UiT The Arctic University of Norway

Norwegian College of Fishery Science, the Faculty of Bioscience, Fisheries and Economics

Chemical and Enzyme-Assisted Extraction of Fucoidan from two Species of Brown Macroalgae (*Ascophyllum nodosum* and *Saccharina latissima*)

Ingrid Maribu

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Abstract

Marine environments contain half of the global biodiversity and are a major source of bioactive compounds. Macroalgae are vital species in marine ecosystems and are known to produce several bioactive compounds and polysaccharides that possesses several bioactivities. These molecules are traditionally extracted using methods that heavily rely on hazardous solvents and heat, that more importantly generates waste and emissions. Sustainability has gained worldwide attention and has led scientists to explore more environmentally friendly extraction methods that have a lower environmental impact. The main goal of this thesis was to compare the fucoidan yield of a conventional method against a new, greener extraction method (enzyme-assisted extraction, EAE) from two species of brown macroalgae commonly found in Norway: *Ascophyllum nodosum* and *Saccharina latissima*. In addition, the polyphenol/phlorotannin, alginate and monomeric sugars content was analyzed. The chemical extraction (CE) method was conducted at 80 °C for 4 hours with 0.1 M HCl. It was tested three different enzymes (Depol692, Depol 793 and Cellulase 13), in addition to a control sample were tested and compared in EAE. The EAE method was conducted at 50 °C for 3 hours with pH 5. The polyphenol/phlorotannin content was analyzed after lyophilization, while the carbohydrate-, L-fucose- and alginate content in the macroalgae were analyzed using two different methods of acid hydrolysis. The CE method resulted in a general higher fucose and carbohydrate yield from *A. nodosum*, indicating higher release of fucoidan and other polysaccharides compared to EAE. However, limited amounts of polyphenols/phlorotannins were detected in the samples after CE, indicating that the process may be harmful for the compounds. In contrast, the extraction yield of polyphenols/phlorotannins after EAE was good. In general, CE resulted in the higher extraction yield of fucoidan, compared to EAE. None of the enzymes used in this study showed a high efficiency compared to the control or CE. However, all enzymes showed potential in degrading the algal cell wall, but further studies are needed to make thorough conclusions on this potential.

Abbreviations

A	Alginate pellet from EAE
ACN	Acetonitrile
$CaCl_2$	Calcium chloride
CE	Chemical extraction
DAD	Diode array detector
DM	Dry matter
DW	Dry weight
EAE	Enzyme-assisted extraction
ESI	Electrospray ionization
EtOH	Ethanol
FA	Formic acid
G block	α-L-guluronic acid
HCl	Hydrochloric acid
HP-SEC	High performance – size exclusion chromatography
HPLC	High performance – liquid chromatography
H_2SO_4	Sulphuric acid
IEX	Ion exchange chromatography
L-FDH	L-fucose dehydrogenase
M block	β-D-mannuronic acid
M_n	Number average molecular weight
M_p	Peak molecular weight
MS	Mass spectrometry
m/z	Mass-to-charge ratio
M_w	Weight average molecular weight
MW	Molecular weight

$NADP^+$ / NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
P	Permeate fraction from EAE
PAD	Pulsed amperometric detector
PGE	Phloroglucinol equivalents
PGU	Phloroglucinol units
QToF	Quadrupole – Time of Flight
R	Retentate fraction from EAE
RID	Refractive index detector
SEC	Size – exclusion chromatography
TFA	Trifluroacetic acid
UPLC	Ultra performance – liquid chromatography

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1 Introduction

1.1 Background

Marine environments contain half of the global biodiversity and has gained worldwide interest as a major source of bioactive compounds (Hunt *et al.*, 2006) and screening marine organisms for novel bioactivity has become a huge field of research. Macroalgae are vital species in marine ecosystems (Barbosa *et al.*, 2019) and are considered to be a sustainable natural resource. Macroalgae have gained a lot of interest for its great potential for different commercial applications, such as functional foods, feeds and nutraceuticals (Wijesinghe *et al.*, 2012). Macroalgae are known for their production of several bioactive compounds such as sulphated polysaccharides and polyphenols (Barbosa *et al.*, 2019). Traditionally, these molecules are extracted using methods that heavily rely on hazardous organic solvents and heat. These methods are both expensive and time consuming, and more importantly – generate waste material that often are released into the environment (Kadam *et al.*, 2013). After the UN´s 17 sustainability goals were approved, the topic of sustainability gained attention worldwide and this global shift has led scientists to explore more environmentally friendly extraction methods that has a lower environmental impact, compared to the conventional methods that are in use in the industry today (Giones *et al.*, 2020; González-Ballesteros *et al.*, 2020). There are several potential novel extraction methods that are being extensively researched. This includes enzyme-assisted, ultrasound-assisted, microwave-assisted, pressurized liquid and supercritical fluid extraction (Kadam *et al.*, 2013).

1.2 Macroalgae

Algae are efficient primary producers and account for approximately 10% of the global primary production (Barbosa *et al.*, 2019) as well as they are fundamental for all food chains in aquatic ecosystems (Pereira, 2015). Algae come in a range of sizes and are divided into two main categories, microalgae and macroalgae, based on their size. Microalgae are small, unicellular organisms, while macroalgae are bigger, multicellular organisms (Machmudah *et al.*, 2020). Macroalgae, commonly known as seaweeds, can be divided into three main groups; red algae (Rhodophyta), green algae (Chlorophyta) and brown algae (Phaeophyceae), depending on their pigmentation (Pereira, 2015). Macroalgae occur in marine, freshwater and estuarine

ecosystems, but are most diverse in marine ecosystems, especially in temperate regions (Macreadie *et al.*, 2017).

Macroalgae are a vast taxonomic group with high diversity with more than 10 000 species recorded worldwide (Barbosa *et al.*, 2019). Even though it is such a vast group of organisms, only 221 species are being exploited globally. In 2015, 30.4 million tons of macroalgae were harvested, where 29.4 million tons were cultivated, and 1.1 million tons were harvested from the wild. The global seaweed industry is worth more than 6 billion USD, where 85% are due to products made for human consumption. The global seaweed industry is also responsible for 40% of the worlds hydrocolloid market with food products that contain carrageenan, agar and alginate (FAO, 2018).

Macroalgae is a nutrient-rich food that has been utilized for centuries in Asian countries. They have pioneered the utilization of seaweeds for both food and medicinal purposes (Kadam *et al.*, 2015a; Padam *et al.*, 2020). Even though macroalgae is a traditional part in Asian diets, the usage as food is still rather new in western countries and steadily increasing (Murray *et al.*, 2018). The consumption of macroalgae has been linked to lower incidences of chronic diseases such as cancer and cardiovascular diseases, when Asian and western diets have been compared. It is believed that these potential health benefits are caused by different concentrations of bioactive compounds present in the macroalgae (Brown *et al.*, 2014). Macroalgae have traditionally been utilized as a source of polysaccharides as well as, minerals and vitamins. In addition, macroalgae are a good source of polyphenols, peptides, pigments, proteins, amino acids, lipids and other bioactive compounds. Despite this, biomolecules from macroalgae are still rather unexploited (Kadam *et al.*, 2015a).

1.3 Brown macroalgae

Brown macroalgae is a group of algae that contain around 2000 species, that are nearly exclusively marine species (Myklestad *et al.*, 2009). The characteristic brown color of brown macroalgae is due to a dominance of the pigment fucoxanthin. Fucoxanthin is a xanthophyll pigment that masks other pigments that are present, such as chlorophyll *a* and *c*, β-carotens and other xanthophylls (El Gamal, 2010).

Brown macroalgae, such as Fucales species dominate the rocky intertidal zones, and Laminariales species dominate the sublittoral zones. Laminariales species form large kelp forests (Myklestad *et al.*, 2009), that thrive in temperate and nutrient rich waters, where they serve as a foundation for ecosystems that supports fish, echinoderms, crustaceans and many

other species (Heckbert *et al.*, 2011). The brown macroalgal species inhabiting these zones are exposed to harsh environmental conditions, such as changes in light, oxygen and salinity levels. In addition, they are also prone to variations in levels of UV-radiation and pollution. As a result, brown macroalgae are more likely to undergo stress, as well as the formation of free radicals and oxidizing agents. Moreover, being photosynthesizing organisms, brown macroalgae are known for their ability to synthesize a wide array of polysaccharides and secondary metabolites to be able to protect themselves from the potentially threatening environmental conditions (Gupta *et al.*, 2011).

1.3.1 *Ascophyllum nodosum*

Ascophyllum nodosum (Linnaeus), also known as rockweed, belongs to the family Fucaceae (Pereira *et al.*, 2020), and is almost exclusively found in the north Atlantic. It dominates the rocky intertidal zones and is easily harvested (Kadam *et al.*, 2017). *A. nodosum* is very efficient in accumulating nutrients and minerals and is commonly harvested for food, fertilizers and animal feed (Pereira *et al.*, 2020). *A. nodosum* is one of the few species that are being harvested from the wild, accounting for less than 5% of the total amount of the global harvested seaweed (FAO, 2018). It is also a good source for different polysaccharides, which can make up to 69.6% of the algal DW. Ascophyllan is a sulphated polysaccharide exclusively produced by *A. nodosum*. Ascophyllan is very similar to other sulphated polysaccharides, like fucoidan, but contain a higher amount of xylose (Pereira *et al.*, 2020).

1.3.2 *Saccharina latissima*

Saccharina latissima (Linnaeus) belongs to the family Laminariaceae and is widely found in polar to temperate regions. It is widely known as sugar kelp due to its high contents of the sugar alcohol mannitol. The algae grow in sheltered regions attached to the seafloor (Lane *et al.*, 2006; Sharma *et al.*, 2018) and thrive in temperatures between 10 and 17 °C. Approximately half of the kelp forests made up of *S. latissima* in the world, is found along the Norwegian coast (Forbord *et al.*, 2020). Laminariaceae is one of the most important families of brown algae in the northern hemisphere and are found abundantly at depths between 8 to 30 m (Tiwari *et al.*, 2015). *S. latissima* is one of the few algae species that are being cultivated extensively for human consumption, but is also harvested from the wild (FAO, 2018). *S. latissima* is a good

source for different carbohydrates and the total carbohydrate composition can vary between 30 to 50% of its DW. Alginate is the most abundant carbohydrate in *S. latissima* (Saifullah *et al.*, 2021) and make up to 23% of the algal DW (Schiener *et al.*, 2017).

1.4 Valuable components from brown macroalgae

In addition to being crucial for the global primary production and aquatic ecosystems (Barbosa *et al.*, 2019), brown macroalgae are known to be rich in polysaccharides, minerals, vitamins and several bioactive compounds (Holdt *et al.*, 2011). The growth of brown macroalgae varies depending on the season where it will grow very little during the dark winter times and then grow fast during summer, which will influence the biochemical composition of the algae (Sharma *et al.*, 2018). Several of the components present in brown macroalgae will undergo seasonal variations and have a peak of maximum levels. In addition, the biochemical composition of brown macroalgae are affected by its environment and maturity (Schiener *et al.*, 2015), as well as it varies between the different parts of the algae (blade, stripe or thallus) (Holdt *et al.*, 2011).

The cell wall of brown macroalgae are made up of a wide array of structural polysaccharides, such as fucoidan, alginate and laminarin (Padam *et al.*, 2020). These polysaccharides can make up to 70% off the algal dry weight (DW) (Afonso *et al.*, 2019), but averages around 50% of its DW (Rioux *et al.*, 2015). Laminarin, together with mannitol serves as a carbohydrate storage and metabolite reserve in the brown macroalgae (El Gamal, 2010; Afonso *et al.*, 2019). Laminarin is made up of (1,3)-β-d-glucose units (Rioux *et al.*, 2015) and is found in high amounts in different species of kelp and can make up to 35% of the DW in *Saccharina latissima*. Laminarin is seasonally dependent and will have a maximum production during summer and autumn (Afonso *et al.*, 2019). Mannitol is also abundantly found in different species of brown macroalgae and can make up 20-30% of its DW. In addition to functioning as a carbohydrate reserve, they are local osmolytes (Rioux *et al.*, 2015).

Brown macroalgae is also a great source of phenolic compounds, such as polyphenols (Santos *et al.*, 2019). In addition, they contain a high abundance of different minerals such as iodine, calcium, magnesium, iron, sodium and potassium. Because of their structural features they have a great ability to accumulate minerals and can contain 30 000 times more iodine than any other edible plant (Afonso *et al.*, 2019). In addition, fucoxanthin has gained a lot of interest due to its promising antioxidant and antidiabetic activity (Gupta *et al.*, 2011; Afonso *et al.*,

2019). Brown macroalgae contain low amounts of lipids compared to other plants, but these lipids contain high amounts of essential fatty acids such as omega-3 (Hamid *et al.*, 2015).

1.4.1 Fucoidan

Fucoidans are a collective term used to describe polysaccharides that contain high amounts of L-fucose and sulphate ester groups. Fucoidans are water soluble polysaccharides that are important constituents in brown macroalgae and can be found in the fibrillar cell walls and extracellular matrix. (Li *et al.*, 2008; Ale *et al.*, 2011; Zayed *et al.*, 2020b). Fucoidans have several vital structural roles in the algae. They act as a cross-link between cellulose and hemicellulose and thereby promote cellular hydration and integrity, which is very important during drought seasons. In addition, fucoidans play important roles in cell-to-cell communication, for example during reproduction and innate immune responses (Zayed *et al.*, 2020b).

Fucoidans are highly heterogenous polysaccharides that have complex structures that varies between species (Zayed *et al.*, 2020a). Their backbone is built up of α(1-3) and α(1-4) linked L-fucopyranose, either alternating or consecutive of one type. They often have various substitutions and some of the most common are sulphate and acetate groups, but also glucose, galactose, xylose and mannose (Ale *et al.*, 2011; Hreggviðsson *et al.*, 2020). Fucoidans are often divided into two groups depending on their source. The first group, which includes *Fucus* and *Ascophyllum* species, are characterized by a backbone of alternating α(1-3) and α(1-4) linked L-fucopyranose residues (Figure 1, structure A) (Carvalho *et al.*, 2020). In this group sulphate ester substitutions are commonly found on C-2, but may also occur on C-4 and rarely on C-3, where SO_3^- is a common substitution on C-2 in *Ascophyllum nodosum* (Zayed *et al.*, 2020a). The second group, which includes *Laminaria* species, consists mainly of a backbone of α(1-3) linked L-fucopyranose residues (Figure 1, structure B) (Carvalho *et al.*, 2020). In this group, sugar substitutions commonly occur on C-2, while sulphate ester groups are commonly found on C-4. Common in *S. latissima* are OSO_3^- substitutions on C-2 and alternating OSO_3^- and H substitutions on C-4 (Zayed *et al.*, 2020a).

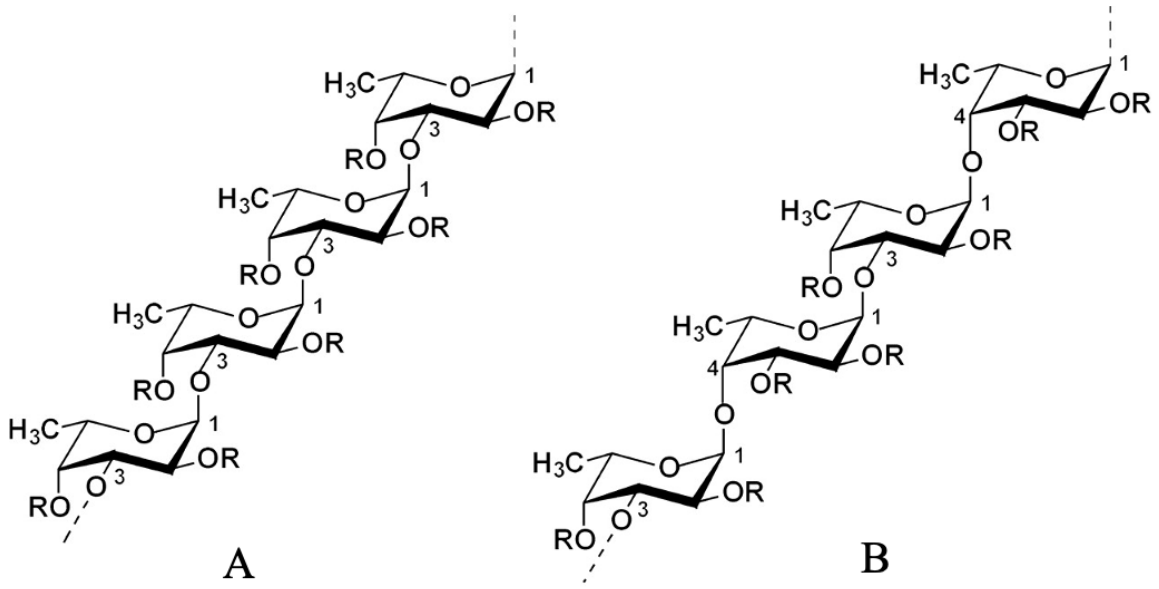

Figure 1. Structural models of the chemical structure of fucoidan retrieved from Cumashi *et al.* (2007).Structure *A* represent a fucoidan molecule with repeating α(1-3) and α(1-4) linked L-fucopyranose with a sulphate ester substitution on C-2. The different R groups represent different substitutions which will vary between species. Structure *B* represents a fucoidan molecule with repeating α(1-3) linked L-fucopyranose with a OSO_3^- (R) substitution on C-2.

Fucoidan has a high molecular weight (MW) ranging from 100 kDa to 16 000 kDa and has attracted a lot of interest due to its wide spectrum of potential bioactivities, such as anticoagulant, antiviral, antioxidant, antitumor, and anti-inflammatory activities (Gupta *et al.*, 2011; Carvalho *et al.*, 2020). The best studied bioactivity of fucoidans is its great anticoagulant activity. Isolated fucoidans have been tested for several types of anticoagulant activities, where all show great potential against activated partial thromboplastin time (APTT) and thromboplastin time (TT) (Li *et al.*, 2008). The great anticoagulant potential of fucoidans are linked to the heparin cofactor II-mediated antithrombin activity (Ale *et al.*, 2011). In addition, the anticoagulant activity is linked to the sulphate content and the position of the sulphate group, as well as the sugar composition of the fucoidan and its MW in general, the higher amounts of sulphate groups, the higher anticoagulant activity (Li *et al.*, 2008).

Fucoidan also show great potential regarding antiviral activities, having low cytotoxicity compared to other antiviral drugs that are being used clinically today. Isolated fucoidans have shown antiviral effect against several virus infections, such as poliovirus III, adenovirus III and herpes simplex virus (Li *et al.*, 2008). Due to the ongoing Covid-19 pandemic, this activity is especially interesting. In a study by Kwon *et al.* (2020), described a potent antiviral activity by means of a tight binding of the virus spike-protein to the tested

fucoidans. Thus, fucoidan can potentially be used as a competitor for the S-protein in SARS-Cov-2, inhibiting binding to host receptors and thereby inhibiting viral infection (Kwon *et al.*, 2020).

1.4.2 Alginate

Alginate, also known as alginic acid, is a linear, anionic polysaccharide, that are naturally found in brown macroalgae (Hreggviðsson *et al.*, 2020). Alginate content can vary from 17 to 45% of the brown macroalgae's DW (Manns *et al.*, 2017) and is an important structural component in the thalli and the cell wall of the macroalgae, as well as it is found abundantly in the intercellular matrix. It provides the macroalgae with flexibility and is involved in the exchange of different ions, such as magnesium and calcium, with seawater (Rioux *et al.*, 2015).

Alginate is a polymer made up of 1-4 linked residues of α-L-guluronic acid (G) and β-D-mannuronic acid (M) that are arranged in a block wise pattern (Jensen *et al.*, 2015). The polymers can either be composed of consecutive G blocks (Figure 2, structure A), consecutive M blocks (Figure 2, structure B), or alternating G and M blocks (Figure 2, structure C) (Lee *et al.*, 2012). The ratio of G and M blocks will vary, depending on the species, season of harvest and geographical location (Jensen *et al.*, 2015). In addition, the G and M ratio can vary in different parts of the algae. For instance, the leaves in *Laminaria hyperborea* contain a higher amount of mannuronic acids, while the stripe contains a higher amount of guluronic acid. Similarly, the fruiting bodies in *A. nodosum* contain a higher amount of mannuronic acids, compared to older tissue that have more guluronic acids (Puscaselu *et al.*, 2020).

Alginate have different solubility depending on species, polymer concentration, pH, as well as the presence of different ions. Some alginates are soluble in hot water, while others are soluble in cold water. Generally, alginic acids are insoluble in acidic conditions as well as in the presence of different ions, such as calcium ions (Ca^{2+}) (Rioux *et al.*, 2015). However, alginate is known for its great gelling abilities in the presence of divalent cations, such as calcium ions (Jensen *et al.*, 2015; Carvalho *et al.*, 2020). The gelling abilities of alginates from different species are dependent on the M/G ratio of the backbone, while the viscosity is determined by the MW. Alginates with a low M/G ratio will form stiff gels, while alginates with a high M/G ratio will from more flexible gels (Jensen *et al.*, 2015). Because of this ability alginate is utilized for several food applications as a potential thickening, gelling and stabilizing agent which is added to several foods, such as ice cream, dressings and beer. Alginate is also

utilized for medicinal purposes as a coagulant and may also be used as an immunostimulatory agent (Kumar *et al.*, 2017).

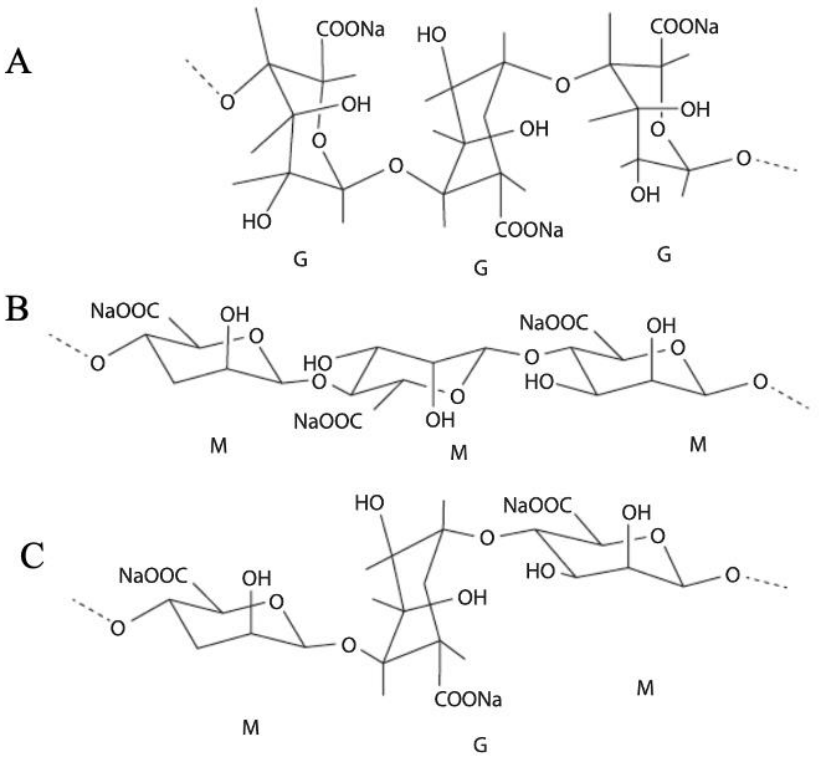

Figure 2. Different structures of sodium alginate, retrieved from Kumar *et al.*, (2017). Structure A represents an alginate made up of consecutive G blocks (α-$_{L}$-guluronic acid). Structure B represents an alginate made up of consecutive M blocks (β-$_{D}$-mannuronic acid). Structure C represents an alginate made up of alternating G and M blocks.

1.4.3 Polyphenols

Brown macroalgae are a good source of different phenolic compounds, that varies from simple polyphenols and flavonoids, to more complex phlorotannins (Santos *et al.*, 2019). Polyphenols play several important roles in the brown macroalgae, ranging from being important structural components in the cell wall to functioning as their defense system against infections, bacteria, grazers and ultraviolet radiation (Afonso *et al.*, 2019; Aminina *et al.*, 2020). The concentration of polyphenols vary greatly between species, where the order Fucales species have the highest total polyphenol content documented in brown macroalgae (Jiménez-Escrig *et al.*, 2012). Some polyphenols are documented to be tightly bonded to cell wall polysaccharides through both hydrophilic and hydrophobic bonds (Wijesinghe *et al.*, 2012). Polyphenols are water soluble

molecules that are commonly extracted using polar solvents (Aminina *et al.*, 2020). Unfortunately, extraction of polyphenols has proven to be problematic, so the presence of these molecules is estimated using a few simple colorimetric methods. Characterization have also proven problematic due to the structural complexity. However several chromatographic methods have been developed for better identification and characterization of polyphenols (Lopes *et al.*, 2018). A study conducted by Koivikko (2008), used chromatographic methods to identify phlorotannins, and reported an absorbance maximum for phloroglucinol at approximately 270 nm.

Phlorotannins are a group of polyphenols that is recognized as the reason for the high abundance of phenolic compounds in brown macroalgae (Santos *et al.*, 2019). Phlorotannins are almost exclusively produced by species belonging to the class of Phaeophyceae and are very abundant in many species (Afonso *et al.*, 2019). Phlorotannins are synthesized though the acetate-malonate pathway and are built up of phloroglucinol units (PGU) (Figure 3, structure A). They are hydrophilic molecules (Murray *et al.*, 2018) that range from 126 Da to 100 kDa in size (Heffernan *et al.*, 2015). Phlorotannins can be divided into several groups depending on the linkages that bind the units together (Ferreres *et al.*, 2012). Fucols represent the simplest group of phlorotannins where the phloroglucinol units are linked together by carbon-carbon linkages, (Figure 3, structure B). The group phlorethols is a result of radical carbon-oxygen couplings and consist of both phloroglucinol units and ether bonding's (Figure 3, structure C). Fucophlorethols include all derivatives that contain both carbon-carbon linkages and carbon-oxygen linkages (Figure 3, structure D). Fuhalols is a more complex group that have an additional hydroxyl group on the phloroglucinol units (Figure 3, structure E). Eckols have a specific intramolecular cyclization, which is formed by dibenzodioxin linkages (Figure 3, structure F) (Kornprobst, 2010). Isofuhalols is a variation of fuhalols (Figure 3, structure F) that only have para and meta oriented ether bonds relative to its substituents (Creis *et al.*, 2018)

Within each of these groups of phlorotannins, it is possible to have both structural and conformational isomers of each phlorotannin (i.e., different compounds with identical elemental composition), making the complexity and variability of these molecules extreme. It is a field of research that is considered to be almost unlimited due to the structural complexity. There are more than 150 phlorotannins identified today from several species of algae (Barbosa

et al., 2019). Phlorotannins have been reported to contain several different bioactivities, such as antioxidant, anticancer and antibacterial activity (Peng *et al.*, 2015).

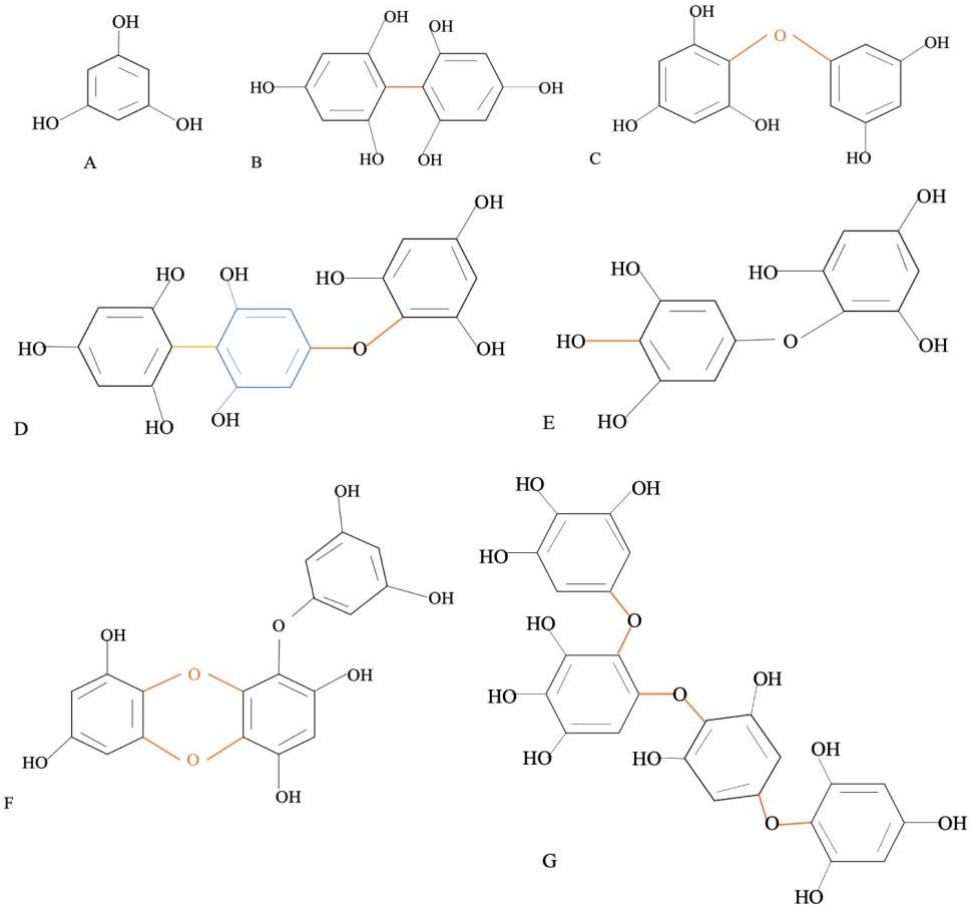

Figure 3. Structural models of the different phlorotannin groups modified from Kornprobst (2010) and Creis *et al.* (2018). Structure A is phloroglucinol, that is the structural unit in all phlorotannins. Structure B is difucol, belonging to the group of fucols. Structure C is diphlorethol, belonging to the group of phlorethols. Structure D is fucophlorethol A, belonging to the group of fucophlorethols. Structure E is bifuhalol, belonging to the group of fuhalols. Structure F is eckol, belonging to the group of eckols. Structure G is tetraisofuhalol, belonging to the group of isofuhalols. The linkages that characterize the different groups are highlighted in orange.

1.5 Extraction of polysaccharides

1.5.1 Conventional extraction methods

The relationship between the chemical structure of polysaccharides and their bioactivity is very important. Harsh extraction methods can influence the structure of the polysaccharide (i.e., thermal degradation) and even the smallest of changes can affect the activity. Therefore, the choice of extraction method is crucial and there are several common extraction methods used

for macroalgae (Garcia-Vaquero *et al.*, 2017). Conventional extraction methods heavily rely on hazardous solvents, such as methanol, acetone, hydrochloric acid (HCl) and ethanol, as well as considerable amounts of heat (Getachew *et al.*, 2020). The main objective of every extraction method is to maximize the yield of the compounds of interest (Garcia-Vaquero *et al.*, 2020).

Chemical extraction of macroalgae will exploit the solubility of the cell wall under different conditions, resulting in the release of components (Nguyen *et al.*, 2020). Distillation techniques have commonly been used to extract polysaccharides from macroalgae, as well as other natural compounds. The method is used to separate multicomponent liquids by using selected boiling points of components present in the liquid, followed by a condensation step. Maceration is another widely used method for extraction of polysaccharides from macroalgae. It is a type of solid extraction that uses the solubility of the desired compound by carefully selecting a solvent with the right polarity and applying heat. The extraction takes a long time and has a low efficiency (Garcia-Vaquero *et al.*, 2020; Nguyen *et al.*, 2020). This method has, for instance, been widely used to extract fucoidan from the algal cell wall (Nguyen *et al.*, 2020). Fucoidan extraction is very often followed by precipitation with ethanol (Ale *et al.*, 2011) and ethanol is considered to be an expensive solvent (Rioux *et al.*, 2015). One of the major drawbacks of conventional extraction methods are the coextraction of other polysaccharides as well as the low extraction yield. In addition, there is a difficulty of upscaling to industrial levels, due to the substantial energy requirements of conventional methods. Several environmental conditions regarding high volume of solvents, emission and storage, also needs to be addressed. And last but not least, it is extremely expensive to generate industrial plants that are able to handle such large quantities of hazardous waste (Getachew *et al.*, 2020).

Despite this, conventional extraction methods are widely used in the industry and by researchers today (Flórez-Fernández *et al.*, 2020; Garcia-Vaquero *et al.*, 2020), however there are several new, greener methods being extensively researched to overcome several of the disadvantages concerning the conventional methods (Kadam *et al.*, 2013; Nguyen *et al.*, 2020).

1.5.2 New, greener extraction methods

Bioactive compounds are often very sensitive and may be altered by harsh extraction methods. Therefore, the choice of extraction method is crucial to avoid modifications of the desired molecules. There are several new, green methods under development to reduce the energy consumption, emissions, cost and increase the safety and quality of the product (Flórez-Fernández *et al.*, 2020; Nguyen *et al.*, 2020). This includes methods such as enzyme-assisted

extraction, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and pressurized liquid extraction (Kadam *et al.*, 2013; Nguyen *et al.*, 2020).

1.5.2.1 Enzyme assisted extraction

Enzyme assisted extraction (EAE) is considered to be a sustainable and efficient alternative to the conventional solvent-based extraction methods that are in use today. EAE uses enzymes and their characteristic properties to carry out a specific reaction (Puri *et al.*, 2012; Nadar *et al.*, 2018). Enzymes are vital proteins that are involved in all metabolic processes and are highly specific catalysts. EAE makes it possible to extract valuable components from seaweed without using denaturizing conditions, high temperatures and organic solvents. It takes advantage of the various enzyme´s disruptive abilities in the molecular environment, which will lead to a higher extraction yield of the targeted molecules (Terme *et al.*, 2020). Enzymes have proven to be useful tools in hydrolysis of macroalgae to facilitate the release of different components (Hreggviðsson *et al.*, 2020).

Macroalgae have cell walls that are made up of complex biomolecules, such as sulphated and branched polysaccharides, that are often associated with lipids, calcium and potassium (Kadam *et al.*, 2013). The cell wall limits the accessibility of bioactive molecules, and it is therefore essential to degrade the various contents of the cell wall. EAE has been successfully employed as an alternative extraction method in macroalgae, as it successfully degrades the algal cell wall with a substantial yield. The choice of enzyme is important as the cell wall is chemically complex and the components are highly connected (Wijesinghe *et al.*, 2012). Hydrolytic enzymes have shown the ability to more efficiently disrupting and/or degrading the cell wall and membranes of the macroalgae compared to other techniques (Flórez-Fernández *et al.*, 2020). EAE will therefore, in theory, result in a higher accessibility of bioactive molecules and result in a higher extraction yield. However, for the extraction to reach its full potential, it is important for the enzyme to work under optimal conditions (Kadam *et al.*, 2013). To obtain the maximum enzymatic reaction rate it is important to maintain the reaction at optimal temperature, pH, enzyme concentration and substrate concentration. Enzymes are temperature sensitive, and the reaction rate often increase parallel with thermal values as the viscosity decrease. EAE is often performed in acidic conditions as it will increase the cellular plasticity by destabilization of hydrogen bonds. The wrong pH can influence the binding capacity of the enzyme to the substrate and negatively influence the enzymatic reaction rate. The enzymatic

concentration is highly correlated to the extraction time. As enzyme activity decrease over time it is important to find the right ratio and the reaction time can often be divided in half by doubling the concentration of enzymes (Muniglia *et al.*, 2014). Compared to conventional extraction methods, EAE is often more cost-efficient as well as it will reduce the extraction time and the use of solvents (Kadam *et al.*, 2013).

1.6 Methods for polymer analysis

1.6.1 Size exclusion chromatography

Size exclusion chromatography (SEC) is a form of liquid chromatography that separates molecules based on their weight and size. This method is also referred to as gel permeation chromatography, where the mobile phase is an organic solvent, or gel filtration chromatography, where the mobile phase is an aqueous solution (Batool *et al.*, 2020). The stationary phase is usually composed of porous particles that act like a "reversed filter" that allows small molecules to penetrate the pores in the particles while larger molecules pass outside of the particles. This allows for larger molecules to elute first, while smaller molecules elute later based on their interactions with the stationary phase (Mansoor, 2005). SEC is commonly used to determine the MW of polysaccharides present in macroalgae (Kadam *et al.*, 2015b). Refractive index detectors (RID) are often used in the characterization of polymer weight distribution (Zhang *et al.*, 2019).

1.6.2 Ion exchange chromatography

Ion exchange chromatography (IEX) separates ions and polar molecules based on their differences in charge. The stationary phase will consist of a charged solid surface (a resin or gel with covalently linked charged molecules) that will interact with molecules with opposite charge in the sample/mobile phase. There are two types of IEX, cationic and anionic. Cationic exchange is used when the molecules of interest are positively charged, and the stationary phase is made up of a negatively charged support. The molecules of interest will elute based on their interaction with the stationary phase and changes of the mobile phase. In anionic exchange, the molecules of interest are negatively charged, and the stationary phase is positively charged (Batool *et al.*, 2020).

Several chromatographic methods have been developed to analyze the carbohydrate content in different samples and ion chromatography is the most widely used for separation of monosaccharides. Once the monosaccharides are separated, they are detected using different detectors, where a RID is often used (Galant *et al.,* 2015). RID is a universal bulk property detector (Swartz, 2010) that measures the difference in refractive index of the sample compared to a reference and is therefore widely used in sugar detections, as native sugars do not contain a chromophore or fluorophore (Galant *et al.*, 2015).

1.6.3 High performance liquid chromatography – mass spectrometry

Mass spectrometry (MS) is a common detection method used in combination with chromatography that operates by converting analyte molecules into an ionized (charged) state. During the ionization process, ions (and fragmented ions) will be analyzed on their mass-to-charge ratio (*m/z*) (Pitt, 2009). The *m/z* ratio is defined as the mass (*m*) of an ion divided by the charge (*z*) of the ion, which can be used to determine the molecular mass of the ion detected (Todd, 1991). The most common ionization method is electrospray ionization (ESI). ESI converts ions into their gaseous phase by using electrical energy before MS analysis (Ho *et al.*, 2003). Diode array detector (DAD) are commonly used in liquid chromatography. It is a type of UV detector that provides measures the absorbance at a number of different wavelengths, providing specificity in quantifying compounds (Senyuva, 2014). Quadruple (Q) analyzer is also commonly used in MS analysis which uses as system of four parallel metal rods that separates ions (based on their *m/z*) using voltage (Ho *et al.*, 2003). The time it takes for the ions to reach the detector can be measured using a time of flight (ToF) analyzer. Each mass has a unique flight time, which is based on the ions´ *m/z* values (Pitt, 2009).

Due to the high complexity of algal phlorotannins, chromatographic methods have been preferred for characterization (Steevensz *et al.*, 2012). Modern High-Resolution ToF-MS instruments have the ability to measure the exact masses of ions and fragmented ions with a high accuracy. Since every element has its own unique mass, it is also possible to generate chemical formulas for the unknown compounds using this technology (Sleno, 2012).

1.6.4 Hydrolysis methods

Carbohydrates from macroalgae have gained attention as a resource for high value carbohydrates. The carbohydrate content in macroalgae is often analyzed after acid hydrolysis.

The hydrolysis will hydrolyze polysaccharides into monosaccharides and the optimal hydrolysis method varies depending on the material and it is therefore no universal method. For materials rich in uronic acids, hydrolysis with HCl or trifluoroacetic acid (TFA) is preferred, while hydrolysis with sulphuric acid is preferred for biomasses rich in lignocellulose. Different hydrolysis methods may influence the chemical composition in the algae, resulting in different results (Manns *et al.*, 2014). Manns *et al.* (2014) states that the use of a two-step sulphuric acid hydrolysis is the best method for quantitative determination of carbohydrates in seaweed, compared to TFA hydrolysis. Megazyme (2018) suggest hydrolysis with 1.3 M HCl, followed by neutralization with 1.3 M sodium hydroxide (NaOH) as a pretreatment to estimate L-fucose in polysaccharides and fibrous plants.

1.7 Aim of the study

The main goal of this study is to compare the extraction efficacy of a new, green method of extraction against a selected conventional chemical extraction method. As a green method, enzyme-assisted extraction (EAE) is chosen, and as a conventional method, chemical extraction (CE) with hydrochloric acid is chosen. The main target compound for comparing the two extraction methods will be fucoidan, but also the yield of alginate, polyphenols and monomeric sugars will be analyzed. The extractions will be performed on two common brown macroalgal species in Norway: *Ascophyllum nodosum* and *Saccharina latissima.*

Sub goals are to:

- Evaluate the efficiency of the different enzymes used in EAE.
- Evaluate the efficiency of fucoidan isolation (precipitation with ethanol *versus* ultrafiltration)
- Evaluate the efficiency of alginate precipitation using $CaCl_2$
- Examine the carbohydrate and polyphenol content after extraction
- Compare two different acid hydrolysis methods

2 Materials and Methods

2.1 Biological material

Sugar kelp, *Saccharina latissima* (L.) (*S. latissima*) (former known as *Laminaria saccharina*) was provided by Seaweed Energy Solutions AS (Trondheim, Norway). The kelp was harvested the 27th of April 2019 and frozen the same day (-30 °C). Rockweed, *Ascophyllum nodosum* (L.) (*A. nodosum*) were collected the 12th of October 2020 in Hamna (Tromsø, Norway) and frozen the same day (-30 °C).

2.2 Enzymes

The enzymes used for extraction were the broad-spectrum enzyme Depol 692L, the multifunctional enzyme Depol 793L and Cellulase 13L, all purchased from Biocatalysts (London, United Kingdom). The optimum conditions and the characteristics for each enzyme is summarized in table 1.

Table 1. Optimum hydrolysis conditions, main activities as well as other activities of the different enzymes used in this study. The information is retrieved from their respective safety data sheet (Biocatalysts, 2015a, 2015b, 2015c)

Enzyme	pH optimum	Temperature optimum (°C)	Main activity	Other activities
Depol 692L	4.0 – 6.0	50 – 60	Cellulase (> 800 U/g); hydrolysis of (1,4) β -D-glucosidic linkages in β-D-glucans Pectinase (endo-galacturonase) (> 535 U/g); hydrolysis of pectin	Ferulic acid esterase Hemicellulase
Depol 793L	4.0 – 7.0 (optimal pH is 6.0)	40-50	β-glucanase (> 5.500 U/g); hydrolysis of β-(1,3)- or (1,4)- linkages in β-D-glucans Pectin lyase (> 5.000 U/g); hydrolysis of pectin Cellulase > (1.200 U/g); hydrolysis of (1,4) β -D-glucosidic linkages in β-D-glucans	Ferulic acid esterase
Cellulase 13L	3.5 – 6.0	50 – 70	Cellulase (> 1.500 U/g); hydrolysis of (1,4) β-D-glucosidic linkages in β-D-glucans	Cellobiase β-glucosidase β-glucanase

2.3 Other chemicals

Ethanol (EtOH) was purchased from (Antibac AS, Norway). Calcium chloride ($CaCl_2$), Folin-Ciocalteus Phenol reagent, sulphuric acid 95-97% (H_2SO_4), glacial acetic acid, sodium hydroxide (NaOH), D(+)-Xylose, sodium carbonate and formic acid (FA) were purchased from Merck KGaA (Dramstadt, Germany). Hydrochloric acid (HCl), phloroglucinol, D-mannitol, α-L(-)-Fucose, fucoidan extracted form *Fucus vesiculosus*, sodium alginate, sodium tetraborate decahydrate and 3,5-Dimetylphenol were purchased from Sigma Aldrich (Saint Louis, MO, USA). D(+)-Glucose anhydrous was purchased from VWR chemicals (Radnor, PA, USA). Acetonitrile (ACN), formic acid (FA) and water of analytical grade were purchased from Honeywell (Charlotte, NC, USA). The L-Fucose assay kit was purchased form Megazyme (Bray, Ireland) and contains an L-fucose standard, buffer (pH 9.5), β-NADP and L-fucose dehydrogenase (L-FDH). Pullulan standards were purchased from PSS-Polymer (Amherst, MA, USA). Distilled water was obtained from ELGA Purelab Chorus 2^+ (Veolia Water, UK).

2.4 Methods

2.4.1 Proximate analysis

The water and dry matter (DM) content in *S. latissima* and *A. nodosum* were determined in triplicates by drying the biomass at 105 °C overnight. The amount of ash was determined by drying the remaining residues in a Carbolite-Gero AAF 11 (Neuhausen, Germany) muffle furnace at 550 °C for 24 hours.

2.4.2 Chemical extraction (CE)

A modified extraction method suggested by Nguyen *et al.* (2020) was followed and Figure 4 shows the workflow of the study. The extraction was performed on homogenized (Polytron PT 45-80 GT, Kinematica AG, Malters, Switzerland) brown macroalgae in the ratio of 50 g DM to 1 L (1:20) 0.1 M HCl, at 80 °C for 4 hours under constant stirring (60 rpm) using an IKA® LR 1000 basic reactor (IKA, China). The pH of the solution was measured to be 1.4 using a Seven Go pH meter (Mettler Toledo, Columbus, OH, USA). After extraction, the solution was centrifuged at 7000 G for 20 min using Avanti JXN-26 (Beckman Coulter, Brea, CA, USA)

and the supernatant was collected. The residual pellet was discarded. Alginate was precipitated from the supernatant by adding 2% $CaCl_2$ (w/v) and incubating at 4 °C overnight. The samples were centrifuged as described above and the supernatant was collected. The alginate residual pellet was dried at 105 °C and stored in a 50 mL Falcon tube (Sigma Aldrich, Saint Louis, MO, USA) at room temperature until hydrolysis with HCl and H_2SO4 and carbohydrate, L-fucose and alginate analysis.

Fucoidan was precipitated and isolated from the supernatant by adding 72% EtOH (v/v), followed by centrifugation at 7000 G for 40 min. The ethanol was evaporated from the supernatant using a BUCHI rotavapor R-215 (Sigma Aldrich, Saint Louis, MO, USA). The precipitated fucoidan pellet and supernatant was lyophilized and stored in 50 mL Falcon tubes at room temperature before MW determination, polyphenol/phlorotannin analysis and hydrolysis with HCl and H_2SO_4.

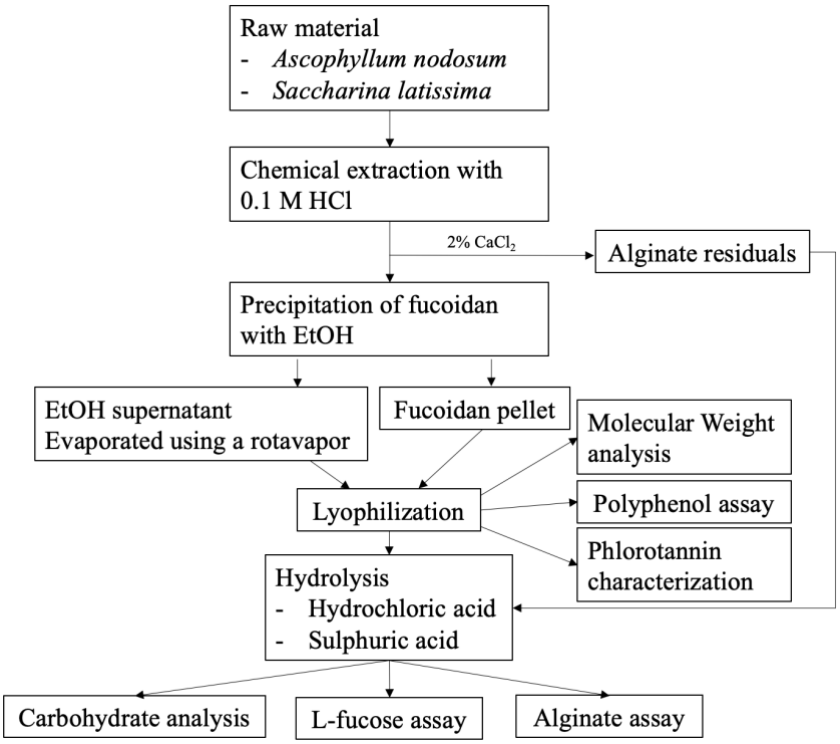

Figure 4. Flowchart for the chemical extraction (CE) method. Extraction was preformed using 0.1 M HCl, followed by alginate precipitation with 2% $CaCl_2$ which was dried at 105 °C. Fucoidan was precipitated from the supernatant with 72% EtOH. The supernatant and fucoidan pellet were lyophilized. Lyophilized material was used to estimate the molecular weight (MW) distribution of polysaccharides in the samples, as well as to estimate the polyphenol content and for phlorotannin characterization. Two hydrolysis methods were performed on the lyophilized material, one with sulphuric acid and one with hydrochloric acid. After hydrolysis, the material was analyzed for monomeric sugars, fucose content and alginate content.

2.4.3 Enzyme assisted extraction (EAE)

A modified extraction method suggested by Nguyen *et al.* (2020) was followed and Figure 5 shows the workflow of the study. Extraction was performed on homogenized *A. nodosum* in the ratio 1 kg wet algae to 3.48 L (1:7) distilled water and homogenized *S. latissima* including its drip loss. The pH of the homogenized brown macroalgae was adjusted to pH 5 using 1 M HCl. The *A. nodosum* homogenate was divided into 4 samples of 750 mL (64.5 g DM) each and the *S. latissima* homogenate was divided into 4 samples of 400 mL (20 g DM) each. The enzymes (Depol 692L, Depol 793L and Cellulase 13L) were added to separate samples constituting 0.25% of the total volume. The fourth sample was used as an untreated control. The enzymatic treatment was conducted at 50 °C for 3 hours under constant stirring (60 rpm) in an IKA® LR 1000 basic reactor. After extraction, the samples were centrifuged at 7000 G for 15 min and the supernatants were collected while the residual pellet was discarded. Alginate was precipitated from the supernatant by adding 2% $CaCl_2$ (w/v) and incubating at 4 °C overnight. The samples were centrifuged as described above and the supernatant of each sample was collected. The alginate residual pellet (A) was dried at 105 °C and stored in 50 mL Falcon tubes at room temperature until analysis.

The collected supernatants were filtered through a Whatman No. 4 filter to remove any particles. The filtrates were further ultra-filtrated using a Millipore Labscale TFF system (Merck Millipore, Brulington, MA, USA) coupled with Millipore Pellicon XL Biomax polyethersulfone membranes. To isolate fucoidan of different sizes, membranes with a molecular cut-off of 100 kDa was used. Both retentates (R) and permeates (P) were collected and lyophilized. The filtrates were stored in 50 mL Falcon tubes at room temperature before MW determination, polyphenol/phlorotannin analysis and hydrolysis with HCl and H_2SO_4.

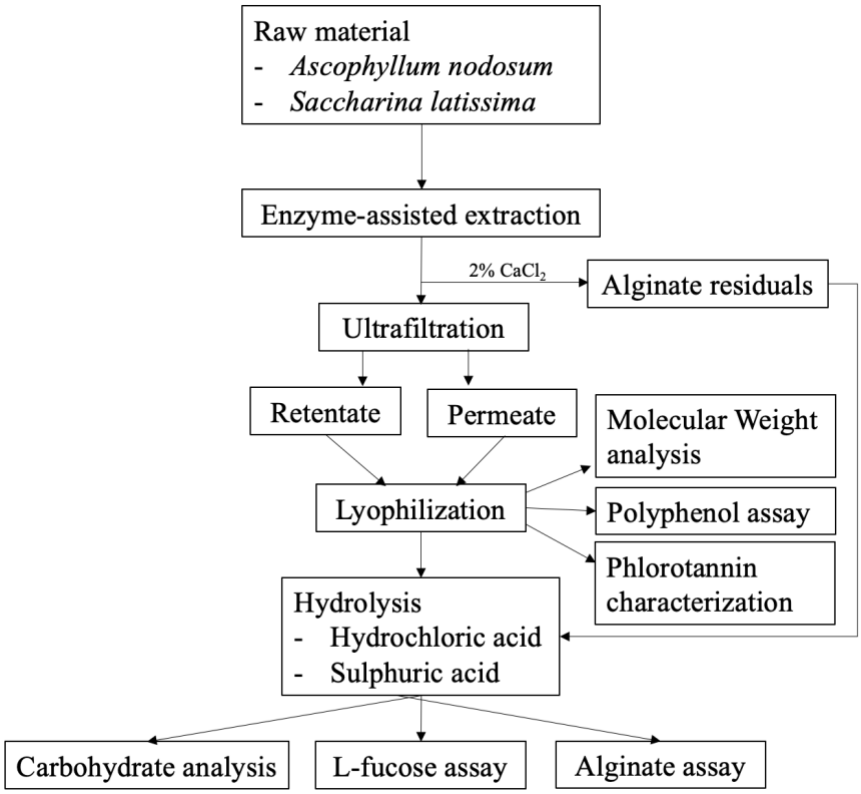

Figure 5. Flowchart of the enzyme assisted extraction (EAE) method. Extraction was performed on homogenized brown macroalgae, using three different enzymes (Depol 692, Depol 793 and Cellulase 13), followed by alginate precipitation with 2% $CaCl_2$ and drying of alginate pellet at 105 °C. Ultrafiltration was performed on the supernatant to separate components based on their molecular weight (MW) using a membrane with molecular cut off of 100 kDa. The retentate and permeate was then lyophilized. Lyophilized material was used to estimate the MW distribution of polysaccharides in the samples, as well as to estimate the polyphenol content and for phlorotannin characterization. Two hydrolysis methods were performed on the lyophilized material, one with sulphuric acid and one with hydrochloric acid. After hydrolysis, the material was analyzed for monomeric sugars, fucose content and alginate content.

2.4.4 Molecular weight analysis of polysaccharides

The MW of polysaccharides present in the lyophilized samples were analyzed with high-performance size exclusion chromatography (HP-SEC) using a LC-20AT apparatus equipped with a CTO-20A column oven, an SPD-M20A diode array detector and a RID-20A refractive index detector (all from Shimadzu, Kyoto, Japan). The MW of the polysaccharides were analyzed using a 300×8 mm Shodex SB-806 HQ GPC column (Shodex™, Japan), fitted with a 50×6 mm Shodex SB-G guard column (Shodex™, Japan). The target MW range of the column is 100 to 20 000 kDa (Shodex, 2020).

Lyophilized material from CE (supernatant and fucoidan pellet) and EAE (retentate and permeate) was dissolved in the mobile phase of 0.1 M Sodium acetate (pH 6) to a final concentration of 25 mg/mL. The analysis was conducted at 40 °C with an injection volume of 50 µL at an isocratic flow rate of 0.5 mL/min for 40 min (Nguyen *et al.*, 2020). Pullulan samples with MW of 1600, 800, 400, 110, 12, 5 and 1 kDa were used as standards. The data was then analyzed using the software GPC Postrun (Shimadzu, Kyoto, Japan).

During HP-SEC, the peaks of Depol 793 retentate and Control retentate from *A. nodosum* were fractionated and collected using an FRC-10A fraction collector (Shimadzu, Kyoto, Japan), and lyophilized. The fractions were stored in a 50 mL Falcon tubes at room temperature before hydrolysis with HCl and H_2SO_4 and carbohydrate, L-fucose and alginate analysis.

2.4.5 Hydrochloric acid and sulphuric acid hydrolysis

Two different methods of hydrolysis were performed and compared. A modified HCl hydrolysis protocol, suggested by Megazyme (2018) for determination of L-fucose in polysaccharides and fibrous plant material was followed. For hydrolysis, 100 mg of lyophilized material from CE and EAE, dried alginate residual pellets and fucoidan standard were dissolved in 1 mL distilled water, in separate tubes. A volume of 5 mL of 1.3 M HCl was added to each sample and the tubes were incubated at 105 °C for 1 hour. The tubes were then cooled to room temperature and an additional 5 mL of 1.3 M NaOH was added. The samples were then frozen until carbohydrate-, L-fucose- and alginate analysis was performed.

A modified two step sulphuric acid hydrolysis based on Sterner *et al.* (2017), Nguyen *et al.* (2020) and Okolie *et al.* (2020) were followed. For this hydrolysis method, 30 mg of lyophilized material from CE and EAE, dried alginate residual pellets, fucoidan standard and fractionated peaks from HP-SEC were individually soaked in 300 µL of 72% (w/w) H_2SO_4. The samples were incubated at 30 °C for 1 hour. In the second step of the hydrolysis, the samples were diluted to 4% (w/w) H_2SO_4 by adding distilled water. This was followed by autoclaving in a TOMY SX-700 E High pressure steam sterilizer (TOMY, San Diego, CA, USA) for 40 min at 121 °C. The samples were then and frozen until carbohydrate-, L-fucose- and alginate analysis was performed.

2.4.6 Carbohydrate analysis

The monomeric sugars (glucose, xylose, mannitol and fucose) present in the different hydrolyzed samples from CE (supernatant, fucoidan pellet and alginate pellet) and EAE (R, P and A) were analyzed by ion-exchange high-performance liquid chromatography using a LC-20AT apparatus equipped with a CTO-20A column oven and a RID-20A refractive index detector (IEX-HPLC-RID) (all from Shimadzu, Kyoto, Japan). The monomeric sugars were separated using a 300×7.8 mm Rezex ROA-Organic acid H+ (8%) analytic column fitted with a Carbo-H 4×3.0 mm guard column (all from Phenomenex, Torrence, CA, USA).

The hydrolyzed material from CE (supernatant, fucoidan pellet and alginate pellet), EAE (retentate, permeate and alginate) and the fractioned peaks from HP-SE was first centrifuged at 1246 G (Eppendorf Centrifuge 5810 R, Sigma-Aldrich, Saint-Louis, MO, USA) for 10 min to remove any particles. The process was then conducted at 65 °C with an injection volume of 10 μL (9.09 mg/mL) of hydrolyzed material, and a mobile phase of 5 mM H_2SO_4 at an isocratic flow rate of 0.6 mL/min for 30 min (Sharma *et al.*, 2018). The HPLC column´s stationary phase is made up of sulfonated styrene-divinylbenzene (Phenomenex, 2017). The sulfonation of the column results in a negatively charged environment allowing neutrally and positively charged molecules to pass though the column, based on their pKa (Transgenomic, 2007). Prior to sample analysis, glucose, xylose, mannitol and fucose standards in concentrations of 2.5, 1.0, 0.5, 0.25 and 0.1 g/L were run to generate standard curves for the different monomeric sugars (Appendix, Figure A1-A4)

2.4.7 L-fucose assay

The hydrolyzed material from CE (supernatant, fucoidan pellet and alginate pellet), EAE (retentate, permeate and alginate) and fractionated peaks from HP-SEC was first centrifuged at 1246 G for 10 min to remove any particles. The L-fucose assay was performed on 10 μL (9.09 mg/mL) hydrolyzed material (two parallels) from both hydrolysis methods according to suggested protocols (Megazyme, 2018). Into each well of a Nunc 96-well microplate, 10μL sample/standard (L-fucose standard solution and fucoidan), 200 μL distilled water, 40 μL buffer solution and 10 μL $NADP^+$ was added. The absorbance was measured at 340 nm using a SpectraMax® i3x microplate reader. A volume of 5 μL of L-FDH was added to each well and the absorbance was then measured every 2 min for 30 min. In this assay, the oxidization of L-

fucose by L-FDH in the presence of $NADP^+$, resulting in the increase of NADPH being measured (Megazyme, 2018). The estimated fucose content was calculated using Formula 1.

Formula 1:

$$mg/mL = \frac{\Delta Asample}{\Delta Astandard} * mg/mL\ standard * F$$

F = dilution factor

Formula was retrieved from (Megazyme, 2018).

2.4.8 Alginate assay

A modified protocol suggested by Usov *et al.* (1995) was followed. Prior to the alginate assay, two solutions were made. A borate solution was made by adding solid NaOH to a 20% suspension of borate in distilled water until complete dissolution. A 3,5-Dimethylphenol solution were made by dissolving 100 mg 3,5-Dimethylphenol in 100 mL acetic acid. Sodium alginate in concentrations of 500, 250, 125, 62.5, 31.25, 15.625 μg/mL were used as standard to generate a standard curve (Appendix, Figure A5).

The hydrolyzed material from CE (supernatant, fucoidan pellet and alginate pellet), EAE (retentate, permeate and alginate) and fractionated peaks from HP-SEC was first centrifuged at 1246 G for 10 min to remove any particles. To separate Eppendorf tubes, 100 μL hydrolyzed material (9.09 mg/mL) or standard (two parallels), 100 μL borate solution and 800 μL H_2SO_4 were added and heated up to 70 °C for 40 min in an Eppendorf Thermomixer compact (Sigma Aldrich, Saint Louis, MO, USA). The tubes were cooled down to room temperature before 20 μL 3,5-Dimethylphenol solution were added. The samples were subsequently incubated for 10 min at room temperature. The absorbance was measured at 450 and 400 nm using a SpectraMax® i3x microplate reader and the difference in absorbance (A450-A400) were used to estimate the alginate content in the different samples.

2.4.9 Polyphenol assay

For estimation of total polyphenol content in algae, a polyphenol assay described by Zhang *et al.* (2006) was followed. Phloroglucinol in concentrations of 100, 50, 25, 12.5 and 6.5 μg/mL were used to generate a standard curve (Appendix, Figure A6). The assay was performed with two parallels of lyophilized material from CE (supernatant and fucoidan pellet) and EAE (retentate and permeate) dissolved in distilled water (100 mg/mL).

In each well of a Nunc 96-well microplate (Sigma Aldrich, Saint Louis, MO, USA), 100 μL of Folin-Ciocalteus reagent was added to 20 μL of sample/standard and incubated for 5 min at room temperature. Then, 80 μL of 7.5% sodium carbonate were added to the wells and mixed. The plate was covered and left in the dark for 2 hours at room temperature. The absorbance was measured at 750 nm using a spectrophotometric microplate reader (SpectraMax® i3x, Molecular Devices, San Jose, CA, USA).

2.4.10 UPLC-DAD-QToF-MS

For characterization of phlorotannins, the samples were analyzed using a 1290 Infinity ultra-performance liquid chromatography (UPLC) system coupled with a 1260 Infinity diode array detector (DAD) and a 6540B Quadruple – Time of Flight Mass spectrometer (QToF-MS) with a dual electrospray ionization (ESI) source (all from Agilent technologies, Santa Clara, CA, USA). The samples were separated using a 2.1×50 mm ZORBAX Eclipse Plus C18 column with a particle size of 1.8 μm (Agilent technologies, Santa Clara, CA, USA).

Lyophilized samples from CE (supernatant and fucoidan pellet) and EAE (retentate and permeate) was dissolved in 1% FA solution (2 mg/mL). The mobile phases used were water of analytical grade with 0.1% FA (A) and ACN + 0.1% FA (B) with a flow rate of 0.3 mL/min. Mobile phase B was run in a gradient from 5 to 100% over 15 min. The data were analyzed using a Resolve Isotope Deconvolution method in the MassHunter Qualitative Analysis B.07.00 software (Agilent technologies, Santa Clara, CA, USA). The Resolve Isotope Deconvolution produces a zero-charge, centroided spectrum from a single MS or MS/MS centroided spectrum.

3 Results

3.1 Proximate composition of fresh macroalgae

The DM and water content of *A. nodosum* in this experiment was 30.3% and 69.7%, respectively. For *S. latissima* the DM and water content were 10.3% and 89.7%, respectively. The ash content of *A. nodosum* was 4.4%, whereas the organic matter content was 25.9% of DM. For *S. latissima* the ash content was 6.9%, whereas the organic matter content was 3.4% of DM, summarized in Table 2.

Table 2. The dry matter (DM), water, ash and organic matter content of fresh *A. nodosum* and *S. latissima*. The DM and water content presented as percent (%) wet weight, whereas ash and organic matter content presented as % dry weight (DM).

	Dry matter	**Water**	**Ash**	**Organic matter**
A. nodosum	30.3	69.7	6.9	25.9
S. latissima	10.3	89.7	4.4	3.4

3.2 Dry matter content of the different samples

After CE the samples were fractionated into three samples, a supernatant, a fucoidan pellet and an alginate pellet. For *A. nodosum*, the supernatant (45.4 g), the fucoidan pellet (4.17 g) and the alginate pellet (0.36 g) gave a yield of 90.8%, 8.34% and 0.72%, respectively, of the start material of 50 g DM. Similarly, the samples after EAE were fractionated into three samples for each enzyme, a retentate (R), permeate (P) and alginate (A). Table 3 summarizes the DM content and the yield of each sample obtained during EAE of *A. nodosum*. The permeates samples provide the largest DM content and yield, followed by the retentate and alginate samples. No major difference was observed between the different enzymes tested, but they all provided slightly higher DM content and yield of the permeates, compared to the control permeate.

Table 3. The dry matter (g DM) content and yield (%) for Permeate (P), Retentate (R) and Alginate (A) sample from enzyme-assisted extraction from *A. nodosum*, based on the start material of 64.5 g DM for each sample

	Dry matter (g)				Yield (%)			
	Permeate (P)	**Retentate (R)**	**Alginate (A)**	**Total**	**Permeate (P)**	**Retentate (R)**	**Alginate (A)**	**Total**
Depol 692L	15.1	3.58	1.42	20.10	23.41	5.55	2.20	31.16
Depol 793L	15.1	3.60	1.51	20.21	23.41	5.58	2.34	31.33
Cellulase 13L	15.3	3.17	1.58	19.79	23.72	4.91	2.45	31.08
Control	14.3	3.86	1.32	19.48	22.17	5.98	2.05	30.20

After CE of *S. latissima*, the supernatant (43.7 g), the fucoidan pellet (0.8 g) and the alginate pellet (0.28 g) gave a yield of 87.4%, 1.6% and 0.56%, respectively, of the start material of 50 g DM. Table 4 summarizes the DM content and the yield of each sample obtained during EAE of *S. latissima*. The permeate samples provides the largest DM content and yield followed by the retentate and alginate samples. No major differences were observed between the different enzymes, but they all provided slightly higher DM content and yield of the retentates compared to the control retentate.

Table 4. The dry matter (g DM) content and yield (%) for Permeate (P), Retentate (R) and Alginate (A) sample from enzyme-assisted extraction from *S. latissima*, based on the start material of 20 g DM for each sample

	Dry matter (g)				Yield (%)			
	Permeate (P)	**Retentate (R)**	**Alginate (A)**	**Total**	**Permeate (P)**	**Retentate (R)**	**Alginate (A)**	**Total**
Depol 692L	8.49	1.50	0.31	10.30	42.45	7.50	1.55	51.50
Depol 793L	7.90	1.65	0.13	9.68	39.50	8.25	0.65	48.40
Cellulase 13L	5.74	1.65	0.24	7.63	28.70	8.25	1.20	38.15
Control	8.46	1.44	0.50	10.40	42.30	7.20	2.50	52.0

3.3 Molecular weight distribution of polysaccharides

The MW distribution of polysaccharides in the samples were analyzed using HP-SEC coupled with a DAD and RI detector. Pullulan samples of different sizes were used as standards for this analysis where Table A1 (Appendix) summarizes the characteristics for each standard sample. Table 5 shows the relative MW distribution of the polysaccharides from *A. nodosum* after EAE and CE. The relative content of the samples was obtained from the peak areas that were based on the total peak area of the sample, collected from GPC Postrun software. For the supernatant

after CE, all polysaccharides were found in the MW range of 180 - 3 x 10^5 Da. In the fucoidan pellet, the highest content of polysaccharides was found in the range of 180 - 3 x 10^5 Da as well, but unlike the supernatant, 12.8% of the polysaccharides were found in the MW range of 3 x 10^5 - 2 x 10^8 Da. The weight average M_w was therefore higher in the fucoidan pellet (M_w 5.8 x 10^5 Da), due to the polysaccharides found in this MW range. The weight average M_w gives an indication of the average size of polysaccharides in the sample. Most of the polysaccharides from *A. nodosum* after CE and EAE were in the MW range of 180 - 3 x 10^5 Da. For Depol 692 and the control, all polysaccharides have a MW distribution of 180 - 3 x 10^5 Da, thus the weight average M_w for these samples were very similar. In Depol 793 retentate and Cellulase 13 retentate, 5.0% and 7.9%, respectively, of the polysaccharides have a MW between 2 x 10^8 - 4 x 10^{10} Da. The weight average M_w were thus much higher for these two samples, where Cellulase 13 retentate have the highest (M_w 2 x 10^9 Da), and much higher than the fucoidan pellet (M_w 5.8 x 10^5 Da) from CE.

Table 5. The molecular weight (MW) distribution in Da of polysaccharides in *A. nodosum* after chemical extraction (CE) and enzyme-assisted extraction (EAE). Each MW range constitutes a percentage of the total sample (Retentate (R), Permeate (P), supernatant and fucoidan pellet). The weight average M_w for each sample, presented in Da.

A. nodosum										
	CE		**EAE**							
Molecular Weight range	**Supernatant**	**Fucoidan pellet**	**Depol 692-R**	**Depol 692-P**	**Depol 793-R**	**Depol 793-P**	**Cellulase 13-R**	**Cellulase 13-P**	**Control-R**	**Control-P**
4 x10^{10} -1 x 10^{13}	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2 x 10^8 - 4 x 10^{10}	0.0	0.0	0.0	0.0	5.0	0.0	7.9	0.0	0.0	0.0
3 x 10^5 - 2 x 10^8	0.0	12.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
180 - 3 x 10^5	100.0	87.2	100.0	100.0	95.0	100.0	92.1	100.0	100.0	100.0
Weight average MW (M_w)	6.4 x 10^4	5.8 x 10^5	6.6 x 10^3	6 x 10^3	1.2 x 10^6	6.4 x 10^3	2 x 10^9	6.3 x 10^3	8 x 10^3	5.6 x 10^3

Table 6 shows the relative MW distribution of polysaccharides from *S. latissima* after CE and EAE. All the polysaccharides present in the supernatant after CE have a MW in the range of 180 - 3 x 10^5 Da. The fucoidan pellet also have the highest percentage of the total polysaccharides in this MW range, but 33.7% of the polysaccharides have a MW between 3 x 10^5 - 2 x 10^8 Da. The weight average M_w was thus higher in the fucoidan pellet compared to the supernatant. Similar to CE, most polysaccharides after EAE have a MW in the range of 180 - 3 x 10^5 Da, whereas all the polysaccharides in the permeate samples have MW in this range.

The weight average M_w in the permeate samples were very similar (M_w 2.5-2.6 x 10^4 Da), but the control samples were slightly higher (MW 2.8 x 10^4 Da). In all the retentate samples there was a small amount of the polysaccharides in the MW range of 4 x 10^{10} - 1 x 10^{13} Da and a small amount between 3 x 10^5 - 2 x 10^8 Da. The retentate sample of Depol 692 was the only sample with polysaccharides with a MW between 2 x 10^8 - 4 x 10^{10} Da. The weight average M_w was highest in the retentate sample of Depol 793 (M_w 2.7 x 10^{11} Da).

Table 6. The molecular weight (MW) distribution in Da of polysaccharides in *S. latissima* after chemical extraction (CE) and enzyme-assisted extraction (EAE). Each MW range constitutes a percentage of the total sample (Retentate (R), Permeate (P), supernatant and fucoidan pellet). The weight average M_w for each sample presented in Da.

S. latissima										
	CE		**EAE**							
Molecular Weight range	**Supernatant**	**Fucoidan pellet**	**Depol 692-R**	**Depol 692-P**	**Depol 793-R**	**Depol 793-P**	**Cellulase 13-R**	**Cellulase 13-P**	**Control-R**	**Control-P**
4 x10^{10} -1 x 10^{13}	0.0	0.0	0.80	0.00	0.04	0.00	0.01	0.00	0.02	0.00
2 x 10^8 - 4 x 10^{10}	0.0	0.0	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3 x 10^5 - 2 x 10^8	0.0	33.7	0.03	0.00	0.05	0.00	0.03	0.00	0.03	0.00
180 - 3 x 10^5	100.0	66.3	96.29	100.00	91.39	100.00	96.91	100.00	95.21	100.00
Weight average MW (M_w)	3.1 x 10^4	2.7 x 10^5	7,7 x 10^{10}	2.5 x 10^4	2.7 x 10^{11}	2.5 x 10^4	4.4 x 10^{10}	2.6 x 10^4	1.9 x 10^{11}	2.7 x 10^4

The HP-SEC chromatograms were very similar for all the retentate and permeate samples after EAE, as well as the supernatant and fucoidan pellet after CE, for both species. Figure 6 shows the HP-SEC chromatogram for Depol 692 retentate (R) and permeate (P) from *S. latissima*, as an example. The main peak observed in the permeate sample, was also observed in the retentate sample and includes all molecules in the MW range of 180 - 3 x 10^5 Da and was the MW range where the highest number of polysaccharides were found in every sample. In the retentate samples, there were detected many high MW compounds that was not observed in the permeate samples. The chromatogram for the retentate sample is focused on this area, which resulted in the entire main peak not being shown. The original chromatogram can be seen in the Appendix, Figure A7. The red baseline gives an indication of how GPC Postrun has integrated the peaks after HP-SEC.

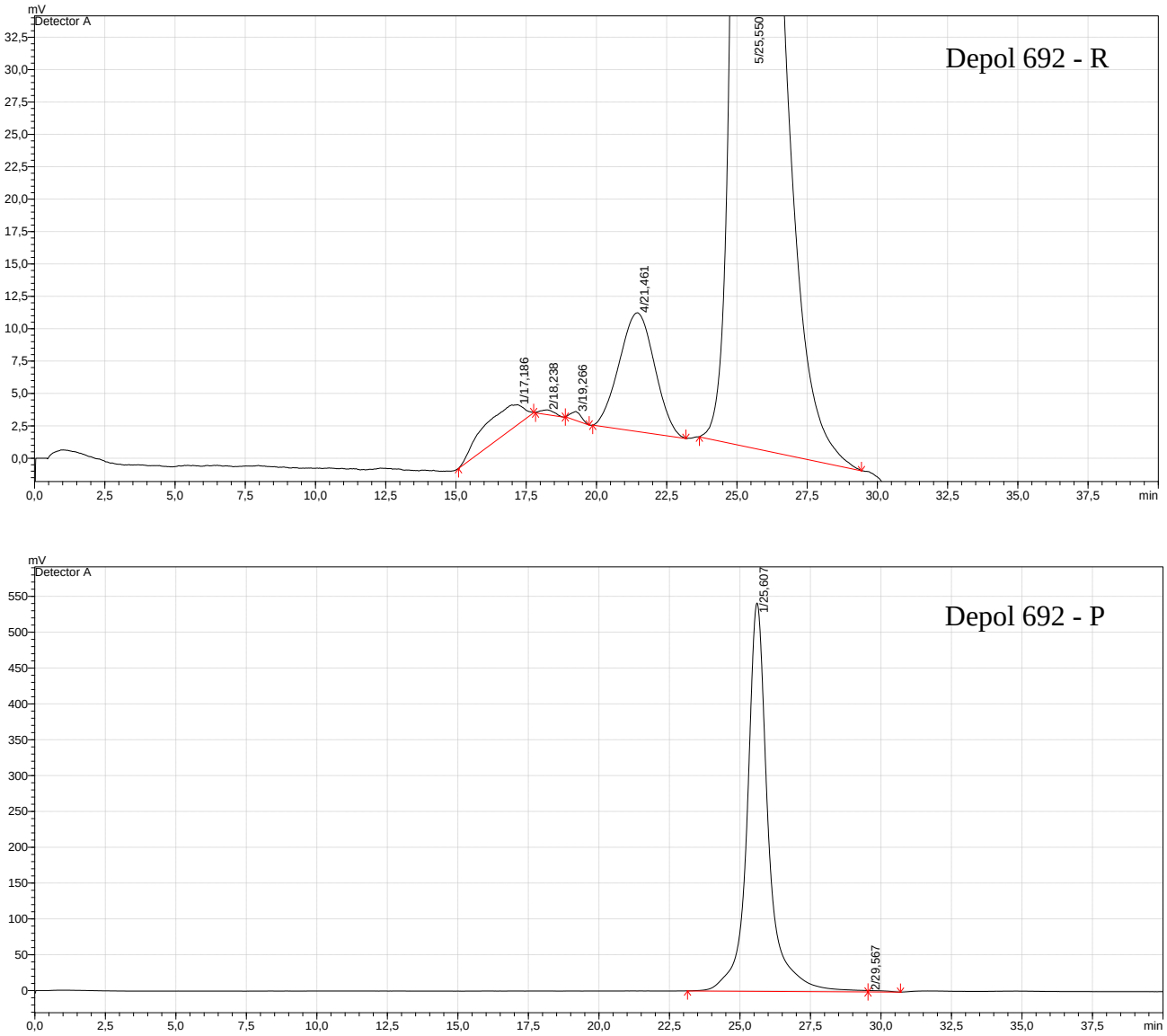

Figure 6. High performance – size exclusion chromatogram (HP-SEC) comparing Depol 692 retentate (R) and permeate (P) samples from *S. latissima*. The main peak in the permeate sample was also observed in the retentate sample, in addition to the smaller peaks ahead of the tallest peak. The x-axis displays the retention time in minutes while the y-axis shows the relative intensity of the refractive index in mV.

3.4 Carbohydrate content

The carbohydrate composition of *A. nodosum* and *S. latissima* after EAE and CE were estimated based on the detection of monomeric sugars after hydrolysis with HCl and H_2SO_4. Analysis was performed using IEX-HPLC-RID. Prior to the analysis, glucose, xylose, mannitol and fucose standards were run, where glucose eluted first (10.7 min), followed by xylose (11.4 min), mannitol (11.8 min) and lastly fucose (13.0 min) (Appendix, Figure A8). The carbohydrate

content varied a lot, depending on the species, extraction method and the hydrolysis method that had been used.

Large differences were observed between the samples obtained after CE when hydrolyzing with HCl and H_2SO_4 in *A. nodosum*, where HCl hydrolysis resulted in approximately 10x higher yield. The supernatant was the only sample after CE with detectable glucose after hydrolysis with HCl and H_2SO_4 in *A. nodosum*. The supernatant contains the highest percentage of mannitol (5.10% DW) and fucose (10.88% DW) after hydrolysis with HCl, (Figure 7, A). The fucoidan pellet contains a slightly lower percentage of mannitol (4.51% DW) and fucose (8.63% DW), although it contains the highest amount of xylose (7.86%DW) after hydrolysis with HCl. The alginate pellet contains a small percentage of xylose (1.80% DW), mannitol (2.21% DW) and fucose (1.97% DW) as well. Hydrolysis with H_2SO_4 (Figure 7, B), resulted in a much lower monomeric sugar yield, compared to hydrolysis with HCl. This is clearly indicated on the y-axis in Figure 7, as the scale is much higher for hydrolysis with HCl (Figure 7, A). Only xylose (1.52% DW) and fucose (1.07% DW) were detected in the fucoidan pellet, however it had the highest content of these two monomeric sugars in the three samples, after hydrolysis with H_2SO_4. In the supernatant all the monosaccharides were detected in small amounts and was the only sample after hydrolysis with H_2SO_4 mannitol (0.51% DW) and glucose (0.17% DW) was detected in. Whereas, in the alginate pellet, only a small amount of fucose (0.26% DW) was present.

Similar to CE, large differences were observed in the samples obtained after EAE when hydrolyzing with HCl and H_2SO_4 for *A. nodosum*. After hydrolysis with HCl, the overall yield of the different monosaccharides was quite low, as well as no enzymes stood out (Figure 8, A). The permeate samples contained similar amounts of glucose and mannitol for all three enzymes and the control. The retentate and alginate samples all contained xylose (approximately 0.35% DW), mannitol and fucose in vary similar amounts for all three enzymes, as well as the control. The retentate samples contained a slightly higher glucose and fucose content than the A samples.

After hydrolysis with H_2SO_4 (Figure 8, B), the monomeric sugar yield was very similar to HCl hydrolysis for all the samples, except Depol 793 retentate. In general, the fucose yield slightly increased, whereas very little glucose was detected using this hydrolysis method. Mannitol was the primary monosaccharide detected in all the permeate samples (<1.1% DW), although it was slightly lower than for HCl hydrolyzed samples. The retentate (except Depol 793 retentate) and alginate samples contained varying amounts of xylose, mannitol and fucose, but were very similar to each other. Depol 793 retentate stood out after hydrolysis with H_2SO_4

with the highest yield for glucose (2.31% DW), xylose (2.57% DW), mannitol (0.59% DW) and fucose (4.21% DW). The mannitol content in this sample was relatively low and was very similar to all the other samples. In general, the overall yield after EAE were approximately 1-3% DW in the different samples, with the exception of Depol 793 which had an overall yield of over 9% DW. This yield was much lower than the samples after CE which were hydrolyzed with HCl. The supernatant and fucoidan pellet had an overall yield of approximately 20% DW.

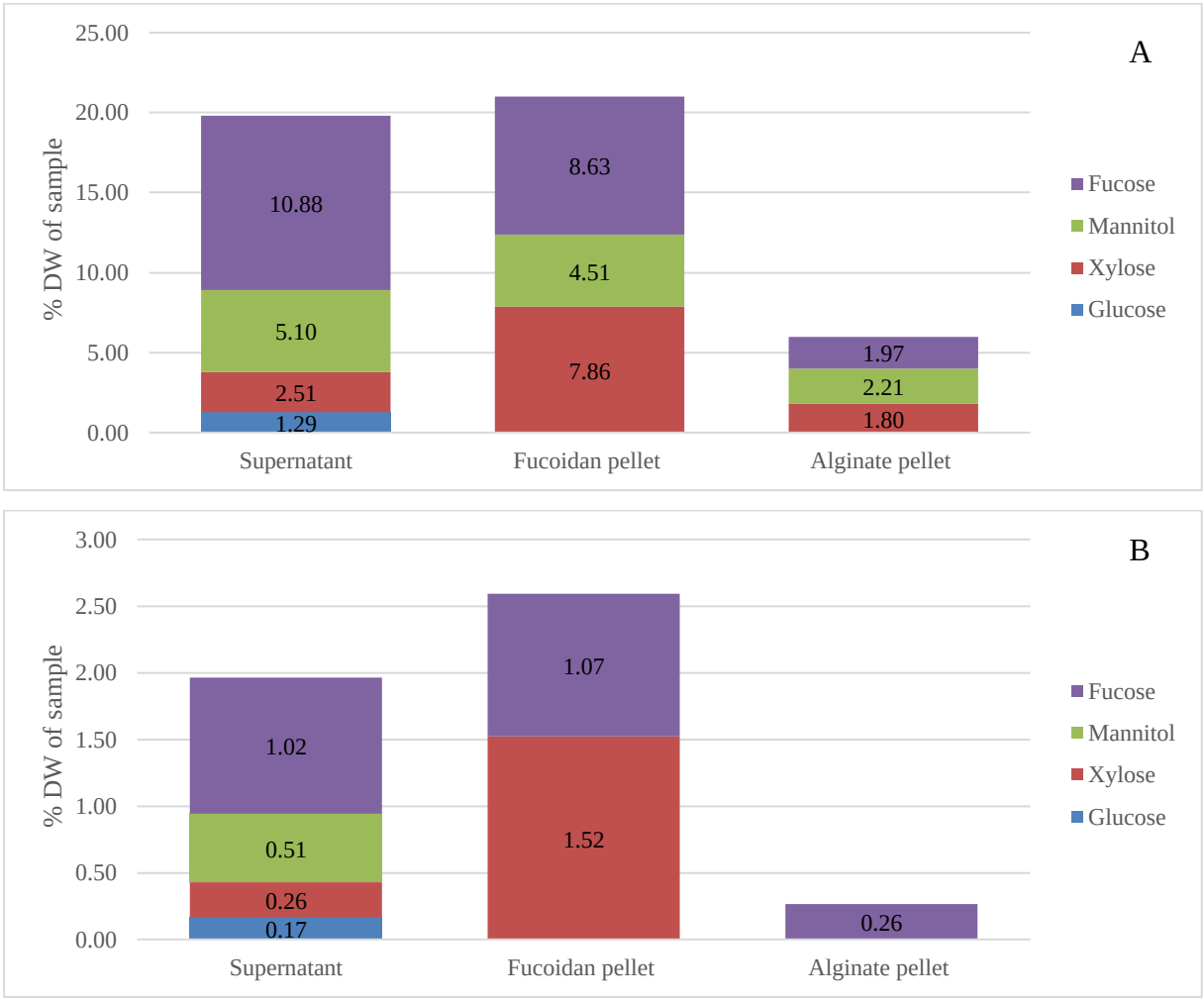

Figure 7. Carbohydrate content in *A. nodosum* after hydrolysis with HCl (A) and H_2SO_4 (B) of the samples obtained after chemical extraction (CE). The content is presented as percent (%) dry weight (DW) of the sample on the y-axis. The % each monomeric sugar make up is included on each respective box. HCl hydrolysis resulted in approximately 10x higher yield than H_2SO_4 hydrolysis. The fucoidan pellet and supernatant contained the highest carbohydrate content. There is very little glucose detected, and only in the supernatant.

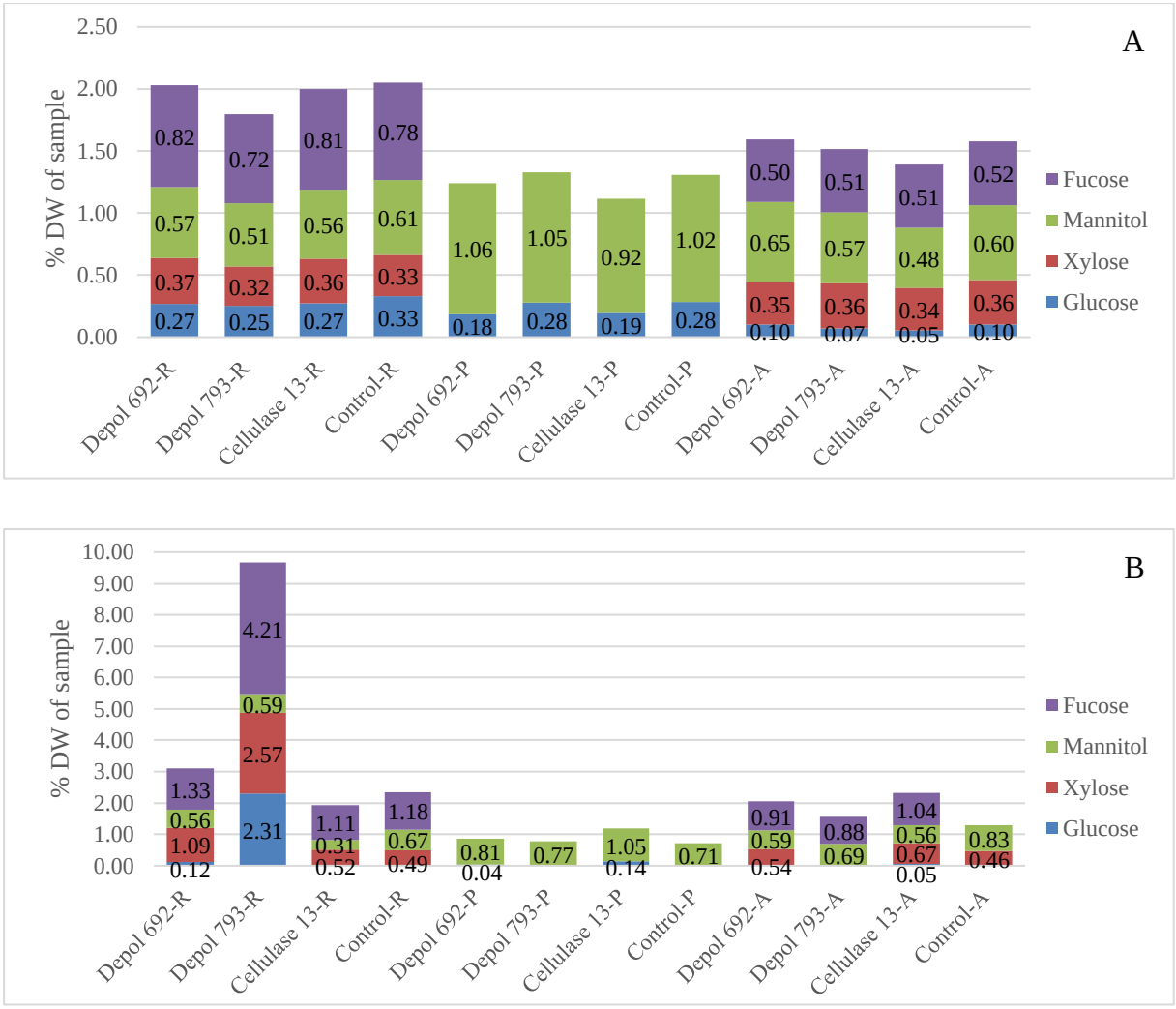

Figure 8. Carbohydrate content in *A. nodosum* after hydrolysis with HCl (A) and H_2SO_4 (B) for the samples obtained after enzyme-assisted extraction (EAE). The content is presented as percent (%) dry weight (DW) of the samples on the y-axis. The % each monomeric sugar makes up are included on each respective box. The samples are grouped together by retentate (R), permeate (P) and alginate (A) for the three different enzymes and the control. The overall monomeric sugar content was approximately 1-3% DW in all the samples, except Depol 793 retentate which had an overall yield of over 9% DW. The retentate samples have the highest carbohydrate content based on the monomeric sugars detected, whereas the permeate samples have the lowest. There is no detection of xylose or fucose in the permeate.

Similar to *A. nodosum*, large differences were observed after CE when hydrolyzing with HCl and H_2SO_4 for *S. latissima*. In contrast to *A. nodosum*, where fucose was the most abundant monosaccharide after CE, mannitol was the most abundant monosaccharide in *S. latissima*. Similarly, HCl hydrolysis resulted in the highest yield of monomeric sugars (Figure 9, A), where the fucoidan pellet (19.55% DW) had the highest yield for *S. latissima*. Glucose and fucose was detected in all the three samples in varying amounts where the fucoidan pellet had the highest content (3.87% DW glucose and 3.37% DW fucose). The supernatant had the

highest content of mannitol (11.44% DW). The alginate pellet contained all four monomeric sugars, but in very small amounts (<1.0% DW for each sugar).

HCl hydrolysis resulted in approximately 8-10x higher monomeric sugar yield compared to hydrolysis with H_2SO_4 (Figure 9, B), and is seen very clearly in Figure 9 where the scale is much higher for HCl hydrolysis. Similarly, glucose and fucose was detected in all three samples and the fucoidan pellet had the highest overall content of monomeric sugars (2.41% DW).

Figure 9. Carbohydrate content in *S. latissima* after hydrolysis with HCl (A) and H_2SO_4 (B) for the samples obtained after chemical extraction (CE). The content is presented as percent (%) dry weight (DW) of the samples on the y-axis. The % each monomeric sugar makes up are included on each respective box. HCl hydrolysis resulted in 8-10x higher monomeric sugar yield compared to hydrolysis with H_2SO_4. The fucoidan pellet contained the highest amount of monomeric sugars and yielded the most glucose, xylose and fucose. Mannitol is the most abundant monomeric sugar present in the samples, where the supernatant had the highest content.

After EAE of *S. latissima*, there is no enzyme that stood out for either HCl or H_2SO_4 hydrolysis. Similar to CE, mannitol was the most abundant monomeric sugar present in the samples. HCl hydrolysis (Figure 10, A) resulted in poor monomeric sugar yields. Fucose was detected in varying amounts in all the samples after HCl hydrolysis, with a slightly higher content in the retentate samples. Cellulase 13 permeates had the highest xylose content (0.94% DW), whereas all the retentate samples and Depol 692 permeate contained a slightly smaller amount. Glucose is detected in all the samples, whereas Depol 692 alginate sample contain a slightly higher amount (0.55% DW).

Hydrolysis with H_2SO_4 (Figure 10, B) resulted in 10x higher monomeric sugar yield, compared to hydrolysis with HCl. This is clearly indicated in Figure 10, as the scale is much higher for hydrolysis with H_2SO_4. In contrast to HCl hydrolysis where fucose was detected in all samples but one, it was limited to the retentate and alginate samples after hydrolysis with H_2SO_4. There was no enzyme that stood out after this hydrolysis method either, however Cellulase 13 retentate had a somewhat higher overall monomeric sugar content (19.17% DW) than the other retentate samples. The permeates primarily contained mannitol, glucose and a minor amount of xylose. Mannitol was the most abundant sugar inn all the samples, where Depol 692 permeate had the highest content (15.79% DW). The control alginate sample had the highest glucose (8.85% DW) and xylose (3.12% DW) content of all the samples, as well as it contained a substantial amount of mannitol (10.72% DW) and fucose (3.00% DW). Hydrolysis with H_2SO_4 resulted in the highest monomeric sugar content in *S. latissima*, compared to CE samples hydrolyzed with HCl. The retentate samples and the control alginate sample had the highest content after hydrolysis with H_2SO_4 which made up over 25% DW of the samples, compared to the fucoidan pellet after CE which made up approximately 20% DW of the sample.

In general, hydrolysis with H_2SO_4 gave the highest yield for all sugars after EAE, while hydrolysis with HCl gave the overall highest yield after CE in both species. For *A. nodosum*, CE estimated the highest carbohydrate content, whereas the carbohydrate content was higher after EAE for *S. latissima*. The estimated carbohydrate content was higher in *S. latissima* compared to *A. nodosum*.

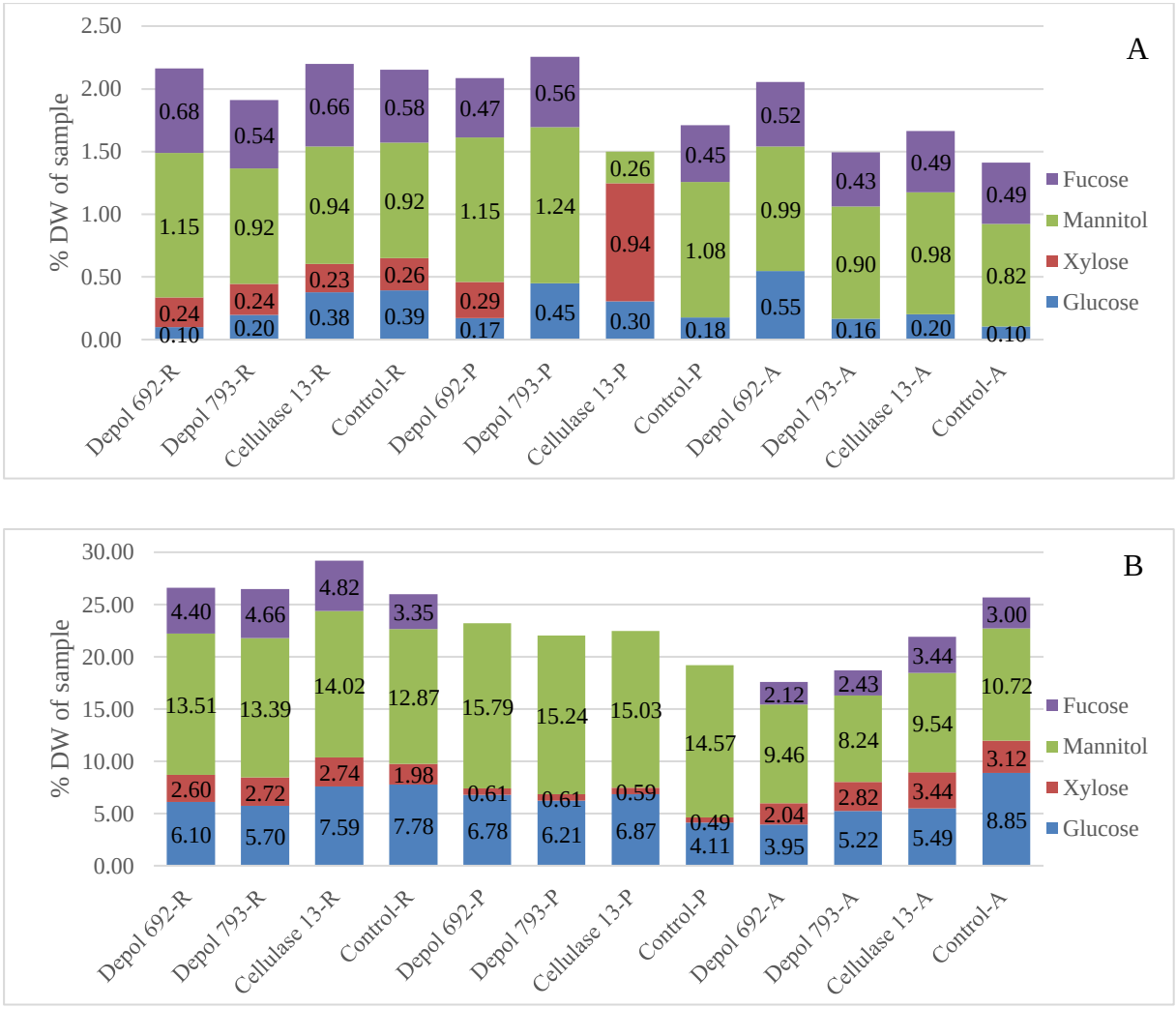

Figure 10. Carbohydrate content in *S. latissima* after hydrolysis with HCl (A) and H_2SO_4 (B) for the samples obtained after enzyme-assisted extraction (EAE) presented as percent dry weight (DW) of the specific sample. The total carbohydrate content in percent DW of the samples can be seen on the y-axis, and the percent for each monomeric sugar make up are included on each respective box. The samples are grouped together by retentate (R), permeate (P) and alginate (A) for the three different enzymes and the control. Hydrolysis with H_2SO_4 resulted in 10x higher monomeric sugar content in the samples, compared to hydrolysis with HCl. The retentate samples and control alginate had the highest content (>25% DW).

3.5 L-fucose content

The L-fucose content in the samples obtained after CE and EAE were estimated using Megazyme's L-fucose kit with L-fucose as standard. The content is only presented for samples hydrolyzed with H_2SO_4 (Figure 11), as samples hydrolyzed with HCl resulted on a low yield and is therefore not presented. After CE, the fucose content in *A. nodosum* and *S. latissima* was very similar in the supernatant and fucoidan pellet, whereas for the alginate pellet, the fucose content was almost absent in *S. latissima*. Overall, there was a higher fucose content in *A.*

nodosum than in *S. latissima*. After EAE, Depol 692 retentate (1.5% DW), control retentate (0.8% DW) and Cellulase 13 permeate (0.85% DW) were the only samples that had a higher estimated fucose content in *S. latissima* than in *A. nodosum*. Cellulase 13 retentate had the highest estimated fucose content (1.13% DW) for *A. nodosum* tightly followed by Depol 793 permeate (1.2% DW) and Cellulase 13 alginate (1.04% DW). The fluctuation of fucose content in the different samples for *S. latissima* was not as substantial, as for *A. nodosum*, where it varied greatly between the samples after EAE.

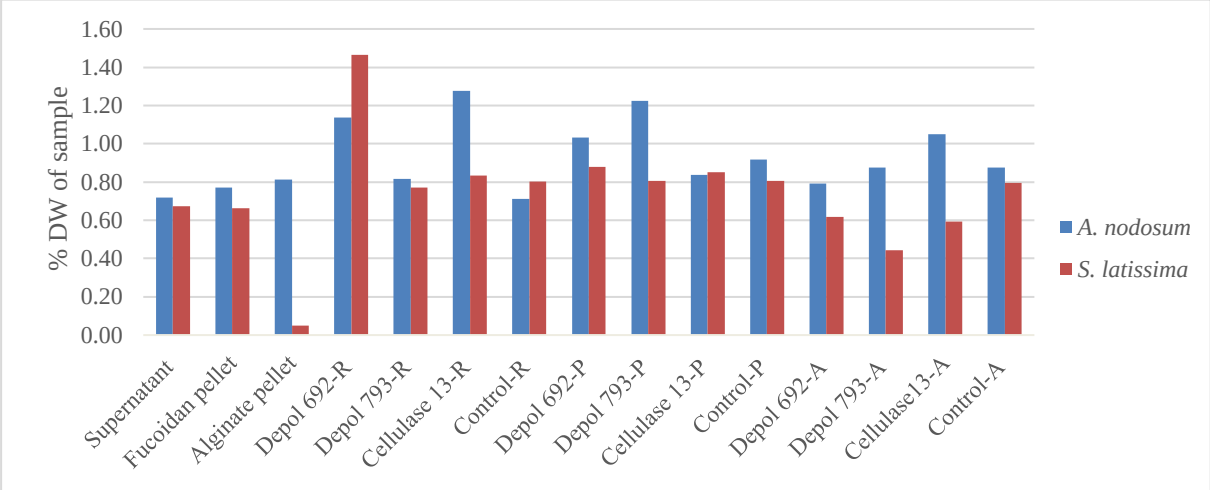

Figure 11. Estimated fucose content after L-fucose assay in *A. nodosum* and *S. latissima* samples obtained after chemical extraction (CE) and enzyme-assisted extraction (EAE). The estimated content is presented as percent (%) dry weight (DW) of the samples and is based on the results from H_2SO_4 hydrolysis. The samples after EAE are grouped together by retentate (R), permeate (P) and alginate (A) samples from Depol 692, Depol 793, Cellulase 13 and the control. The estimated L-fucose content is in general higher in *A. nodosum* compared to *S. latissima*. EAE resulted in the highest release of L-fucose compared to CE, where the Cellulase 13 retentate sample had the highest L-fucose content (1.2% DW) for *A. nodosum*. Depol 692 retentate sample contained the highest L-fucose content (1.5% DW) for *S. latissima*.

Commercially available fucoidan was added as an additional standard to this assay to estimate the amount of fucoidan present and its degree of purity compared to the standard. As there was a high degree of uncertainty regarding the results, the data was not presented.

3.6 Alginate content

The alginate content in the different samples were estimated using the difference in absorbance (A450-A400). Samples hydrolyzed with HCl and H_2SO_4 methods were analyzed, however as

HCl hydrolysis resulted in a higher alginate content (Figure 12), data from H_2SO_4 hydrolyzed samples are not presented. After CE, the alginate pellet had the highest alginate content (9.22% DW), followed by the fucoidan pellet (4.73% DW), and lastly the supernatant (2.18% DW) in *A. nodosum*. However, the highest alginate content was estimated in the control alginate sample (3.31% DW) after EAE. After EAE, alginate was almost exclusively detected in the alginate samples, with the exception of Depol 793 permeate, where a minor amount of alginate was estimated (0.66% DW). Compared to *A. nodosum*, the estimated alginate content was much lower in *S. latissima.* After CE, the fucoidan pellet had the highest alginate content (4.79 % DW), whereas a smaller amount of alginate was detected in the alginate pellet (2.56% DW). The Depol 793 alginate sample had the highest estimated alginate content (1.69% DW) after EAE. In addition to estimated alginate content in the alginate samples, a minor amount of alginate was estimated in the retentate (0.24% DW) and permeate (0.05% DW) samples of Depol 692, as well as the retentate samples for Depol 793 (0.13% DW) and Control (0.16% DW). In general, CE resulted in the highest alginate content, compared to EAE for both species.

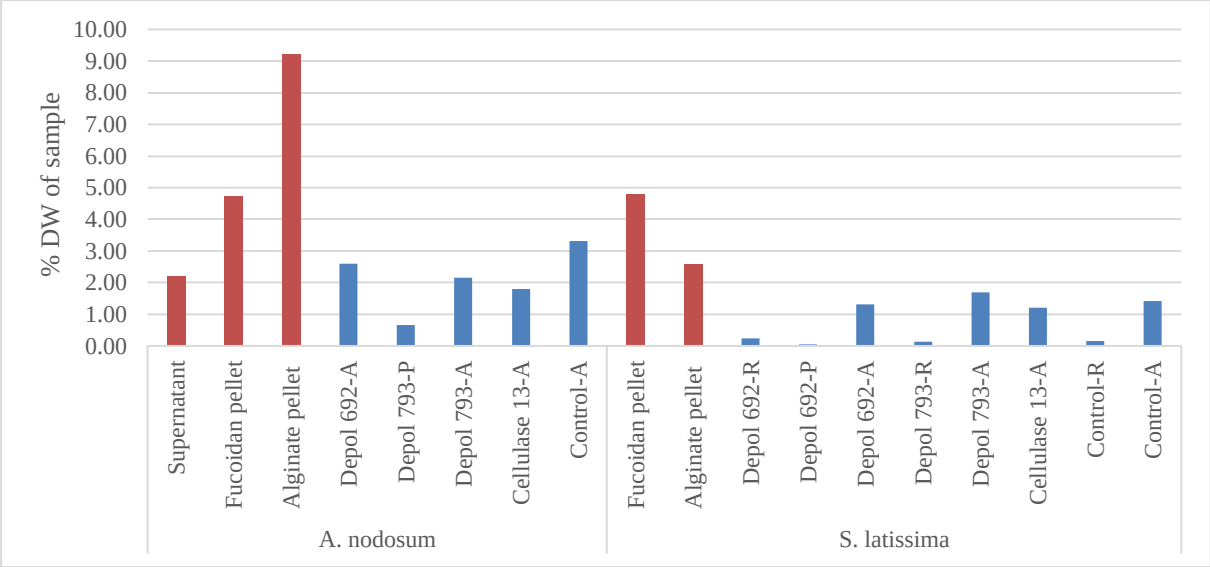

Figure 12. Estimated alginate content in *A. nodosum* and *S. latissima* after chemical extraction (red bar) and enzyme-assisted extraction (blue bar). The data is presented as percent (%) dry weight (DW) of the specific sample on the y-axis and is based on the results form HCl hydrolysis. CE resulted in the overall highest alginate content in both species.

3.7 Fractionated Depol 793 and control retentate from *A. nodosum*

During HP-SEC, the peaks of Depol 793 retentate and control retentate, form *A. nodosum* was fractionated and collected. Depol 793 retentate was chosen due to the high carbohydrate yields

(Figure 8, B) and the high MW of the polysaccharides present in the sample (Table 5). Therefore, the peaks were collected and analyzed more closely to determine in which fraction the different valuable components were located. The control retentate was also fractionated for comparison. Three peaks were observed in the samples after HP-SEC and were collected, where peak 1 contain molecules with a MW between 2 x 10^8 - 4 x 10^{10} Da, whereas peak 2 and 3 contain molecules with a MW between 180 - 3 x 10^5 Da (Table 5). Alginate analysis was performed on the collected peaks, but no alginate was detected (data not shown). There was a much higher amount of fucose detected using the L-fucose assay, compared to carbohydrate analysis with IEX-HPLC-RID, as seen in Figure 13. After L-fucose assay, the highest fucose content was estimated in peak 3 of Depol 793 (0.89% DW) and peak 1 of the control (1.04% DW), however the estimated fucose content was only slightly lower in the remaining peaks for both Depol 793 and control. In contrast, the fucose content was <0.01% DW of all the peaks after carbohydrate analysis with IEX-HPLC-RID. For both Depol 793 and control, fucose was detected in peak 1 (0.05% DW of both samples), indicating the fucose present had MW between 2 x 10^8 - 4 x 10^{10} Da. Xylose is detected in peak 2 for both Depol 793 (0.02% DW) and control (0.02%DW), in addition to a small amount of fucose, which have a MW between 180 - 3 x 10^5 Da. In peak 3 for both samples, glucose (0.01% DW for both samples), mannitol (0.03% DW for both samples) is detected, as well as xylose is detected in Depol 793 (0.02% DW), which have a MW between 180 - 3 x 10^5 Da.

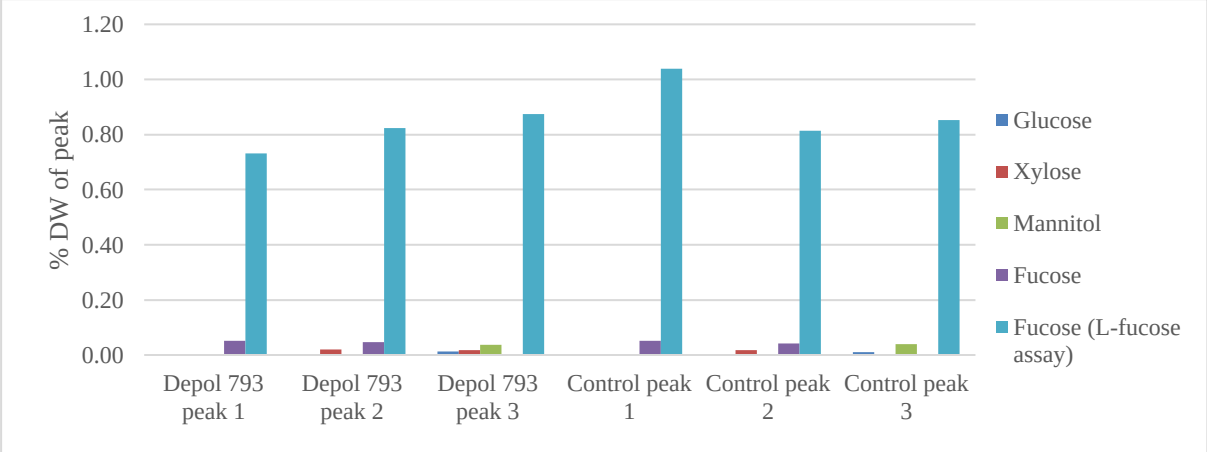

Figure 13. The carbohydrate content in the collected peaks during high performance-size exclusion chromatography (HP-SEC) of Depol 793 sample from *A. nodosum*. The data is presented as percent (%) dry weight (DW) of the peak. Glucose, xylose, mannitol and fucose was estimated using ion exchange-high performance liquid chromatography (IEX-HPLC-RID), while the second fucose was estimated using L-fucose assay. Peak 1 contain molecules in the MW range of 2 x 10^8 - 4 x 10^{10} Da, whereas peak 2 and 3 contain molecules in the MW range between 180 - 3 x 10^5 Da.

3.8 Polyphenol content

The polyphenol content in the samples was determined using colorimetric detection of phloroglucinol equivalents (PGE) at 750 nm. In general, the estimated polyphenol content was much higher in *A. nodosum* samples compared to samples from *S. latissima*. The Depol 692 retentate sample was the only sample from *S. latissima* showing detectable polyphenol content. The estimated polyphenol content constituted only 0.004% DW of the sample from *S. latissima*. For *A. nodosum*, the estimated polyphenol content was higher in the samples after EAE than for CE. The retentate sample treated with the enzyme Cellulase 13 yielded the highest estimated PGE content (1.08% DW) after EAE, followed by the Depol 692 retentate (0.71% DW), control retentate (0.59%) and Depol 793 retentate (0.54% DW) samples (Figure 14). PGE´s were most abundantly found in the retentates samples after EAE, however, small amounts were detected in the permeate samples as well. After CE, the highest amount of PGE was found in the fucoidan pellet (0.05% DW), but it was less than for the control permeate (0.08% DW). Whereas very little PGE were detected in the supernatant (0.01% DW).

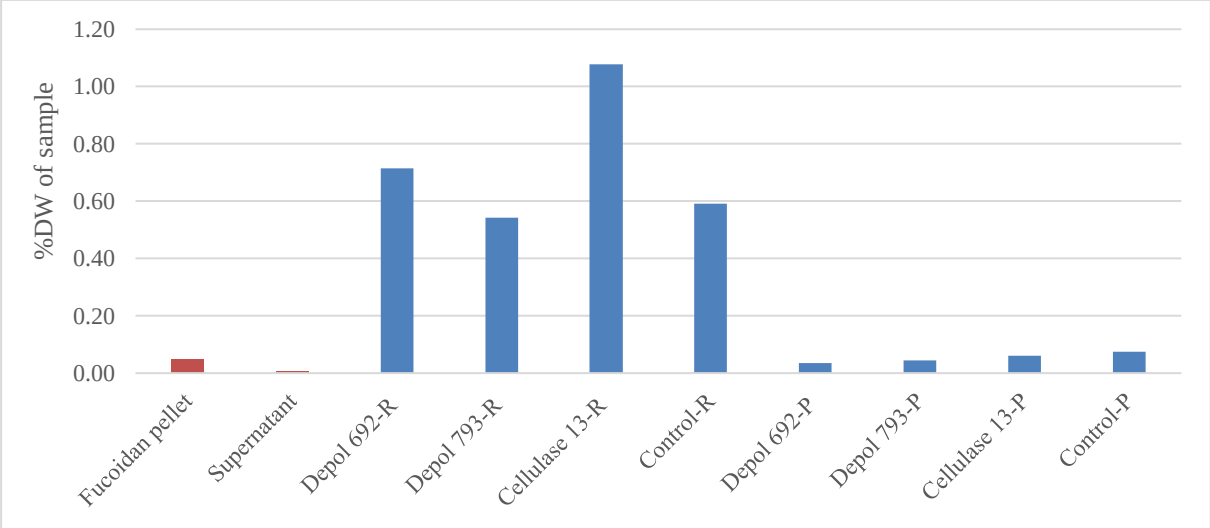

Figure 14. Estimated phloroglucinol equivalents (PGE) in the different samples for *A. nodosum* after chemical extraction (red bar) and enzyme assisted extraction (blue bar) The data is presented as percent (%) PGE of the dry weight (DW) for each sample on the y-axis.

3.9 Phlorotannin characterization

As an additional analysis, it was chosen to run UPLC-DAD-QToF-MS on the samples there was detected PGE´s in, using the polyphenol assay, for characterization of phlorotannins. Se samples were analyzed using both positive and negative ionization mode. However, as negative

ionization mode gave the best results, positive ionization mode is not presented. Figure 15 shows the UV-chromatogram of each samples. After UPLC-DAD-QToF-MS it was clear that the highest amount of polyphenols was found in the retentate samples, where Depol 692 retentate showed the highest polyphenol content after this analysis. The permeate samples from EAE as well as the supernatant and fucoidan pellet from CE had a much smaller content of polyphenols compared to the retentate samples. The Depol 692 retentate sample from *S. latissima* had the lowest polyphenol content of all the samples analyzed using UPLC-DAD-QToF-MS, in accordance with the colorimetric detection (Figure 14). The polyphenol content peaks elute approximately after 5.4 min, whereas the supernatant and fucoidan pellet after CE elute some seconds later.

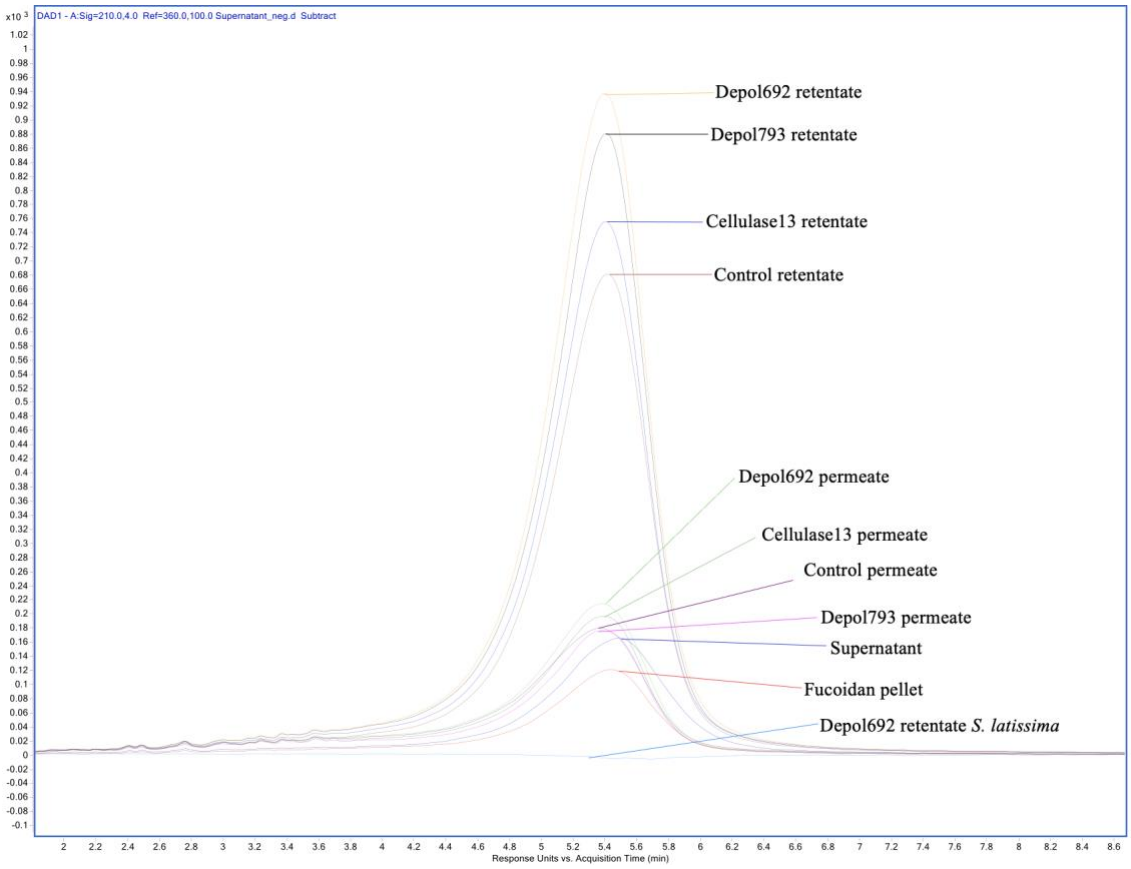

Figure 15. UV-chromatogram at 210 nm showing the polyphenols content of the different samples after analysis using UPLC-DAD-QToF-MS. All samples are from *A. nodosum* with the exception of Depol 692 retentate from *S. latissima*. The retentate samples have the highest polyphenol content of the samples, where the permeates have a much lower polyphenol content. However, the supernatant and fucoidan pellet from chemical extraction (CE) have the lowest polyphenol content from *A. nodosum*. The x-axis shows the retention time in minutes, while the y-axis shows the intensity of the signals detected by diode array detector (DAD). The peaks have an intensity of 0.94 x 10^3 at the highest (Depol 692 – retentate), and the polyphenol peaks were eluting at approximately 5.4 min for all the samples, with the exception of the supernatant and fucoidan pellet after CE, which elutes a few seconds later.

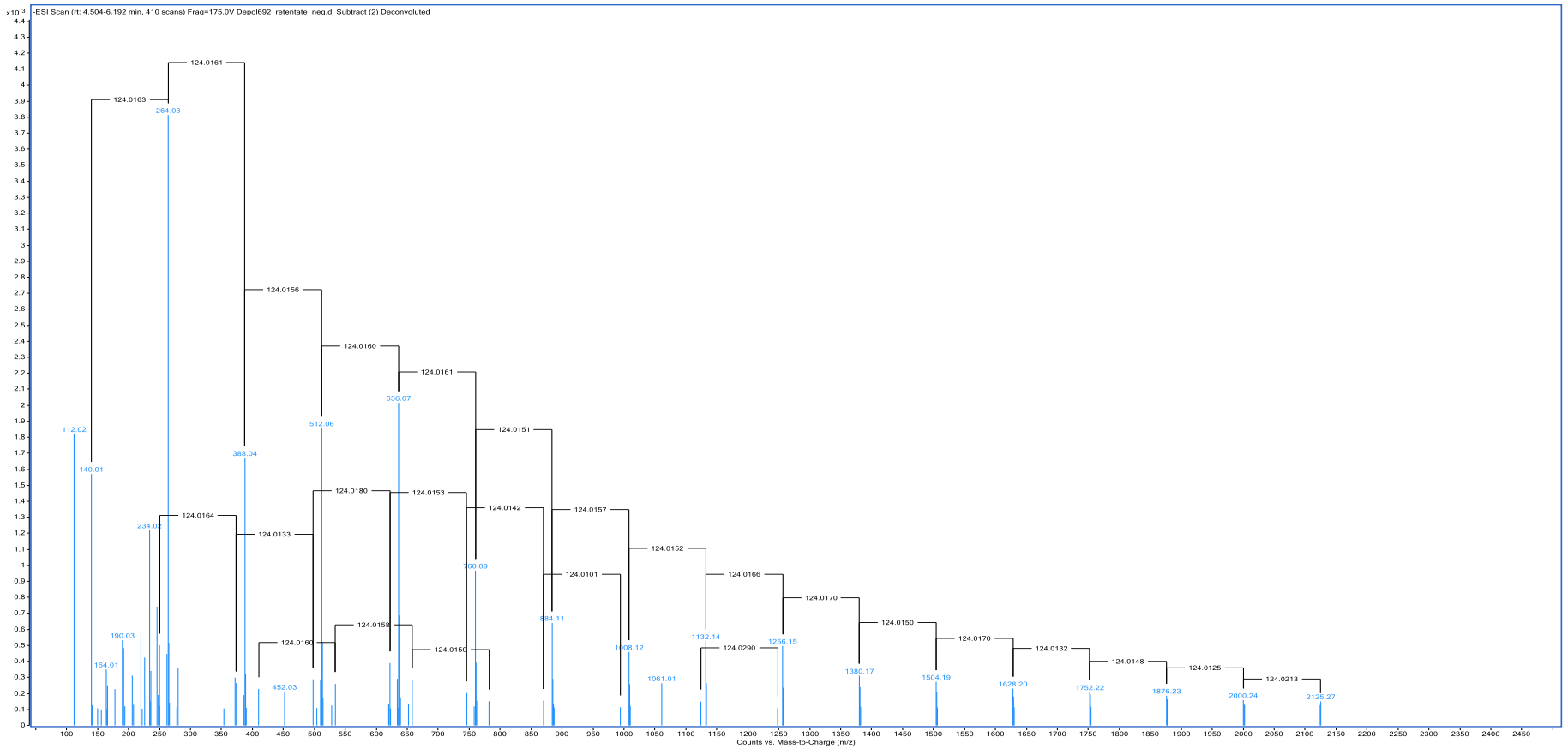

Figure 16. Deconvoluted ESI mass spectrum showing phlorotannins detected in the Depol 692 retentate from *A. nodosum*. The x-axis shows the mass of different compounds, whereas the y-axis shows the intensity (ion counts) of each mass. Delta 124 between the different mass peaks represents a phloroglucinol unit (PGU, M-2H) indicating the increase of one PGU.

Figure A9 (Appendix) shows the isolated UV-chromatogram of Depol 692 retentate sample at 210 nm. The UV-chromatogram shows the absorbance maximum of the sample at approximately 211 nm and 277 nm. The ESI Deconvoluted mass spectrum of Depol 692 retentate (Figure 16) clearly indicate the increase of one PGU (M-2H) through Delta 124 Da between the measured masses.

The mass spectra (Figure 16) of Depol 692 retentate, as well as the mass spectra of the remaining samples gives an indication of the variation of phlorotannins in the samples, where Delta 124 Da clearly indicates the increase of one PGU unit between the measured masses. Based on this data, a range of phlorotannins in a range of sizes was identified in the samples. Figure 17 shows the number of phlorotannins detected in the samples and the retentate samples were most phlorotannins rich. Depol 692 retentate have the highest phlorotannin content, with 28 detected phlorotannins. Slightly less phlorotannins were detected in Cellulase 13 retentate compared to the control retentate. In general, approximately twice as many phlorotannins was detected in the retentates as in the permeates. Depol 692 permeate and Cellulase 13 permeate have the highest amount of 15 phlorotannins. The supernatant and fucoidan pellet after CE have the lowest amount of detected phlorotannins with 6 and 5, respectively. Table A2 (Appendix) shows the variation of phlorotannins in Depol 692 retentate, with phlorotannins ranging from 2-18 PGU. In addition, a suggested molecular formula based on the measured monoisotopic mass were included. Based on already identified phlorotannins from MarinLit database (http://pubs.rsc.org/marinlit), a suggested phlorotannin were proposed for some of the measured masses.

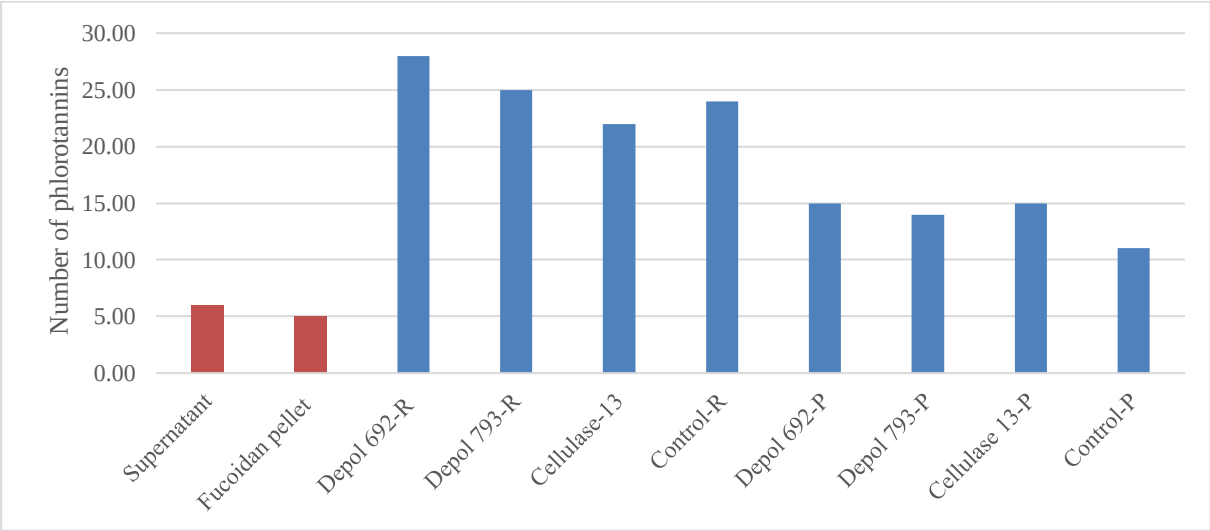

Figure 17. Number of detected phlorotannins in the analyzed samples. The retentate samples have the highest detected amounts with Depol 692 with 28 phlorotannins, followed by the permeate samples from enzyme-assisted extraction (blue bar). The supernatant and fucoidan pellet after chemical extraction (Red bar) have the lowest detected amounts of phlorotannins with 6 and 5 phlorotannins respectively.

3.10 Total yield of valuable components

The chemical composition of the algae varied with the extraction method used for both species. Figure 18 compares the overall yield from the start material after CE (supernatant, fucoidan pellet and alginate pellet combined) (50 g DW) and EAE (retentate, permeate and alginate combined) (64.5 g DW) in *A. nodosum*. After CE, the total carbohydrate content accounted for 19.7% of the total DW in *A. nodosum*. This was distributed in 1.2% glucose, 3.0% xylose, 5.0% mannitol and 10,6% fucose. The L-fucose assay resulted in a fucose yield of 0.72% DW of the total algal biomass. The total estimated PGE constituted 0.01% DW, whereas alginate constituted 2.8% DW of the total algal biomass. The total carbohydrate content in *A. nodosum* after EAE accounted for 0.66% of the total DW using Depol 793. This enzyme gave the highest carbohydrate yield in *A. nodosum*. This was distributed in 0.13% glucose, 0.14% xylose, 0.11% mannitol and 0.28% fucose. Compared to the carbohydrate analysis with IEX-HPLC-RID, the L-fucose assay gave a much higher yield of fucose with the highest being 0.35% DW of the total algal biomass after EAE with Depol 793. The total estimated PGE constituted 0.07% of the total DW with Cellulase 13. The estimated alginate content constituted 0.2% DW of the total algal biomass.

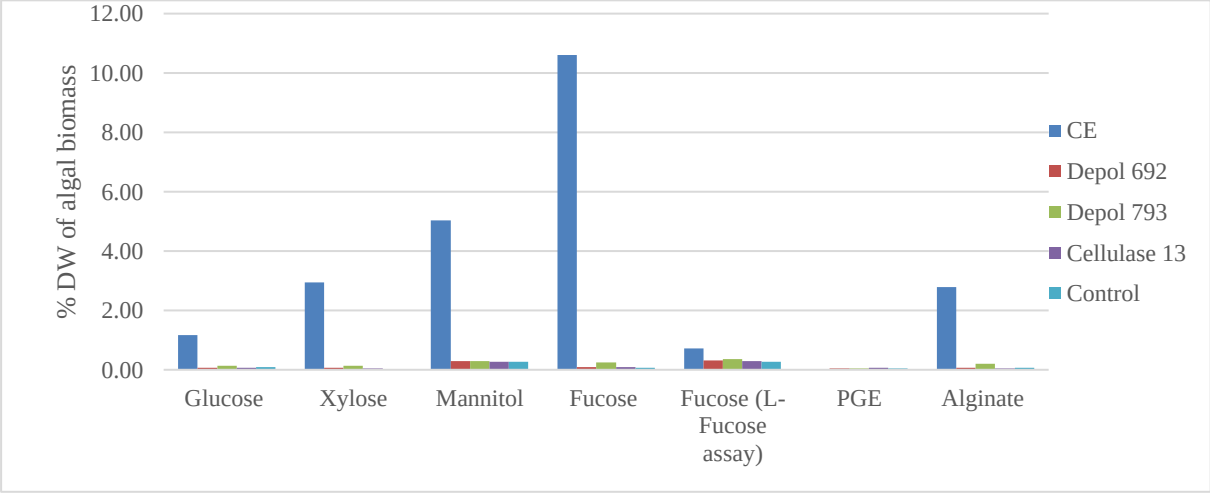

Figure 18. Total yield of the different valuable components from *A. nodosum* after the various analysis methods. The content is presented as total percent (%) dry weight (DW) of the total algal biomass on the y-axis. The hydrolysis method with the highest yield, mentioned in the various sections was used to calculate the % DW of algal biomass. Glucose, xylose, mannitol, fucose from IEX-HPLC after EAE were hydrolyzed with H_2SO_4 for the EAE, while for CE the data was based on HCl hydrolyzed material. The L-fucose data was hydrolyzed with H_2SO_4 while the alginate data was based on hydrolysis with HCl for both extraction methods. Overall, CE resulted in the highest yield of the different valuable components, except for phloroglucinol equivalents (PGE), which was higher for EAE.

Figure 19 compares the overall yield of the start material after CE (supernatant, fucoidan pellet and alginate pellet combined) (50 g DW) and EAE (R, P and A combined) (20 g DW) in *S. latissima*. After CE, the total carbohydrate content accounted for 12.9% DW of the total algal biomass, distributed in 1.6% glucose, 0.03% xylose, 10.2% mannitol and 1.1% fucose. The L-fucose assay resulted in a fucose yield of 0.6% DW of the total algal biomass. The estimated PGE and alginate content after CE constituted <0.01% DW of the total algal biomass. The total carbohydrate content in *S. latissima* after EAE accounted for 12.11% DW of the total algal biomass with Depol 692, distributed in 3.4% glucose, 0.5% xylose, 7.9% mannitol and 0.4% fucose. Compared to carbohydrate analysis using IEX-HPLC-RID, L-fucose assay resulted in fucose constituting 0.15% DW of the total algal biomass in *S. latissima* after EAE with Depol 692. The estimated PGE and alginate content in *S. latissima* constituted <0.01% DW of the total algal biomass.

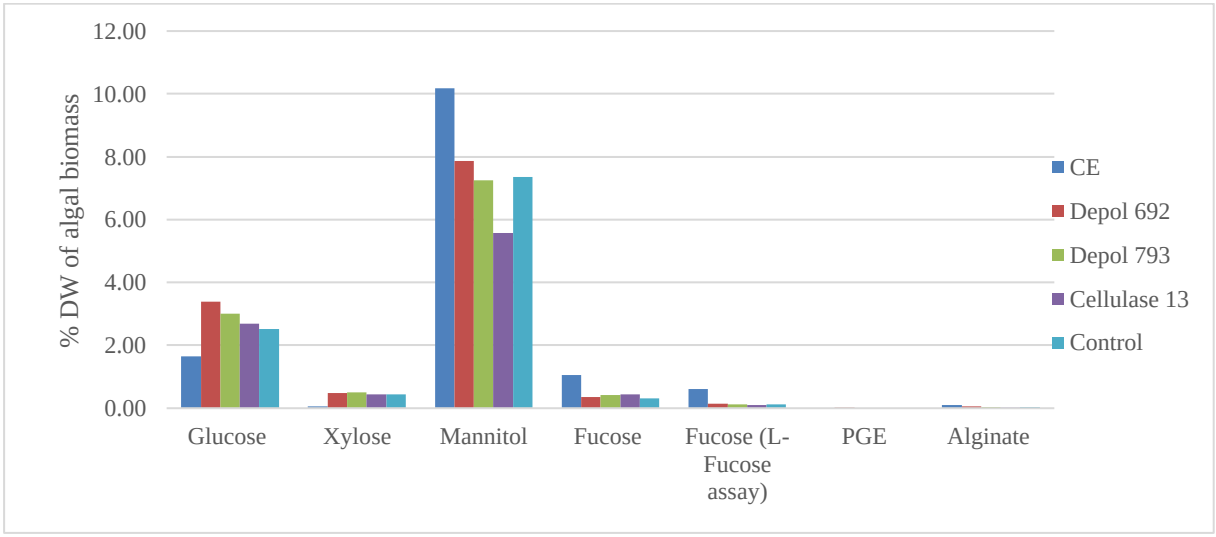

Figure 19. The total yield of the different valuable components from *S. latissima* after the analysis methods used. The content is presented as total percent (%) dry weight (DW) of the total algal biomass on the y-axis. The data presented was based on the hydrolysis method with the highest yield. Glucose, xylose, mannitol, fucose from IEX-HPLC after EAE were hydrolyzed with H_2SO_4 for the EAE, while for CE the data was based on HCl hydrolyzed material. The L-fucose data was hydrolyzed with H_2SO_4 while the alginate data was based on hydrolysis with HCl for both extraction methods.

4 Discussion

There is a growing interest in utilizing valuable components from brown macroalgae, such as polyphenols, fucoidan and different carbohydrates (Schiener *et al.*, 2015). The increasing focus on sustainability in today's society has led to a high focus on developing more sustainable methods for extraction. The major aim of this study was to compare the efficiency of a chemical extraction (CE) method against a greener, enzyme-assisted extraction (EAE) method, performed on two species of brown macroalgae; *Ascophyllum nodosum* and *Saccharina latissima*. One important subgoal was to evaluate the efficiency of the three enzymes used in this study: Depol 692, Depol 793 and Cellulase 13, as well as the efficiency of alginate precipitation and fucoidan isolation. The polyphenol content in the samples were analyzed, in addition the carbohydrate content was analyzed after two methods of acid hydrolysis. As additional analyses the MW distribution of polysaccharides and characterization of phlorotannins in the samples were added to the study. There is observed large differences in the extraction yield after the two extraction methods, as well as the hydrolysis methods used. The results observed in this study indicates that hydrolysis with HCl resulted in higher carbohydrate yield after CE in both species, whereas H_2SO_4 hydrolysis resulted in the highest carbohydrate yield after EAE. Therefore, the following discussion are based on these results unless otherwise stated.

Both CE and EAE methods ended up with fractionating the samples into 3; supernatant, fucoidan pellet and alginate pellet after CE and retentate, permeate and alginate after EAE. It was chosen to analyze all three fractions from both extraction processes to get an overview of where the different valuable components end up in the process. This was favored as the process can be optimized for the extraction of several valuable components from the same raw material, as the methods used in this study is focused primarily on the extraction of fucoidan.

4.1 CE *versus* EAE

4.1.1 Evaluation of CE

The CE method used in this study was chosen as it was reported to result in the highest yield of fucoidan compared to other CE methods (Fletcher *et al.*, 2017). Fucoidans are very sensitive molecules and are therefore easily influenced by different extraction parameters, such as acid concentration and temperature (Ale *et al.*, 2013). Ale *et al.* (2013) reports that an acid

concentration of only 0.2 M HCl, broke the structural integrity of fucoidan molecules, resulting in loss of fucose and bioactivity. However, an acid concentration of 0.03 M HCl, resulted in the best preservation of the fucoidan molecules. This suggests that extraction with 0.1 M HCl, used in this study, might result in loss of integrity and activity of the fucoidan molecule. As fucoidan molecules are water soluble, it can be discussed how necessary it is with acid extraction, and if a lower acid concentration can be favorable for the purpose of extraction bioactive fucoidan (Ale *et al.*, 2013). In this study, CE resulted in the highest extraction yield of all valuable components analyzed in this study, with the exception of polyphenols for both species studied. (Figure 18 and 19). However, there are indications that the CE method might have influenced the structure of the polysaccharides present in the samples, as the average M_w observed in the samples after CE are lower than what is observed after EAE.

4.1.2 Evaluation of Depol 692, Depol 793 and Cellulase 13

The most important considerations that needs to be made in an EAE method, is the extraction conditions. It is important to ensure optimal conditions for the enzyme, to achieve optimal enzymatic rate. There were three different enzymes used in this study; Depol 692, Depol 793 and Cellulase 13. Whereas Cellulase 13 contained only cellulase activity as its main activity, Depol 692 and Depol 793 contained additional carbohydrases (Table 1). As three different enzymes were studied, it would have been favorable to optimize the extraction process for each enzyme. However, this would require a separate control for each extraction process. Due to practical reasons, it was chosen to study all three enzymes, but use a common control, hence one generalized extraction process. The enzymes and the control were incubated at 50 °C at pH 5.0 for 3 hours. The pH optimum and temperature optimum for the three enzymes varied, where the temperature used in the extraction process are in the outer range for all three enzymes. A generalized extraction process proved to be only semi-optimal as none of the enzymes had remarkable results compared to the control, which can be considered to be a mild chemical extraction (Table 3 and 4). After extraction of *A. nodosum*, the control sample resulted in the lowest yield of the start material, however the enzymes only yielded approximately 1% more of the start material. After extraction of *S. latissima*, the control sample resulted in the highest yield of the start material compared to the enzymes. However, this indicates that the enzymes used in this study might not be efficient enough in degrading the algal cell wall. Nguyen *et al.* (2020) stated that cellulase enzymes, often in combination with other enzymes, are efficient in degrading the algal cell wall. Based on the results observed in this study, no enzyme can be

recommended for extraction of valuable components from macroalgae, due to the low efficiency compared to the control. However, further analyzes of the enzymes under optimal conditions are needed to make thorough conclusions. In addition, it might be favorable to analyze the enzymes under optimal conditions, for optimal enzymatic activity, as well as exploring other enzymes.

Despite this, some interesting finds were made in the study. Depol 793 is a multifunctional enzyme that possesses several activities including ferulic acid esterase activity. This activity has proven to be essential in opening up the structures in the cell wall, allowing for the degradation of the cell wall and the release of components (Biocatalysts, 2015b). For instance, Depol 793 resulted in a high yield of carbohydrates in *A. nodosum* (Figure 18). Similarly, Depol 692 possessed this activity and showed the greatest potential in degradation of the cell wall in *S. latissima*, resulting in the overall highest carbohydrate yield (Figure 19). However, Cellulase 13 yielded a slightly highest carbohydrate content in the retentate samples in *S. latissima* (Figure 10). In addition, Cellulase 13 possesses the most potent cellulase activity of the three enzymes and showed the greatest potential in efficiently extracting polyphenols (Figure 14) from the raw material during the extraction process. In contrast, UHPLC-DAD-QToF-MS, showed Depol 692 to contain the highest polyphenol content (Figure15). These results indicate that all three enzymes show some potential in cell wall degradation in macroalgae, as polysaccharides and polyphenols often are tightly bonded in the cell wall (Wijesinghe *et al.*, 2012; Padam *et al.*, 2020). However, the yield is only a little higher than the control. For the following discussion, Depol 793 will be the basis for discussion of *A. nodosum*, while Depol 692 will be the bases for discussion of *S. latissima*, unless otherwise stated.

4.1.3 Evaluation of alginate precipitation using $CaCl_2$

Fucoidan is often coextracted with alginate due to the complex cell wall in macroalgae. $CaCl_2$ is therefore often added after the extraction to precipitate alginate and to obtain purer fucoidan extracts. Alginate was precipitated from the samples with 2% $CaCl_2$ (w/v) in both extraction methods. This resulted in low extraction yields of alginate, constituting 2.8% and 0.2% DW of the algal biomass after CE and EAE, respectively in *A. nodosum* and 0.06% and 0.09% DW in *S. latissima*, respectively. Schiener *et al.* (2017) reported that alginate can make up to 20% and 23% of the total DW in *A. nodosum* and *S. latissima*, respectively. Both extraction methods were performed in acidic conditions, where alginate is generally insoluble, and low levels of alginate in the extracts were therefore expected. Following the $CaCl_2$ precipitation, a

considerable amount of alginate was detected in the fucoidan pellet after CE, indicating coextraction of fucoidan. However, there was no alginate present in the retentate or permeate samples after EAE, indicating efficient removal of alginate from the samples after EAE. This confirms that the addition of $CaCl_2$ to the samples after extraction will efficiently remove extracted alginate. $CaCl_2$ will bind to the guluronic acid rich high MW alginates, resulting in the formation of a gel. However, low MW alginates will not precipitate with $CaCl_2$, due to the dominance of mannuronic acid in the alginates, however low MW alginates can easily be removed from the samples by IEX. This suggests that there are more low MW alginates present in the samples from *A. nodosum*, especially after CE, as a substantial amount are present in the supernatant and fucoidan pellet, indicating a partial insufficient precipitation of alginate in these samples. In addition to adding $CaCl_2$ it is possible to add alginate lyase to the samples to purify fucoidan, as alginate lyase efficiently degrades alginate (Nguyen *et al.*, 2020). Although precipitation with $CaCl_2$ is efficient in precipitating extracted alginate, it will not be efficient in extracting alginate from the raw material as alginates are relatively insoluble in the presence of calcium ions (Rioux *et al.*, 2015). In addition, the left over $CaCl_2$ used in this precipitation step might influence the weight, resulting in a higher DM content in the supernatant and permeate samples after CE and EAE (Table 3 and 4).

4.1.4 Evaluation of fucoidan isolation with ethanol *versus* ultrafiltration

Fucoidan is often precipitated with ethanol, as it was done in CE. After IEX-HPLC-RID and L-fucose assay, both the supernatant and alginate pellet contained a substantial amount of fucose (Figure 7, 9 and 11), in addition to the fucoidan pellet, where it was expected the largest portion of fucose was going to be. This indicates an insufficient isolation and precipitation of fucoidan, as a substantial amount were left in the supernatant. The fucose content in the alginate pellet suggests some coextraction of fucoidan and alginate may have occurred during the alginate precipitation.

As a greener alternative to precipitation with ethanol, ultrafiltration with membranes with 100 kDa cut off was used to isolate fucoidan after EAE. This resulted in fucose rich high MW retentates and fucose-deprived low MW permeates. Similar to CE, the alginate pellet after alginate precipitation in the EAE samples also contained a substantial amount of fucose, suggesting that coextraction of fucoidan and alginate might have occurred during alginate precipitation. When the fucose content was measured in the retentates and permeates samples,

conflicting results were obtained. According to the carbohydrate analysis using IEX-HPLC-RID, the ultrafiltration worked efficiently in isolating fucoidan, as no fucose was detected in the permeate samples in any of the two species studied. However, according to the L-fucose assay, a substantial amount of fucose was detected in the permeate samples in addition to the retentate and alginate sample for both species. These conflicting results indicates limitations of the analysis methods, as to how the same material can give such different result depending on the analysis method.

4.1.5 Evaluation of IEX-HPLC-RID *versus* L-fucose assay

RID are commonly used in quantitative estimation of carbohydrates, since native sugars do not contain a chromophore or fluorophore (Galant *et al.*, 2015). The conflicting results observed in this study, especially between CE and EAE for *A. nodosum*, has suggested limitations of the two analysis methods. RID are in general highly sensitive detectors and even the slightest change in temperature, pressure and mobile phase can cause baseline shifts or movements (Galant *et al.*, 2015). However, RID are being replaced by other detectors such as light scattering detector and pulsed amperometric detector (PAD), due to the high sensitivity of RID, difficulties with reproducibility and incompatibility with gradients (Swartz, 2010). This suggests that several factors could have influenced the analysis. The samples from each extraction method were run as a series over several hours. The slightest change in temperature or pressure in the column could have affected the results. However, this is unlikely as the equipment (including the column oven, LC-apparatus and RID) is considered to be up to standard. A difference in the viscosity of the samples could have occurred, hindering the full 10 μL being injected onto the column. In addition, it is possible that remains from previous runs could have eluted together with the samples causing a higher detection of monosaccharides.

There are also limitations to the L-fucose assay regarding the specificity of L-FDH. The assay is based on the rapid conversion of L-fucose by L-FDH. Whereas Megazyme (2018) states that L-FDH will also convert L-galactose and D-arabinose, however at a slower rate. This suggests that higher NADPH values measured in the samples might not specifically be due to the conversion of L-fucose, as it might be influenced by the conversion of these two sugars as well.

However, IEX-HPLC-RID might still be preferrable, as RID gives good detection of compounds that are of relatively small amounts in the samples. However, RID does not provide

the same sensitivity as other detectors, such as PAD (Galant *et al.*, 2015). It can thus be favorable to replace RID with a different detector or add an additional analysis to support the findings by this method in future projects.

4.1.6 Fucose content

The fucoidan yield after extraction is often measured as the % fucose obtained of the total fucose present in the macroalgal DW (Ale *et al.*, 2013). Therefore, fucose can be used as an indirect measurement of fucoidan. The fucose content in the samples were analyzed using IEX-HPLC-RID and the L-fucose assay. After CE, IEX-HPLC-RID measured the highest % fucose in both species studied. Whereas after EAE, L-fucose resulted in the highest % fucose obtained in *A. nodosum* (Figure 18) and IEX-HPLC-RID resulted in the highest fucose content in *S. latissima* (Figure 19).

The measured fucose content in *A. nodosum* between the two analysis methods varied greatly after CE. IEX-HPLC-RID estimated 10.61% DW fucose of the total algal biomass, which is approximately 10x higher than the estimated fucose content after L-fucose assay. The L-fucose assay measured the fucose content to be 0.72% DW fucose of the total algal biomass (Figure 18). It is likely that the samples had been contaminated during the extraction process, as it is only observed for *A. nodosum* after CE. However, as the material used for IEX-HPLC-RID and L-fucose assay are from the same extraction process, this is considered unlikely. Although, it is very possible that something might have influenced both analysis methods, such as difference in viscosity or remains from previous runs, resulting in the increased detection of fucose. In addition, the same trend is observed for the Depol 793 retentate sample (Figure 8), where it yielded approximately 8x higher carbohydrates than the rest of the samples after IEX-HPLC-RID for *A. nodosum*, whereas the same trend is not observed for the L-fucose assay.

Regardless, CE measured a higher amount of fucose in *A. nodosum* after both IEX-HPLC-RID and the L-fucose assay, compared to Depol 793. After IEX-HPLC-RID, Depol 793 had released 0.26% DW and 0.35% DW of the total algal biomass after the L-fucose assay. According to Schiener *et al.* (2017), a fucose content of 0.9% DW in biorefined *A. nodosum* was detected. This further confirms the uncertainty regarding the results obtained after IEX-HPLC-RID for this species after CE, as it is 10x higher than what was reported in the algae. However, this indicates a relatively good extraction of fucose after CE as the measured fucose content after L-fucose is high. Whereas the release of fucose after EAE were lower, indicating a higher release of fucoidan after CE compared to EAE in *A. nodosum*.

The measured fucose content in *S. latissima* after IEX-HPLC-RID and the L-fucose assay was not as extreme as observed for *A. nodosum*, thus IEX-HPLC-RID measured the highest fucose content after both CE and EAE. After CE, the measured fucose content was 1.1% DW fucose of total algal biomass after IEX-HPLC-RID and 0.60% DW fucose of total algal biomass (Figure19). Similar to *A. nodosum*, CE resulted in the highest extraction of fucose compared to EAE in *S. latissima* as well. Depol 692 resulted in the extraction of 0.36% DW fucose of the total algal biomass according to IEX-HPLC-RID, whereas L-fucose measured 0.15% DW fucose of the total algal biomass. According to Schiener *et al.* (2017), the fucose content in biorefined *S. latissima* is 0.7% DW of the algal biomass. This indicates a complete extraction of fucose in CE and a relatively good extraction of fucose with Depol 692 according to IEX-HPLC-RID. This suggests a high extraction of fucoidan after CE from *S. latissima* as well, compared to EAE.

4.1.7 Carbohydrate content

The carbohydrate content in the samples were measured by the detection of monomeric sugars (glucose, xylose, mannitol and glucose) with IEX-HPLC-RID. CE resulted in the extraction of 19.7% DW carbohydrates of the total algal biomass in *A. nodosum*, whereas Depol 793 resulted in the extraction of 0.66% DW carbohydrates of the total algal biomass. Tabassum *et al.* (2016) reported a carbohydrate content in *A. nodosum* harvested in October at approximately 70% DW of algal biomass. This indicates a very low extraction yield of carbohydrates for both CE and EAE in *A. nodosum*. The carbohydrate content after CE was distributed in 1.17% DW glucose, 2.95% DW xylose, 5.03% DW mannitol and 10.61% DW fucose of the total algal biomass. The carbohydrate content after EAE was distributed in 0.13% DW glucose, 0.14% DW xylose, 0.29% DW mannitol and 0.26% DW fucose of the total algal biomass (Figure 18). In contrast, Schiener *et al.* (2017) reported the carbohydrate content to be 5.4% DW glucose, 9.0% DW xylose/mannose/galactose, 0.9% DW mannitol and 0.9% DW fucose in biorefined *A. nodosum* harvested in February and November. This indicates a low extraction yield of carbohydrates after both CE and EAE. The *A. nodosum* studied was harvested in October, when carbohydrates are reported at a high, due to the accumulation of carbohydrates during summer and autumn in the species (Tabassum *et al.*, 2016). This was not reflected in this study. However, storage carbohydrates such as mannitol and laminarin accumulate during winter and will be at a low during summer and autumn (Tabassum *et al.*, 2016). Nor this is reflected in the results from this study. Mannitol is the carbohydrate with the highest extraction yield after EAE in *A.*

nodosum, as well as there is a high extraction yield of mannitol after CE compared to Schiener *et al.* (2017) study.

Compared to *A. nodosum*, there was detected more monomeric sugars in *S. latissima*, as expected. *S. latissima* is known as sugar kelp due to its high contents of different carbohydrates, especially mannitol (Sharma *et al.*, 2018). After CE of *S. latissima* the total carbohydrate content was 12.9% DW of the total algal biomass, whereas after Depol 692 the carbohydrate content was 12.11% DW of the total algal biomass. This is considerably lower than 63.1% DW of algal biomass, reported by Schiener *et al.* (2015) in *S. latissima*. This indicates a low extraction yield of carbohydrates after CE and EAE in *S. latissima* as well. After CE, the carbohydrate content was distributed in 1.65% DW glucose, 0.07% DW xylose, 10.17% DW mannitol and 1.1% DW fucose of the total algal biomass. The carbohydrate content after EAE was distributed in 3.40% DW glucose, 0.49% DW xylose, 7.86% DW mannitol and 0.36% DW fucose of total algal biomass. The extraction yields from CE and EAE are very similar for *S. latissima*. This strengthens the theory that the samples after CE from *A. nodosum* could have been contaminated, as well as the results could have been influenced by the analysis method. . Schiener *et al.* (2017) reported the carbohydrate composition in biorefined *S. latissima* harvested February and November was 6.3% DW glucose, 4.5% xylose/mannose/galactose, 3.6% DW mannitol and 0.7% DW fucose of the total algal biomass. Compared to Schiener *et al.* (2017) study, the carbohydrate extraction yield after CE and EAE are relatively good. The *S. latissima* studied were harvested in April, when carbohydrates are reported to be at a maximum, whereas storage carbohydrates such as mannitol and laminarin are at a low (Schiener *et al.*, 2015). However, the extraction yield of mannitol is high compared to Schiener *et al.* (2017) study. The low extraction yield of glucose is reflected in the low amounts of laminarin and cellulose present in the species in April.

4.1.8 MW distribution of polysaccharides

There is no doubt that the extraction process effects the MW of the polysaccharides in the samples, as the MW distribution was quite different after CE compared to EAE in the species two species studied. There were no polysaccharides present in the CE samples higher than the MW range of 3×10^5 - 2×10^8 Da. This fact suggest that the acid treatment might lead to the degradation of the polysaccharides, in addition to efficiently degrading the algal cell wall. For instance, Phyo *et al.* (2019) states that acidic pH will contribute to the spatial separation of cell wall polysaccharides, supporting this thought.

In contrast to CE, there were found polysaccharides in the MW range between 2 x 10^8 - 4 x 10^{10} Da for both species after EAE. In addition, in *S. latissima*, polysaccharides in the MW range of both 3 x 10^5 - 2 x 10^8 Da and 4 x 10^{10} -1 x 10^{13} Da were detected. This indicates that the ultrafiltration had been efficient in isolating and concentrating high MW compounds to the retentate samples. It also suggests that EAE are better at preserving the structural integrity of the compounds and that the selected enzymes do not degrade the polysaccharides to the same degree as it was observed with acid. However, the target MW of the column was 20 000 000 Da, meaning that these findings are outside the target MW range of the column and therefore separation of the molecules might not be optimal.

As an additional analysis, the peaks of Depol 793 and control retentates (obtained from *A. nodosum*) were fractionated during HP-SEC, to further examine the location of different valuable components in the samples. As these high MW peaks were only observed in the retentate samples (Figure 6), it was suggested that they might contain compounds of interest. After IEX-HPLC-RID and the L-fucose assay of the fractionated peaks from Depol 793 and control from *A. nodosum* there was detected both xylose and fucose in the first peak (Figure 13), representing polysaccharides inside MW range of 2 x 10^8 - 4 x 10^{10} Da. There was detected glucose, xylose, mannitol and fucose in peak 2 and 3 as well, indicating polysaccharides with MW between 0 - 3 x 10^5 Da fucose. These results indicates that there is material of interest inside this MW range 2 x 10^8 - 4 x 10^{10} Da, even though it is outside the standards used in this study, as well as the target MW of the column. As there were identified polysaccharides in the MW range of 4 x 10^{10} -1 x 10^{13} Da it might be favorable to fractionate a sample from *S. latissima* as well, to analyze the polysaccharides present in this MW range. Due to time limitations, this was not included in this study.

These results raise the question if the selected enzymes were efficient enough in degrading the cell wall and releasing the polysaccharides. In addition, they might imply a complex of polysaccharides, due to the high MW observed in the samples. In general, the HP-SEC results shows a generally higher MW of the polysaccharides after EAE compared to CE, in both species (Table 5 and 6).

4.1.9 Polyphenol content

The estimated polyphenol content in this study was analyzed using a quantitative colorimetric method where Folin-Ciocalteus reagent react with phenolics present forming a blue complex that can be measured (Blainski *et al.*, 2013). Almost no polyphenols were detected in the

samples from for *S. latissima* using this method. In general, the phenolic composition in *S. latissima* is much lower than for *A. nodosum* (Schiener *et al.*, 2017). However, the estimated polyphenol content varied between the samples obtained after CE and EAE. The lowest amounts of polyphenols were detected in the fucoidan pellet and supernatant after CE, suggesting that the high acid concentrations during CE negatively influence the extraction of polyphenols. The CE is also performed under a high temperature which can result in the destruction of polyphenols, as they have shown to be temperature sensitive (Mekinić *et al.*, 2019). The polyphenol content after EAE is much higher and the highest content is found in the retentate samples for all enzymes, where Cellulase 13 provided the highest estimated content (1.08% DW of the sample) (Figure 14). This suggests a high extraction yield of polyphenols after EAE, compared to the findings from Schiener *et al.* (2017), reporting 1.13% DW polyphenol in biorefined algae.

4.1.10 Phlorotannin characterization

As an additional analysis, UPLC-DAD-QToF-MS was added to this study to support the findings after the polyphenols assay, which was a quantitative colorimetric method. This method would also provide insight into the diversity of phenolic compounds in the samples. As the polyphenol content in macroalgae have proven to be problematic, due to the structural complexity of the molecules, quantitative colorimetric assays are often used (Lopes *et al.*, 2018). A disadvantage with these types of assays, are that they only give an indication of the total polyphenol content and provide no information about the diversity of compounds (Blainski *et al.*, 2013). Preferably all samples should have been further analyzed, but due to time limitations, only the samples with positive results from the polyphenol assay were analyzed. UPLC-DAD gave a quantitative estimation of the total polyphenol content (Figure 15), that correlated quite well with what was observed in the polyphenol assay (Figure 14). The retentate samples after EAE contained the highest amounts of polyphenols, where in contrast Depol 692 had the highest polyphenol content whereas Cellulase 13 was the enzyme with the lowest polyphenol yield, though it was still higher than the control.

As the Depol 692 retentate sample showed the highest polyphenol content after UPLC-DAD (Figure 15), it will be the basis for the phlorotannin characterization presented in this study and thus the basis for the following discussion. The UV-spectra (Figure 15) of the different samples are at 210 nm, whereas the isolated UV-spectra of Depol 692 retentate sample are presented in the Appendix, Figure A9. The isolated UV-spectra show a maximum

absorbance at approximately 211 and 277 nm. This is very similar to the absorbance maximum of phloroglucinol, reported by Koivikko (2008), which indicates the presence of phlorotannins in the samples.

Table A2 (Appendix) summarizes the monoisotopic masses measured during UPLC-DAD-QToF-MS (Figure 16) and presents the suggested molecular formula of the measured masses. Based on the molecular formula the amount of PGU in the phlorotannin and can be determined. A total of 28 (Figure 17) phlorotannins, ranging from 2-18 PGU were found in the sample. The measured monoisotopic mass of 388.04 Da has a suggested molecular formula of $C_{18}H_{12}O_{10}$ (theoretical mass of 388.04 Da) and can therefore potentially be 7-hydroxyeckol, made up by 3 PGU, reported by Glombitza *et al.* (1985). Another example is the measured monoisotopic mass of 636.07 Da that can potentially be triphlorethohydroxycarmalol, reported by Li *et al.* (1991), having a molecular formula of $C_{30}H_{16}O_{16}$ (theoretical mass of 636.08 Da). However, this molecular formula is made up of 5 PGU, indicating that there are some uncertainties regarding its identity, as several phlorotannins can have the same molecular composition, and therefore the same mass.

However, there are some limitations to this method. It is only able to detect masses of polymers of phenols (phlorotannins) up to ca. 2500 Da. Everything that is bigger will not be shown in the mass spectra. This is unfortunate, as there is detected compounds with a much higher MW in the samples analyzed. Moreover, it is a surprise that the retentate samples contain the highest polyphenol content, as the observed masses are lower than the molecular cut of off the membranes (100 kDa) used under ultrafiltration. This further confirms an insufficient ultrafiltration in concentrating low MW compounds to the permeate samples, observed after HP-SEC (Figure 6, Table 5 and 6). In addition, due to the high complexity of phlorotannins, the more PGU´s they are made of – the more different couplings might occur, leading to several phlorotannins having the exact same mass and elemental composition. Therefore, the bigger the phlorotannin is, the harder it is so characterize.

4.2 Hydrolysis with HCl *versus* H_2SO_4

Different hydrolysis methods have been reported to influence the biomass differently. The reason for including HCl hydrolysis in this experiment was because it was suggested as a pretreatment of the material before L-fucose assay by Megazyme (2018). Manns *et al.* (2014), stated that materials rich in uronic acids were better hydrolyzed with HCl, as a two-step sulphuric acid hydrolysis is unable to completely hydrolyze the β-(1,4) linkages present in

alginate. HCl hydrolysis was therefore included to see how the different hydrolysis methods would influence the yield of valuable components. In this study the alginate content was estimated using a colorimetric method, based on the reaction between concentrated H_2SO_4 and uronic acids in the absence of water, that further reacted with 3,5-Dimethylfenol, producing a red-pink colored chromogen that was measured (Manns *et al.*, 2014). Hydrolysis with HCl resulted in higher alginate yields, compared to hydrolysis with H_2SO_4, confirming Manns *et al.* (2014) observations.

However, hydrolysis with H_2SO_4 have proven to be the better hydrolysis method for quantitative estimation of carbohydrates (Manns *et al.*, 2014). In this study, the estimated monomeric sugar content in both species varied substantially depending on the hydrolysis method used in both species. Hydrolysis with H_2SO_4 resulted in the highest amount of fucose in both species using the L-fucose assay (HCl data not shown). H_2SO_4 hydrolysis resulted in the highest amount of monomeric sugars in the samples obtained after EAE in both species. In contrast, an opposite trend was observed in the samples after CE, where hydrolysis with HCl resulted in the highest yield of monomeric sugar compared to hydrolysis with H_2SO_4. This implies that the optimal hydrolysis method can also vary depending on the choice of extraction method, as well as type of biomass. This suggests that a combination of hydrolysis methods can be favorable, however it is dependent on the material for extraction.

4.3 Limitations and further work

There are several limitations to the study. Every experiment in the study was only conducted once and must be repeated to make any concrete conclusions. As the study was considered to be preliminary, only two parallels were used for the different analyzes. As a result of this, no standard deviations or statistical analysis are included in the thesis.

Due to practical reasons, a generalized extraction method was used for all three enzymes used in this study, so only one control was needed for the process. Ideally, the extraction processes should have been optimized for each enzyme with a control for each process. As none of the enzymes analyzed in this study resulted in remarkable yields, it might be favorable to include several enzymes in a future study to explore if any other enzyme will work better for this specific purpose.

As all analysis methods used in this study were being established along the way, it was hard to keep a streamline process of the experiments. This led to unnecessary storage, freezing and thawing of the samples. As a result, it is reasonable to think that this might have led to

precipitation in the samples, that can potentially affect the results. It might therefore be advantageous to perform future projects in a streamline process to avoid this. This was especially the case for the samples obtained after EAE, as this extraction process was conducted at an earlier stage and had to be stored for a longer period of time. In contrast, CE was conducted relatively late in the process and it was thus not necessary to store.

Due to the conflicting fucose results obtained after IEX-HPLC-RID and L-fucose assay, as well as big differences in the estimated carbohydrate content between CE and EAE samples after IEX-HPLC-RID, it raised the question of the sensitivity of the methods. As a result, it might be an idea to replace RID with PAD or similar detectors, to avoid some of the limitations observed with RID. It might also be favorable to analyze the carbohydrate content using another method such as gas chromatography in addition to IEX-HPLC-RID to be able to compare the results. This would give an indication if the carbohydrate analysis gave trustworthy results. Due to time limitations this was not possible for this project but can be a suggestion for future projects.

The first natural step after this study would be to optimize the extraction process for one selected enzyme that proved to be efficient for this purpose, as a generalized method was not optimal. The next step would be to would be to purify the isolated fucoidan to remove all impurities, as it is still considered crude type. This can for example be done by using IEX, SEC and affinity chromatography (Zayed *et al.*, 2020b). After the fucoidan molecules has been purified structure characterization with NMR can be done to determine sulphate content and substitutions. As fucoidans are bioactive molecules, different bioactivity test could be performed to determine the activity of the isolated fucoidan.

5 Conclusion

The main goal of this project was to compare the efficiency and yield of a new green method of extraction (EAE) against a selected conventional method (CE), where the main target compound for comparison was fucoidan. CE resulted in the highest extraction yield of fucoidan in both species studied. However, compared to literature values, the extraction yield from *A. nodosum* is considered to be low. In general, CE resulted in the highest extraction yield of all valuable components analyzed in this study, except for polyphenols.

Based on the results obtained in this study, none of the three enzymes used in the EAE method can be recommended for extraction of valuable components from macroalgae, due to low efficiency/yield of selected target molecules compared to the untreated control and CE. However, all enzymes showed a potential in degrading the algal cell wall, but further studies are needed to make thorough conclusions on this aspect.

Alginate was precipitated from the samples with $CaCl_2$ in both extraction methods, and this method proved to be efficient in removing alginate from the samples.

Different methods for fucoidan isolation were used in the two extraction protocols. Ethanol was used for fucoidan precipitation in the samples after CE, and this method proved to be inefficient in isolating fucoidan. Ultrafiltration, which was used as a greener alternative for isolation of fucoidan from the samples, resulting in fucose rich retentate samples from EAE. This method proved to be efficient, to some extent, in isolating fucoidan. However, substantial amounts of fucose were also detected in the alginate samples, indicating coextraction of fucoidan with the alginate.

The extraction yield of carbohydrates was in general low for both species studied, using both extraction methods. However, mannitol was successfully extracted from *S. latissima* after both CE and EAE. The extraction yield of polyphenols in the samples after EAE from *A. nodosum* was good, compared to CE, where very little or no polyphenols were detected.

Finally, two different acid hydrolysis methods were compared in this study. The results obtained indicate that the choice of hydrolysis methods vary depending on the extraction method, as well as the type of biomass. This suggest that a combination of hydrolysis methods can be favorable for extraction of valuable components from macroalgae.

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Appendix

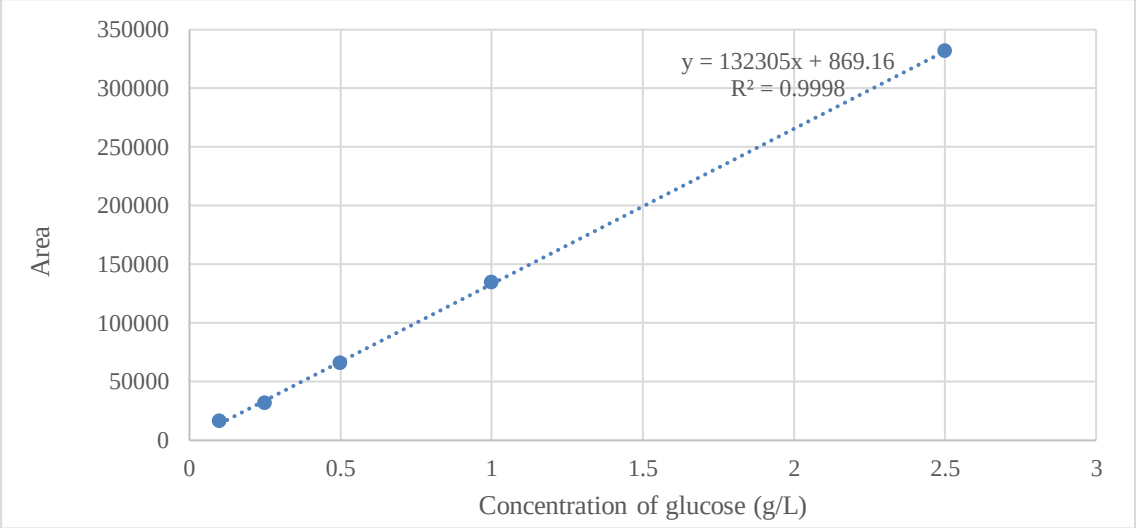

Figure A1. Standard curve for glucose at concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 g/L obtained after carbohydrate analysis using ion exchange – high performance liquid chromatography – refractive index detector (IEX-HPLC-RID). The equation for the standard curve was used to estimate the total glucose content in the samples.

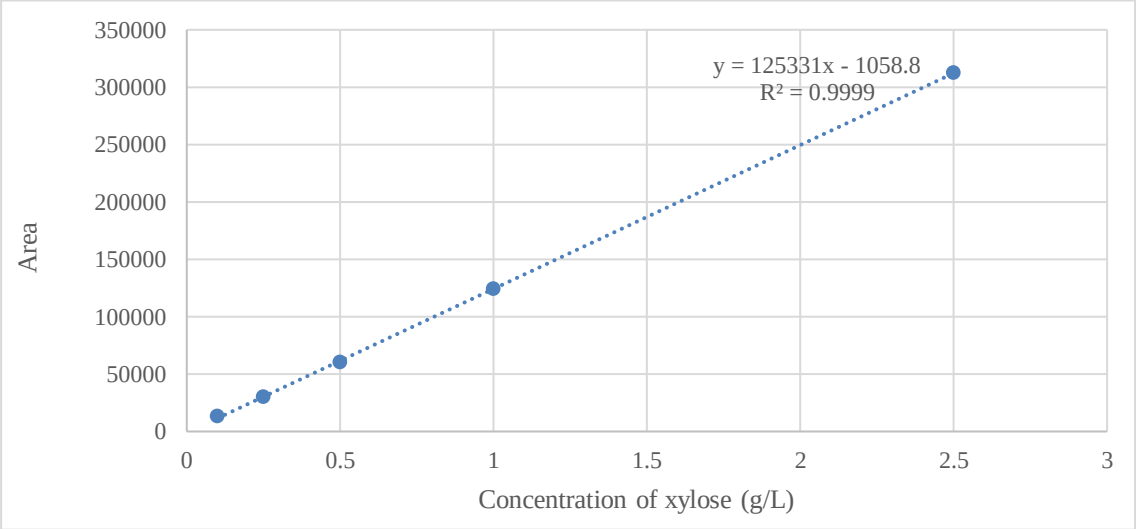

Figure A2. Standard curve for xylose at concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 g/L obtained after carbohydrate analysis using ion exchange – high performance liquid chromatography – refractive index detector (IEX-HPLC-RID). The equation for the standard curve was used to estimate the total xylose content in the samples.

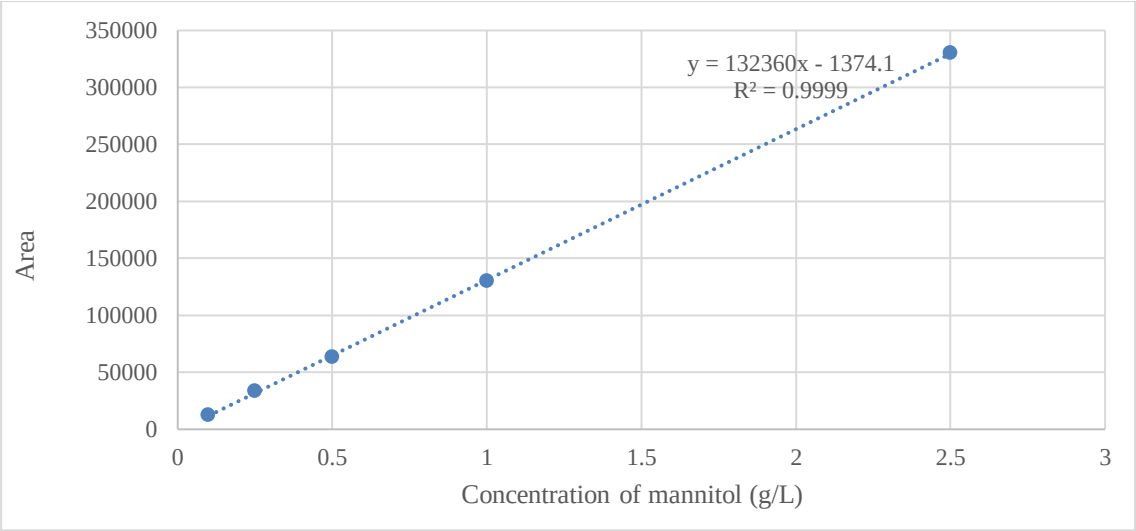

Figure A3. Standard curve for mannitol at concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 g/L obtained after carbohydrate analysis using ion exchange – high performance liquid chromatography – refractive index detector (IEX-HPLC-RID). The equation for the standard curve was used to estimate the total mannitol content in the samples.

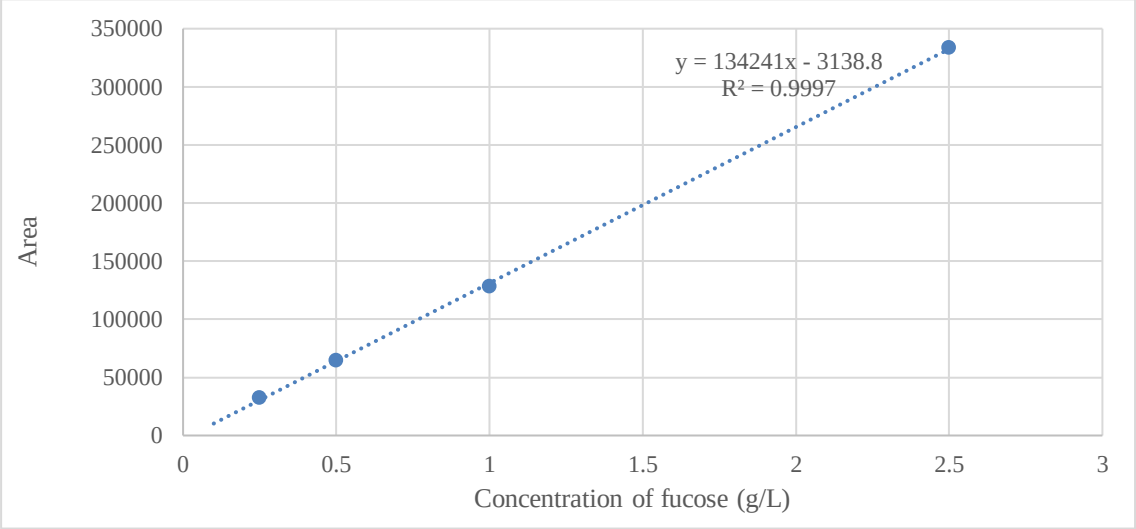

Figure A4. Standard curve for fucose at concentrations of 0.1, 0.25, 0.5, 1.0 and 2.5 g/L obtained after carbohydrate analysis using ion exchange – high performance liquid chromatography – refractive index detector (IEX-HPLC-RID). The equation for the standard curve was used to estimate the total fucose content in the samples.

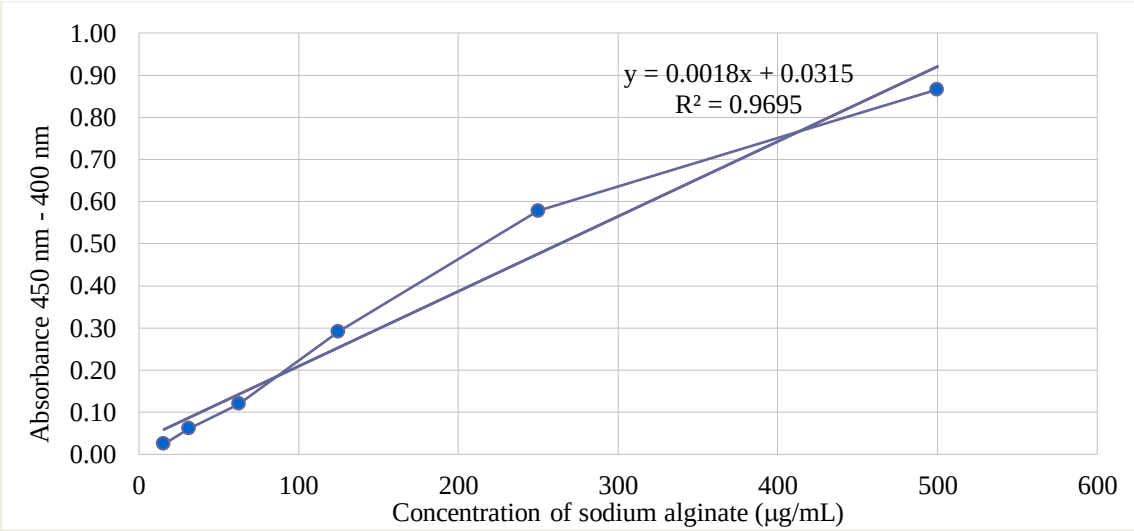

Figure A5. Standard curve for sodium alginate at concentrations of 15.6, 31.2, 62.5, 125, 250 and 500 µg/mL obtained after alginate assay. The equation for the standard curve was used to estimate the total sodium alginate content in the samples.

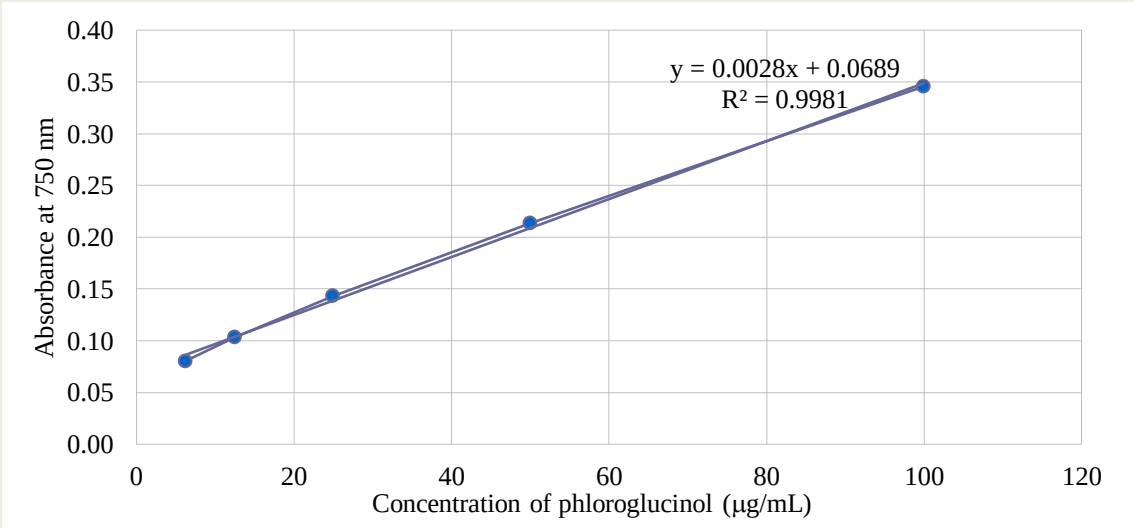

Figure A6. Standard curve for phloroglucinol at concentrations of 6.5, 12.5, 25, 50 and 100 µg/mL obtained after polyphenol assay. The equation for the standard curve was used to estimate total polyphenol content in the samples.

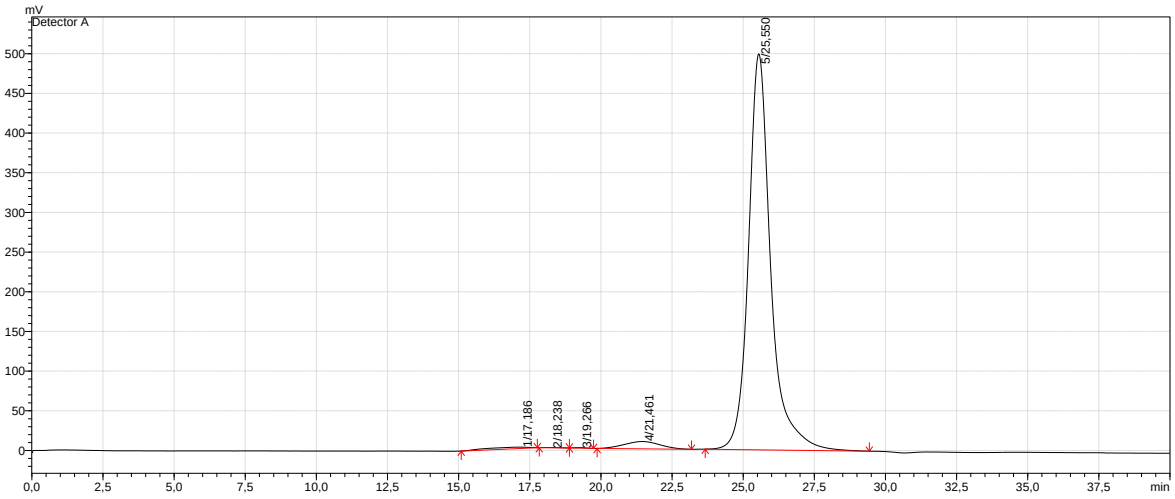

Figure A7. High performance – size exclusion chromatography (HP-SEC) chromatogram of Depol 692 retentate (R) from *A. nodosum*. The x-axis displays the retention time in minutes while the y-axis shows the relative intensity of the refractive index in mV.

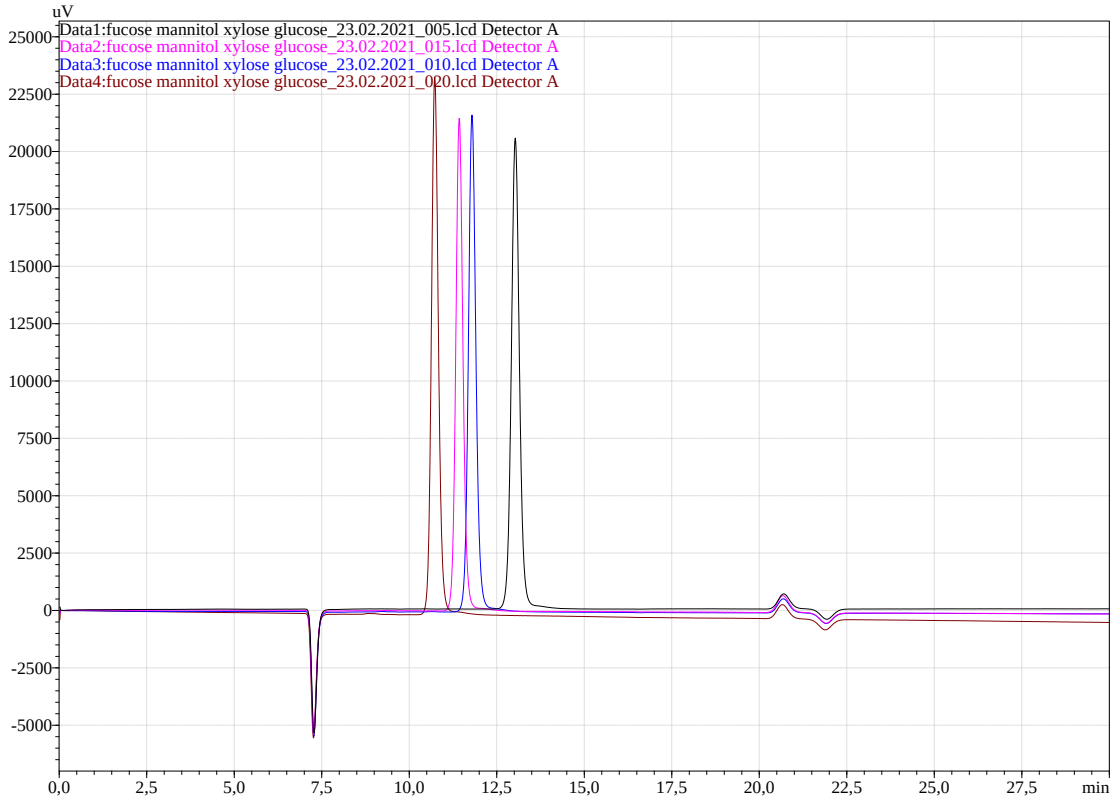

Figure A8. Overlaying chromatograms showing the retention times of the different standards, glucose (black), xylose (pink), mannitol (blue) and fucose (brown), all run at the highest concentration of 2.5 g/L. The x-axis displays the retention time in minutes while the y-axis shows the relative intensity of the refractive index in uV.

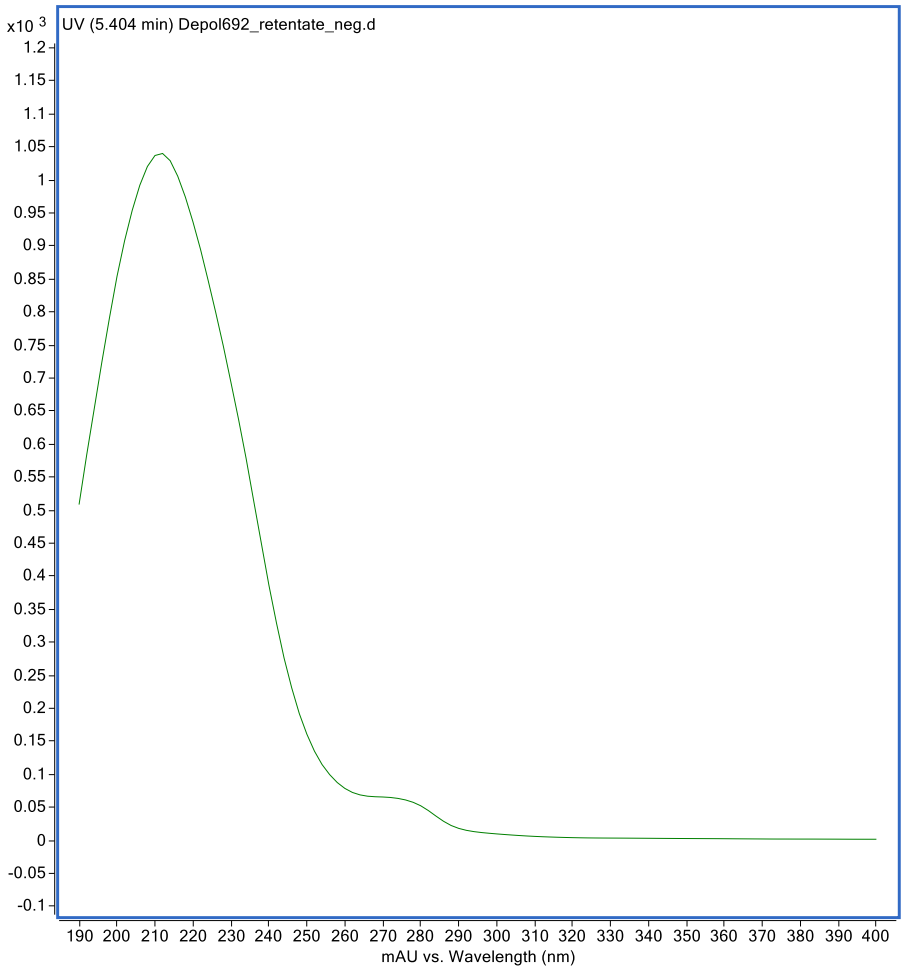

Figure A9. UV-spectrum of the phlorotannin-rich area observed after HPLC-DAD analysis of the Depol 692 retentate from *A. nodosum*. The spectrum is recorded at retention time 5.404 min. The y-axis shows the absorbance (mAU), while x-axis shows the wavelength (nm).

Table A1. Summary of the characteristics of each pullulan samples used as standard for analysis of molecular weight (MW) distribution in the samples. M_p = Peak molecular weight, M_w = weight average molecular weight, and M_n = number average molecular weight.

Pullulan sample	**M_p**	**M_w**	**M_n**	**Retention time (min)**
Pullulan NP0	1600000	1600000	1600000	21.9
Pullulan NP1	710000	788000	641000	22.4
Pullulan NP2	380000	404000	358000	23.3
Pullulan NP4	106000	112000	100000	24.8
Pullulan NP7	11200	11800	10700	26.2
Pullulan NP8	5600	5900	5400	26.4
Pullulan NP9	1080	1320	1080	26.9

Table A2. Potential phlorotannins detected using ultra performance – liquid chromatography Quadruple Time of Flight (UPLC-QToF), in the Depol 692 retentate sample from *A. nodosum*. The number of phloroglucinol units (PGU) of the potential phlorotannin is listed as well as suggestion of potential phlorotannins based on already characterized phlorotannins from the MarinLit database (http://pubs.rsc.org/marinlit).

Potential phlorotannin	Suggested Molecular Formula	Measured Monoisotopic Mass	Number of PGU
diphlorethol	C12H10O6	250.05	2
	C12H8O7	264.03	2
triphlorethol-A	C18H14O9	374.06	3
7-hydroxyeckol	C18H12O10	388.04	3
tetraphlorethol-A	C24H18O12	498.08	4
diphlorethohydroxycarmalol-A	C24H16O13	512.06	4
difucodiphlorethol-A	C30H22O15	622.10	5
triphlorethohydroxycarmalol	C30H20O16	636.07	5
bisfucotriphlorethol-B	C36H26O18	746.11	6
	C36H24O19	760.09	6
trifucotriphlorethol A	C42H30O21	870.12	7
	C42H28O22	884.11	7
octaphlorethol A	C48H34O24	994.13	8
	C48H32O25	1008.12	8
	C54H36O28	1132.14	9
bisfucoheptaphlorethol-A	C60H42O30	1242.17	10
	C60H40O31	1256.15	10
	C66H44O34	1380.17	11
	C72H48O37	1504.19	12
	C78H54O39	1614.22	13
	C78H52O40	1628.20	13
	C84H60O42	1740.26	14
	C84H56O43	1752.22	14
	C90H64O45	1864.27	15
	C90H60O46	1876.23	15
	C96H68O48	1988.29	16
	C96H64O49	2000.24	16
	C102H72O51	2112.30	17
	C102H68O52	2125.27	17
	C108H74O54	2234.30	18