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Searching for efficient methods of extraction and characterization of protein from *Saccharina latissima* and *Alaria esculenta*.

Monika Katarzyna Kopczyk

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Supervisor: Turid Rustad, IBT

Norwegian University of Science and technology
Department of Biotechnology and Food Science

Abstract

Growing population and alteration of the environment due to climate change and human impact, water scarcity and reduction of the available arable land demand search for alternative food sources. To meet the increasing demand for food, more resources must come from seas and oceans. Exploring possibilities of using seaweed as food and feed is one way of increasing the amount of food from the ocean and it is an important path in meeting the increasing demand for nutrient-rich food and feed. Of the three main nutrients: protein, fat and carbohydrates, the need for protein is the most difficult to satisfy, therefore there should be an increased focus on exploring new protein sources. Successful extraction of macroalgal protein might lead to sustainable production of protein concentrates and other types of food and feed additives rich in protein.

The purpose of this thesis was to examine how different conditions influence the extraction of protein from two brown macroalgae: *Saccharina latissima* and *Alaria esculenta*. Both contain all the essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine

A two steps protein extraction was conducted. First, a standard extraction in distilled water was performed. Then, the pellets from this extraction were resuspended in one of the following: 0.1M NaOH, 0.4M NaOH, 0.1M HCl, 0.4M HCl or distilled water. Further, samples were divided into three groups and were exposed to: no treatment, ultrasound bath and sonicator. then, protein content was measured using Lowry method. Total soluble FAA content and soluble FAA profile were determined with HPLC analysis.

Protein concentration *Saccharina latissima* was the highest in samples extracted in alkali and treated with ultrasound. For the first set of samples (originating from sample SL0.1) extracted in 0.1M NaOH, protein content increased from 302.5 µg/mL in SL2.1 (no treatment) to 443.5 µg/mL in SL9.1 (sonicator) and 428.6 µg/mL in SL5.1 (ultrasound bath). For the same extraction from samples originating from SL0.2, a decrease in extracted protein yield was observed. Protein content decreased from 446.6 µg/mL in an untreated sample SL1.2, to 354.5 µg/mL in the sample SL9.2 treated in the sonicator and to 287.8 µg/mL in SL5.2 treated in the ultrasound bath. For the samples extracted in 0.4M NaOH a similar tendency was observed. While protein yield increased with applied ultrasound treatments in samples originating from SL0.1 to 443.5 µg/mL in SL10.1 (sonciator) and 428.7 µg/mL in SL5.1 (ultrasound bath), it decreased in samples originating from SL0.2 to 424.7 µg/mL in SL10.2 (sonicator) and SL 5.2 (ultrasound bath).

However, these values were still significantly higher than those of corresponding samples extracted in 0.1M NaOH and H_2O.Using HCl as an extraction solvent did not contribute to the increased yield of the extracted protein.

For *Alaria esculenta* samples where NaOH was used, protein content generally increased after the ultrasound treatment was applied with the exception of sample AE10.1, originating from AE0.1 and extracted in 0.4M NaOH in sonicator. Here the protein content decreased to 69.8 µg/mL. The corresponding sample from the set originating from AE0.1, sample AE9.2 had much higher protein score, 131.7 µg/mL and it was also higher in comparison to the sample that did not undergo any treatment, AE2.2. In samples AE9.1 and AE9.2, where the extraction was performed in 0.1M NaOH with treatment with sonicator the protein content increased to 165.3 µg/mL and 176.3 µg/mL, respectively. Protein contents of the samples extracted in the same solvent concentration, where the treatment in the ultrasound bath was applied were higher, 182.3 µg/mL for the sample AE5.1 and 212.4 µg/mL for AE5.2. Protein content in samples extracted in HCl was generally lower than the ones extracted in NaOH and slightly lower than the ones extracted in water (control).

Several analyses of content of free amino acids (FAA) with HPLC were performed. However, due to the time limitations of this project only a selection of samples were analyzed.

For *S. latissima* the highest total soluble FAA yield was 74.6 µg/mL and it was measured in sample SL2 that was extracted in 0.4M NaOH and did not undergo ultrasound treatment. Second highest total soluble FAA yield was 57.5 µg/mL and it was measured in sample SL1 that was extracted in 0.1M NaOH and did not undergo ultrasound treatment. The lowest total soluble FAA yield was 16.1 µg/mL measured in sample SL3 that was extracted in 0.1M HCl and did not undergo any ultrasound treatment. Generally, samples SL1 and SL extracted in 0.1M NaOH and 0.4M NaOH that did not undergo any ultrasound treatment had total soluble FAA yield significantly higher than samples extracted in the same solvents and treated with ultrasound bath or with sonicator.

Total FAA content of *Alaria esculenta* supernatants was measured only for extractions in water. During the first extraction, total soluble FAA content was 174.1 µg/mL in AE0.1 and 221.1 µg/mL in AE0.2. Further the results showed that the second extraction with water did not contribute to a significant increase in the FAA content in the supernatant. Sample AEW3, treated with sonicator was the highest, however only slightly higher than the two other and it was 42.6 µg/mL.

The analysis of the soluble FAA profile of *Saccharina latissima* showed that the three amino acids alanine, glutamic acid and aspartic acid are dominant among FAA found in samples extracted in both solvents. Samples extracted in alkali and in acid had generally the same soluble FAA proportions with the exception of Gly/Arg that had significantly higher peak for the extraction in NaOH. FAA were most abundant in sample SL2 (0.4M NaOH, no ultrasound treatment) and in sample SL1 (0.1M NaOH, no ultrasound treatment). Samples SL5 (0.1M NaOH) and SL6 (0.4M NaOH) treated in the ultrasound bath and samples SL9 (0.1M NAOH) and SL10 (0.4M NaOH) treated in sonicator had similar FAA content and it was lower than in untreated samples. FAA after extraction in HCl were most abundant in samples SL4 (no ultrasound treatment), SL7 (ultrasound bath) and SL11 (sonicator) extracted in 0.4M HCl and FAA and it did not increase after ultrasound treatment. The lowest FAA content was found in sample SL3 that was extracted in 0.1M HCl and did not undergo ultrasound treatment.

In the analysis of the soluble FAA profile of *Alaria esculenta* for samples from the first extraction in water (AE0.1 and AE0.2) glutamic acid dominates, followed by a high content of leucine and slightly lower content of alanine. For samples extracted in water for the second time no significant increase of soluble FAA content was measured, however it was shown that sample treated with sonicator (AEW3) had the highest content of FAA.

Preface and acknowledgments

This study is a master thesis in 2 years international master program in Biotechnology at the Norwegian University of Life Science and Technology, NTNU. The research was performed at the biotechnology department at NTNU. The research was carried out from Fall-2018 to Winter 2020 as part-time studies and counts for 60 credit points.

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List of figures

List of contents

1 Introduction.

Last year (2019) the world population reached 7,7 billion people. This is one billion more than in 2007 and 2 billion more than in 1994. According to the estimations in United Nation's newest World Population Prospects, the size of the global population will reach 8,5-8,6 billion in 2030, 9,4-10,1 billion in 2050 and as much as 9,4-12,7 billion in 2100. The most intensive population growth is estimated to take place in Sub-Saharan Africa [1].

Climate change is going to have a dramatic impact on agriculture in the future. Temperature on Earth is assumed to increase by a range from 1,8^{o}C to 4,0^{o}C by the year 2100 [2]. This will not only affect food production but will also demand development of new methods of food storage. Current agriculture practices led to diminishing biodiversity of plants and animals providing food.

Growing population and alteration of the environment due to climate change and human impact demand development of improved agricultural methods and searching for alternative food sources. Another challenge that must be faced is water scarcity that is likely to worsen due to climate change. Furthermore, we cannot expect to increase the available arable land, quite the opposite, this is more likely to be reduced. The available arable surface will decrease both due to the climate change and increasing population that will demand more living space.

Figure 1. Earth (Getty Images).

Salt water is a huge and relatively unexploited resource when it comes to farming. Approximately 71% of the Earth's surface is covered by oceans and seas and they make up 96,5% of the total global water. Freshwater only accounts for 2,5% of the total (Wikipedia). According to FAO, 4,3 billion people rely on fish for 15% of their animal protein intake, and 10% of world's population depends on fisheries for their livelihoods (FAO).

To meet the increasing demand for food, more resources must come from seas and oceans. Exploring possibilities of using seaweed as food and feed is one way of increasing the amount of food from the ocean and it is an important path in meeting the increasing demand for nutrient-rich food and feed. It could also contribute to reduction/eradication of hunger and malnutrition.

1.1 Macroalgae-an overview.

1.1.1 What are macroalgae.

Macroalgae, also known as seaweed or kelp, are oxygen-producing, photosynthetic unicellular and multicellular organisms. They are also called marine plants. Together with microalgae (phytoplankton) and aquatic seed plants, they create the aquatic primary biomass. Although they mostly grow under water, some species can also be found in the tidal zone, where part of the seaweed is floating on the surface of the sea [3].

Asian countries such as the Republic of Korea, Japan and China were the pioneers of macroalgae farming and use. Nowadays they are both the biggest producers (99% of the global production) and consumers of edible seaweed. Ireland, Iceland and Canada also have tradition of eating seaweed, although this market is still under development [4]. In Europe, kelp is farmed in Denmark, Portugal, Spain, Faeroe Islands and in Norway [5].

In Asia, 99,9% of macroalgae is cultivated, while in Europe seaweed cultivation is still in the developmental phase and only 0,1% is cultivated [5].

In Norway, wild seaweed has been used as an addition to animal feed and as a fertilizer. Nowadays, possibilities of cultivation are developed. With a very long shore line and good water quality, Norway has an opportunity to become an important seaweed producer. There are many species of macroalgae growing in Norwegian sea: 175 species of brown algae, 200 species of red algae and 100 species of green algae [5]. Both *Saccharina latissima* and *Alaria esculenta* that were the subject of research for the purpose of this master thesis are cultivated in Norway.

1.1.2 Why should we study macroalgae?

Macroalgae have several features that make them an interesting research subject:

- they grow in the sea, they use sunlight energy and extract nutrients from the ocean, therefore they neither have need for farmland nor fresh water (as opposed to terrestrial plants grown for food);
- they incorporate CO_2 in the biomass;
- they can be grown in all geographical latitudes and on seaweed cultivation farms;
- they are one of the fastest growing plants in the world and can be grown year-round with biomass harvest in a period of 2-5 months;
- harvest with a high content of carbohydrates and proteins can be obtained [5].

Since seaweed takes up nutrients and reduce plankton biomass in the sea, it is essential to develop cultivation strategies that will eliminate harmful effects on the sea environment [5].

1.1.3 Classification of seaweed.

Macroalgae can be divided in three taxonomic groups: brown, red and green, depending on the pigment that they produce. Botanical names of these groups are respectively: Phaeophyceae, Rhodophyceae and Chlorophyceae [4]. They differ in size and occurrence and they contain different substances of interest for industry. Detailed comparison of these three groups can be found in Table 1.

Brown seaweed

Brown seaweed, with 1800 known species, produce the pigments chlorophyll a and c, fucoxanthin and carotenoids. They are mostly used as whole food or for alginate production [4]. Both *Saccharina latissima* and *Alaria esculenta* are brown macroalgae. They are the biggest and heaviest macroalgae, with a maximum size up to 50 meters and weight up to 50 kilograms.

Table 1. Macroalgal classification and key compounds[3, 4].

	Brown algae	**Red algae**	**Green algae**
Pigments	Chlorophyll a+c Fucoxanthin o.a. Carotenoids	Chlorophyll a Phycobilines Div. carotenoids	Chlorophyll a+b Div. carotenoids
Number of Species	1800	5000	9000
Habitat	Marine Most benthic	Marine 90% Benthic	Limnic 90% Marine 10%
Storage Carbohydrates	Laminaran (β-1,3 glucan) Mannitol	Floridean starch (α-1,4 glucan) Floridosid	Starch (α-1,4 glucan)
Structural Polysaccharides	Alginate Fucoidan (Cellulose)	Galactanes (agar, carrageenan) (cellulose)	Cellulose Mannose Ulvan
Morphology	Macrophytes None single celled	Macrophytes Rarely single celled	Single celled Colonies Macrophytes
Size/ weight	50 m/200 kg	1 m/50 kg	1 m/2 kg
Bioactive Compounds	Phlorotannines (polyphenols)	Halogenated organic Compounds	Few known
Examples	 *Laminaria hyperborea*	 *Porphyra umbilicalis*	 *Ulva lactuca*

Red seaweed

Red seaweeds are significantly smaller, from a few centimetres to a maximum size up to 1 meter and maximal weight 20 kilograms. They produce chlorophyll a, phycobilines and diverse carotenoids, which give them colours. However, red algae are not always red - they can also be purple, or brownish red. There are approximately 5000 known species of red seaweed. They are mostly used to produce agar, carrageenan and food [3].

Green seaweed

Green algae are considerably different from the two other groups. First of all, they live mostly in waters with low concentration of salt, such as lakes or ponds. Only 10% of 9000 known species prefer the marine environment. They produce diverse carotenoids and chlorophyll a and b, responsible for their green colour. They are also the smallest of all macroalgae, with maximum size up to 2 meters and maximum weight of only 2 kilograms. In contrast to brown and red seaweeds, green algae are often unicellular and they create colonies [4].

1.1.4 Chemical composition of macroalgae.

Seaweeds contain vitamins, minerals, proteins, polysaccharides and fiber. This makes them nutritionally important [6]. They consist mostly of water, which is 70-90% of the total weight. The quantity of the rest of the compounds varies between the three macroalgae groups, but also within them, between species. Significant seasonal variations occur as well. Protein is intensively produced during winter, while carbohydrate production is at maximum during the summer period [4]. In general, seaweed have high ash and fiber content, and a relatively low content of proteins and fatty acids [6]. An overview over the chemical composition of seaweed can be found in Table 2, where the percentage value of particular compounds (except water) refers to the dry mass.

Table 2. Chemical composition of seaweed [3].

	Brown algae	**Red algae**	**Green algae**
Water (% of fresh weight)	70-90% ww	80-90% ww	Approx. 80% ww
Ash	15-35% dw	10-25% dw	10-20% dw
Carbohydrates	50-60% dw	40-50% dw	40-50% dw
Fiber	5-8% dw	2-7% dw	4-5% dw
Proteins	5-15% dw	10-45% dw	15-25% dw
Lipids	2-7% dw	0,5-3% dw	0,7% dw
Polyphenols	1-10% dw	0% dw	0% dw
Iodine	0,01-1,1% dw	0,0005% dw	n.d.

Ash.

Most seaweeds have a high ash content, which indicates a high content of various minerals. Mineral content varies from 10% in red and green algae up to 45% for *Laminaria* and *Saccharina* [4, 6]. There are also strong seasonal variations in mineral content with a maximum in the spring (March) and the lowest level during summer (July). Ions that dominate in seaweed are sulphate (SO_4^{2-}), chlorine (Cl^-) and potassium (K^+). Brown algae, especially *Laminaria* and *Saccharina*, are good sources of iodine [4]. This is further discussed later in this chapter.

Protein and amino acids.

Protein content varies significantly between macroalgae groups. In brown algae protein content is 5-15% , while in green algae it varies but usually is between 15 and 25%. The highest content of protein can be found in red algae, where it can reach up to 45% of the dry mass. This makes them an important source of protein. There is a large variation in protein content of seaweed that depends upon seasonal and environmental growth conditions with maximum of production during winter time [6]. Protein content of macroalgae will be further discussed in section 1.2.

Carbohydrates.

Seaweeds are especially valued for their high content of carbohydrates, that is common for all species. In *Laminaria* and *Saccharina* carbohydrates constitute 40-70% of the dry weight. Carbohydrate content vary depending on the season and environmental growth conditions, with maximum during the summer time. The carbohydrate fraction consists of structural (agar, carrageenan, alginate, fucoidan) and storage (laminaran, mannitol) polysaccharides. Major sugar components of macroalgal polysaccharides are galactose, mannose and glucose. Polysaccharides with the highest industrial interest are agar, carrageenan and alginate due to their gel forming properties [5]. The main producers of alginate are United States of America (70% of the annual alginophytes production) and Norway [7]. Alginate is the only compound currently isolated from macroalgae in Norway. It is obtained from *Laminaria hyperborea,* a wild species of brown algae, that has high content of high quality alginate [5].

Fiber.

Dietary fiber is a complex material that is not digested in the human small intestine. Eating food rich in fiber is health beneficial as it helps to control weight (reduces calorific value of diets),

reduces the absorption of cholesterol, glucose and toxins from food. Alginate, agar and carrageenan are food fibers obtained from seaweeds [6].

Lipids.

Marine macroalgae contain low amounts of lipids, from 0,5% in red seaweed to 7% in brown macroalgae. Seaweeds are source of polyunsaturated fatty acids (PUFA), with high level of omega-3 fatty acids and nutritionally perfect omega-6/omega-3 free fatty acid ratio, however some species have more of omega-3 fatty acids [6].

Other

Seaweeds are also a good source of iodine, however have to be consumed carefully, to avoid overdose. While nori can be safely eaten every day, kombu should not be eaten more often than once a month [3]. They are also source of antioxidants. Brown algae, especially *Fucus* spp. and *Ascophyllum* are rich in phenolic compounds (12-14% of dry mass). Phenolic compounds have antioxidative, antibacterial and antifungal properties. Fucoxanthin, a pigment occurring in brown algae is an antioxidant and has potential anticancer effect [5]. Kelps in general contain high amount of the trace element iodine and contents as high as 6,500 mg · kg^{-1} dw have been found in European sugar kelp. Kelps in general contain high amount of the trace element iodine[6] and contents as high as 6,500 mg · kg^{-1} dw have been found in European sugar kelp. Comparing the iodine content of seaweed to sea foods that contain up to 30 mg · kg^{-1} dw which are known to a rich iodine source, the iodine content of sugar kelp is extremely high.

1.1.5 Various applications of seaweed.

Directly as whole food.

Macroalgae have been an important part of diet in Asian countries such as China, Japan and the Republic of Korea since prehistoric times. On account of human migration and new trade markets, eating seaweeds becomes more and more popular in other parts of the world [4]. Fresh seaweed is used, for instance, as a lettuce replacement in salads, burgers, taco or sandwiches. Dried seaweed can be used in sushi (Nori) or as an addition to salads. Nori can be also used to make soup, salad dressing or mayonnaise.

Nori, also called purple laver (*Porphyra* spp) is a seaweed used in sushi. It is cultivated in Japan, the Republic of Korea and China. Nori is rich in proteins (30-50%), and about 75% of this is digestible. It also contains significant amounts of Vitamins A_1, B_1, B_2, B_6, B_{12}, C, niacin

and folic acid. Sugar and sodium content are very low. The characteristic taste of nori originates from presence of large amounts of three amino acids: alanine, glutamic acid and lysine [4].

Aonori, also called green laver (*Monostroma* spp. and *Enteromorpha* spp) are green seaweed cultivated in Japan. Both have an average protein content of 20% and contain useful vitamins and minerals [4].

Kombu or **haidai** (*Laminaria japonica*). Kombu is a Japanese name for a dried seaweed derived from a mixture of *Laminaria* species. Haidai is a Chinese name for *Laminaria japonica*. *Laminaria* species are rich in iodine. They contain approximately 10% protein, 2% fat and significant amounts of vitamins and minerals (however still low, compared to nori) [4].

Wakame (*Undaria pinatifida)* is a brown seaweed, cultivated mainly in the Republic of Korea, but also in Japan and China. Wakame has a significantly higher content of fiber than nori of kombu. It is also a good source of B vitamins, manganese, copper, cobalt, iron, nickel and zinc [4].

Other types of seaweed used as food are: hiziki (*Hizikia fusiforme*), green caviar (*Caulerpa lantillifera*), mozuku (*Cladosiphon okamuranus*), dulse (*Palmaria palmata*) and irish moss (*Chondrus chrispus*) [4].

Hydrocolloids extracted from seaweeds have a broad use in food product development. An overview over them is presented in Table 3. They also known for their health beneficial properties. When used as dietary fiber they stimulate the immune system, reduces intestinal absorption, increases satiety, reduces glycaemic index value of food, modulates colonic microflora and elevates colonal barrier function [6].

Animal and fish feed.

Seaweeds have always been a part of diet of farm animals that live in coastal areas. Norway was one of the first producers of seaweed meal, using *Ascophyllum nodosum.* This macroalgae, on account of its accessibility, has become the main raw material for seaweed meal. It contains useful amounts of minerals such as: potassium, phosphorus, magnesium, calcium, sodium, chlorine and sulphur. It also contains vitamins and trace elements (Table 4). Seaweed is prone to mould infestation, therefore if used for meal production it must be freshly cut [4].

Table 3. Chosen applications of seaweed hydrocolloids in food industry[6].

Products	Polysaccharide and action
Fish products: surimi, fish meat gel, burgers, sausages.	Carrageenan and alginate enhance yield, hardness and bind strength, texture and fiber content.
Red meat products: turkey, restructured beef products, low-fat meat balls, beef burgers.	Carrageenan increases yield, improved visual appearance, sliceability and rigidity, and decreases expressible juice.
Bakery products	Carrageenan enhances loaf volume and water absorption and improves crumb grain score
Dairy products	Carrageenan, agar, alginates act as stabilizing, thickening and gelling agents.
Fruit juices	Carrageenan inhibits browning.
Vegan/Muslim and Jew-friendly products.	Agar is used as a gelatine substitute.
Beer	Alginate acts as foam stabilizer.
Fruit preserves such as jams, marmalades, and fruit sauces.	Alginate gives ideal viscosity and prevents crystallization and shrinkage.

Table 4 Valuable nutrients in seaweed meal [3]

	In algae-dry weight	Daily need adult	g flour to cover daily need
Iodine	0,9 mg/g	0,15 mg	0,2 g
Selenium	0,08 µg/g	40 µg	500 g
Vitamin E	350 µg/g	10 mg	30 g
Vitamin C	1 mg/g	60 mg	60 g
Vitamin B_{12}	0,001 µg/g	2 µg	

Pharmacy and medicine.

Agar is used as a culture medium for practically all pathogenic and non-pathogenic bacteria. None of the pathogenic bacteria is known to digest it. Mixed with other substances, agar and alginate serve as the ideal dental impression materials. After oral intake, agar can absorb water and expand considerably, thus increasing the bulk and stimulating peristalsis of intestine, helping with waste elimination and serving as laxative preparation. Agarose is an ideal gel matrix for diffusion and electrokinetic movement of biopolymers, and its gel is an anti-convection medium, which is biologically inactive and with controlled ionic properties, therefore it is broadly used in medical, pharmaceutical and biotechnological research. Alginate is also broadly used in medicine and pharmacy: in surgical lubricants, in suspension agents for drugs, in wound dressings [7].

Cosmetics

Hydrocolloids are used to improve texture of cosmetics, especially those used in face creams, and body creams/lotions. Milled seaweed is also used as an active ingredients in many cosmetics [4].

Biogas

Seaweed can become a renewable source of methane, however more research is needed in this field. Macroalgae are suitable for biogas production, as they are easier to hydrolyse and convert (better than wood since the macroalgae do not contain lignin and contain low amounts of cellulose). Production of methane from seaweed, that have high content of carbohydrates might be increased by adding nitrogen-rich contents, such as fish or household wastes [4, 5].

Fertilizers

Coastal people have been using macroalgae as a fertilizer for a long time. Seaweed was either used fresh, mixed with sand or sun dried. Nowadays, seaweeds are processed first. It is common to use dried and milled seaweed meal, made of brown algae species: *Ascophyllum, Ecklonia* and *Fucus.* It can be used both as a soil conditioner and fertilizer. It has good nitrogen and potassium content; however it is lower in phosphorus than animal manures. Seaweed extracts and suspensions are also produced from *Ascophyllum* and *Ecklonia maxima*. They are sold in concentrated form, are easy to dilute, transport and they act more rapidly than the fertilizer derived from seaweed meal [4].

Wastewater treatment.

Macroalgae have two potential applications in wastewater treatment: reduction of nitrogen- and phosphorous- containing compounds and removal of toxic metals from industrial wastewater. Enrichment of waters with nitrogen- and phosphorous- containing materials (eutrophication) leads to the excessive growth of unwanted matter such as marine plants or particular types of algae. Seaweeds can solve this problem. Many species take up ammonium for their growth and might also take up more phosphorous thank it is actually required for the maximal growth. The ability to absorb and accumulate heavy metals (such as copper, nickel, lead, zinc and cadmium) make algae excellent candidates for the treatment of industrial wastewater [4].

1.2 Macroalgal protein.

The challenge of meeting the increasing demand for food and feed in the future was presented in the beginning of this thesis. Of the three main nutrients: protein, fat and carbohydrates, the need for protein is the most difficult to satisfy, therefore there should be an increased focus on exploring new protein sources. Successful extraction of macroalgal protein might lead to sustainable production of protein concentrates and other types of food and feed additives rich in protein.

1.2.1 Why should we extract algal proteins?

Macroalgae contain several antinutritional components that have a detrimental effect on protein digestibility and extractability and these include polysaccharides and phenolic molecules, the phlorotannins [8]. Algal polysaccharides act as soluble or insoluble fibers. Polysaccharides from brown macroalgae that act as soluble fiber show a strong inhibitory influence on pepsin (*in vitro* test) and thus, lead to a significant decrease in protein digestibility [9]. In addition to antinutritional compounds, such as phlorotannins and large amounts of fiber, *S. latissima* contains high levels of iodine and *A. esculenta* may contain sufficient amounts of arsenic to be potentially harmful [10]. Consideration of all the above factors could lead to the conclusion that protein extract obtained from brown seaweed might be of better use in food and feed than using the whole plant.

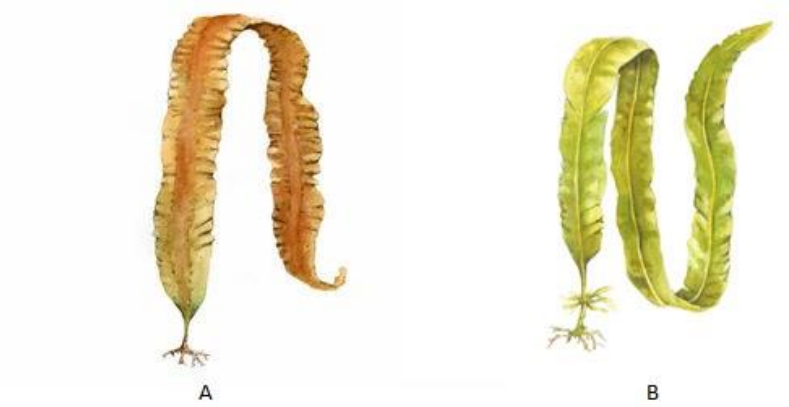

Figure 2. Macroalgae *Saccharina latissima* (A) and *Alaria esculenta* (B) (Stein Mortensen).

1.2.2 Amino acid composition.

	Mix depths		2m		5m		8m	
Amino acid	Average	St.dev.	Average	St.dev.	Average	St.dev.	Average	St.dev.
Taurin	0,1	0,1	0,02	0,02	0,07	0,00	0,04	0,00
Aspartic acid + Asparagine	1,7	0,0	0,65	0,16	1,22	0,08	1,28	0,04
Threonine	0,6	0,0	0,27	0,04	0,45	0,04	0,42	0,01
Serine	0,9	0,0	0,35	0,09	0,66	0,04	0,70	0,02
Glutamic acid + Glutamine	2,4	0,1	1,29	0,33	2,34	0,12	2,88	0,02
Proline	0,5	0,0	0,20	0,10	0,33	0,05	0,40	0,02
Glycine	0,8	0,0	0,31	0,08	0,59	0,04	0,59	0,02
Alanine	1,9	0,0	0,94	0,29	1,75	0,02	2,12	0,01
Cystine (Cys-Cys)	0,5	0,1	0,21	0,12	0,28	0,07	0,22	0,03
Valine	0,9	0,0	0,36	0,10	0,69	0,08	0,62	0,03
Methionine	0,3	0,0	0,09	0,02	0,18	0,02	0,16	0,00
Isoleucine	0,6	0,0	0,26	0,07	0,47	0,03	0,46	0,02
Leucine	1,1	0,0	0,43	0,12	0,79	0,06	0,79	0,03
Tyrosine	0,5	0,1	0,19	0,05	0,35	0,05	0,33	0,01
Phenylalanine	0,8	0,1	0,43	0,07	0,64	0,07	0,59	0,04
Histidine	0,3	0,0	0,15	0,04	0,25	0,01	0,24	0,01
Lysine	0,9	0,0	0,39	0,10	0,71	0,04	0,67	0,03
Tryptophan	0,2	0,0	0,12	0,04	0,19	0,01	0,17	0,01
Arginine	0,6	0,0	0,24	0,06	0,46	0,04	0,47	0,02
Sum (AA)	15,7	0,5	6,9	1,9	12,4	0,9	13,1	0,4

Figure 3. Amino acid profile of *Alaria esculenta* harvested in June 2016[11]

	Mix depths		2m		5m		8m	
Amino acid	Average	St.dev.	Average	St.dev.	Average	St.dev.	Average	St.dev.
Taurin	0,1	0,0	0,01	0,01	0,05	0,01	0,09	0,00
Aspartic acid + Asparagine	1,2	0,0	0,69	0,10	0,77	0,04	1,09	0,15
Threonine	0,4	0,0	0,19	0,01	0,29	0,01	0,45	0,02
Serine	0,6	0,0	0,33	0,07	0,39	0,02	0,60	0,03
Glutamic acid + Glutamine	1,4	0,0	0,79	0,15	0,93	0,00	1,24	0,05
Proline	0,4	0,1	0,26	0,06	0,24	0,01	0,33	0,01
Glycine	0,7	0,0	0,37	0,09	0,41	0,04	0,64	0,03
Alanine	1,2	0,1	0,68	0,19	0,75	0,05	1,03	0,06
Cystine (Cys-Cys)	0,2	0,1	0,06	0,03	0,16	0,06	0,18	0,00
Valine	0,7	0,0	0,39	0,10	0,43	0,03	0,63	0,03
Methionine	0,2	0,0	0,12	0,04	0,14	0,01	0,20	0,01
Isoleucine	0,5	0,0	0,29	0,08	0,33	0,03	0,46	0,03
Leucine	0,9	0,0	0,51	0,14	0,59	0,06	0,83	0,05
Tyrosine	0,3	0,0	0,18	0,05	0,20	0,00	0,29	0,01
Phenylalanine	0,6	0,0	0,43	0,09	0,45	0,02	0,64	0,03
Histidine	0,2	0,0	0,15	0,03	0,15	0,01	0,20	0,01
Lysine	0,6	0,0	0,36	0,09	0,40	0,04	0,58	0,03
Tryptophan	0,2	0,0	0,00	0,00	0,07	0,07	0,13	0,04
Arginine	0,4	0,0	0,27	0,08	0,32	0,04	0,45	0,03
Sum (AA)	11,0	0,5	6,1	1,4	7,1	0,5	10,1	0,6

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Figure 4. Amino acid profile of *Saccharina latissima* harvested in June 2016[11].

As mentioned earlier, in brown macroalgae, which have been the subject of research for this project, protein content usually varies between 5 to 15% of dry mass [12]. Protein in brown macroalgae has not been studied to a large degree, possibly due to relatively low protein content in comparison to red seaweed.

Both *Saccharina latissima and Alaria esculenta* contain all the essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine [10, 13]. The major amino acids found in macroalgae are glycine, arginine, alanine and glutamic acid [10, 13]. Alanine is the most abundant amino acid in *Saccharina latissima,* followed by significant amounts of aspartate and glutamate [14] that contribute to the umami taste.

1.2.3 Digestibility

Earlier research suggested good protein digestibility, however it was usually performed *in vitro,* using proteins extracted in strong alkaline condition [8]. Macroalgae as whole plants have generally poor digestibility in the raw, unprocessed form. In addition to that, it was shown that consumption of whole macroalgae has a negative influence on protein uptake from food [15].

1.2.4 Challenges in protein extraction.

Protein extraction yield is highly dependent on the availability of protein molecules. In seaweed, protein extractability is low generally because of the high viscosity and ionic interactions of polysaccharides both the intracellular and those polysaccharides that are part of the cell wall. After cell disruption, these polysaccharides remain in the extraction medium as hydrocolloids and increase its viscosity. This further leads to limited access to protein [16]

In brown seaweed, alginate and phlorotannins have a negative effect on protein extraction [8, 15]. Alginate (Figure 2) is an anionic structural polysaccharide of the cell-wall in brown algae and also contributes to viscosity. It contains three kinds of polymer units, that consist of D-mannuronic acid (M), L-glucoronic acid (G) and alternates of M and G units. Alginate can absorb 200-300 times its own weight in water and it needs calcium ions to form gels [3, 6]. Algin or alginate is a generic name for the alginic acid salts (sodium, potassium, ammonium, calcium, propylene glycol alginates).

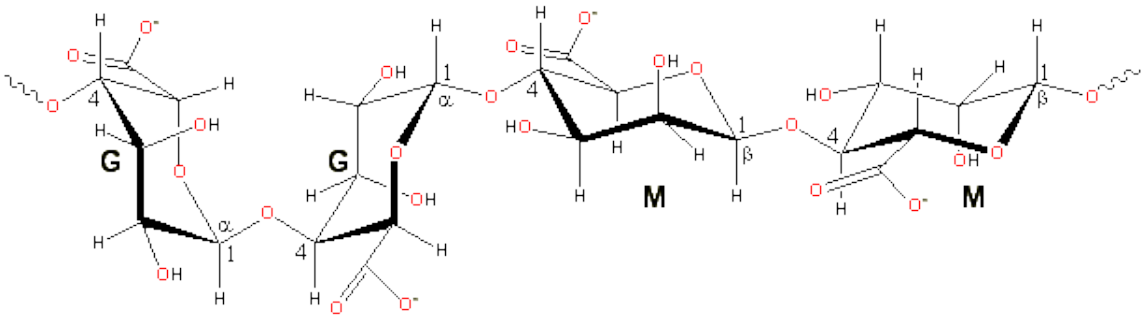

Figure 5. Structure of. G: α-L-guluronate. M: β-D-mannuronate.

Phlorotannins (polyphloroglucinols) are polyphenolic compounds found in algae. They are powerful antioxidants, taking part in protection against UV rays and dissuading animals. Their concentration in brown algae varies from 2% up to as much as 30%. and they exist in soluble or insoluble form. Soluble form is stored in membrane-bound vesicles (physodes). Phlorotannins become insoluble when they form complex with alginates, present in cell walls [17]. Several phlorotannins have the ability to oxidize and form covalent bonds with some proteins that decrease their accessibility and thus digestibility and extractability[18].

1.3 Protein extraction from brown seaweed.

Macroalgal proteins have been successfully extracted by means of solvent extraction, physical extraction, enzyme-assisted extraction and by combination of the above[15] has been studied.

However some challenges still exist regarding yield. There is still need to find an optimal method that will allow to extract all the protein (or nearly all) that an algae contains.

1.3.1 Solvent extraction

In 2016, Lyne Hovednak Lyng examined how different extraction conditions affect protein extraction from *S. latissima, A. esculenta* and *Palmaria palmata.* One of the extraction procedures for brown algae involved a combined acid and alkaline treatment. For this, 0,1M formic acid and 0,12M NaOH were used. Of these extraction methods , extraction with 0,12M NaOH was the most effective [14].

1.3.2 Enzyme-assisted extraction.

Another experiment conducted by Lyng was an enzyme-assisted extraction with polysaccharidases alginate lyase and cellulase. Enzymatic treatment did not increase the protein yield [14]. This could be due to too low amounts of enzymes or wrong/ not optimal conditions.

1.3.3 Ultrasound assisted extraction.

To achieve a good yield of extracted proteins from macroalgae it is necessary to find a method to degrade or break the cell wall. One of the cell-rupturing methods, an ultrasound treatment, has been applied to brown algae, *Ascophyllum nodosum* for different extraction solutions [19]. Acid (HCl), alkali (NaOH), combined acid-alkali together with and without ultrasound pretreatment were used. Combined acid-alkali treatment was proven the most effective method for protein extraction, followed by treatment with only NaOH.

1.4 Ultrasound assisted extraction (UAE).

1.4.1 What is ultrasound?

Ultrasound can be defined as high-frequency sound waves. Ultrasound can be defined as high-frequency sound waves. In a liquid medium, these accelerate its particles to high velocities, which results in pressure differences between regions of the liquid. These pressure differences cause a phenomena called cavitation - the formation of vapour bubbles within a liquid that implode on the surface of the vegetal tissue (Figure 3). This results in several physical processes that together affect the extraction of compounds from the vegetal matrix: fragmentation, erosion, capillarity, detexturation and sonoporation [20].

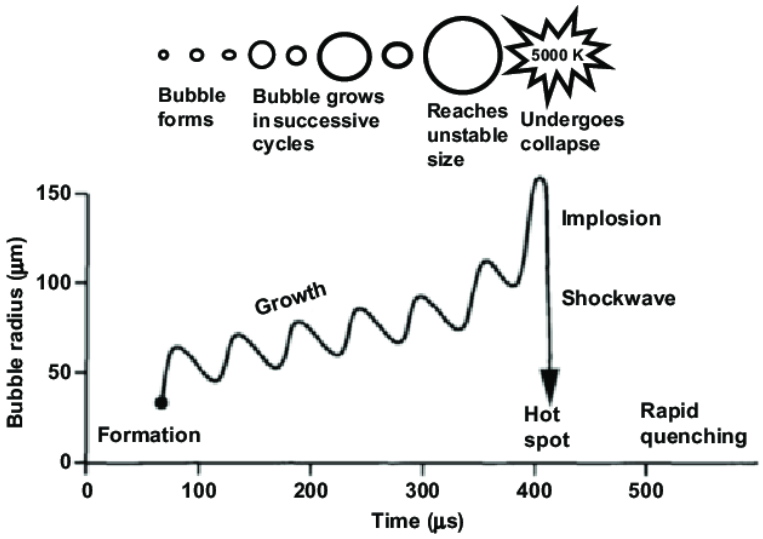

Figure 6. Cavitation caused by ultrasound.

1.4.2 Why UAE?

The extraction of components from plants in various solvents has been used since ancient times. With the development of chemical research (and civilization), awareness of potential impact on health and environment arose. This applies not only to the chemicals used in industry but also to the industrial process itself. Energy consumption, greenhouse gas emission, utilization of hazardous waste are only examples of current concerns regarding food industry. Another factor is the potential impact of chemicals used in food and feed production for health, followed by more and more demanding law regulations. Ultrasound treatment allows increasing extraction yield as a 'green' alternative to use of harmful solvents. It also gives rapid results, decreasing time and energy consumed by the extraction procedure/

1.4.3 Mechanism of action.

Fragmentation.

Raw plant material suspended in a liquid medium may undergo rapid fragmentation due to ultrasound action. This results in reduced particle size and increased surface area of the solid, further leading to higher mass transfer and improved extraction yield. Fragmentation is caused by physical processes that are caused by ultrasound treatment: inter-particle collisions and shockwaves created by collapsing cavitation bubbles [20].

Erosion.

Erosion of the raw plant material leads to enhanced accessibility of the extraction solvent to the plant tissue, improving extraction and solubilization. Erosion is believed to be caused by implosion of cavitation bubbles on the surface of the plant tissue [20].

Sonocapillary effect.

Sonocapillary effect, also known as ultrasound capillary effect (UCE) is the increase of depth and velocity of penetration of liquid into canals and pores in under sonication. This leads to swelling and rehydration of the plant tissue, and, as a consequence, improves mass transfer, improving also extraction yield. [20]

Sonoporation.

Sonoporation is a phenomenon of pore formation in the plant tissues caused by cavitation, releasing desirable content to the extraction medium [20].

Local shear stress

Shear forces are generated locally and they arise from oscillation and collapse of cavitation bubbles within the fluid[20].

1.5 The purpose of this thesis.

The purpose of this thesis was to examine how different conditions influence the extraction of protein from two brown macroalgae: *Saccharina latissima* and *Alaria esculenta*. Two concentrations of NaOH and HCl were used as extraction solvents to determine how pH and these two particular solvents affect protein extraction yield. In addition, a part of the samples underwent treatment in either sonicator or an ultrasound bath to determine if the ultrasound influence on the cell matrix contributes to the improved protein extraction yield.

2 Materials and methods.

2.1 Raw material.

Two species of brown macroalgae were used in the experiments: *Saccharina latissima* and *Alaria esculenta*. Both were grown by Seaweed Energy Solutions AS in Trondheim. *S. latissima* was harvested 8.03.18, received fresh and then and freeze-dried at NTNU 13.04.18 *A. esculenta* was harvested 12.03.19, received fresh and then and freeze-dried at NTNU 03.04.19

2.2 Experimental design.

Figure 5. presents an overview over the work on this thesis. Details will be presented in the following sections. A two steps protein extraction was conducted. First, a standard extraction in distilled water was performed. Then, the pellets from this extraction were resuspended in one of the following: 0,1M NaOH, 0,4M NaOH, 0,1M HCl, 0,4M HCl or distilled water. Further, samples were divided into three groups and were exposed to: no pre-treatment, ultrasound bath and sonicator.

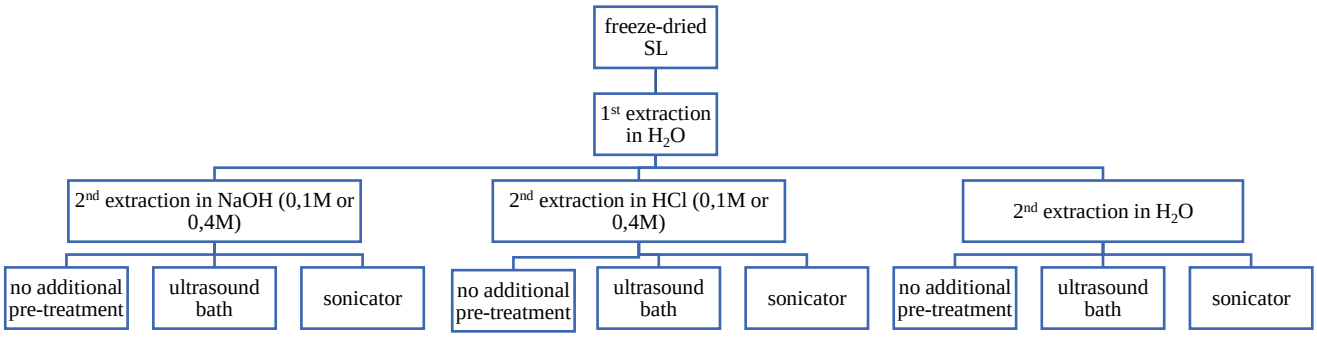

Figure 7. An overview over work during this thesis.

The goal of the second extraction was to examine whether the yield of the extracted protein can be increased by using solvents within various pH ranges and by applying ultrasound pre-treatment. Samples prepared with water are <control> and were prepared to examine whether the

yield of extracted protein can be increased only by applying ultrasound treatment. After the pre-treatment has been applied, samples were centrifuged for 1 hour in 4°C by 5500 rpm. Supernatants were separated from pellets. Both fractions were weighed and the volume of the pellets was noted.

Further, the content of soluble proteins was measured using the Lowry method. This method was chosen above others due to its sensitivity. It allows to detect protein at very low concentrations.

Free amino acid (FAA) profile was determined by high-pressure liquid chromatography (HPLC) method.

2.3 Extraction of soluble protein.

2.3.1 First extraction-in water.

4 g freeze-dried and milled macroalgae (*S. latissima and A. esculenta*) was suspended in 80 mL distilled water. Two parallels of each sample were prepared (in total 4 samples-2 parallels per species), further called SL0.1, SL0.2 (for *S. latissima*) and AE0.1, AE0.2 (for *A. esculenta*). Samples were incubated with stirring on an orbital shaker (PSU-10i, Grant-bio) first for 1 hour at room temperature (20°C), and further for 16 hours at 4°C. After 16 h, samples were centrifuged at 12 000 x g for 20 min at 4°C. After centrifuging, supernatants were separated from pellets, then both fractions were weight.

2.3.2 Second extraction: NaOH, HCl and H_2O.

Pellets SL0.1, SL0.2 AE0.1 and AE0.2 were resuspended in one of the following solvents: 0.1M NaOH, 0.4M NaOH, 0.1M HCl, 0.4M HCl or in distilled water (control) in a pellet-solvent weight ratio 1:15. 3 parallels per solvent were prepared (54 samples in total).

All samples were incubated with stirring on an orbital mixer for 1 hour in 4°C. After the incubation, samples were divided in three groups, as shown in the Table 6. The first group did not undergo any treatment and was centrifuged straight after incubation. at 5500 rpm for 60 min at 4°C. The second was placed in the ultrasound bath and the last group underwent treatment with sonicator.

2.4 Ultrasound treatment.

After 1 hour incubation in the solvents (NaOH, HCl and water) in 4ºC, 2 types of ultrasound pre-treatment were applied to maximize protein extraction: ultrasound bath and sonicator.

Table 6. An overview over treatments each sample was exposed to.

S.latissima			***A. esculenta***		
Sample ID	Solvent	Treatment	Sample ID	Solvent	Treatment
SL1.1	0,1M NaOH	none	AE1.1	0,1M NaOH	None
SL1.2			AE1.2		
SL2.1	0,4M NaOH		AE2.1	0,4M NaOH	
SL2.2			AE2.2		
SL3.1	0,1M HCl		AE3.1	0,1M HCl	
SL3.2			AE3.2		
SL4.1	0,4M HCl		AE4.1	0,4M HCl	
SL4.2			AE4.2		
SL5.1	0,1M NaOH	ultrasound bath	AE5.1	0,1M NaOH	ultrasound bath
SL5.2			AE5.2		
SL6.1	0,4M NaOH		AE6.1	0,4M NaOH	
SL6.2			AE6.2		
SL7.1	0,1M HCl		AE7.1	0,1M HCl	
SL7.2			AE7.2		
SL8.1	0,4M HCl		AE8.1	0,4M HCl	
SL8.2			AE8.2		
SL9.1	0,1M NaOH	sonicator	AE9.1	0,1M NaOH	sonicator
SL9.2			AE9.2		
SL10.1	0,4M NaOH		AE10.1	0,4M NaOH	
SL10.2			AE10.2		
SL11.1	0,1M HCl		AE11.1	0,1M HCl	
SL11.2			AE11.2		
SL12.1	0,4M HCl		AE12.1	0,4M HCl	
SL12.2			AE12.2		
SLW1	H_2O	none	AEW1	H_2O	none
SLW2		ultrasound bath	AEW2		ultrasound bath
SLW3		sonicator	AEW3		sonciator

2.4.1 Treatment in an ultrasound bath.

For this pre-treatment, samples were placed in a simple Ultrasonic Cleaner USC-T from VWR for 30 minutes.

Figure 8. Ultrasound bath (to the left) and sonicator (to the right) used in the extraction.

2.4.2 Pre-treatment in sonicator.

Another type of pre-treatment was performed on a parallel set of samples with use of Sonic Dismembrator, Ultrasonic Liquid Processor from Fisher Scientific (Figure 6). To minimalize time required and maximalize protein extraction, amplitude 40% was used (it was the maximum amplitude available for the tip used). Samples were treated for 30 seconds with intervals of pulse for 2 seconds and break for 1 second.

2.5 Lowry protein quantitation analysis.

2.5.1 Principle.

Lowry's assay is a spectroscopic method that relays on the reaction of divalent copper ions with peptide in the protein under which Cu^{2+} are reduced to Cu^{+}. Simultaneously, residues of amino acids, mostly tyrosine and tryptophan and to a lesser extent cysteine and histidine are involved. Under alkaline conditions, their radical groups react with Folin-Ciocalteu phenol to produce an unstable product that becomes reduced to molybdenum/tungsten blue. For the lower protein concentrations (as expected in this project), absorbance is read at the peak at 750 nm. Absorbance at 550 nm is used to determine higher protein concentrations. Addition of the Folin phenol reagent increases absorbance between 550-750 nm [21-23].

To calculate the exact concentration of protein in the examined sample, it is necessary to use a standard curve with known protein concentration and dilution. For this experiment, bovine serum albumin (BSA) was chosen due to similarities in amino acid composition of BSA and algae [24].

2.5.2 Procedure.

A detailed procedure is given in Appendix. In the experiments, supernatants obtained from the first extraction SL0.1, SL0.2, AE0.1 and AE0.2 were diluted 1:5 times, due to their relatively high protein concentration in comparison to the supernatants obtained from the second extraction. These were originally diluted 1:2 times. All were diluted in corresponding solvents, meaning water, NaOH or HCl, depending on the solvent used during the extraction.

As mentioned earlier, reactions that give colored products in Lowry's assay need an alkaline pH. If extraction is performed in water, there is no need for pH adjustment of diluted supernatants because the pH of the solution is either neutral or close to neutral. During this project however, both water, NaOH and HCl were used, giving a pH range of supernatants between 1.5 and 11.5. First, an attempt was made to perform protein measurement without previous pH adjustment. Results were chaotic and inconclusive and therefore a pH adjustment to neutral/close to neutral was necessary.

2.6 Free amino acid content determination with HPLC.

The determination of the amount and composition of free amino acids (FAA) in the CPH was performed as described by Osnes and Mohr (1985). S. *latissima/A. esculenta* solution (2 mg/mL, 1 mL) and 10 % sulphosalicylic acid (0.25 mL) was thoroughly mixed in an Eppendorf tube before the samples were left in a cold room (2^{o}C) for 30 minutes. Two parallels were prepared. Samples were centrifuged for 15 minutes at 4500 rcf. The liquid solution was decanted and the precipitation discarded. To check that all the protein in the samples had precipitated sulphosalicylic acid (0.25 mL) was added to 1 mL of the supernatant of one of the parallels and mixed as before. Following the complete precipitation of protein, the supernatant was diluted (1:25) using distilled water. The diluted sample was filtered through a 0.22 μm filter. Sample (0.205 mL) was transferred into glass vials, which were delivered for running on HPLC, as previously described .

3 Results and discussion.

3.1 Extraction of water soluble protein.

3.1.1 Water soluble protein content of *Saccharina latissima*.

Samples SL0.1 and SL0.2 were prepared by incubating 4g of freeze dried macroalga *Saccharina latissima* in 80 mL distilled H_2O over 16 h in 4°C. These were further used as base samples for two independent lines of base/acid/water extraction with or without the use of ultrasound treatment.

Table 5. Amount of water soluble protein extracted from *Saccharina latissima*.

Sample number	Solvent	Soluble protein content	Extracted protein fraction
SL0.1	H_2O	702.3±8,1 µg/mL	9.3±0,1 % DW
SL0.2	H_2O	700.6±9,3 µg/mL	9,1±0,1 % DW

3.1.2 Water soluble protein content of *Alaria esculenta*.

Samples AE0.1 and AE0.2 were prepared by incubating 4g of freeze dried macroalga *Alaria esculenta* in 80 mL distilled H_2O over 16 h in 4°C. These two independent samples gave the same results in terms of water soluble protein content (Table 8): 6.6±0.1 % DW for AE0.1 and 6/72±0,1 % DW for AE0.2. They were further used as base samples for two independent lines of alkali/acid/water extraction with or without the use of ultrasound treatment.

Table 8. Amount of water soluble protein extracted from *Alaria esculenta.* Results are given in µg/mL extract and extracted protein in % of DW of macroalgae. Results are presented an average of three parallels with the standard deviation.

Sample number	Solvent	Soluble protein content	Extracted protein fraction
AE0.1	H_2O	500.4±10,1 µg/mL	6.6±0,1 % DW
AE0.2	H_2O	507.4±6,6 µg/mL	6.7±0,1 % DW

3.2 Second protein extraction-extraction in HCl or NaOH.

3.2.1 General observations

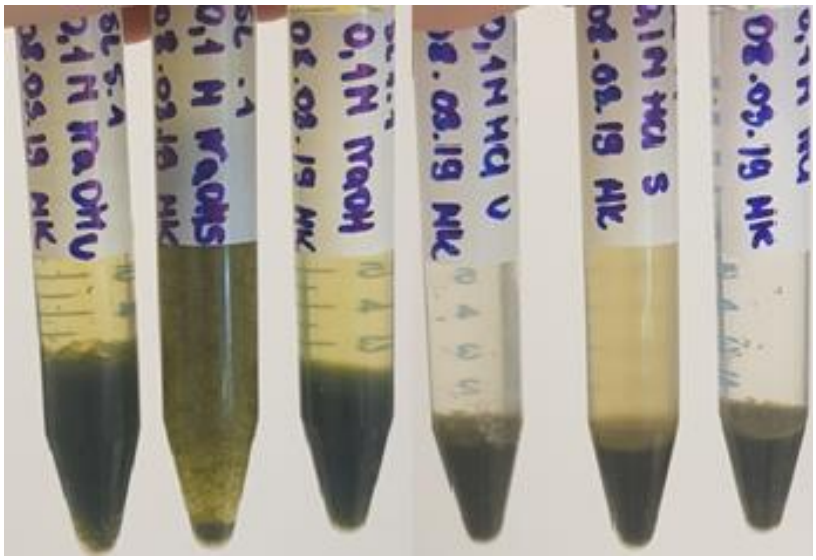

Figure 9. A visual comparison of samples extracted in alkali (left) and acid (right) that di or did not undergo ultrasound treatment.

After resuspending the pellet in NaOH, the color of the solution turned green. Alkaline pH preserved chlorophyll. Sample extracted in NaOH that was treater in sonicator formed a gel, likely due to a high content of extracted carbohydrates.

Table 6. An overview over samples names and treatments each sample was exposed to.

S.latissima			***A. esculenta***		
Sample ID	Solvent	Treatment	Sample ID	Solvent	Treatment
SL1.1	0,1M NaOH	None	AE1.1	0,1M NaOH	None
SL1.2			AE1.2		
SL2.1	0,4M NaOH		AE2.1	0,4M NaOH	
SL2.2			AE2.2		
SL3.1	0,1M HCl		AE3.1	0,1M HCl	
SL3.2			AE3.2		
SL4.1	0,4M HCl		AE4.1	0,4M HCl	
SL4.2			AE4.2		
SL5.1	0,1M NaOH	ultrasound bath	AE5.1	0,1M NaOH	ultrasound bath
SL5.2			AE5.2		
SL6.1	0,4M NaOH		AE6.1	0,4M NaOH	
SL6.2			AE6.2		
SL7.1	0,1M HCl		AE7.1	0,1M HCl	
SL7.2			AE7.2		
SL8.1	0,4M HCl		AE8.1	0,4M HCl	
SL8.2			AE8.2		
SL9.1	0,1M NaOH	sonicator	AE9.1	0,1M NaOH	sonicator
SL9.2			AE9.2		
SL10.1	0,4M NaOH		AE10.1	0,4M NaOH	
SL10.2			AE10.2		
SL11.1	0,1M HCl		AE11.1	0,1M HCl	
SL11.2			AE11.2		
SL12.1	0,4M HCl		AE12.1	0,4M HCl	
SL12.2			AE12.2		
SLW1	H_2O	None	AEW1	H_2O	none
SLW2		ultrasound bath	AEW2		ultrasound bath
SLW3		sonicator	AEW3		sonciator

3.2.2 Soluble protein extracted in alkali or acid from *Saccharina latissima*.

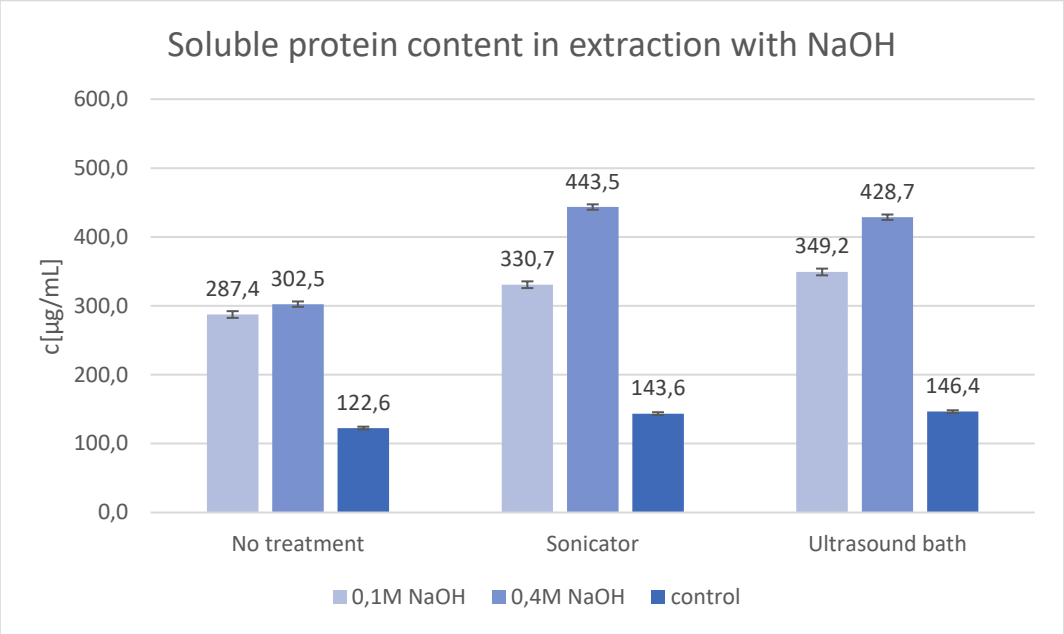

Figure 10. Alkali soluble protein content extracted from sample SL0.1 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

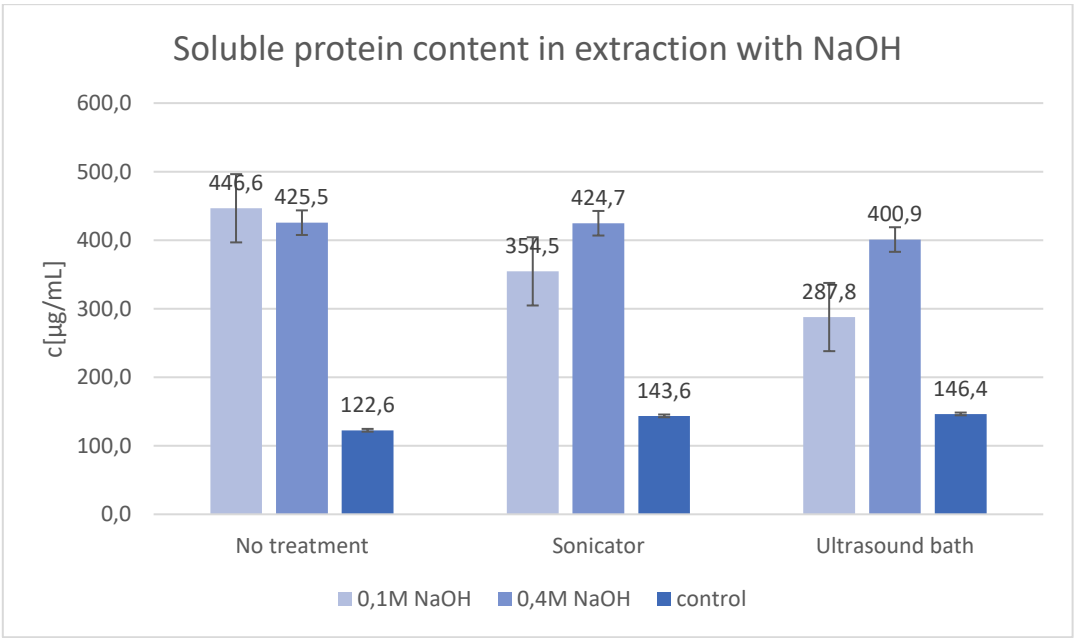

Figure 11 Alkali soluble protein content extracted from sample SL0.2 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

Figures 10 and 11 present the results of the protein extraction from pellets obtained during the first extraction (in water) for the samples SL0.1 and SL0.2, respectively. Pellets from the first extraction were resuspended in 0.1M NaOH or 0,4M NaOH or H_2O (control) in the pellet-solvent weight ratio 1:15 and a final volume of the prepared sample below 13 mL (due to equipment limitations). The prepared samples underwent no treatment or treatment in the sonciator for 30 seconds or treatment in the ultrasound bath for 30 minutes.

Samples extracted without ultrasound treatment.

Of all the supernatants, samples extracted in water (control) have the lowest protein content, 122.6 μg/mL for the sample SLW1 (water, no ultrasound treatment) and this is significantly lower than the protein content of samples extracted in 0.1M NaOH, where the extracted protein content was 287.4 μg/mL for sample SL1.1 and 446.6 μg/mL for sample SL1.2 or 0.4M NaOH where the extracted protein content was 302.5 μg/mL sample SL2.1 and 42.5 μg/mL for sample SL2.2. This shows that extracting in water several times do not increase the yield of the extracted protein. At the same time, the amount of extracted protein increases when 0.1M NaOH or 0.4M NaOH was used as the solvent. However, based on these results, it is not conclusive which concentration of NaOH gives the highest protein extraction yield.

Ultrasound assisted extraction.

Protein concentration in the supernatant during ultrasound assisted extraction has been increased for all the solvents used. For the extraction in H_2O (control), the concentration of the extracted protein increased from 122.6 μg/mL in SLW1 that did not undergo any additional treatment to 143.6 μg/mL in SLW3 treated with sonicator and to 146.4 μg/mL in SLW2 treated in ultrasound bath.

For the first set of samples (originating from sample SL0.1) extracted in 0.1M NaOH, protein content increased from 302.5 μg/mL in SL2.1 (no treatment) to 443.5 μg/mL in SL9.1 (sonicator) and 428.6 μg/mL in SL5.1 (ultrasound bath). For the same extraction from samples originating from SL0.2, a decrease in extracted protein yield was observed. Protein content decreased from 446.6 μg/mL in an untreated sample SL1.2, to 354.5 μg/mL in the sample SL9.2 treated in the sonicator and to 287.8 μg/mL in SL5.2 treated in the ultrasound bath. These findings are consistent with the findings of Lyng [14] and [19] that NaOH used as a solvent contributes to increased protein extraction.

For the samples extracted in 0.4M NaOH a similar tendency was observed. While protein yield increased with applied ultrasound treatments in samples originating from SL0.1 to 443.5 µg/mL in SL10.1 (sonciator) and 428.7 µg/mL in SL5.1 (ultrasound bath), it decreased in samples originating from SL0.2 to 424.7 µg/mL in SL10.2 (sonicator) and SL 5.2 (ultrasound bath). However, these values were still significantly higher than those of corresponding samples extracted in 0.1M NaOH and H_2O.

Since different results were obtained for these two sets of samples, it needs to be discussed what could be a possible cause. First, it is important to mention the lack of homogeneity of the raw material, that is the freeze-dried macroalgae. Another contribution to the lack of homogeneity is that the samples SL0.1 and SL0.2 have not been milled with homogenizer after hydration (first extraction in water). As mentioned earlier in the characteristic of macroalgal proteins, they are often bound to phenolic compounds, phlorotannins. It is not clear how ultrasound treatment influences the plant matrix. If phlorotannins are still bound to proteins after the extraction, they can limit the access of reagents used in the Lowry analysis to the protein, hence decrease the color intensity of the reaction, giving lower results than expected. Seaweed are rich in polysaccharides, that might undergo breakage to smaller, optically active units due to ultrasound treatment. These might also cause 'noise' in the results. Moreover, the experiments were conducted on a small scale, therefore it is possible that there were significant differences in the chemical composition of fractions of pellets SL0.1 and SL0.2 used in the second extraction. Further, for the set obtained from SL0.2 and extracted in 0.1M NaOH, the protein content decreases in samples treated with ultrasound in comparison to the untreated one. Last but not least, there is a significantly higher value of the standard deviation for Lowry analysis of these samples, especially the ones extracted in 0.1M NaOH in comparison to the standard deviation values calculated for the first set. Ultrasound treatment generally contributes to an increased protein extraction from *S. latissima* and the extraction is the most effective when 0.4M NaOH is used as a solvent (samples SL10.1 and SL6.1)

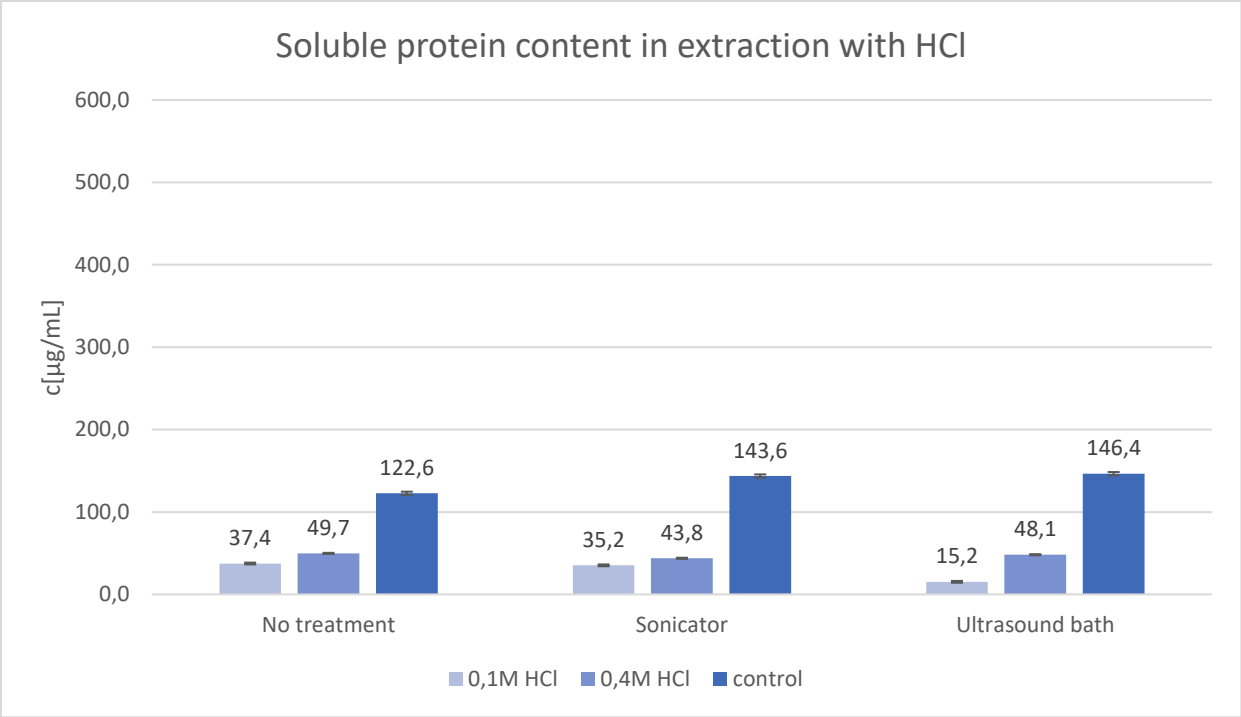

Figure 12. Acid soluble protein content extracted from sample SL0.1 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

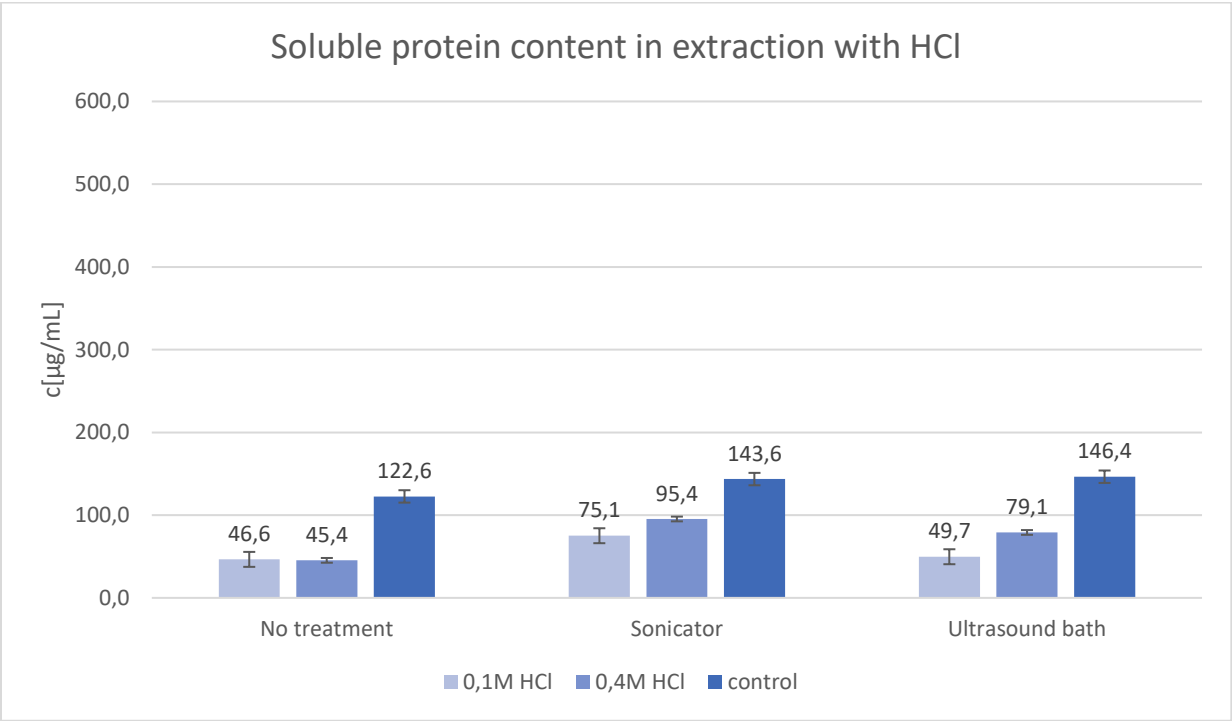

Figure 13. Acid soluble protein content extracted from sample SL0.2 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

Samples extracted without ultrasound treatment.

Figures 12 and 13 present the results of protein extraction from samples SL0.1 and SL0.2 performed in 0.1M HCl or 0.4M HCl or H_2O (control). Of the samples that did not undergo any treatment, the highest protein content was measured for the SLW1 (control). 0.1M HCl was used as solvent for samples SL3.1 and SL3.1 and protein content in these two was only 37.4 μg/mL and 46.6 μg/mL, respectively. For the samples where 0,4M HCl was used, measured protein content was 49.7 μg/mL for SL4.1 and 45.4 μg/mL for SL4.2. It is slightly higher than for samples extracted in 0.1M NaOH, however the difference is not significant. These findings are also consistent with results of research conducted by Lyng [14] og Kadama [19] which showed that low pH solvent does not contribute to the increase of the extracted protein fraction.

Ultrasound assisted extraction.

For samples originating from SL0.1and extracted in 0.1M HCl, protein content of 35.2 μg/mL in SL11.1 (sonicator) and 15.2 μg/mL for SL 7.1 (ultrasound bath) was measured and it was lower compared to the protein content in untreated samples. A similar trend was observed in samples originating from SL0.1 and extracted in 0.4M HCl, where protein content decreased to 43.8 μg/mL in SL12.1 (sonicator) and 48.3 μg/mL. In case of samples originating from SL0.2 and extracted in 0.1M HCl, protein content increased in SL.11.2 (sonicator) to 75.1 μg/mL and to 49.7 μg/mL. For the higher concentration of acid (0.4M HCl), protein concentration increased to 95.4 μg/mL in the sample treated in sonicator (SL 12.2) and to 79.1 μg/mL in the sample treated in the ultrasound bath (SL8.2).

Application of the ultrasound to the plant material can cause changes in the plant matrix that can lead to a poorer extraction of protein (and other compounds). This is possibly the reason for lower protein content measured in one set of samples after treatment with ultrasound and at the same time increased in the other set of samples. It is also clear that 0.1M NaOH used either alone or in a combination with ultrasound is a poor solvent. It does not contribute to the increased solubility/extractability of protein from the macroalgal tissue in comparison to extraction in water. The same conclusion can be drawn for 0.4M HCl, in spite of the higher protein content of samples extracted in this concentration compared to when 0.1M HCl is used and it is still much lower than when only water was used.

3.2.3 Soluble protein extracted in alkali or acid from *Alaria esculenta.*

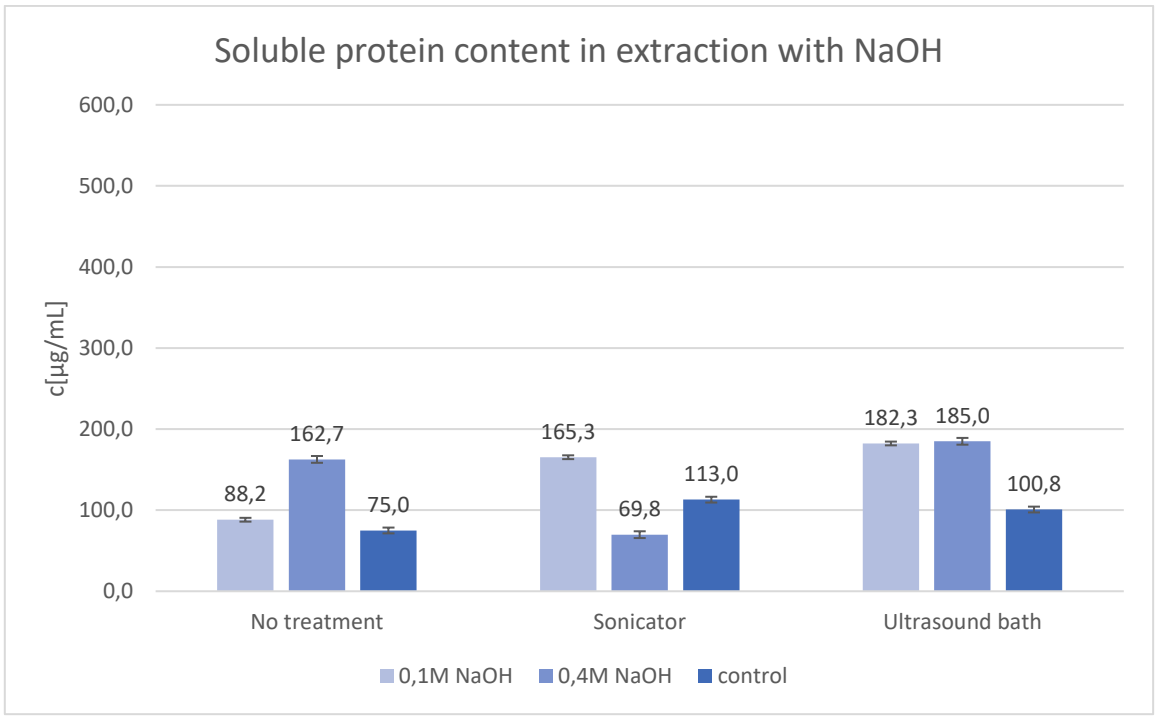

Figure 14. Alkali soluble protein content extracted from sample AE0.1 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

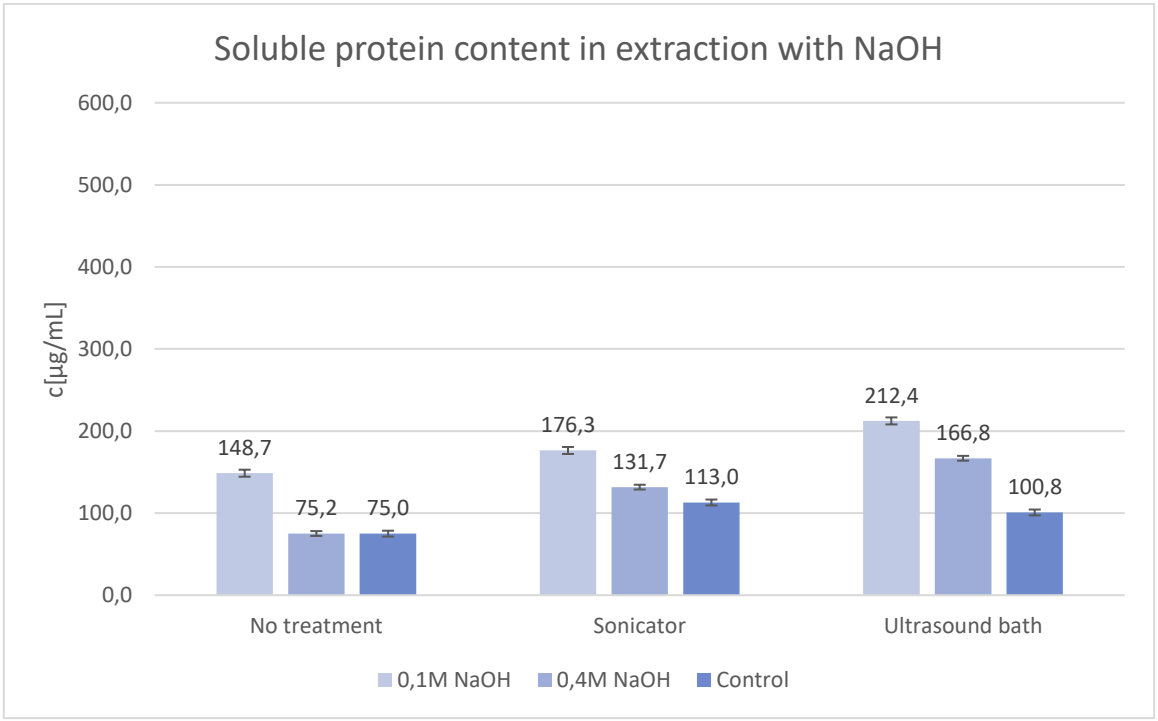

Figure 15. Alkali soluble protein content extracted from sample AE0.2 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

Figures 14 and 15 present the results of protein extractions from *Alaria esculenta* for samples originating from AE0.1 or AE0.2, conducted in 0.1M NaOH or 0.4M NaOH without any treatment or with ultrasound treatment with sonicator or the ultrasound bath.

Samples extracted without ultrasound treatment.

Of the samples that did not undergo any treatment, the sample AE2.1, extracted in 0.4M NaOH had the highest protein content, 162.7 μg/mL. A corresponding sample AE2.2, extracted in the same solvent had a protein content of 75.2 μg/mL and the control extracted in water 75.0 μg/mL. Protein content of the samples extracted in 0.1M NaOH was 88.2 μg/mL and 148.7 μg/mL for the samples AE1.1 and AE1.2, respectively. Results vary significantly, however analysis lead to the general conclusion that one can achieve a higher protein extraction yield than extracted in water when NaOH is used as a solvent.

Ultrasound assisted extraction.

Protein content was increased in all the control samples and reached a maximum of 113.0 μg/mL sample AEW3 (sonicator) compared with 100.8 μg/mL for sample AEW2 (ultrasound bath). For samples where NaOH was used, protein content generally increased after the ultrasound treatment was applied with the exception of sample AE10.1, originating from AE0.1 and extracted in 0.4M NaOH in sonicator. Here the protein content decreased to 69.8 μg/mL. The corresponding sample from the set originating from AE0.1, sample AE9.2 had much higher protein score, 131.7 μg/mL and it was also higher in comparison to the sample that did not undergo any treatment, AE2.2. In samples AE9.1 and AE9.2, where the extraction was performed in 0.1M NaOH with treatment with sonicator the protein content increased to 165.3 μg/mL and 176.3 μg/mL, respectively. Protein contents of the samples extracted in the same solvent concentration, where the treatment in the ultrasound bath was applied were higher, 182.3 μg/mL for the sample AE5.1 and 212.4 μg/mL for AE5.2.

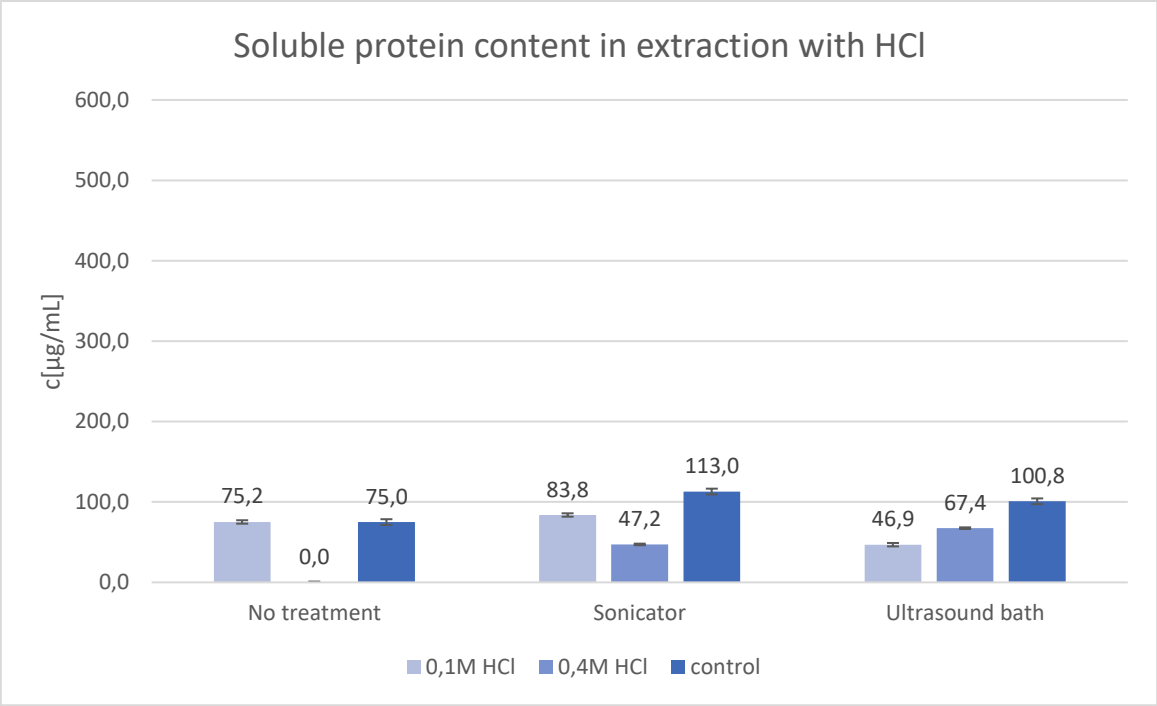

Figure 16. Acid soluble protein content extracted from sample AE0.1 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

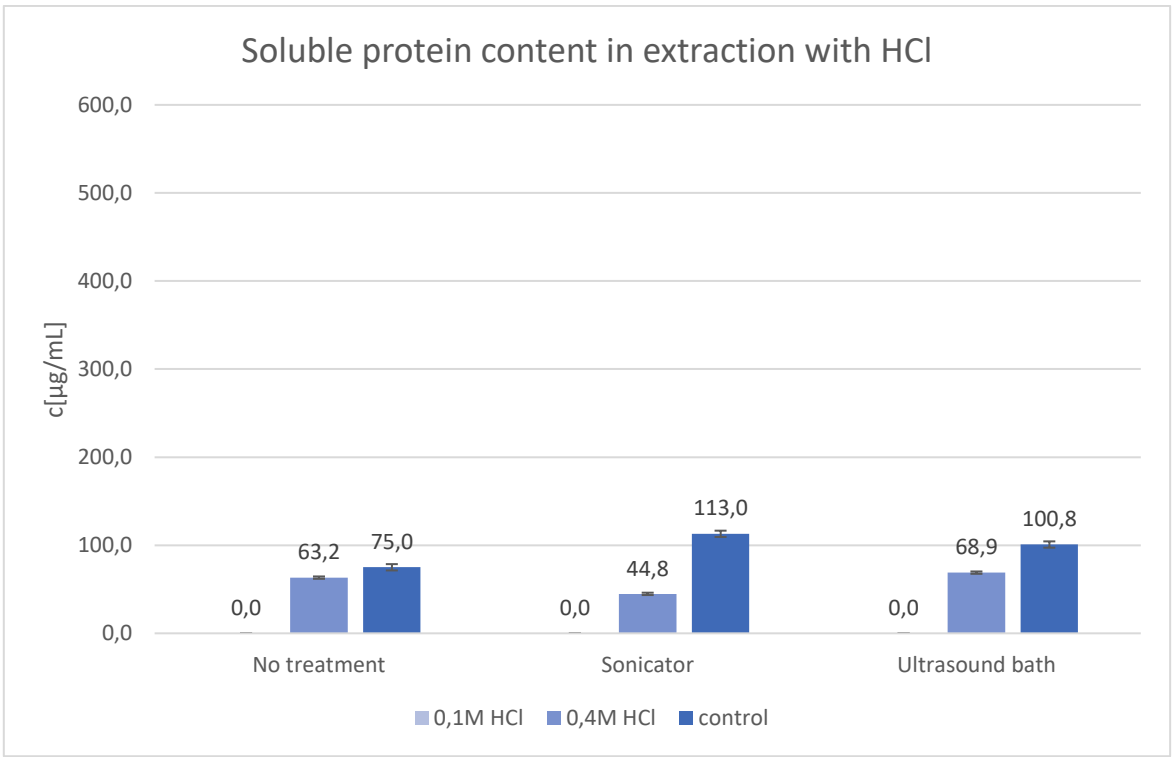

Figure 17. Acid soluble protein content extracted from sample AE0.2 during the second extraction, compared with control extracted in water. Results are presented as an average of three parallels with the standard deviation.

Samples extracted without ultrasound treatment.

Protein content in samples extracted in HCl was generally lower than the ones extracted in NaOH and slightly lower than the ones extracted in water (control). In samples extracted in 0.1M HCl that did not undergo ultrasound treatment it was 75.2 μg/mL in AE3.1 (originating from AE0.1) and below the detection limit for AE3.2 (originating from AE0.2). These values, compared to the 75.0 μg/mL extracted in water (AEW1) and 88.2 μg/mL in sample AE1.1 (0.1M NaOH) and 148.7 μg/mL in AE1.2 (0.1M NaOH), suggest that both 0.1M HCl and water are poor solvents for the second extraction in case of *A. esculenta*. Similar to 0.1M HCl, 0.4M HCl did not contribute significantly to the increased extracted protein content. It was below the detection limit for sample AE4.1 (originating from AE0.1) and 63.2 μg/mL for sample AE4.2 (originating from AE0.2).

Ultrasound assisted extraction.

Protein contents measured in samples extracted in 0.1M HCl that underwent treatment in the sonicator were 83.8 μg/mL for AE11.1 (originating from AE0.1) and below the detection limit for AE11.2 (originating from AE0.2). Protein content for these samples extracted in 0.4M HCl, with the same ultrasound treatment were 47.2 μg/mL and 44.8 μg/mL for samples AE12.1 and AE12.2, respectively. All of the above had lower values for protein content than the protein content obtained in the control sample AEW3 (water used as solvent), where it was 113.0 μg/mL. As for the samples treated in the ultrasound bath, a similar tendency was observed. Once again, the sample extracted in 0.1M HCl and originating from AE0.2 (AE7.2) had a score below the detection limit. Protein content measured in sample AE7.1 (originating from AE0.1 and extracted in 0.1M HCl) was 46.9 μg/mL. Samples extracted in 0.4M HCl and treated in the ultrasound bath had protein content of 67.4 μg/mL in sample AE8.1 (originating from AE0.1) and 68,9 μg/mL in sample AE8.2 (originating from AE0.2). This is higher than the protein content obtained with 0.1M HCl as a solvent, however still less than when water was used (100.8 μg/mL for AEW2).

The results presented above indicate that both 0.1M NaOH and 0.4M NaOH, combined with ultrasound treatment in the ultrasound bath contribute to the best protein extraction yield in *A. esculenta.* Protein content of the original samples from the first extraction, AE0.1 and AE0.2 was 500.4 μg/mL and 507.4 μg/mL, respectively. Protein content of the samples AE5.1 (extracted in 0.1M NaOH) and AE 6.1 (extracted in 0.4M NaOH) originating from sample AE0.1, was 182.3 μg/mL and 185.0 μg/mL, respectively. Protein content of the samples AE5.2

(extracted in 0.1M NaOH) and AE 6.2 (extracted in 0.4M NaOH) originating from sample AE0.1, was 212.4 μg/mL and 166.8 μg/mL, respectively. Generally it could be concluded that the ultrasound treatment (whether it is treatment in the ultrasound bath or in the sonicator) is efficient and gives the best protein yield when NaOH is used as a solvent.

Slight differences in results between the parallel sample sets were again observed, however not as dramatic as in case of the extraction from *S. latissima*. These differences might again be explained by the lack of the homogeneity of the raw material (crushed seaweed). It is also possible that several components that interfere with Lowry analysis were extracted. These could be phenolic compounds bound to protein that block access of reagents used in Lowry reactions to the protein molecule and a high content of optically active sugars that interfere with spectrophotometer reading.

3.3 Content of free amino acids (FAA).

Several analyses of content of free amino acids (FAA) with HPLC were performed. However, due to the time limitations of this project only a selection of samples were analyzed. Samples obtained during the extraction in water (both during the first extraction and the control samples) were analyzed for *A. esculenta* and for the *S. latissima* samples extracted in NaOH or HCl during the second extraction were analyzed. Samples were prepared by mixing supernatants of the corresponding samples obtained from AE0.1 and AE0.2 for *A. esculenta* and SL0.1 and SL0.2 for the *S. latissima.*

3.3.1 Total soluble FAA content in *Saccharina latissima.*

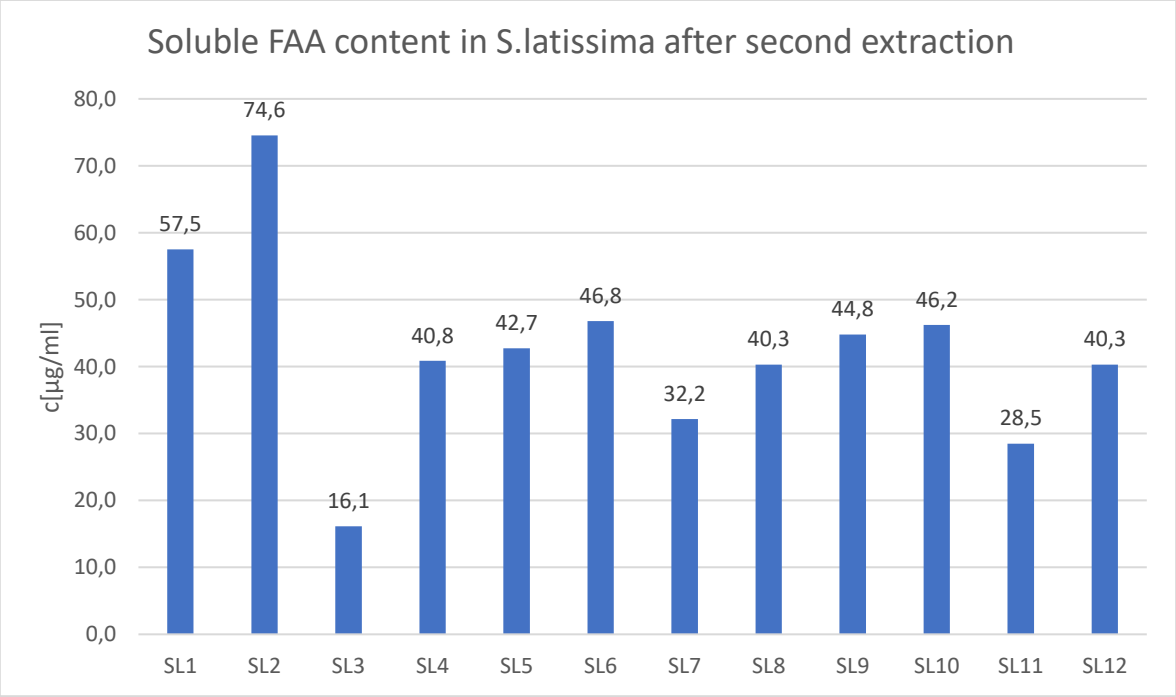

Figure 18. Total FAA content of *Saccharina latissima* supernatants after extraction with NaOH or HCl, with and without use of ultrasound treatment. Values are expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

Figure 18 presents an overview over the total soluble FAA content extracted during the second extraction from *S. latissima.* The highest total soluble FAA yield was 74.6 µg/mL and it was measured in sample SL2 that was extracted in 0.4M NaOH and did not undergo ultrasound treatment. Second highest total soluble FAA yield was 57.5 µg/mL and it was measured in sample SL1 that was extracted in 0.1M NaOH and did not undergo ultrasound treatment. The lowest total soluble FAA yield was 16.1 µg/mL measured in sample SL3 that was extracted in 0.1M HCl and did not undergo any ultrasound treatment. Generally, samples SL1 and SL extracted in 0.1M NaOH and 0.4M NaOH that did not undergo any ultrasound treatment had total

soluble FAA yield significantly higher than samples extracted in the same solvents and treated with ultrasound bath or with sonicator. Sample SL5 (0.1M NaOH, ultrasound bath) had total soluble FAA content 42.7 μg/mL and sample SL9 (0.1M NaOH, sonicator) had total soluble FAA content 44.8 μg/mL. Samples extracted in 0.4M NaOH had total soluble FFA content 42.7 μg/mL for sample SL6 (ultrasound bath) and 46.2 μg/mL for sample SL10 (sonicator). Samples extracted in 0.4M HCl had only slightly lower total soluble FFA content than there extracted in 0.4M NaOH and it was 40.8 μg/mL in SL4 (no ultrasound treatment), 40.3 μg/mL in SL8 (ultrasound bath) and also 40.3 μg/mL in SL12 (sonicator). These results suggest that 0.4M NaOH used as an extraction solvent without ultrasound treatment contributed to the highest total soluble FAA content in *S. latissima*.

3.3.2 Total water soluble FAA content in *Alaria esculenta*.

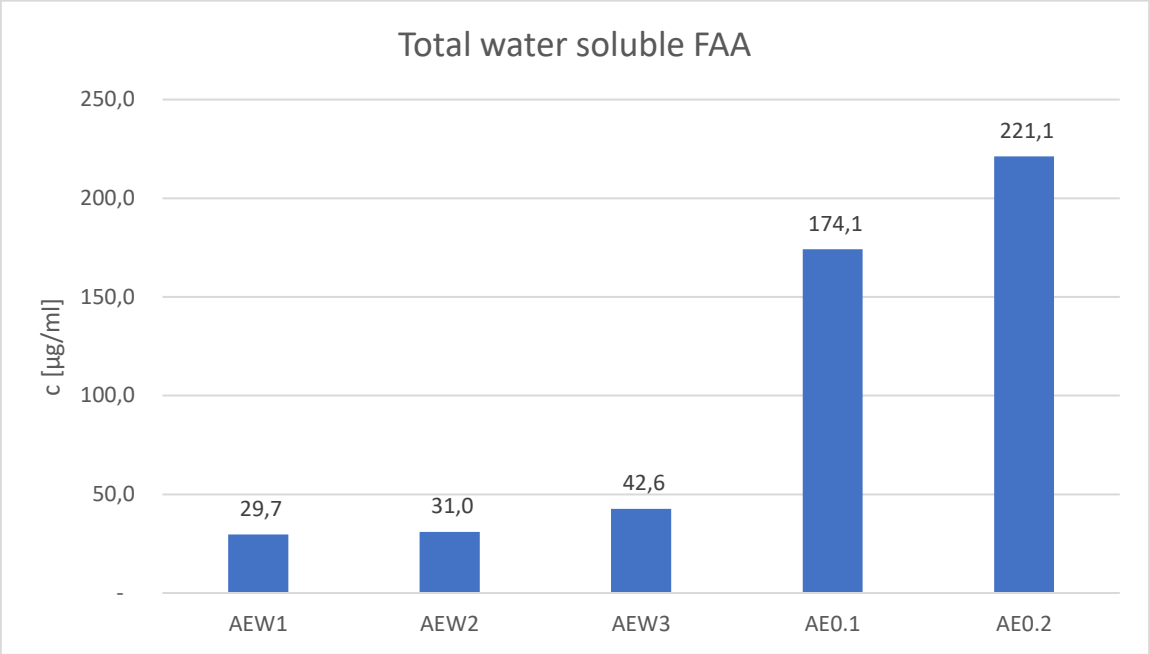

Figure 19. Total FAA content of *Alaria esculenta* supernatants after first extraction with water and after second extraction in water with and without ultrasound treatment. Values are expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

Total FAA content of *Alaria esculenta* supernatants was measured only for extractions in water. During the first extraction, total soluble FAA content was 174.1 µg/mL in AE0.1 and 221.1 µg/mL in AE0.2. The difference of FAA content between these two samples is rather interesting, considering the fact that both supernatants had a very similar protein content and it was 500.4 µg/mL and 507.4 µg/mL, respectively. Further the results show that second extraction with water does not contribute to a significant increase in the FAA content in the supernatant. Sample AEW1 that did not undergo any ultrasound treatment had the lowest FAA content and it was only 29.7 µg/mL. Sample AEW2 treated in the ultrasound bath had a very similar FAA content and it was 31.0 µg/mL. Total soluble FAA content of the last sample, AEW3, treated with sonicator was the highest, however only slightly higher than the two other and it was 42.6 µg/mL. This could either be due to inhomogeneous sample or to the fact that treatment in the sonicator is more intensive than in the ultrasound bath and leads to more protein damage and thus, higher content of FAA:

3.3.3 Soluble FAA profile in *Saccharina latissima*.

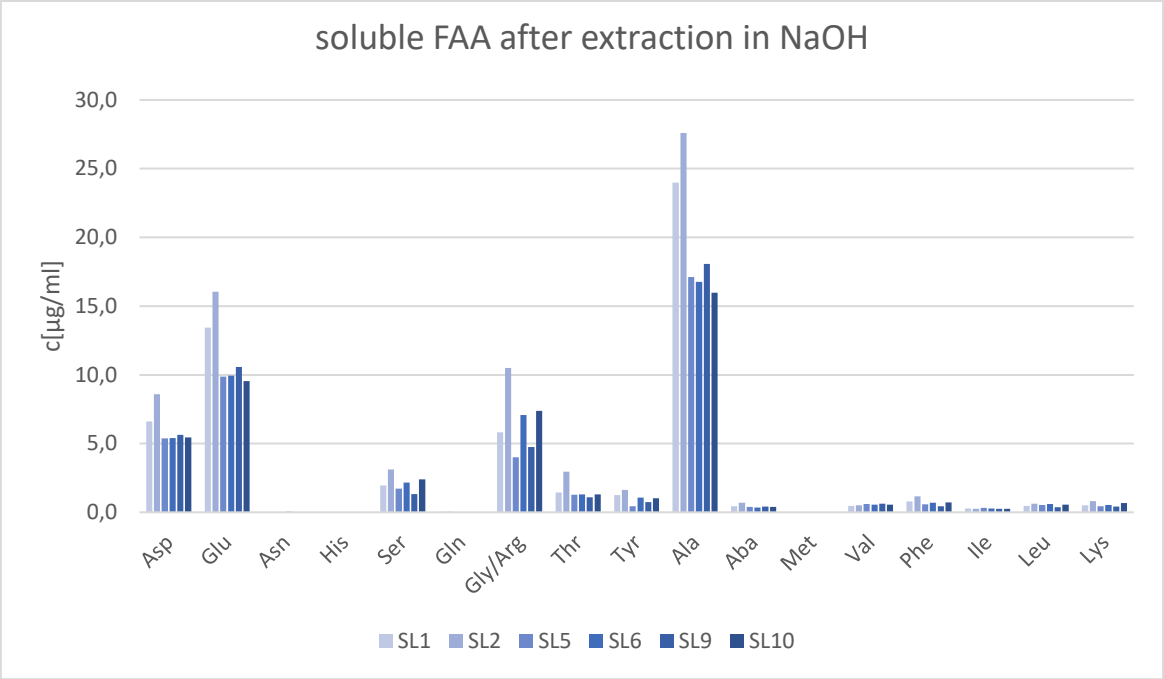

Figure 20. Soluble FAA profile of *Saccharina latissima* supernatants after extraction with NaOH, with and without use of ultrasound treatment. Values are expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

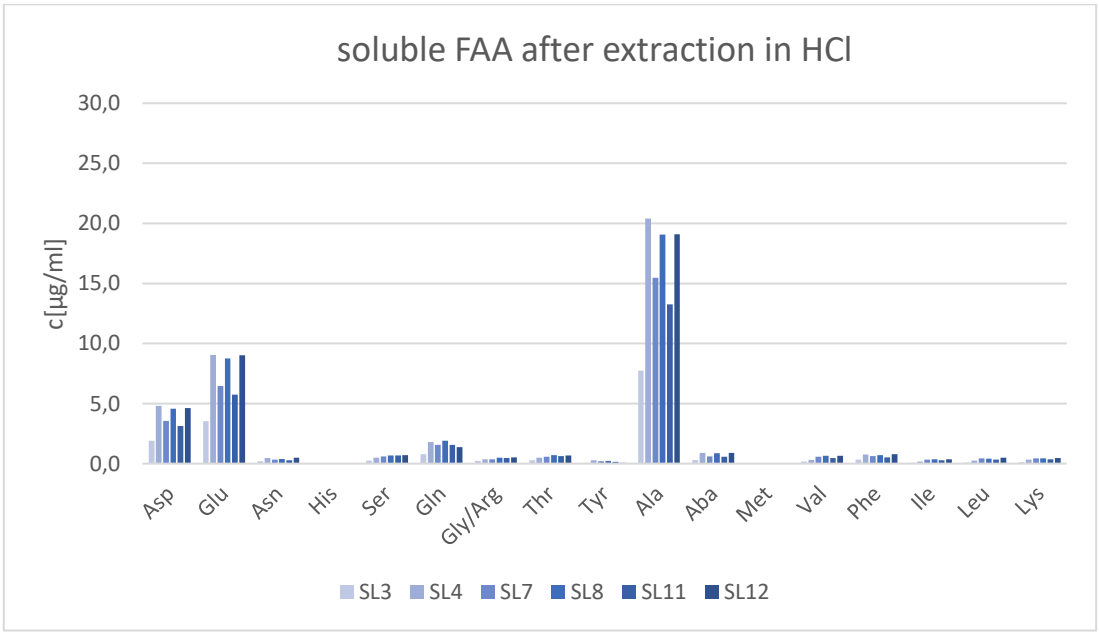

Figure 21. Soluble FAA profile of *Saccharina latissima* supernatants after extraction with HCl, with and without use of ultrasound treatment. Values expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

Figures 20 and 21 present soluble FAA profile of *Saccharina latissima* supernatants after extraction with 0.1M NaOH or 0.4M NaOH (Figure 20) or 0.1M HCl or 0.4M HCl (Figure 21) with or without ultrasound treatment.

The three amino acids alanine, glutamic acid and aspartic acid are dominant among FAA found in samples extracted in both solvents. The 14 remaining FAA accounted for a small portion compared to the 3 most abundant FAA. This outcome shows similarities with the results of previous studies for FAA profile and AA profile of *Saccharina latissima* conducted [11, 14] with the difference in the proportions between Asp and Glu. While previous studies show that the content of Asp is higher than Glu, in this studies results are the opposite and a slightly higher content of Glu than Asp was reported.

Samples extracted in alkali and in acid had generally the same soluble FAA proportions with the exception of Gly/Arg that had significantly higher peak for the extraction in NaOH. FAA were most abundant in sample SL2 (0.4M NaOH, no ultrasound treatment) and in sample SL1 (0.1M NaOH, no ultrasound treatment). Samples SL5 (0.1M NaOH) and SL6 (0.4M NaOH) treated in the ultrasound bath and samples SL9 (0.1M NAOH) and SL10 (0.4M NaOH) treated in sonicator had similar FAA content and it was lower than in untreated samples.

FAA after extraction in HCl (Figure 21) were most abundant in samples SL4 (no ultrasound treatment), SL7 (ultrasound bath) and SL11 (sonicator) extracted in 0.4M HCl and FAA and it did not increase after ultrasound treatment. The lowest FAA content was found in sample SL3 that was extracted in 0.1M HCl and did not undergo ultrasound treatment.

Presented results lead to the conclusion that the extraction in 0.4M NaOH and 0.1M NaOH without any ultrasound treatment give the highest yield of FAA: It could be the consequence of the influence that an ultrasound has on a cellular matrix or on the protein or even amino acid itself that leads to various degrees of protein degradation. Moreover, alkalic and acidic pH influence amino acid/protein charge and solubility.

3.3.4 Soluble FAA profile in *Alaria esculenta.*

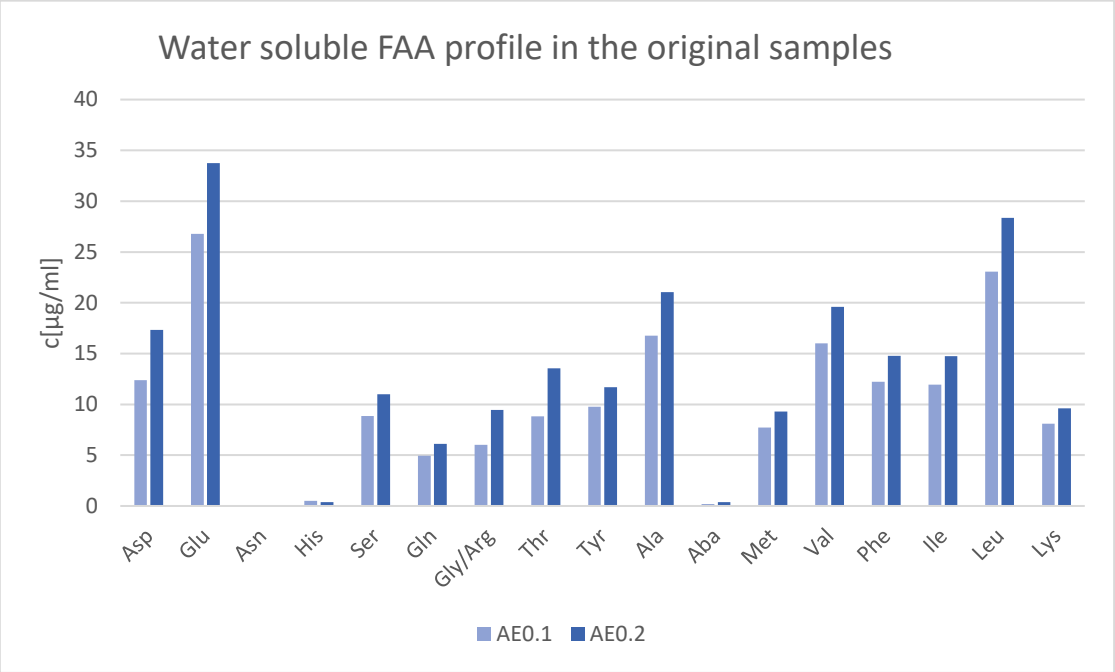

Figure 22. Water soluble FAA profile of *Alaria esculenta* supernatants after first extraction with water. Values are expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

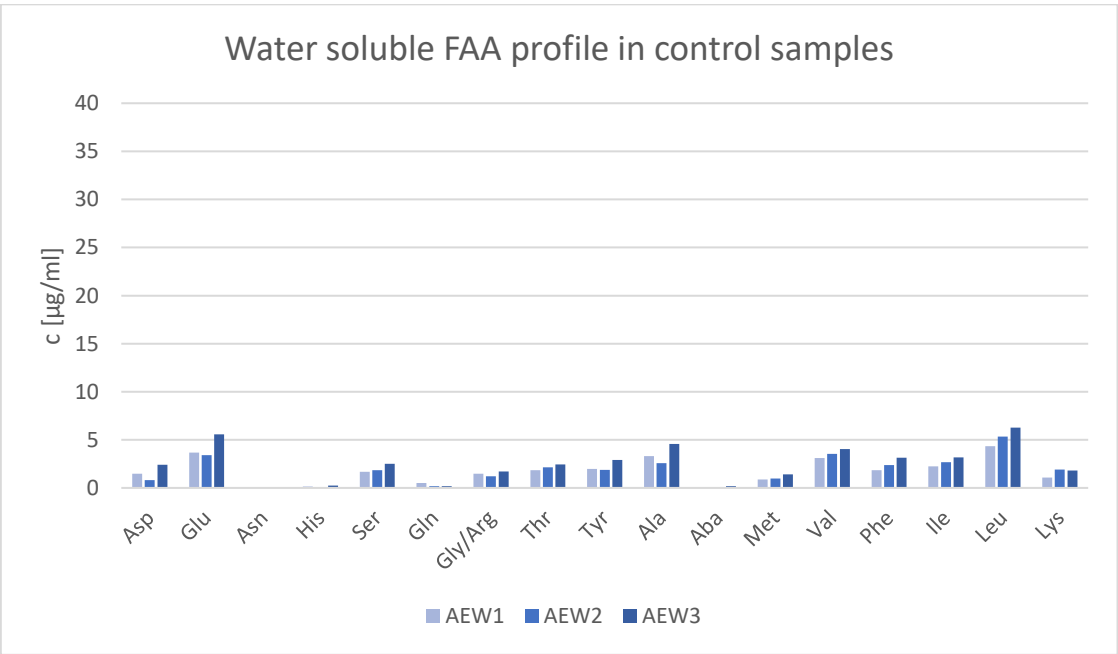

Figure 23. Water soluble FAA profile of *Alaria esculenta* supernatants after second extraction in water with and without ultrasound treatment. Values are expressed as an average of 2 parallels from the HPLC method and given in µg/mL.

Figures 22 and 23 present the soluble FAA profile in *Alaria esculenta* for samples from the first extraction in water (AE0.1 and AE0.2) and for samples from the second extraction in water (AEW1, AEW2 and AEW3), respectively. In samples AE0.1 and AE0.2, glutamic acid dominates, followed by a high content of leucine and slightly lower content of alanine. These results are different than those presented in Lyng's and Reissiger's work, where alanine was the most abundant FAA (or AA) followed by a significantly lower content of all other FAA: It could be due to seasonal variation, since their seaweed was harvested in June.

For samples extracted in water for the second time no significant increase of soluble FAA content was measured, however it was shown that sample treated with sonicator (AEW3) had the highest content of FAA.

For both species the dominating FAA are non-essential, however both species contain almost all the essential amino acids as well. This is relevant for their value in feed and food production, although the total amino acid content is more crucial regarding this aspect, as well as the bioaccessibility (potential of proteins to be absorbed in the the body). Furthermore, FAA, especially glutamic acid contribute to taste.

4 Conclusion.

Ultrasound treatment increases the amount of protein extracted from brown seaweed *Saccharina latissima* and *Alaria esculenta*. The highest protein yield was extracted using NaOH and ultrasound treatment, however it was not conclusive which concentration of the alkali is a better choice. It was also shown that HCl is not an optimal solvent for protein extraction from these two macroalgae neither alone nor in the combination with the ultrasound treatment. Ultrasound treatment slightly increases the extracted protein yield, however this value is still lower that the one measured for control samples extracted in water.

Short treatment in sonicator and long in ultrasound bath give similar results and it is up to individual assessment which one is most appliable and cost efficient for the need.

Ultrasound treatment does not increase the amount of extracted free amino acids in supernatant and it did not cause any changes in the FAA profile. On the contrary, the amount of FAA was significantly higher in samples where no ultrasound treatment was applied than in samples treated in the ultrasound bath or sonicator. It was also measured that the extraction in 0.4M NaOH gives a significantly higher FAA in *S. latissima* than other solvents.

In A. *esculenta* glutamic acid dominated, followed by a high content of leucine and slightly lower content of alanine. Other FAA were present in similar amounts. The three amino acids alanine, glutamic acid and aspartic acid are dominant among FAA found in samples extracted from *S. latissima* followed by relatively low amounts of other FAA. For both species the dominating FAA are non-essential, however have a significant contribution to taste.

5 Future work.

Microscopy research is necessary to determine the influence of ultrasound treatment on the plant matrix and the quality of the extracted protein. Moreover, large scale experiments are needed to assess the opportunities of using ultrasound treatment on an industrial scale and at the same time assess the sustainability of this technology.

In terms of solvents, an interesting direction of research is to determine the optimal concentration of NaOH for the ultrasound assisted extraction. Combination of NaOH and other solvents should be examined, as well as a possible combination of ultrasound treatment and enzymatic treatment.

When the best extraction conditions for seaweed protein is found, research should focus on determining digestibility and purity of the extracted protein. Further, it is necessary to determine how this extracted protein may be commercialized and this means what applications it has and if it is profitable.

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7 Appendices

Appendix A Detailed protocol for the Lowry method

Detailed protocol: Solution A was made by dissolving 20g Na2CO3 in 1l 0.1 M NaOH while dissolving 1g of CuSO4 x 5H2O in 100 ml water for solution B. Solution C requires 2g of potassium sodium tartrate in 100 ml distilled water. A mixture with the 3 listed substances with a ratio 1:1:1000 of B:C:A contributes to solution D which only was made on the performing day. Solution E includes 2N Folin –reagents stirring with distilled water ratio 1:2 respectively which was made daily. Finally, solution F is the standard 1000 µl/ml BSA. The experiment started with preparing all the mentioned solutions. Then, 7 standard solutions with different concentrations were prepared as 125 µL, 250 µL, 500 µL, 1000 µL, 1500 µL, 2000 µL and 3000 µL per ml. For the testing samples, 0.5 ml of each 1: 10 dilution with 1ml of seaweed extraction into 9 ml distilled water was made and poured into a test tube. Adding 2.5 mL solution D came after with mixing thoroughly and those mixtures were left at room temperature in 10 minutes precisely. The final step before measuring spectrophotometer absorbance was adding 0.25 mL solution E with mixing through. The absorbance was evaluated from spectrophotometer at OD 750nm.

A1. Determination of the total soluble protein level by Lowry method

Lowry method was used to determine the total soluble protein content in testing samples by relating the absorbance level of samples to a stock solution with a known concentration. To achieve that, a standard curve based on the stock solution concentration was built. A linear standard curve is constructed by the horizontal axis is the stock solution BSA concentration (µg/ml) and absorbance (at OD 750nm). Data from the Lowry experiments were recorded in Excel 2016 generating the standard curve as the formula: y = ax + b, a is the intercept of x-axis and b is the slope. The absorbance at OD 750nm of the testing samples will be measured three times.

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