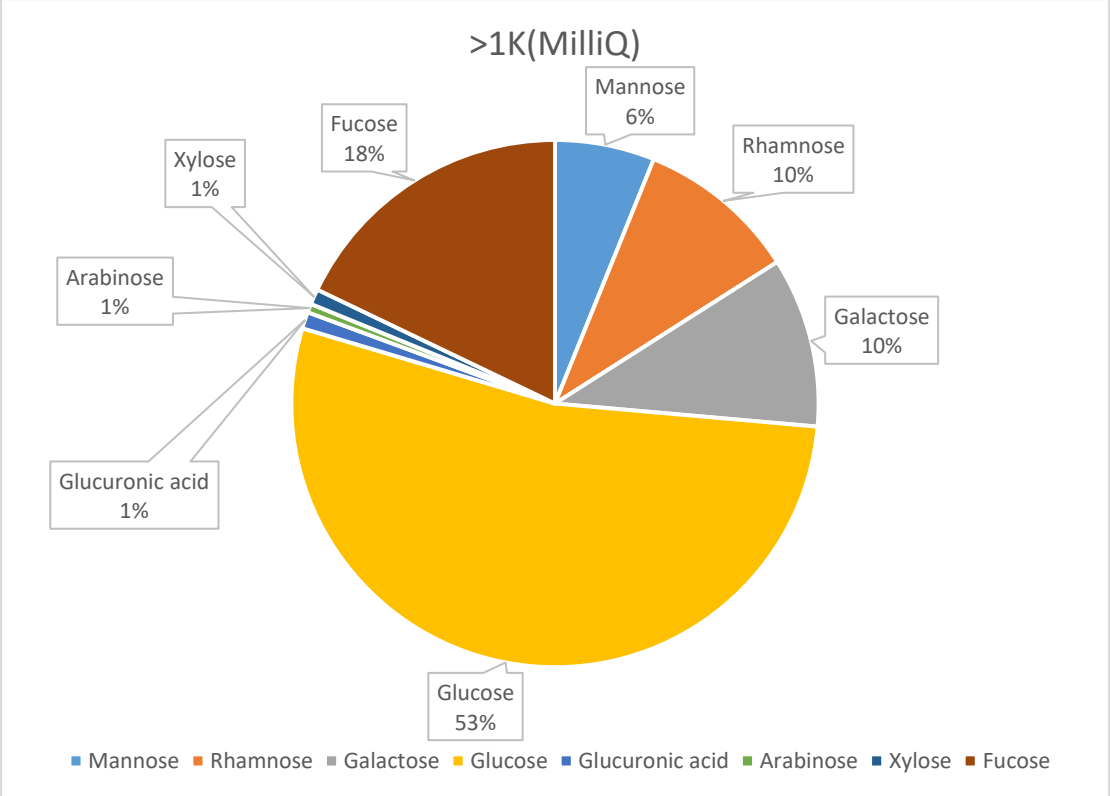

>3K(MilliQ)
Mannose 6%
Rhamnose 13%
Galactose 14%
Glucose 41%
Glucuronic acid 2%
Arabinose 1%
Xylose 1%
Fucose 22%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose

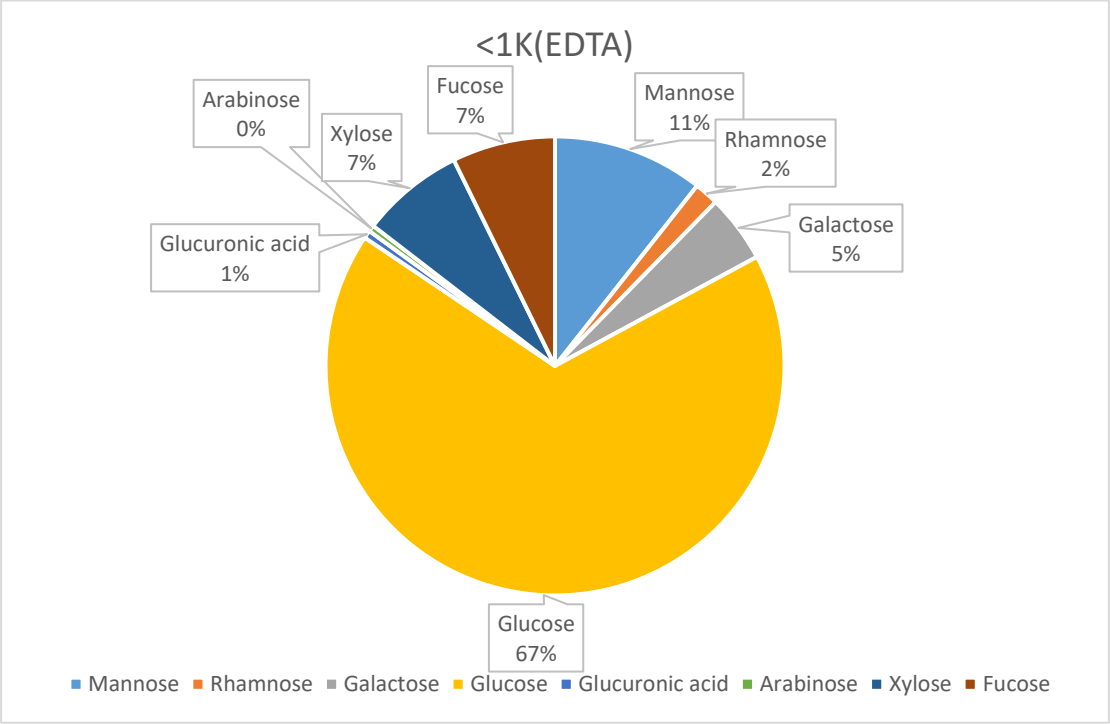

>3K(EDTA)
Mannose 6%
Rhamnose 9%
Galactose 17%
Glucose 39%
Glucuronic acid 1%
Arabinose 1%
Xylose 1%
Fucose 26%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose


<3K(EDTA)
Mannose 13%
Rhamnose 0%
Galactose 14%
Glucose 31%
Glucuronic acid 0%
Arabinose 13%
Xylose 6%
Fucose 23%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose


<3K(MilliQ)
Mannose 17%
Rhamnose 19%
Galactose 13%
Glucose 13%
Glucuronic acid 0%
Arabinose 12%
Xylose 10%
Fucose 16%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose


>1K(EDTA)
Xylose 1%
Fucose 10%
Mannose 5%
Arabinose 1%
Rhamnose 3%
Glucuronic acid 0%
Galactose 5%
Glucose 75%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose


>1K(MilliQ)
Mannose 6%
Fucose 18%
Rhamnose 10%
Xylose 1%
Arabinose 1%
Galactose 10%
Glucuronic acid 1%
Glucose 53%
Mannose
Rhamnose
Galactose
Glucose
Glucuronic acid
Arabinose
Xylose
Fucose


<1K(EDTA)
Arabinose 0%
Fucose 7%
Mannose 11%
Xylose 7%
Rhamnose 2%
Galactose 5%
Glucuronic acid 1%
Glucose 67%
Mannose Rhamnose Galactose Glucose Glucuronic acid Arabinose Xylose Fucose

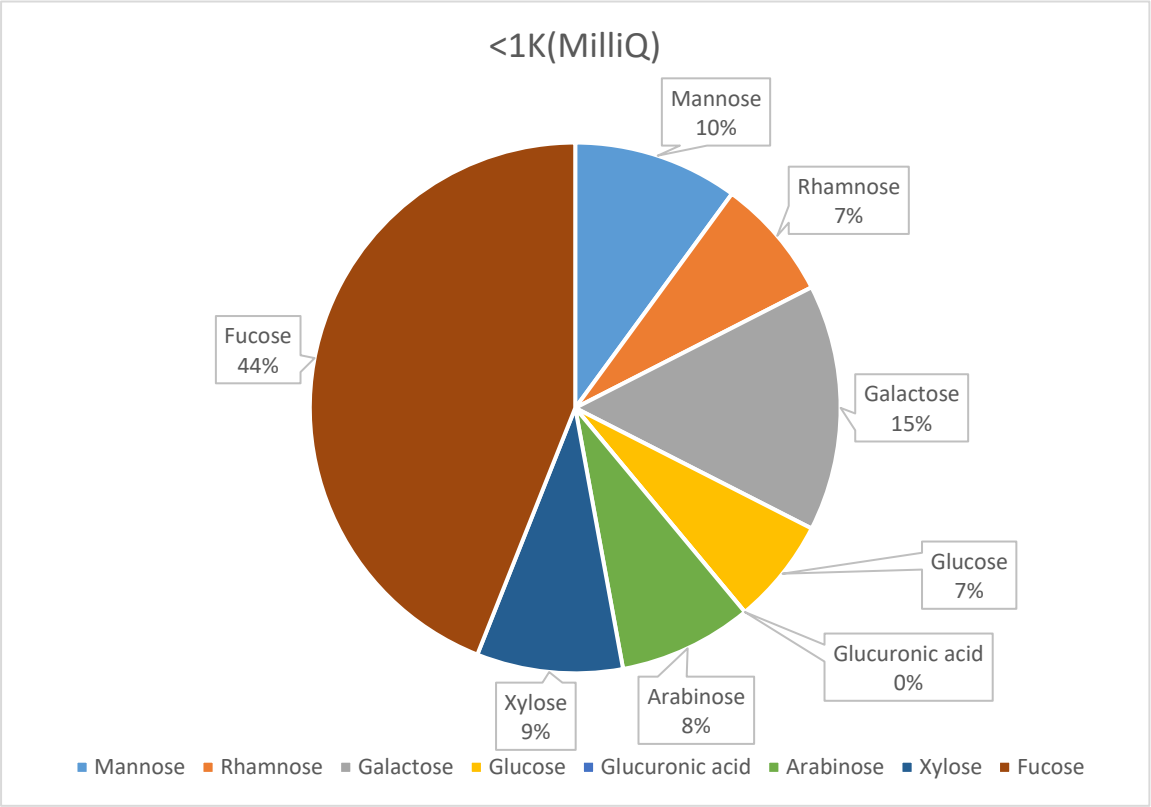

<1K(MilliQ)
Mannose 10%
Rhamnose 7%
Fucose 44%
Galactose 15%
Glucose 7%
Glucuronic acid 0%
Xylose 9%
Arabinose 8%
Mannose Rhamnose Galactose Glucose Glucuronic acid Arabinose Xylose Fucose

Table 6. Total amount of 8 monosaccharide of fucoidan (mannose, xylose, fucose, glucuronic acid, galactose, glucose, rhamnose and arabinose)

Molecular Weight	EF/10K	MF/10K
Single Sugar Amount in 1000PPM Total Solution (PPM)	1.15ppm	3.02ppm
Molecular Weight	>3K	>3K
Single Sugar Amount in 1000PPM Total Solution (PPM)	40.43ppm	55.31ppm
Molecular Weight	<3K	<3K
Single Sugar Amount in 1000PPM Total Solution (PPM)	0.072ppm	0.108ppm
Molecular Weight	>1K	>1K
Single Sugar Amount in 1000PPM Total Solution (PPM)	4.50ppm	3.01ppm
Molecular Weight	<1K	<1K
Single Sugar Amount in 1000PPM Total Solution (PPM)	7.999ppm	0.145ppm

The standard solution was duplicated to make stand curve. The occupation percentage was posted on the above of pie chat and table. The origin fucoidan (10kDa MF) consisted of 50% Glucose, 18% fucose, 12% Rhamnose, 11% galactose, 7% Mannose, 1% xylose and 1% Glucuronic acid. After treatment with EDTA-Na_2, the major change was that arabinose occurred, 3%; fucose increased by 10% and glucose dropped by 22% percent, the other monosaccharides concentrations only changed a little. After degradation, the upper fucoidan fraction (>3kDa) of EF and MF had the same proportion order. The content of glucose was the highest, followed by fucose, then Galactose, Rhamnose and Mannose, the other monosaccharides only represented a small percentage. Below 3kDa Mw fraction, EF mainly contained 31% glucose and 23% fucose. MF was more equally distributed (~15%) and was comprised of all the expected sugars except glucuronic acid. In low Mw fractions, glucose was a major component of the total extraction, exhibiting over 50%. The <1kDa MF was an exception as it exhibited high fucose content of 44% instead of glucose which only represent 7% of the sample. In addition, glucuronic acid returned a very low proportion around 0%~1%.

4.5 Total sugar content

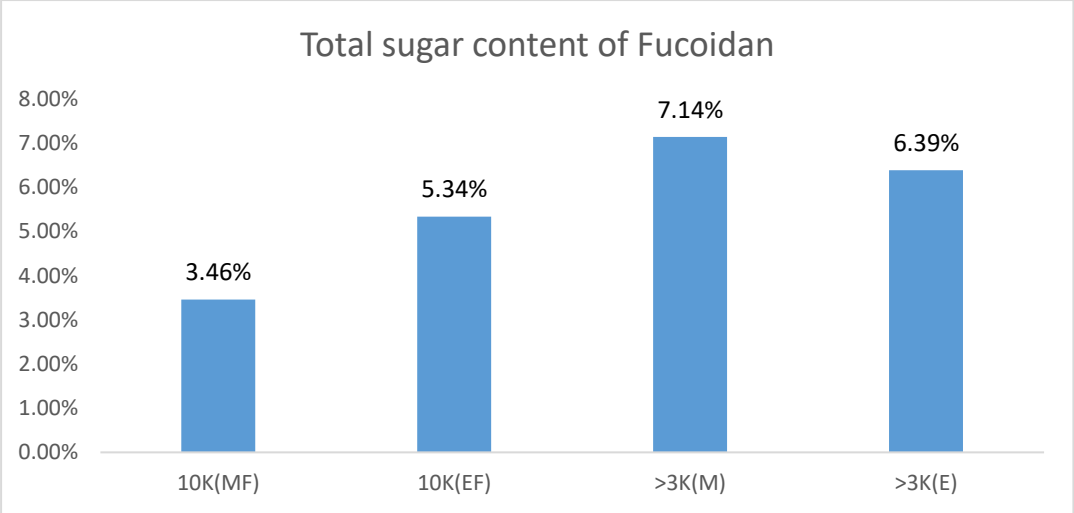


Table 7. total sugar content of 10kDa and 3kDa fucoidan

Fucoidan can be hydrolyzed in acidic conditions and combined with phenols by addition of concentrated sulfuric acid. The fucoidan/phenol derivates are yellow and the absorbance can be detected at 490nm using a spectrophotometer. The total sugar content in crude EF was higher than MF. However, after degrading by treatment with EDTA-Na_2, the upper matter of EF (>3kDa) had lower content than MF (Table 7).

Chapter 5 Discussion

Compared with the previous study on content of *U. pinnatifida* species fucoidan, Mak et al 2013 pointed out that the major composition of fucoidan in each Mw fraction from (171kDa to 22kDa) was fucose, yet the content of fucose did not correlate with the change in Mw. In this study, glucose was the main monosaccharide in 10kDa original fucoidan before processed by EDTA-Na_2. The difference in monosaccharide compositions might be caused by different extraction methods, that would also explain the reason for the solubility of this fucoidan.

Crude fucoidan processed by EDTA-Na_2 showed higher solubility than Milli-Q water base fucoidan, which meant that EDTA-Na_2 was responsible for redissolving the precipitate. Meanwhile, the total content of metal and arsenic increased, especially the content of potassium. The types of monosaccharides present also changed, fucose and galactose increased, whereas glucose decreased. It was indicated that the main component of the insoluble fraction of crude fucoidan was fucose, galactose, arsenic and potassium ion. As mentioned before, the *U. pinnatifida* contained complex of fucose and galactose, which as galactofucan may form separate blocks and intersperse in one polymer backbone (Yang et al., 2018). Meanwhile, in early study, Smidsrod (1967) indicated that divalent metals form gels with alginate and potassium ions which could form gels with carrageenan. They also explained that sulfated groups in polysaccharides had strong binding affinities with metals and a high degree of ion pair formation. This would decrease the effective charge on the polymer and decrease solubility. As fucoidan is extracted from a brown seaweed and is a typical sulfated polysaccharide, it may have the same mechanism of gel formation.

After detecting the metal content and component of crude fucoidan, compared with EF and MF, the increased solubility by EDTA-Na_2 may indicate that in 10kDa Mw Milli-Q base fucoidan, there might exist galactofucan gel with potassium which prevent the

dissolution from water.

Processed fucoidan contained more potassium and its monosaccharide content changed. Although EDTA-Na_2 would not be able to chelate with alkaline metals, it would indirectly dilute the degree of ion pair formation within the polysaccharide. This might lead to break the gel balance, allowing precipitates to redissolve. Inorganic arsenic ion was normally found in algae cells, strongly bound with proteins and polysaccharides (Shigeru et al., 1987). The higher arsenic content might explain why fucoidan exhibited a higher Mw. With degradation of fucoidan, less polysaccharide presented, instead, the polymer cleaved to monosaccharide and no affinity to arsenic anymore. The arsenic content was found mostly in the 3kDa and 10kDa fucoidan samples.

EDTA-Na_2 exerted great effectiveness in chelating mercury and magnesium because of its characteristic to chelate divalent metal ions. The centrifuged fucoidan below 3kDa combined with the EF solution contained huge amount of mercury and magnesium metals. Alkali metals can co-crystallize with sugars (Yano and Otsuka, 1996), hence I predicted that the conification shape would be generated after freeze drying, which might interfere the solubility of fucoidan. However, this interference did not happen, conversely the >3kDa freeze dried fucoidan fraction of MF was easier to redissolve. It seems that the compound of 3D configuration in fucoidan structure was very weak to inhibit dissolving.

Compared with all fraction fucoidans processed by EDTA-Na2 and Milli-Q water, the sugar component of each fucoidan sample had certain content differences. Transition metal ions like zinc and mercury could interact with sugars for example, Zn(II) are six-coordinated, binding to two arabinose moieties on O(3), O(4) of the first and O(1), O(5) of the second sugar molecule as well as to two H_2O molecules. Mercury(II) can bind to two sugar molecules in a similar way as Zinc which lead to a coordination number of four. The same phenomena would occur on glucose as well, Zinc would bind to one D-glucose molecule, possibly via the 1-OH and 2-OH groups with 4 H_2O molecules, to get a six-coordinate metal cation. While mercury had the same condition, as sulfate

groups was anion charge, they could possibly have interaction to form compounds (Yano and Otsuka, 1996). For fucoidan, the coordination would result in metals remaining into fucoidan structure and would not pass through the membrane. As EDTA-Na_2 has strong affinity to coordinate the transition metal ions, there would be a competition between EDTA coordination of transition metal ions and transition metal ions coordination of the monosaccharides. As shown in Table 3, there was 15ppm difference in unit (Table 5). On one hand, over 3kDa EF contained less monosaccharides than the same class of MF. Indicating that MF may bind more free monosaccharides or its residues around the backbone to form over 3kDa polymers. On the other hand, the affinity of EDTA was stronger than fucoidan polymer and diminished the activity of transition metals, which inhibited the interaction of metal and polysaccharide. In addition, arsenic also has good adsorption of cations (Yoshitake, 2003), the final fraction of MF contained no arsenic and little free monosaccharides ~0.145ppm per unit. The >3kDa MF samples contained roughly equal arsenic amount to <3kDa MF's, with the monosaccharide total amount below 1kDa MF which was very low in comparison (Table 5). This could be in response to the interaction between metal and sugar. The metal activity was diminished by EDTA, hence, the content of arsenic existed in every class of EF and more free monosaccharide or maybe residue groups.

It is well known that polysaccharides over 10 units of sugar have a Mw that is normally over 1.5kDa. Hence, the portion over 1kDa is mix of polysaccharide and smaller substances and below 1kDa represents main of mono-, di-, oligo- saccharides and smaller residue groups instead of an entire fucoidan polysaccharide structure hereby the discussion for this part was less meaning for structure and content of application of fucoidan.

Interestingly, the difference in composition between the two fucoidan treatments fucoidan was unexpected. The reason of the difference could not only about the above interpretation. As cyclic sugars have free reducing group could react with primary and secondary amines under mild circumstance to generate N- glycosides, this is regarded

as the first step of Millard reaction. 1-phenyl-3-methyl-5-pyrazolone a derivatization agent and the primary amines derivative, can react with sugars become to N-glycoside ligands. In previous studies, the observed phenomena were that transition metals, such as cobalt, N-glycoside-coordinated cobalt could induce chiral inversion around a metal center by interaction of sugars and tetrahedral oxo anions, such as SO_4^{2-} and PO_4^{3-} (Ansell, 1982). As the marine environment is getting more and more contaminated and large amounts of heavy metals are being released into ocean and adsorbed by algae, there possibility that this kind of sugar reconfiguration is occurring, disrupting fucoidan structure and interfering with experimental detection.

Design of this experiment is improvable, after deeper studying, more analysis could be applied . The metal detection range could be enlarged to include more metals, to reflect the current condition of marine contamination. Desulphated fucoidan and well-characterized fucoidan samples could be added for comparison to further validate our findings. According to the interaction of sugar, metal and anionic groups, EDTA-salt could be changed to inert EDTA or benzene-EDTA which would introduce further metal salts and bias. Further investigation using wider methodologies could be applied, like NMR detection, zeta potential (ZP) experiments and HMQC. Which, NMR could detect the known and unknown compounds, as well as the molecular conformation; ZP could help to compare the stability of EDTA-metal coordination and polysaccharide-metal coordination, to further determine the affinity strength of EDTA and polysaccharide; HMQC is an advanced NMR-related analysis, it could show the clear relationship of directly connected hydro-carbon compound. Take advantage of more and wide analytical methods, the interaction of metal and fucoidan could be interpreted more convincing.

The organic system is very complicated sugars, anionic groups and metals can form known and unknown complexes. Even now, the chemistry of metal-sugar interactions are relatively unexplored in many aspects. However, it is certain that metals participate in biological and chemical activities. The is an issue that confused researchers for

several decades, is that fucoidan is a heterogeneous polysaccharide and a single factor can change the composition of fucoidan, especially the extraction method. Here, removal of metals from fucoidan before extraction might be helpful to reduce the disparity among fucoidans. However, this study requires further investigation which would yield essential and meaningful results.

Chapter 6 Conclusion

Fucoidan samples have metal relevant sediment. EDTA as an economical product that has been widely used in many fields. In this research, EDTA-Na_2 exhibited the function to solubilize the sediment, it also showed good efficacy of removal metals, like transition metals and heavy metals, in fucoidan. Meanwhile, as marine product, fucoidan might contain wide range of metals which could bend, injure, or transform the structure of fucoidan. EDTA could act as a protective agent to diminish metallic activity. Without interaction with metal, fucoidan structure could show less disparity allowing scientists to resolve some of the controversies.

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