Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Nora Gotaas Buøen

Polyphenol extraction and antioxidant activity of *Saccharina latissima* and *Alaria esculenta*

The effect of different drying methods and extraction solvents

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Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Turid Rustad
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Abstract

The interest in cultivation, production and utilization of seaweeds has increased over the last decade in the Western World. The rapid population growth in the world increases the demand for food, feed and fuel, and it is therefore important to utilize the un-exploited resources the sea and land can provide, such as seaweed biomass. Seaweeds contain a variety of valuable compounds and components which can be used as food, feed, functional ingredients and fertilizers etc. Moreover, the compounds and components in seaweeds possess important biological activities that benefits human health and may prevent different diseases.

In the present study, five different screening methods for measuring potential antioxidant activity of freeze-dried, oven-dried and wet *Saccharina latissima* and *Alaria esculenta*, extracted in water, 70% acetone and 70% ethanol were performed. Significant differences between pre-treatment methods, extraction solvents and the two macroalgae were observed.

The five different screening methods used in the present study were; the Folin-Ciocalteu assay, which measures the total phenolic content (TPC) in the macroalgae. Two free radical scavenging assays were performed, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). A metal ion-chelating ability assay was also utilized, which measures the seaweeds compounds ability to chelate to transition metals, and lastly the ferric reducing antioxidant power, FRAP-assay, measuring the reducing capacity of the components extracted from seaweeds.

The results for total phenolic content and antioxidant activity demonstrated that freeze-drying preserves phenolic compounds and exhibit higher antioxidant activity compared to oven-drying, except for metal chelating ability. Wet samples showed significant differences between *A. esculenta* and *S. latissima* indicating that *A. esculenta* is a high-phenolic species and has better antioxidant potential. Correlations were found between total phenolic content and the two radical scavenging activity assays, indicating that polyphenols contribute to antioxidant activity. However, no correlations were found between total phenolic content and metal-chelating activity, and ferric reducing antioxidant power which means that other compounds may also contribute to the antioxidant activity.

Sammendrag

Interessen for dyrking, produksjon og utnyttelse av tang og tare har økt det siste tiåret i den vestlige verden. Den raske befolkningsveksten i verden gjør at etterspørselen etter mat, fôr og drivstoff øker, og det er derfor viktig å utnytte de ressursene som sjø og land kan gi, som ikke utnyttes til det fulle i dag, for eksempel biomasse fra tang og tare. Tang inneholder en rekke verdifulle forbindelser og komponenter som kan brukes som mat, fôr, funksjonelle ingredienser, gjødsel osv. I tillegg har forbindelsene og komponentene i tang viktige biologiske aktiviteter som kan forbedre menneskers helse og kan forhindre forskjellige sykdommer.

I dette studiet har fem forskjellige screeningmetoder blitt utført for å måle potensiell antioksidantaktivitet av frysetørket, ovnstørket og våt *Saccharina latissima* og *Alaria esculenta*, ekstrahert i vann, 70% aceton og 70% etanol. Det ble observert signifikante forskjeller mellom behandlingsmetoder, ekstraksjonsløsemidler og de to makroalgene.

De fem forskjellige screeningmetodene som ble brukt i denne studien var Folin-Ciocalteu-analysen som ble brukt for å måle det totale fenolinnholdet (TPC) i makroalgene. Det ble utført to metoder som måler inhibering av frie radikale, DPPH (2,2-diphenyl-1-picrylhydrazyl)-analyse og ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid))-analyse. En jernbindingsevne analyse ble utført og denne måler forbindelsene i tang sin evne til å binde seg til overgangsmetaller, og sist ferric reducing antioxsidant power, FRAP-analyse, som måler reduksjonsevnen til komponentene ekstrahert fra tang.

Resultatene av totalt fenolinnhold og antioksidantaktiviteten demonstrerte at frysetørking bevarer fenolforbindelser og utviser en høyere antioksidantaktivitet sammenlignet med ovntørking, bortsett fra ved jernbindingsevne analysen. De våte prøvene viste signifikante forskjeller mellom *A. esculenta* og *S. latissima* som indikerer at *A. esculenta* er en høyfenolisk art og har bedre antioksidantpotensial. Korrelasjoner ble funnet mellom totalt fenolinnhold og de to metodene som måler inhibering av frie radikaler (DPPH og ABTS), noe som indikerer at polyfenoler bidrar til antioksidantaktiviteten i tang. Imidlertid ble det ikke funnet korrelasjoner mellom totalt fenolinnhold og jernbindingsevne, og ferric reducing antixoidant power, som betyr at andre forbindelser også kan bidra til antioksidantaktiviteten.

Preface

This master thesis is submitted to the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) and constitutes the completion of the Master of Science in Chemical Engineering and Biotechnology. The work was carried out from January 2020 to June 2020, and the experiments were performed at the Food Chemistry laboratory at NTNU. Campus and laboratories were closed due to the the COVID-19 pandemic, and limited the experimental work this semester.

First of all, I would like to give a major thanks to my supervisor Turid Rustad, for great discussions, feedback, hours of proofreading and good support throughout the whole process, and especially for still being available at all time, positive and helpful when NTNU was closed due to COVID-19. I would also thank the laboratory engineer Siri Stavrum for the practical help and advice in the lab.

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Finally, I would like to thank my family and friends for all the love and support. A special big thanks to my good friend Katarina Stiff Aamlid for being a great laboratory and reading partner, and good support through our five year master's study and for the discussions and feedback during the master project this last semester.

Specialization Project

The present master thesis is a continuation of the TBT4500 Biotechnology, Specialization project, delivered autumn 2019. The specialization project investigated freeze-dried *S. latissima* and *A. esculenta* extracted in water and 70% acetone. The master thesis experiments is extended with oven-dried and wet samples, together with one more extraction solvent; 70% ethanol. The introduction part will therefore show some similarity in the theories discussed, however it is rewritten and contains additional theories as well. In addition, the methods for measuring dry matter, total phenolic content, DPPH radical scavenging activity and metal ion-chelating ability is performed in the exact same way in the materials and methods part.

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Abbreviations

ABTS	=	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
A. esculenta	=	*Alaria esculenta*
AOA	=	antioxidant activity assay
D(40)	=	algae dried in convective oven at 40°C for 76 hours
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
dw	=	dry weight
FCR	=	Folin-Ciocalteu phenol reagent
FD	=	freeze-dried
OD	=	oven-dried
PG	=	propyl gallate
PGE	=	propyl gallate equivalent
RSA	=	radical scavenging activity
SD	=	standard deviation
SES	=	Seaweed energy solution
TPC	=	total phenolic content
TPTZ	=	2,4,6-tris(2-pyridyl)-s-triazine
S. latissima	=	*Saccharina latissima*
ww	=	wet weight

Chapter 1

Introduction

1.1 Cultivation and production of seaweed

It is estimated a global population growth from today's 7.7 billion people to 9.8 billion people in 2050, and there will therefore be a large increase in the demand for food, feed and fuel. With the global climate challenges the world is facing, a transition from fossil-based industries towards a more sustainable production, low carbon footprints and environmentally friendly industries will be necessary (Skjermo et al., 2014). Seaweeds is one of the largest un-exploited biomass resources, and have the ability to produce great amounts of biomass with a high content of valuable compounds (Stévant et al., 2017). Cultivation of seaweeds is more sustainable compared to land-based agriculture, because the seaweeds do not need fresh water, chemical fertilizer, farmlands and pesticides. Moreover, seaweeds take up nutrients from the seawater which makes seaweeds rich in valuable and beneficial nutritional compounds. The marine plants, especially sea-grasses and macroalgae, has a large potential for incorporating the anthropogenic CO_2 emission, because of their large biomass and turnover time of about 1 year. This turnover time is relatively long compared to phytoplankton's turnover time (1 week). The ocean take up 25-35% of the emissions from fossil-fuel and tropical deforestation, with this seaweeds contribute to counteract pollution (Jiang et al., 2013) (Tiwari and Troy, 2015). However, after a year the carbon would be released to the atmosphere because of decomposition of the macroalgae, and the effect of marine plants on the carbon cycle is therefore temporary. In contrast, seaweed production would give a more long term effect on the carbon cycle, because the cultivated

seaweed will absorb CO_2 which which is later removed from the ocean by harvesting, and not returned until consumed (Jiang et al., 2013). The use of seaweed biomass has a large potential for different applications such as food, feed ingredients, pharmaceuticals, fertilizers and biofuel (Skjermo et al., 2014).

Asian countries, especially China, have a long history within the cultivation and production of seaweeds, and marine algae has been used as food for human and animal consumption for decades, as well as in folk medicine. The interest for cultivation of macroalgae has increased over the last years in the Western World, because of the high content of valuable components with a bio-active potential (Stévant et al., 2017).

The cultivated production of aquatic plants, mostly seaweeds, increased from 19 millions tons in 2010 to right above 30 millions tons in 2016, with China and Indonesia as major cultivation producers. The Asian countries stood for 97.5% of the global cultivated algae production of seaweed in 2016, and this is a slight decrease from 2010, where the production in Asia were 99.0%, this is due to a higher cultivation production in the Western World (Fao, 2012), (Fao, 2018). 83% of this biomass is used directly in human consumption, while the remaining parts are used as components in fertilizers, animal feed, cosmetics and for medical and biotechnological applications (Barbier et al., 2019). Chile and Norway were in 2015 the top harvesters of wild seaweed outside Asia and stood for respectively 345 704 tons and 147 391 tons of the seaweeds (Ferdouse et al., 2018), and over the last years the interest and cultivation of seaweeds has increased also in Norway.

1.2 Norwegian brown macroalgae

Norway has a long tradition of harvesting from the sea and is internationally leading within salmon farming. The country does also possess first-class technology related to the aquaculture sector, and therefore the preconditions for industrial seaweed cultivation are good. The long coastline and large nutritious sea areas, together with the cold-temperature and arctic zones gives Norway a great potential for cultivation of seaweed at an industrial scale. The coastal areas of Norway are a rich source for valuable macroalgae with 175 brown, 200 red and 100 green algae (Skjermo et al., 2014). Norway has used seaweeds as fertilizers, food and feed for hundreds of years. The industrial harvesting and utilization of macroalgae began in the 18th century. The seaweeds were burned to ash and one of the

first large costumers were the glass-works (Østgaard and Indergaard, 2017). For many decades, Norway has harvested wild seaweed and isolated the biopolymer, alginate, from the brown algae. Most of the alginate, around 40%, are used as thickening of color paste for textile printing. The remaining parts are used in the food industry for example as an thickening agent in ice-cream and soups because of its good gelling and stabilizer properties. Other industries such as pharmaceuticals, feed and cosmetics uses alginate in the form of alginic acid (Holdt and Kraan, 2011). The first commercial licence for cultivation of seaweeds in Norway came in 2014, and the number of licenses has increased from 8 to 47 from 2014 to 2018. The total production of cultivated seaweed in Norway was 60 tons wet weight in 2016 for a total area of 540 ha, this area has a maximum production potential at 60 000 tons wet weight (Hancke et al., 2018). Henriksen et al. (2012) has estimated a total production of cultivated seaweed to around 20 million tons in 2050, which is far from where Norway was in 2016. In 2018 the total production of cultivated seaweed in Norway was 178 tons (Fauske, 2019), which is almost three times higher than for 2016, but there is still a long way to fully utilize the production potential.

Fast growth, large individuals, low loss of valuable components and resistance against epiphytic fouling and other diseases are an important set of properties for macroalgae if the production and utilization should be economic beneficial (Skjermo et al., 2014). The two brown algae *Saccharina latissima* and *Alaria esculenta* have a high content of valuable bio-active compounds and have the ability to reach high biomass yield, and are therefore interesting for a large-scale cultivation in Europe, and then especially Norway (Stévant et al., 2017).

Saccharina latissima is one of the fastest growing species among the kelps, and has the highest carbohydrate content, and also a high iodine content. The macroalgae grows down to 30 meter depth and at more sheltered areas like further into the fjords compared to other brown macroalgae in Norway. *S. latissima* can grow to a length of 3-4 meters and have a lifespan of 2-4 years (Indergaard, 2010). To reach high biomass yield for *S. latissima* the cultivation is favorable in the upper 10 meters. Here it would be stronger water currents which gives higher nutrient supply per time compared to the sheltered areas (Skjermo et al., 2014). On the other hand, *Alaria esculenta* grows down to 8 meters depth and have the most favorable growth conditions at highly exposed areas, at rocky shores, exposed to waves and wind. This could cause difficulties in sampling the algae, and to find the optimum time for harvesting to obtain minimum loss of biomass. The loss of biomass is a result from the presence of fouling organisms (Walls et al., 2017). *A. esculenta* can

be 1.5 -2.5 meters, and have a lifespan of 12-14 years (Indergaard, 2010). The algae is a great source for production of biofuel, especially bio-ethanol, because of the high content of sugar (Schiener et al., 2015). The cost and profit of the use of biomass as bio-fuel is described in a later paragraph.

(a) *Saccharina latissima*   **(b)** *Alaria esculenta*
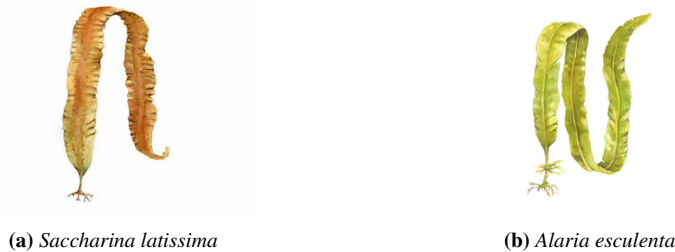
Figure 1.1: The two brown algae used in the present study, photo: Stein Mortensen (miljolare.no)

1.3   Bioactive compounds in seaweed

Seaweeds has a good potential for reaching high biomass yield, and can therefore be a good contributor as a sustainable renewable energy resource. The algae biomass is a valuable source for the production of bio-ethanol, which is expected to reduce the dependence on fossil resources in the future. However, there are still technology challenges and the costs for cultivation, harvesting and production of bio-fuel from biomass are too high to make it profitable compared to today's fossil resources in Norway (Alvarado-Morales et al., 2013), (Gosch et al., 2012).

Macroalgae is a rich source of different valuable components, and the nutritional content and bio-active potential may give beneficial health effects. Seaweeds can be used as functional foods or as functional ingredients in food for human consumption, which is used to improve food and would give specific health benefits beyond basic nutrition. Functional food can also provide a reduced risk of chronic diseases and improve the life quality (Holdt and Kraan, 2011), (Amorim et al., 2020). Marine algae contain high amounts of polysaccharides, which are utilized in various products such as thickeners, emulsifiers, food, feed, stabilizers, beverages etc. Furthermore, macroalgae contain bio-active substances and nutritional elements like proteins, lipids, pigments, phytochemicals like polyphenols, vitamins, minerals and trace elements, which all contribute to maintaining human health and also play a therapeutic role in disease prevention (Ganesan et al., 2019).

Table 1.1: Chemical composition of seaweed (Østgaard, 2017)

	Brown algae	**Red algae**	**Green algae**
Water (% of fresh weight)	70-90% ww	80-90% ww	approx. 80% ww
Ash	15-25% 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

The ash content in brown algae is especially high and varies from 15-35% dw. *Laminaria* and *Saccharina* can have a ash content up to 45% dw. For red and green algae, the ash content lies between 10-25% dw, as shown in table 1.1. The ash content includes the minerals in the algae, such as sodium, potassium, calcium and magnesium, as well as the trace elements, iron, zink, manganese, iodine and cobber. These elements are necessary for important processes in the human body and works as building blocks (Schiener et al., 2015). In general, the protein content are higher in green and red algae compared to brown, and the protein concentration has a seasonal variation, where *Saccharina* species and *A. esculenta* demonstrated a maximum concentration during February to May. In addition to providing essential amino-acids, proteins and peptides may possess biological activities like anti-inflammatory, anti-HIV, anticancer, anti-tumor, anti-diabetic and antioxidant properties (Holdt and Kraan, 2011). Vitamins are considered as the most essential micronutrients for generating many metabolic pathways, seaweeds are a rich source of vitamin A, C and B12. Organisms can not synthesise vitamins, and need to receive these through the diet (Ganesan et al., 2019).

The phytochemical, polyphenols, plays an important role of the antioxidant capacity of the algae, and also possess other biological activities, anti-inflammatory, anti-diabetic, anti-HIV and anti-Alzheimer's. Pigments, such as carotenoids and fucoxanthin has also been demonstrated to contribute to the antioxidant activity in the seaweed (Holdt and Kraan, 2011).

Some compounds in seaweed are undesirable, and may give a negative effect on the human health. Accumulation or high concentration of different heavy metals could be toxic if

consumed in large amounts. The concentrations or accumulation of heavy metals depends on the environment surrounding the algae and there can be local variations, it is therefore important to perform experiments for undesirable compounds before use as feed or food (Holdt and Kraan, 2011).

S.latissima is reported to have a iodine content up to 6568 mg/kg dw (Stévant et al., 2018) which exceeds the French food safety authority on 2000 mg/kg dw. Therefore an over-consumption of *S. latissima* will be unhealthy. A small amount (150 μg per day) of iodine is important for human nutrition and is a part of the metabolic hormones thyroxine and triodothyronine which are produced in the thyroid gland. On the other hand, an intake of iodine over 600 μg per day is not recommended and could lead to either hyper- or hypothy-roidism and goitre (Wells et al., 2017). Lüning and Mortensen (2015) demonstrated that the high iodine content could be reduced by boiling *S. latissima*, and also by reduced sea-water turnover in the tanks. In addition, Nielsen (2018) investigated, in her master thesis, the effects of steam and water blanching on the iodine content in *S. latissima*. The results demonstrated a significantly reduced iodine content in water blanching experiments with a reduced concentration from 5 739$\pm$105 mg/kg dw to 830$\pm$184 mg/kg dw, which means a decrease of 85$\pm$3%, but only a slight decrease in iodine content with steam blanching were observed.

1.4 Phenolic compounds and antioxidants

Athukorala et al. (2006) and other studies have observed a high positive relationship be-tween the phenolic content and the antioxidant activity in the macroalgae, and polyphenols are therefore said to be one of the main contributor to the antioxidant capacity.

1.4.1 Phenolic compounds

Phenols are a group of heterogeneous chemical compounds, and consists of one or more benzene rings attached to one or more hydroxyl group (-OH). Phenolic compounds can be divided into three major classes; *simple phenols*, consisting of one phenol unit, *flavonoids*, consisting of two phenol units, and *tannins*, consisting of three or more phenol units. The two latter is called polyphenols, and the three major groups is further divided into subgroups. *Flavonoids*, with subgroups; flavones, flavonols, flavanols and isoflavones,

are major contributors to the antioxidant activity in plants. These *flavonoids* are usually present as glycosides, which make them more soluble and less reactive towards free radicals (Rice-Evans et al., 1997). All the subgroups consists of two aromatic rings associated with a 3 C oxygenated heterocycle, an example of one subgroup, flavones, is shown in table 1.2. *Phenolic acids* (hydroxybenzoic acids and hydroxycinnamic acids) is another phenol group, and is characterized by possessing one carboxylic acid group. Hydroxybenzoic acids and hydroxycinnamic acids and their derivatives exhibit high antioxidant activity (Ratnavathi, 2019).
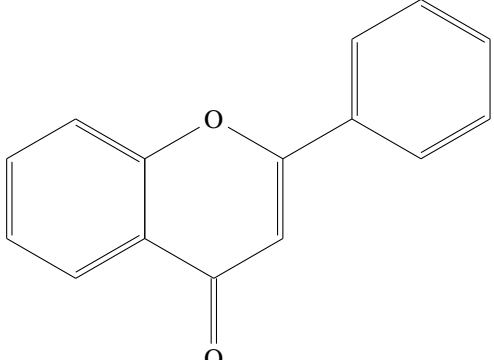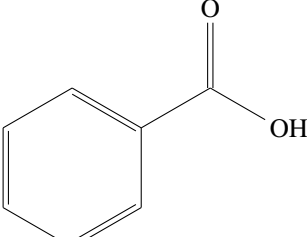
Class - subgroup/name	Structure
Flavonoids - flavones	
Phenolic acids - Hydroxybenzonic acid	

Table 1.2: General structure of flavones, a subgroup of *flavonoids*, and general structure of a *phenolic acid* group, hydroxybenzonic acid, which contributes to antioxidant activty in plants.

The antioxidant effect is related to the phenol rings which act as electron traps by scavenging free radicals compounds such as peroxy, superoxide-anions and hydroxyl radicals. The more interconnected rings, the more potent the compound is to scavenge free radicals (Wang et al., 2009). Earlier studies has also demonstrated that polysaccharides, carotenoid pigments, including fucoxanthin and astaxanthin, possess high antioxidant potential. These are all compounds found in brown macroalgae (Maeda et al., 2008), (Rupérez et al., 2002), (Zhao et al., 2008). The phenolic compounds from seaweed can act as antioxidants in various ways; reducers (electron donors), proton donors, free radical

scavengers and forming metal complexes (Sappati et al., 2019). Polyphenols are rich in micronutrients which is important for our diet, and besides the antioxidant effect, polyphenols possess anti-inflammatory and anticancer effect (Liaudanskas et al., 2017). Several studies have demonstrated a correlation between consumption of phenols and a reduced risk for cardiovascular and neurodegenerative diseases (Spencer, 2010), (Weichselbaum et al., 2010).

1.4.2   Natural antioxidants and antioxidant activity

The interest for natural antioxidants, obtained from natural sources, instead of the use of synthetic antioxidants has increased over the years. The use of synthetic antioxidants in food or medicine components could lead to problems for human health because of the suspected toxicity. Scientist are therefore searching for natural antioxidants without side effects which could be used as oxidation inhibitors (Ahn et al., 2007). Antioxidant activity is defined as the ability of a compound to prevent or slow down oxidative deterioration. Antioxidants are for example used as an additive to slow-down lipid-oxidation in muscle food, fish and meat. Lipid-oxidation is induced by oxygen and initiated by heat, free radicals, light photosensitizing agents and metal ions. Lipid-oxidation occurs in three different reaction pathways (Laguerre et al., 2007);

1. Non-enzymatic chain autoxidation mediated by free radicals.

2. Non-enzymatic and non-radical photooxidation.

3. Enzymatic oxidation.

Lipid-oxidation in muscle food will lead to quality deterioration under storage and processing, which further give bad taste and odors of the meat or fish, change in color and texture, and a reduction of nutritional content (Wang et al., 2009). Oxidative stress is a result of free radicals complex formation, an imbalance between the antioxidant system and the production of oxidants, including a over-production of reactive oxygen species (ROS) or a decrease in antioxidant defense (Laguerre et al., 2007). Oxygen is ubiquitous and necessary for an aerobic organisms' oxidative metabolism, therefore oxidative stress response is a common process. To maintain the oxidative homeostasis (redox balance) in the cell and cell survival, the toxicity of oxygen needs an effective defense system, hence antioxidants (Augustyniak et al., 2010). Several diseases such as heart disease, respiratory diseases, cancer, arthritis, stroke, immune deficiency, emphysema, Parkinson's disease, is partly due to oxidative stress. Hence, antioxidants could prevent these diseases

by scavenging free radicals and inhibit oxidation. Antioxidants can also be used as an additive in the manufacturing of rubbers and plastic to delay the oxidation. The antioxidant molecule can be defined as a substance able to prevent or delay another molecule, such as proteins, nucleic acids, polyunsaturated lipids, sugars, from undergoing oxidative damage (San Miguel-Chávez, 2017).

Furthermore, antioxidants could be classified as **primary antioxidants** and **secondary antioxidants** according to their mechanism of action.

Primary antioxidants, also called chain-breaking antioxidants, are free radical scavengers. They react with lipid radicals and convert them to more stable compounds, which slow down or prevent oxidation. The two ways primary antioxidants can slow down lipid-oxidation is by; (i) inhibiting the initiation phase of lipid peroxidation by scavenging free lipid radicals, or (ii) inhibiting the propagation phase of lipid peroxidation by scavenging lipid alkoxyl or lipid peroxyl radicals. Phenolic compounds, carotenoids and Ascorbic acid are considered as effective free radical scavengers (Gordon, 1990). The antioxidant efficiency is a measurement of the compounds ability to transfer a hydrogen atom to a free radical. The two reaction mechanisms of hydrogen donation is hydrogen-atom transfer (HAT) and single-electron transfer (SET) (Wright et al., 2001). These reaction mechanisms are further described in chapter 1.7.

Secondary antioxidants, also named preventive antioxidants. Through a variation of processes, different from converting free radicals to more stable products, secondary antioxidant reduce the rate of autoxidation of lipids. The variation of actions includes compounds binding to metal ions, deactivation of singlet oxygen, reducing agents that convert hydroperoxide into stable components in a non-radical way, oxygen scavenging or absorb ultraviolet radiation. For secondary antioxidants, it is common that another minor component is present in the sample for exhibition of antioxidant activity. This feature is seen in cases of sequestering agents, such as metal chelators, and reducing agents such as ascorbic acid (Gordon, 1990). In the present study, the metal ion-chelating ability assay is performed, which are based upon binding metal ions to reducing pro-oxidant effect, this is described more in chapter 1.7.

1.4.3   Synthetic antioxidants, propyl gallate

Synthetic antioxidants are made through chemical processes and were developed to prevent or slow down oxidation of oils and fats (Sanhueza et al., 2000). Synthetic antioxidants stabilize bulk fats and oils or lipid rich food, and are usually more effective than natural antioxidants as the natural ones are less soluble in lipid phase. They extend shelf-life of food, reduce wastage and nutritional losses, and inhibit oxidation. Since synthetic antioxidants are 100% pure and most natural antioxidants are not, a lower concentration of synthetic antioxidants is necessary for giving the same prevention effectiveness, 0.02% lipidic fraction compared to 0.1-0.5% (Pokornỳ, 2007). There are restrictions for the use of different synthetic antioxidants because of the potential toxicity. Within the food industry BHA(E-320), BHT(E-321), TBHQ(E-319) and Propyl Gallate(E-310) are the most commonly used synthetic antioxidants. In this study Propyl Gallate is used for comparison and measurements, it occurs as a white crystalline powder. Propyl-3,4,5-trihydroksybenzonic acid (Popyl gallate) is an ester, formed by a condensation between gallic acid and propanol (Medina et al., 2013). PG is certified for the use as synthetic antioxidant additive in foods and cosmetics to prevent oxidation of fats and oil. Its fat-soluble properties makes PG excellent for scavenge DPPH radicals and ABTS$^{\bullet +}$. The solubility in water is poor and limits PG's application, and therefore unsuitable for water-soluble food (Li et al., 2018).

1.5   Effects of drying

Freeze-drying, oven-drying and sun-drying are the most common drying processes used for seaweed. It is important to dry seaweed because fresh biomass from seaweed contains high amounts of water which would give an increased volume and weight of the material, removal of water would also slow down the growth of microorganisms. Another reason is for storage of raw algae material, less water give easier storage opportunities, and especially when the macro-algae, in the present study, is harvested once a year. Enzymatic and/or non-enzymatic processes may occur in all three drying methods, and these may affect the chemical composition of phytochemicals and antioxidant properties (Amorim et al., 2020), (Capecka et al., 2005). However, it is also demonstrated that drying processes would prevent decomposition, increase shelf life and help the extraction of some chemical compounds (Ito and Hori, 1989). Sun-drying is common and the cheapest method, but the quality of the product could be affected by weather and microbial attack, together with contaminations by dust, insects and birds (Uribe et al., 2019). Anyhow, sun-drying of seaweed in Norway is probably not very effective, because of the cold climate. The

problem with sun-drying is eliminated when seaweeds are dried in the oven or freeze-dried. Freeze-drying provides high production costs, low through-puts and high energy consumption, but it is still considered the best method for high-quality dried products (Uribe et al., 2019). Wong and Cheung (2001) indicated that freeze-drying was better than oven-drying for maintaining the nutritional composition of three brown algae, *Sargassum*. In contrast, oven-drying was more appropriate for extraction of proteins from the same three species. Oven-drying at 40°C have been demonstrated to provide high values of phytochemicals and possess better scavenging and reducing ability compared to oven-drying at 80°C, freeze-, sauna-, sun-, hang-, shade-drying (Ling et al., 2015).

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1.6   Extraction principles

After sample handling, here freeze-drying, oven-drying or air-drying, extraction is the main step for isolating and recovering of bio-active phytochemicals from the macroalgae. Extraction is a commonly used method for separating one or a few organic compounds from a matrix using a suitable solvent. For analysis of polyphenolics and simple phenolics in plants, liquid-liquid and solid-liquid extraction is the most common procedures. The solvent for extraction should be chosen with regard to the chemical structure of the compound of interest, solvent and compound should have the same polarity. Alcohols (methanol, ethanol), acetone, diethyl ether and ethyl acetate are commonly used extraction solvents for phenols. However, very polar phenolic acids such as benzoic and cinnamic acids would not be extracted by pure organic solvents, therefore a mixture of water and ethanol or acetone are recommended for extraction (Stalikas, 2007). Wang et al. (2009) demonstrated a higher extracted content of phenolics, from different macroalgae, using 70% acetone extract compared to water extract.

1.7   Antioxidant Activity Assay

There are several different antioxidant activity assays (AOA). The methods used in the present study are described in the sections below. The deactivation of radicals by antioxidants can be divided into two major reaction mechanisms; hydrogen atom transfer (HAT) and single electron transfer (SET), but with the same end results (Prior et al., 2005). The different AOA are therefore separated into HAT based assay and SET based assays, some

AOA methods are utilizing both mechanisms.

The HAT mechanism is when a hydrogen atom from an antioxidant (AH) is removed by the free radical ($X^\bullet$), so the antioxidant it self becomes a radical.

$$X^\bullet + AH \rightarrow XH + A^\bullet \tag{1.1}$$

In evaluating the antioxidant action the bond dissociation enthalpy (BDE) is an important parameter. A lower BDE of the H-donating group in the antioxidant will lead to an easier reaction of free radical inactivation (Liang and Kitts, 2014). Some example of HAT based assays are; oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and total radical-trapping antioxidant parameter (TRAP) (Prior et al., 2005).

The SET mechanism is based on the ability of an antioxidant to transfer one electron to reduce a compound, e.g metals, carbonyls and radicals (Prior et al., 2005). The transfer of the single electron from the nucleofile to the substrate provides a radical intermediate, which further give the same net results as for HAT mechanism, as demonstrated in figure 1.2. The antioxidant become a radical cation when providing an electron to the free radical (Liang and Kitts, 2014).

$$X^\bullet + AH \rightarrow X^- + AH^{\bullet+} \text{(electron transfer)} \tag{1.2}$$

$$AH^{\bullet+} + H_2O \rightleftharpoons A^\bullet + H_3O^+ \text{(deprotonation equilibrium)} \tag{1.3}$$

$$X^- + H_3O^+ \rightarrow XH + H_2O \text{(hydroperoxide formation)} \tag{1.4}$$

The net result from the equations above is therefore the same as for the HAT mechanism; $X^\bullet + AH \rightarrow XH + A^\bullet$ (Prior et al., 2005), (Wright et al., 2001). For evaluating the antioxidant action in SET mechanism, the most important energetic factor is the ionization potential (IP). The electron abstraction will be easier with a lower ionization potential. DPPH, ABTS, FRAP and Folin-Ciocalteu are examples of SET-based assay (Liang and Kitts, 2014).
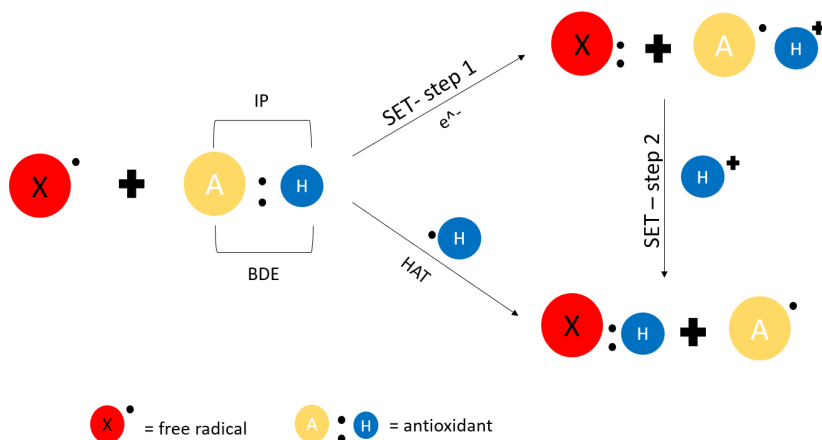
Figure 1.2: Schematic drawing of the two mechanisms of antioxidants reacting with free radical, SET - single electron transfer, HAT - hydrogen atom transfer. Ionization potential (IP) most important energetic factor in SET mechanism, bond dissociation enthalpy (BDE) most important parameter in HAT mechanism.

Metal Chelators or sequestering agents, binds metal ions and alter the pro-oxidative activity, and the action is categorized under secondary antioxidants. Lipids contain trace amounts of metal ions, and transition metals possessing two or more valency states (e.g Co, Cu, Fe, Mn, etc.) promote the oxidation of lipids (Gordon, 1990). Three different mechanisms of lipid oxidation by metals are suggested;

- Interaction with unsaturated fatty acids

- Interaction with hydroperoxides (Fenton reactions, probably the most common)

- Activation of ground state molecular oxygen to its excited state, singlet oxygen.

The ferrous ion-chelating ability assay is based on preventing the Fenton reaction, which happens by a reaction between hydrogen peroxide and ferrous salts, and gives rise to reactive species able to oxidize e.g lipids (Winterbourn, 1995).

Further, the metal chelators need to be ionized to alter oxidation, and can inhibit the metal ions activities in three ways, (i) occupation of all metal coordination sites, (ii) formation of insoluble metal complexes, and (iii) steric hindrance of interaction between metals and lipids or oxidation intermediates (hydroperoxides). Typical metal chelators found in food are compounds that contain multiple carboxylic acid groups or phosphate groups.

Compounds using the two first inhibition pathways ((i), (ii)) are transferrin, ferritin and lactalbumin. Citric acid, EDTA, polyphosphates, phytates, phenolic acids and flavonoids also exhibit good chelators capacity by the third inhibition pathway (Laguerre et al., 2007).

1.7.1 The Folin-Ciocalteu assay

The Folin-Ciocalteu method is used to determine the total phenolic content (TPC), here in *S. latissima* and *A. esculenta*, and is based on a chemical reduction of the Folin-Ciocalteu reagent. The metal oxide reduction products will have a blue color with absorption maximum at 765 nm (Rover and Brown, 2013). The blue color is obtained when an electron is transferred by an antioxidant from a phenolic compound in alkaline medium to phosphomolybdic/phosphotungstic acid complexes (FC reagent), and phenolic antioxidant is oxidized. The FC colorimetric assay is simple, good and widely used for measuring the TPC in plant extracts. However, the FC method is nonspecific, which means it can be affected by nonphenolic organic substances, such as adenine, alanine, aminobenzonic acid, ascorbic acid, benzealdehyde and more. Also some inorganic substances may react with the F-C reagent and provide elevated apparent phenolic concentration, hydrazine, iron sulfate, manganese sulfate, potassium nitrite etc. Sugar aromatic amines, sulfur dioxide are some examples on interfering substances to the F-C method. These factors needs to be considered to obtain rationally comparable results for the total phenolic content (Prior et al., 2005) (Dasgupta and Klein, 2014).

1.7.2 DPPH radical scavenging activity assay

The DPPH radical scavenging assay is a widely used method, for measuring the antioxidant capacity in plant extract and is based upon the compounds ability to scavenge free radicals (Dasgupta and Klein, 2014). The DPPH radical have a single electron at the nitrogen atom, and are one of a few stable organic nitrogen radicals and have a deep violet color with max absorption at 517 nm. The DPPH assay measures the loss of the radical's violet color after reaction with an antioxidant. The deactivation of free radicals is mainly done by the SET reaction mechanism, but can also be done by the HAT reaction mechanism. For strong hydrogen-bond accepting solvents, like methanol and ethanol, the SET reaction mechanism is predominant (Prior et al., 2005).

1.7.3 ABTS radical scavenging activity assay

The ABTS radical scavenging activity assay is based on the hydrogen atom transfer (HAT) reaction mechanism, but also the single electron transfer (SET) mechanism. The mechanisms can change with pH and during reactions of slowly reacting antioxidants, this is an important disadvantage for the ABTS assay. The basis for the assay is followed by the reaction below;

Probe + electron from antioxidant $\rightarrow$ reduced probe + oxidized antioxidant

The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation, $ABTS^{\bullet+}$, is produced directly through a reaction between ABTS and potassium persulfate, and the reaction allows ABTS to be oxidized by peroxyl radicals. The reaction solution has a deep green/blue color, and addition of antioxidants will decolorize the mixture, because of the reduction of the $ABTS^{\bullet+}$ radical cation to ABTS, with max absorption wavelengths at 645 nm, 734 nm and 815 nm (Re et al., 1999), (Dasgupta and Klein, 2014). The addition of antioxidants after the radical cation is formed will minimize the interference of compounds with oxidants and also prevent the possible overestimation. $ABTS^{\bullet+}$ react quickly with antioxidants and it is soluble in both organic and aqueous solvents (Prior et al., 2005).

1.7.4 Ferric reducing antioxidant power, FRAP

The ferric reducing antioxidant power assay (FRAP) is measuring the antioxidant potential of the seaweed extracts by their ability to reduce Fe^{3+}-TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) to Fe^{2+}-TPTZ (reducing capacity), and is a single electron transfer (SET) reaction mechanism. At low pH (3.6) when Fe^{3+} is reduced to Fe^{2+} a deep blue color is formed with max absorption at 593 nm (Dasgupta and Klein, 2014). The color development occurs when there are antioxidants (a reductant) present (Benzie and Strain, 1996). FRAP is a good screening method for the ability to maintain redox potential in cells or tissues because of the reaction conditions (pH 3.6), which also decreases ionization potential that drives electron transfer. The degree of hydroxylation and conjugation in polyphenols is related to the reducing power. The FRAP assay is speedy, the redox reactions proceed so rapid that all reactions are complete within 4-6 min, it is simple and inexpensive (Prior et al., 2005).

1.7.5 Metal ion-chelating ability assay

The metal ion-chelating ability assay is based on preventing the production of reactive oxygen species by minimizing ferrous ion, Fe^{2+}, and chelate transition metals. Fe^{2+} is known to produce reactive hydroxyl radicals when reacting with a competitive chelator of metal ions, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acids)-1,2,4-triazine (ferrozine), and the reaction is called Fenton reaction. This reaction gives rise to a deep red color (Dasgupta and Klein, 2014). By adding a compound that have the ability to conjugate to transition metal and inhibit the metal-induced free radical complex formation will reduce the red color intensity. This inhibition will prevent the formation of free radicals, which further leads to oxidation. The binding ability of the substances to iron will therefore make Fe^{2+} unavailable as a pro-oxidant (Aparadh et al., 2012).

1.8 The aim of the present study

The aim of the present master thesis was to investigate how different drying methods and extraction solvents would influence the extraction of polyphenols and differences in the antioxidant activity for two different brown macroalgae, *S. latissima* and *A. esculenta*. Two different drying methods were tested, freeze-drying, oven-drying and frozen control samples, that were thawed (referred to as wet in the rest of the study). For the two drying methods and the wet control samples, three different solvents were used for the extraction; water, 70% ethanol and 70% acetone. Five different antioxidant activity assays with a variety of reaction pathways were performed and a correlation between polyphenols and antioxidant activity was also determined.

Chapter 2

Materials and Methods

2.1 Seaweed materials

Saccharina Latissima was harvested 29.04.2019 by Seaweed Energy Solution and frozen directly in the HitraMat plant. *S. latissima* was received from SES at 18.11.2019 and stored at -20°C. *Alaria Esculenta* was received from Seaweed Energy solution and harvested 06.05.19 outside Frøya. Further, *A. esculenta* were vacuum packed, frozen and stored at -20°C.

2.2 Dry matter and ash content

Approximately 2.0 grams of wet *S. latissima* and *A. esculenta* were weighed and dried at 105°C for 24 h. The crucibles with the algae were placed in a desiccator and cooled down to room temperature. Afterwards the dried samples were weighed and dry matter was calculated.

Crucibles with the dried content were further placed in a cold muffle furnace, to determine the ash content. The oven was heated to 550°C and samples were ashed for 24 h. After 24 h the oven was turned off, leaving the samples inside for 30 minutes, and further 30 minutes with the door open, crucibles were transferred to desiccator and cooled down to room temperature, and weighed.

2.3   Flow sheet

Figure 2.1 is a schematic presentation of performed experiments with freeze-dried, wet and oven-dried *S. latissima* and *A. esculenta*.
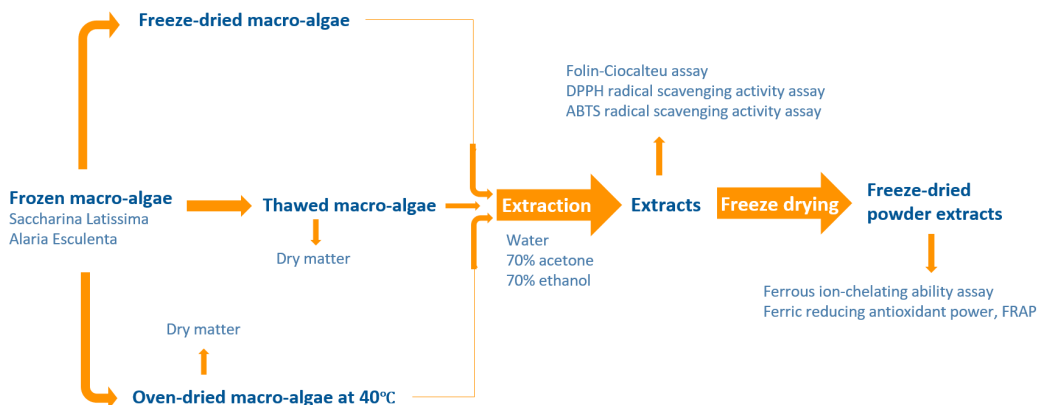
Figure 2.1: Schematic drawing of the two drying methods and wet control samples, and the experiments performed on the two macroalgae, *S. latissima* and *A. esculenta*.

2.4   Preparation of sample extracts

2.4.1   Sample extracts - Freeze-dried algae

300 grams of frozen *S. latissima* and *A. esculenta* were freeze-dried at -58°C and with a pressure at 0.20 mbar for 48 hours. The six extracts were prepared with 10 grams of freeze-dried *S. latissima* and *A. esculenta*, respectively, mixed with 200 ml of distilled water, 70% acetone or 70% ethanol and stirred at room temperature for 24 h. Further, the extracts were decanted using a funnel and glass wool. The extracts were used for the determination of total phenolic content, DPPH-assay and ABTS-assay. Afterwards, the six different extracts were freeze-dried to algae powder, and used for FRAP-assay and metal ion-chelating ability assay.

2.4.2   Sample extracts - Oven-dried algae at 40 °C

250 grams of each frozen macroalgae, *S.latissima* and *A. esculenta*, were dried in a convective oven at 40°C for 76 h. Afterwards, the dried *S.latissima* and *A. esculenta* were

prepared in the same manner as for the freeze-dried samples. The dry matter of the dried samples were determined as described in section 2.2.

2.4.3 Sample extracts - Wet algae

100 grams of frozen *S. latissima* and *A. esculenta* were thawed in a cool room at 4°C for 24 h. The thawed *S. latissima* and *A. esculenta* were cut and prepared in the same way as for the freeze-dried and oven-dried samples. The dry matter were determined as described in section 2.2.

2.5 Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteau method as described by Singleton et al. (1999), with some modifications. 0.5 ml of suitably diluted extract were mixed with 0.5 ml of Folin-Ciocalteu phenol reagent and 5 ml distilled water in test tubes. After exactly 3 minutes, 1 ml of 20% sodium carbonate was added to the tubes, followed by an addition of 3 ml distilled water. The test tubes were vortexed, covered and incubated for 1 h at room temperature. The blank was prepared in the same way, with 80% methanol instead of extract. The absorbance was measured at 725 nm, with water as reference. A standard curve with a dilution series of propyl gallate solution, ranging from 0.5 mM to 2 mM, was used for calibration. All the measurements were performed in triplicate.

2.6 DPPH radical scavenging activity assay

A protocol described by Thiansilakul et al. (2007), with slight modifications, was used to determine the DPPH radical scavenging activity. One day before analysis a solution of 0.15 mM 2,2-diphenyl-1-picryllhydrazyl (DPPH) in 96% ethanol was prepared and stirred in the dark at 4°C overnight. 1.5 ml of suitably diluted extracts were mixed with 1.5 ml DPPH, vortexed, covered and left in the dark for 30 minutes. The blank was prepared in the same way, but 80% methanol was used instead of extract. The absorbance was measured at 517 nm with ethanol as a reference, and a standard curve with propyl gallate solutions was used for calibration (ranging from 10 μM to 30 μM). The radical scavenging activity was determined in triplicate (n = 3). DPPH radical scavenging was calculated by the equation below:

$$\text{Radical scavenging activity}[\%] = (1 - \frac{A_{sample}}{A_{blank}}) * 100\% \qquad (2.1)$$

Where A_{sample} was the absorbance of the sample, and A_{blank} was the absorbance of the blank.

2.7 ABTS radical scavenging activity assay

The ABTS radical scavenging activity assay was performed as described by Nenadis et al. (2004) with modifications. The day before analysis, 25 ml of 7 mM ABTS solution was mixed with 440 μl of 140 mM $K_2S_2O_8$, and covered with aluminium foil. The reaction mixture was diluted with methanol until the absorbance was 0.75 ± 0.05 at 734 nm, using water as reference. All the sample extracts were diluted 1:20 with methanol. 2 ml of the diluted ABTS reaction was mixed with 200 μl of extract, methanol (blank) or standards, the mixtures were vortexed and incubated for 6 minutes. A standard curve with propyl gallate solution was used for calibration, with concentration ranging from 10 μM to 50 μM. The absorbance was read at 734 nm with water as reference, and the measurements were done in triplicate. The antioxidant activity was expressed as percent radical scavenging activity:

$$\text{Radical scavenging activity}[\%] = (1 - \frac{A_{sample}}{A_{blank}}) * 100\% \qquad (2.2)$$

Where A_{sample} was the absorbance of the sample, and A_{blank} was the absorbance of the blank.

2.8 Metal ion-chelating ability assay

The metal ion-chelating ability was determined according to the method of Klompong et al. (2008) with minor modifications. 0.5 grams of freeze-dried water extracted algae powder was mixed with 10 ml distilled water, and 0.5 grams of freeze-dried acetone and ethanol extracted algae powder was mixed with 10 ml ethanol. The sample extract concentration was therefore 0.05 g/ml and all extracts were further diluted 1:5 with water for water extracted algae powder and ethanol for acetone and ethanol extracted algae powder. One milliliter of each extract was mixed with 3.7 ml distilled water, and the mixture re-

acted with 0.1 ml 2 mM $FeCl_2$ for 20 minutes and further mixed with 0.2 ml of 5 mM Ferrozine. The control was prepared in the same way, but with distilled water instead of the extract. The blank was also prepared in the same manner, but with distilled water instead of the iron solution. The absorbance was measured at 562 nm, with water as reference, and all measurements were done in triplicate. The chelating activity was determined as follows:

$$\text{Chelating activity } [\%] = (1 - \frac{A_0 - A_1}{A_2}) * 100\%$$ (2.3)

Where A_0 was the absorbance of the extract, A_1 was the absorbance of the blank and A_2 was the absorbance of the control.

2.9 FRAP assay

The ferric reducing antioxidant power was determined as described by Nenadis et al. (2007), with some modifications. The FRAP reagent was prepared freshly before analysis by mixing 2.5 ml of 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_2.6H_2O$ and 25 ml of 0,3 M acetate buffer, pH 3.6. The FRAP reagent was pre-warmed at 37°C. Water extracted algae powder was mixed with water, and acetone and ethanol extracted algae powder were mixed with ethanol. In addition, a series with different concentrations were made of each extract (0.0125 g/ml, 0.025 g/ml, 0.0375 g/ml, 0.05 g/ml). A dilution series of propyl gallate with concentration from 0-150 μl was prepared for calibration. 1680 μl of pre-warmed FRAP reagent was mixed with 120 μl of extracts, methanol (blank) or standards, and incubated for 4 minutes at 37°C. Absorbance was measured at 593 nm with water as reference. Final concentration of propyl gallate was 0-10μM (final dilution 1/15). The concentration of 0.025 g/ml for all the sample extracts was chosen for further determination of FRAP value, using the the calibration curve.

Chapter 3

Results

In this section the results are presented. First a table of dry matter and ash content of fresh/wet *Saccharina latissima* and *Alaria esculenta*, and dry matter of oven-dried algae at 40°C (OD), is outlined. The differences in total phenolic content and antioxidant activity for *S.latissima* and *A. esculenta* with different pre-treatment methods, freeze-dried (FD), wet and OD, and extraction solvents are presented in the next section. The last section shows correlation between total phenolic content and the antioxidant activity assays. The wet algae is used as a control to see if the two drying methods affects the antioxidant activity.

3.1   Dry matter and ash content

Table 3.1 shows the dry matter of wet *A. esculenta* and *S. latissima* and the ash content of the dry weight of the two algae. *A. esculenta* and *S. latissima* was harvested early May 2019 and late in April 2019, respectively. *S. latissima* demonstrate the highest ash content. In table 3.2 the dry matter of oven-dried algae is shown.

Table 3.1: Dry matter and ash content for wet *S. latissima* and *A. esculenta*, measured in % of wet weight and % of dry weight, respectively, SD is given and n=3.

Algae	Dry matter [% ww]	Ash content [% dw]
Saccharina latissima	10.7±0.6	41.4±0.1
Alaria esculenta	9.5±2.5	37.8±1.0

Table 3.2: Dry matter for oven-dried *S. latissima* and *A. esculenta* with standard deviation.

Algae	Dry matter oven-dried [% of D40 weight]
Saccharina latissima	39.0 ± 3.4
Alaria esculenta	25.6 ± 1.0

3.2 Antioxidant activity

In the sections below the results from the five different antioxidant activity assays is outlined. All measurements were done in a triplicate, and the standard deviation is shown by the error bars. In some cases, a measurement is neglected due to large deviations from the other two measurements (shown in appendix). Total phenolic content performed by Folin-Ciocalteau assay is presented as [mg propyl gallate equivalents (PGE)/g dried algae]. The FRAP-value is given in mgPGE/dry extracts, ABTS and DPPH is given in percentage inhibition, or percentage radical scavenging activity (RSA [%]), and metal ion-chelating activity is given in percentage metal chelating ability. Statistically significant difference between *S. latissima* and *A.esculenta* and between pre-treatment methods are determined by t.test in excel with a probability of $p < 0.05$ considered as significant.

The results of each antioxidant method is presented in two graphs, the first one shows the three different drying methods, wet, freeze-dried and oven-dried with *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol as bars. The second graph shows the six different algae extracts with drying methods as bars (blue = freeze-dried, orange = wet, grey = oven-dried). The graphs uses the abbreviations;

S.L water	=	*Saccharina latissima* extracted in water
A.E water	=	*Alaria esculenta* extracted in water
S.L acetone	=	*Saccharina latissima* extracted in 70% acetone
A.E acetone	=	*Alaria esculenta* extracted in 70% acetone
S.L ethanol	=	*Saccharina latissima* extracted in 70% ethanol
A.E ethanol	=	*Alaria esculenta* extracted in 70% ethanol

3.2.1 Folin-Ciocalteau assay, total phenolic content

The total phenolic content of *S. latissima* and *A. esculenta* are presented in figure 3.1 and 3.2. Significant differences were observed between the two algae for extraction from the wet experiments. *A. esculenta* extracted in water, 70% acetone and 70% ethanol exhibited a significantly higher amount of phenols compared to the *S. latissima* samples. For the oven-dried samples the extracted amount of phenols were generally lower for both algae

and the three extraction solvents. Freeze-dried *A. esculenta* in 70% acetone exhibit an outstanding amount of phenols compared to the other freeze-dried extracts. The highest amount of extracted phenols determined with the Folin-Ciocalteau method was found in wet *A. esculenta* extracted in 70% acetone with 23.67±0.24 mgPGE/g dried algae, and the lowest amount were found in freeze-dried *S. latissima* extracted in 70% ethanol with 2.24±0.07 mgPGE/g dried algae. No significant differences were observed between wet, oven-dried and freeze-dried *S. latissima* extracted in water, acetone or ethanol, figure 3.2.
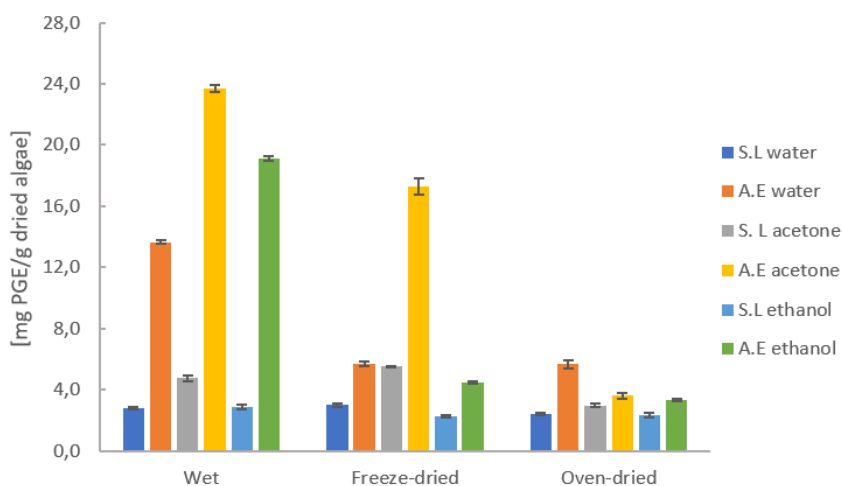
Figure 3.1: Total phenolic content of the wet, freeze-dried and oven-dried *A. esculenta* and *S. latissima* extracted in water, 70% acetone and 70% ethanol. Expressed in propyl gallate equivalents, mgPGE/g dried algae. (n = 3 and error bars are standard deviation (SD)).
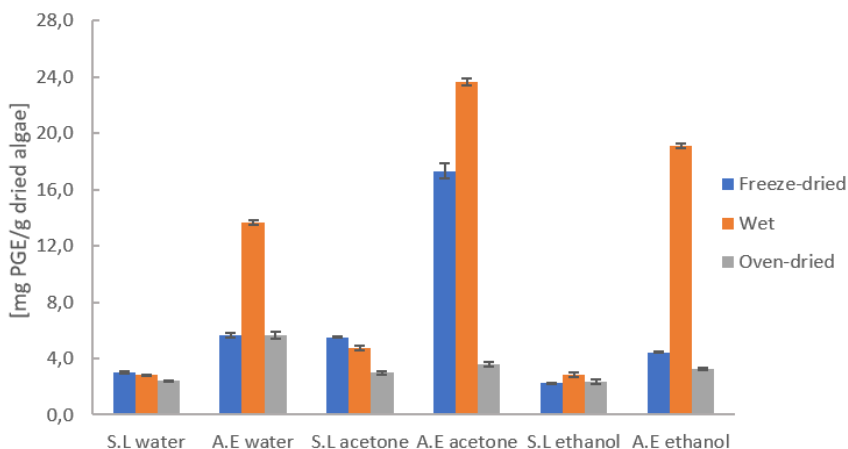
Figure 3.2: Total phenolic content of the wet, freeze-dried and oven-dried *A. esculenta* and *S. latissima* extracted in water, 70% acetone and 70% ethanol. Expressed in propyl gallate equivalents, mgPGE/g dried algae. (n = 3 and error bars are standard deviation (SD)).

3.2.2 DPPH radical scavenging activity

From the DPPH radical scavenging activity assay (figure 3.3 and 3.4), the freeze-dried algae samples generally showed a higher radical scavenging activity compared to oven-dried and wet algae. In the inhibition of free radicals, freeze-dried *A. esculenta* extracted in 70% acetone exhibited the highest activity with 68.6±1.2%, followed by freeze-dried *S. latissima* extracted in water with 62.8±4.4%. For the wet samples, *A. esculenta* extracted in 70% acetone and 70% ethanol demonstrated a significantly better radical scavenging potential compared to the other wet algae extracts. Wet *S. latissima* extracted in water showed the lowest scavenging activity of 5.2±0.01%. Wet *S. latissima* extracted in 70% ethanol and oven-dried *S. latissima* extracted in 70% acetone and 70% ethanol, and *A. esculenta*, *S. latissima* extracted in 70% acetone and 70% ethanol all demonstrated a low radical scavenging activity below 10%.
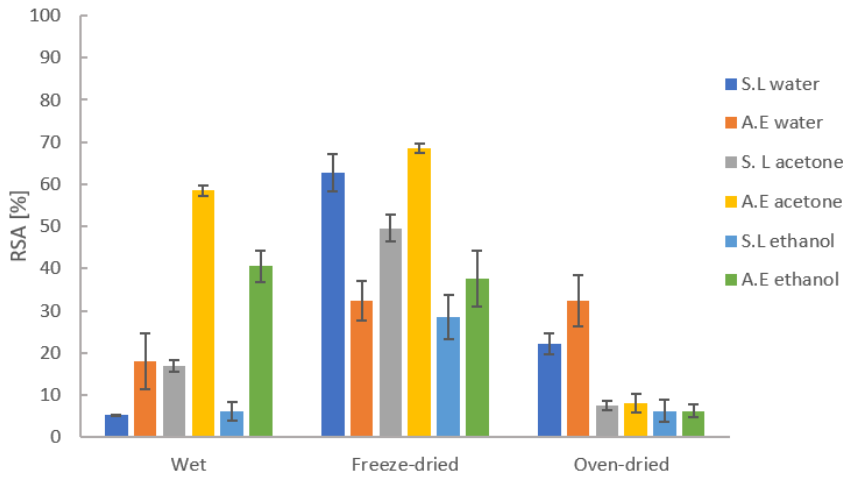
Figure 3.3: DPPH radical scavenging activity, of the wet, freeze-dried and oven-dried algae extracts, expressed in percentage inhibition of free radicals. (n = 3 and error bars are standard deviation (SD)).
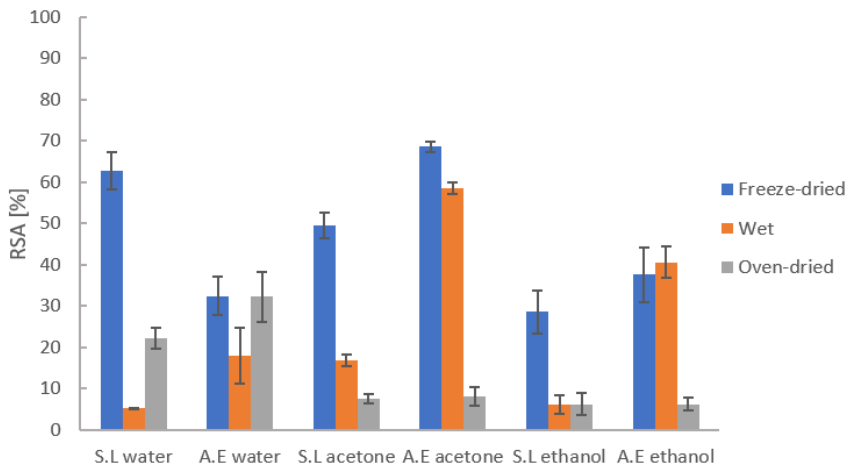
Figure 3.4: DPPH radical scavenging activity, of *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol, expressed in percentage inhibition of free radicals. (n = 3 and error bars are standard deviation (SD)).

3.2.3   ABTS radical scavenging activity

For the ABTS assay, figure 3.5 and 3.6, the freeze-dried algae again exhibited a generally higher antioxidant activity, as for the DPPH free radical inhibition, compared to wet and oven-dried algae. Freeze-dried *A.esculenta* extracted in 70% acetone demonstrated the strongest radical scavenging activity with 83.8±8.5%. No significant differences were found between the wet and oven-dried samples, or between the extracts, with a relatively low radical scavenging activity for all of the algae extracts.
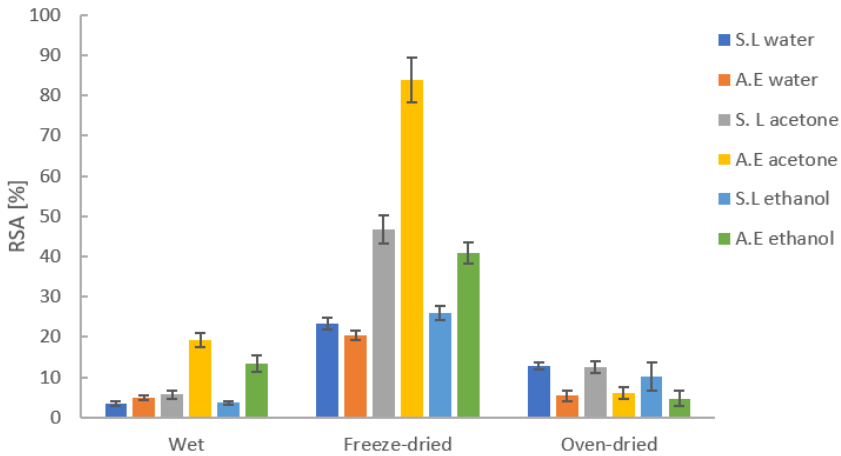
Figure 3.5: ABTS radical scavenging activity, of the wet, freeze-dried and oven-dried algae extracts, expressed in percentage inhibition of free radicals. (n = 3 and error bars are standard deviation (SD)).
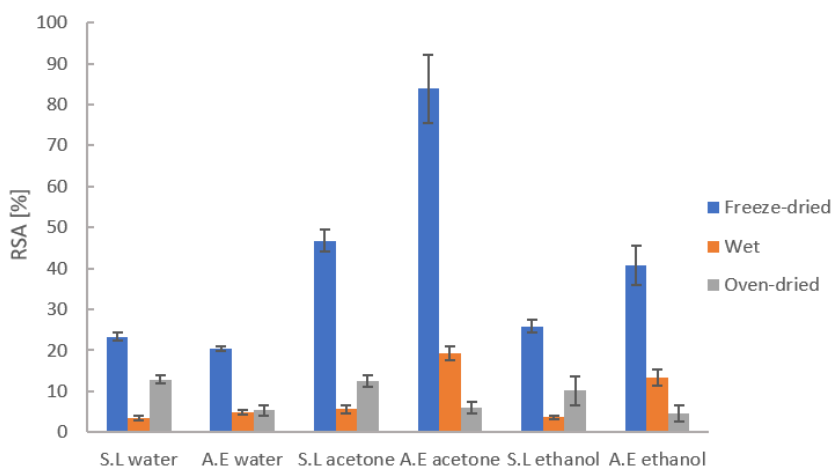
Figure 3.6: ABTS radical scavenging activity, of *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol, expressed in percentage inhibition of free radicals. (n = 3 and error bars are standard deviation (SD)).

3.2.4 Metal ion-chelating ability

In the metal ion-chelating activity assay, differences were found between treatments and extraction solvents. The oven-dried samples of *A. esculenta* extracted in water, 70% acetone, 70% ethanol and wet *A. esculenta* extracted in water demonstrated the highest metal chelating abilities, 90.8±0.8%, 94.2±1.2%, 93.9±1.2% and 92.9±7.0%, respectively. Both for the wet and freeze-dried samples, *A. esculenta* extracted in water showed a relatively high activity compared to the other extraction solvents and *S. latissima*. In general, the freeze-dried sample extracts exhibited the lowest metal chelating activity, with *S. latissima* in 70% acetone as the lowest, 10.1±2.9%.
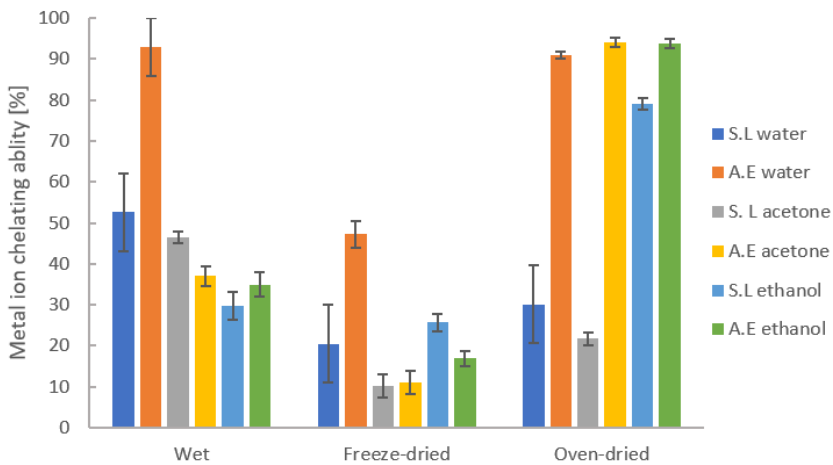
Figure 3.7: Metal ion-chelating activity of the three different pre-treatments methods, wet, freeze-dried and oven-dried, expressed as percentage of metal chelating ability (n = 3 and error bars are standard deviation (SD)).
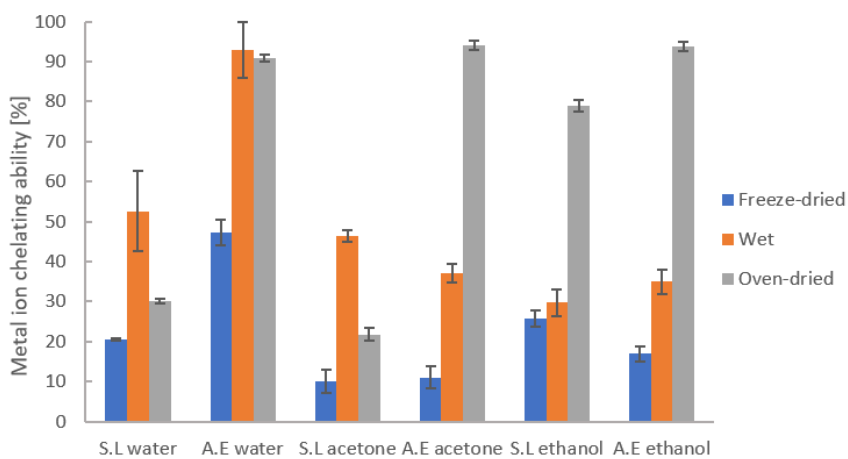
Figure 3.8: Metal ion-chelating activity of *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol, expressed as percentage metal chelating ability. (n = 3 and error bars are standard deviation (SD)).

3.2.5   FRAP assay, reducing capacity

In figure 3.9 differences between wet *A. esculenta* and *S. latissima* is observed. Wet *A. esculenta* extracted in 70% ethanol exhibit the highest reducing capacity with 0.39±0.03 mgPGE/g dry extract, followed by wet *A. esculenta* extracted in 70% acetone and water with an antioxidant reducing potential of 0.23±0.02 mgPGE/g dry extract and 0.14±0.01 mgPGE/g dry extract, respectively. No significant differences were observed for wet and freeze-dried *S. latissima*. All the oven-dried samples demonstrated a generally low reducing capacity.
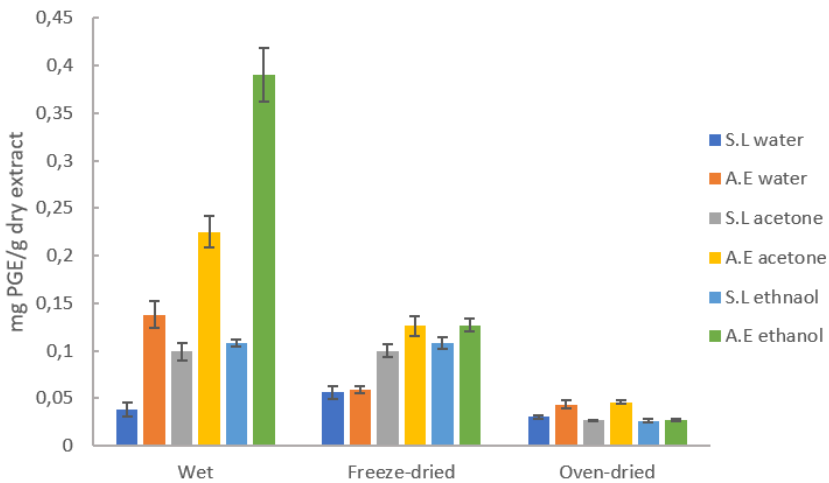
Figure 3.9: The figure shows the ferric reducing antioxidant power for the two different drying methods and the wet control samples for all algae extracts, measured in mgPGE/g dry extract. (n = 3 and error bars are standard deviation (SD)).
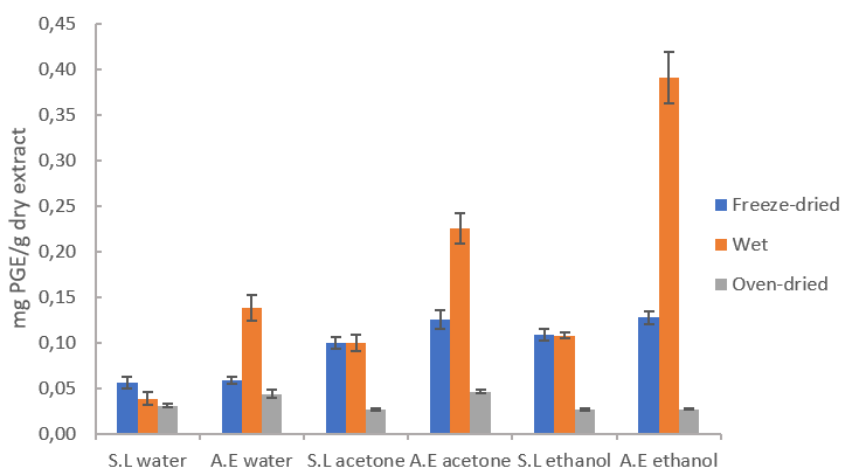
Figure 3.10: Ferric reducing antioxidant power, FRAP, for each algae powder extract of the three pre-treatments methods. It is measured in mgPGE/g dry extract. (n = 3 and error bars are standard deviation (SD)).

3.3 Correlation between total phenolic content and antioxidant activities

Pearson's correlation analysis was performed for determination of the relationship between TPC and antioxidant activity assays for the six algae extracts. Figure 3.11 shows a positive correlation between total phenolic content and ABTS radical scavenging activity of freeze-dried *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol. The correlation coefficient was determined to r = 0.91, with a probability lower than 5% (p = 0.014) for given measurements (N = 6), which indicates high positive correlation.
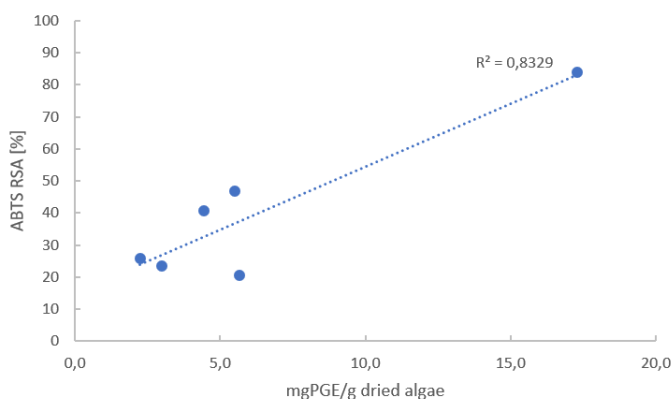
Figure 3.11: Correlation graph, for freeze-dried *S. latissima* and *A. esculenta* algae extracts, between total phenolic content (TPC) [mgPGE/g dried algae] and ABTS radical scavenging activity [%] (r = 0.91, p < 0.05).

The two figures below (3.12 and 3.13) are correlation graphs of wet *S. latissima* and *A. esculenta* extracted in water, 70% acetone and 70% ethanol between total phenolic content and DPPH radical scavenging activity (3.12), and total phenolic content and ABTS radical scavenging activity (3.13). These graphs indicates high positive correlations and correlation coefficients were calculated to r = 0.95 (p < 0.05) and r = 0.91 (p < 0.05) for TPC against DPPH, and TPC against ABTS, respectively.
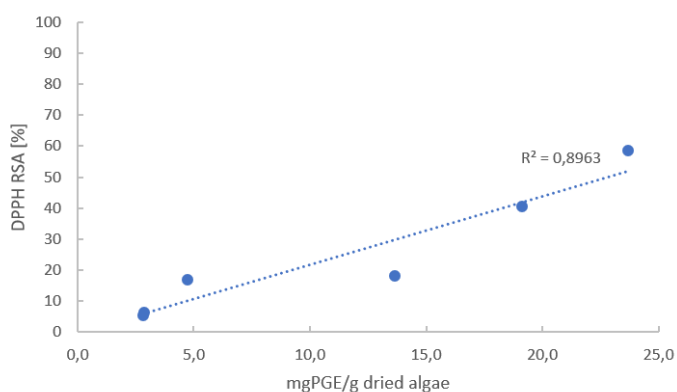
Figure 3.12: Correlation graph, for wet *S. latissima* and *A. esculenta* algae extracts, between total phenolic content (TPC) [mgPGE/g dried algae] and DPPH radical scavenging activity [%] (r = 0.95, p < 0.05).
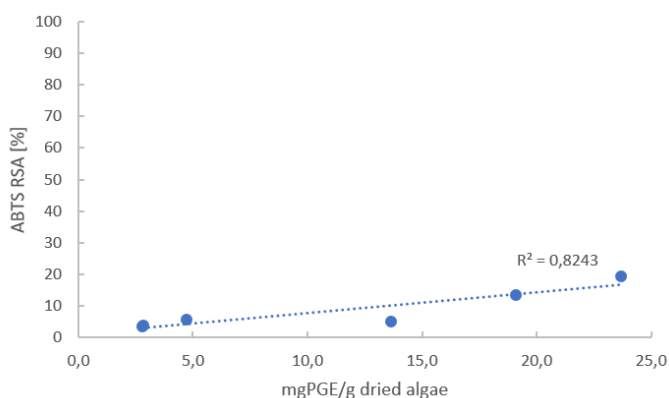
Figure 3.13: Correlation graph, for wet *S. latissima* and *A. esculenta* algae extracts, between total phenolic content (TPC) [mgPGE/g dried algae] and ABTS radical scavenging activity [%] (r = 0.91, p < 0.05).

Low correlations and high probabilities (p > 0.05) for the antioxidants activity methods to be uncorrelated were found for the oven-dried *S. latissima* and *A. esculenta* experiments. No correlation was indicated between total phenolic content and metal chelating activity for any of the drying methods (wet, freeze-dried and oven-dried). Low correlation coefficients were determined between TPC and ferric reducing antioxidant power, FRAP, with FD; r = 0.43, wet; r = 0.78, and OD; r = 0.71, all with p > 0.05, indicating low or no correlation. The correlation graphs for these methods are therefore not included in this section.

Chapter 4

Discussion

4.1 Dry matter and ash content

The dry matter for *Saccharina latissima* and *Alaria esculenta* was calculated to 10.7±0.6% of ww and 9.5±2.5% of ww, respectively. From the literature it has been indicated that dry matter can vary both with seasonal differences when harvesting and between species (Schiener et al., 2015). Schiener et al. (2015) reported a dry matter content of 15.1±2.9% for *S. latissima* and 14.5±2.5% for *A. esculenta*, which are average values from algae harvested regularly throughout a whole year. Comparing to the experimental dry matter values in this study the literature values are higher and can be explained by different harvesting location and several harvesting times, and the dry matter values from the present study is only determined by a one-time harvesting, late April for *S. latissima* and early May for *A. esculenta*. The determined ash content were 41.4±0.1% dw and 37.8±1.0% dw for *S. latissima* and *A. esculenta*, respectively. In the study from Schiener et al. (2015) the ash content was lower, with 31.7±7.6% dw for *S. latissima* and 25.3±5.8% dw for *A. esculenta*. Through a whole year the ash levels varied from 20-40% dw for *S.latissima* and 20-25% dw for *A. esculenta*. It is reported, through metal-analysis, that high ash contents could be due to accumulation of potassium and sodium ions which are high during the winter months and reduces in the autumn (Schiener et al., 2015), and in the present study the two brown algae where harvested late April and start of May in the transition between winter and summer time.

Stévant et al. (2017) determined a dry matter content of 17.2±0.8% and 16.1±1.2% for *A. esculenta* and *S. latissima*, respectively, which also is a higher dry matter value than the values found in this study. In addition, the ash contents were lower and more similar to Schiener et al. (2015), with 24.2±1.4% dw for *A. esculenta* and 26.2.7±2.6% dw for *S. latissima*. The two brown algae in Stévant et al. (2017) were harvested in France. Location and ecological conditions can affect the ash content, and general nutrient contents, due to environmental factors such as salinity, light, nutrients, water temperatures and water currents (Marinho-Soriano et al., 2006). In general, the dry matter was lower and the ash content was higher for *S. latissima* and *A. esculenta* in the present study compared to the literature values.

4.2 Antioxidant activity of *S. latissima* and *A. esculenta*

4.2.1 Total phenolic content, Folin-Ciocalteau assay

Differences were found between both *A. esculenta* and *S. latissima*, and between drying methods. For the wet samples the differences between extraction solvents are not significant compared to differences between the two algae, *A. esculenta* extracted in acetone exhibited the highest amount of phenols. For all of the extraction solvents and drying methods, *A. esculenta* generally demonstrated a higher amount of extracted polyphenols. *A. esculenta* can be classified as a high-phenolic species. Van Alstyne and Paul (1990) divided the seaweed species in two classes based upon the amount of total phenolic content in the seaweed, low-phenolic species ($< 2\%$ dw) and high-phenolic species ($>2\%$ dw). Roleda et al. (2019) reported the highest amount of phenolic compounds extracted from *A. esculenta* and *S. latissima* to 6.1% dw and 1.5% dw, respectively, which indicates that *A. esculenta* is a high-phenolic species and *S. latissima* is a low-phenolic species. This statement is consistent with the observation in figure 3.1 for the wet samples.

For the freeze-dried (FD) samples, *A. esculenta* extracted in acetone demonstrated a significant higher content of phenolic compounds compared to all the other FD extracts. This agrees with the results in the specialization project, TBT4500, done autumn 2019. Roleda et al. (2019) found a much higher content of phenolic compounds for freeze-dried *A. esculenta* in 80% acetone (36.86±2.69 mgPGE/g dried algae) compared to *S. latissima* in 80% acetone (8.07±0.51 mgPGE/g dried algae), the values are averages of samples harvesting in the spring, summer and autumn. Compared to the present study, the phenolic

content was determined to 5.55±0.05 mgPGE/g dried algae and 17.30±0.53 mgPGE/g dried algae for freeze-dried *S. latissima* and *A. esculenta* extracted in 70% acetone, respectively, which is lower than the literature values. The lowest phenolic content, reported by Roleda et al. (2019), was recorded in spring, April-May, with 5.09 mgPGE/g dried algae and 14.10 mgPGE/g dried algae for *S. latissima* and *A. esculenta*, respectively, which better aligns with the values in this study. From Schiener et al. (2015) the phenolic content was determined in % dw, and the values for freeze-dried *S. latissima* and *A. esculenta* harvested in May, extracted in an acetone/water mixture, was reported to 0.46±0.01% dw and 1.49±0.04% dw, respectivley. In comparison, the total phenolic content in the present study for freeze-dried *S. latissima* and *A. esculenta* extracted in 70% acetone were determined to 0.55±0.004% dw and 1.73±0.053% dw, respectively.

The use of extraction solvents is reported to have an effect on the output on the polyphenol content (Wang et al., 2009). For extraction of polyphenols, organic solvents are recommended, such as ethanol and acetone, because phenols will dissolve better in solvents less polar than water (Wang et al., 2009). In addition, it is reported that acetone increases the polyphenol yield by inhibiting the complex formation of polyphenols and proteins, or by breaking hydrogen bonds (Hagerman, 1988). For the present study, 70% acetone extracts exhibits the highest amount of phenolic compounds. The use of only water as extraction solvent will lead to extraction of other water soluble compounds such as polysaccharides, proteins and organic acids (Chirinos et al., 2007). The results in the present study indicate a little higher amount of extracted polyphenols for *S. latissima* extracted in water compared to 70% ethanol of all the drying methods, and for *A. esculenta* of the oven-dried and freeze-dried samples, this agrees with Koivikko et al. (2005). Koivikko et al. (2005) compared different extraction solvents for soluble polyphenols of the brown algae (*Fucus vesiculosus*) and reported highest amount of polyphenols in 70% acetone extracts followed by water and 80% ethanol.

The differences in the drying methods for the extracted amount of polyphenols where not significant, except for the wet *A. esculenta* extracted in water, 70% acetone and 70% ethanol, and freeze-dried *A. esculenta* extracted in acetone which demonstrated a significantly higher amount of phenols. The oven-dried samples had a generally lower amount of polyphenols for all the algae extracts. Amorim et al. (2020) and Le Lann et al. (2008) reported a lower phenolic content in oven-dried samples compared to wet and freeze-dried, this reduced amount could be due to degradation of phenolic compounds at high temperatures starting at 40°C. Several studies have reported different mechanisms why high

temperatures decrease the phenolic content: (i) due to breaking of linkages, some phenolic compounds would be released from the cell wall, (ii) a partial degradation of the cell wall, due to changes in the aromatic rings of phenolic compounds or changes in the oxidation levels in side chains, and (iii) a thermal degradation of polyphenols by oxidative enzymes (Lim and Murtijaya (2007) and Schoenwaelder and Clayton (1998), referred by Le Lann et al. (2008)). Lastly, as mentioned in the introduction, Folin-Ciocalteu assay is a SET (single electron transfer) based assay, and this is not specific for polyphenols. This non-specificity could lead to detection of interfering compounds when measuring the absorbance to the extracts (Prior et al., 2005).

4.2.2 Radical scavenging activity, DPPH and ABTS assay

The radical scavenging activity (RSA) was measured by two methods; 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, which both mainly are based upon single electron transfer mechanism (SET). The results demonstrated a generally higher radical scavenging activity for all the freeze-dried extracts in both assays compared to the wet and oven-dried samples. The same DPPH radical scavenging activity outcome was observed by Badmus et al. (2019), where they investigated five different drying methods, on four different brown seaweeds, and freeze-drying and oven-drying at 40 °C were two of them, and freeze-drying exhibited the highest RSA.

For the DPPH radical scavenging activity some differences were observed for the different extraction solvents, and between *S. latissima* and *A. esculenta* in the FD samples. For the OD samples, *S. latissima* and *A. esculenta* extracted in water showed a higher RSA compared to aqueous ethanol and acetone. These findings correspond to observations done by Norra et al. (2016), where water as extraction solvent for oven-dried brown seaweed (*Sargassum sp*) exhibited a higher RSA compared to aqueous ethanol. However, for the ABTS assay differences between the FD samples were observed, whereas wet and OD sample showed approximately no radical scavenging activity, and significantly differences between the FD samples and the OD samples, and the FD samples and the wet samples are indicated. The generally low DPPH and ABTS radical scavenging activity of the OD samples can be explained by a oxidation of molecules with antioxidant activity which can occur at high temperatures (Amorim et al., 2020). Le Lann et al. (2008) observed a lower antioxidant activity for the oven-dried brown algae compared to freeze-dried samples as observed in the present study as well, and concluded with the mechanisms for a loss in

phenolic compounds described in section 4.2.1. In addition, freeze-drying is a vacuum process, this means absence of oxygen and therefore prevention of oxidation reactions and degradation of valuable components (Murgatroyd, 1997). The higher DPPH- and ABTS RSA in the FD samples compared to the wet samples, observed in the present study, agrees with results from Zhang et al. (2009), and can be explained by operations such as cutting and slicing which induce a rapid enzymatic oxidation of natural antioxidants and cause depletion of antioxidants.

Water, 70% acetone and 70% ethanol extracts differed in their DPPH and ABTS radical scavenging activities, indicating that the choice of extraction solvent may influence the antioxidant activity. *A. esculenta* extracted in 70% acetone clearly exhibited the highest radical scavenging activity in both the ABTS assay and the DPPH assay for the freeze-dried and wet samples, in comparison to the other algae extracts for the same drying methods. This observation correlates with the results observed for total phenolic content, and may indicate that polyphenols contribute to the antioxidant activity, as mentioned in the introduction. At the same time, there may not be a correlation between TPC and radical scavenging activity for all the algae-extracts. According to Wang et al. (2009) the use of 70% acetone demonstrated a higher RSA compared to water for freeze-dried *A. esculenta* and *S. latissima*. However, 70% acetone is non-specific, which means that other classes of antioxidant compounds, e.g fucoxanthin and sterols could be extracted together with the polyphenols and contribute to the overall antioxidant activities (Wang et al., 2009).

Because of different sample pre-treatments, extraction methods, macroalgae and determinations of measurements from various studies on antioxidant activity in brown seaweeds, direct comparison with the results in the present study is not feasible.

4.2.3 Metal ion-chelating ability

For the metal chelating ability, OD samples demonstrated the highest capacity to chelate transition metals, and was clearly most effective for *A. esculenta* extracted in all the extraction solvents. FD, OD and wet samples and all the algae extracts demonstrated metal chelation, which means that compounds in the two algae are capable to capture metal ions before Ferrozine. Common for wet, FD and OD is a high chelating ability for *A. esculenta* extracted in water, these observation agrees with the results from the specialization project performed autumn 2019 and Wang et al. (2009) which reported a higher ability to chelate

transition metals for *A. esculenta* and *S. latissima* extracted in water compared to 70% acetone. The same trend was observed for *S.latissima* for both the drying methods and wet samples. These trends are probably due to the fact that water may extract out water soluble compounds such as polysaccharides, proteins and peptids, which are demonstrated to be capable of metal ion-chelating (Saiga et al., 2003). Wang et al. (2009) obtained a chelating ability of approximately 75% for both freeze-dried *S. latissima* and *A. esculenta* extracted in water. For the 70% acetone extracts *S. latissima* showed a metal chelating ability on 15%, while *A. esculenta* only obtained 23% (values are read from a graph in the study by Wang et al. (2009) and therefore no standard deviations and approximately answers). In comparison with the present study, the values were lower with a metal chelating capacity of 20.5±0.3% and 47.2±3.1% for water extracts of FD *S. latissima* and *A. esculenta*, respectively. The 70% acetone extracts demonstrated an ability to chelate metal ions of 10.1±2.9% for *S. latissima* and 11.6±2.8% for *A. esculenta*.

Farvin and Jacobsen (2013) investigated different brown, red and green seaweeds, and observed a significantly higher metal ion chelating activity for water extracts compared to ethanolic extracts. The same study reported that other highly polar components could be extracted by water and affect the Fe^{2+} chelating capacity, such as proteins, polysaccharides and peptides. It must however be noted that Farvin and Jacobsen (2013) used pure ethanol extracts and not an aqueous solution. However, differences between 70% ethanol and water as the most effective extraction solvent for the different algae and drying methods were observed for the present experiment. Several studies have demonstrated that proteins, polysaccharides and peptides exhibit high ability to chelate metal ions, and thus being greater contributors to the metal chelating activity than polyphenols (Saiga et al. (2003), Toth and Pavia (2000), Toyosaki and Iwabuchi (2009)). In the literature, there are inconsistent reports regarding polyphenols ability to chelate transition metals, some studies claims that polyphenols from brown macroalgae are potent as metal chelators (Chew et al., 2008). In contrast, Rice-Evans et al. (1996) showed that metal chelation of some polyphenols play a minor role in the overall antioxidant activity.

The variations between wet, OD and FD can be explained by the higher water content in the wet and OD samples compared to freeze-dried before extraction. Since, water extraction demonstrated the most effective chelating ability, the higher water content in *S. latissima* and *A. esculenta* could lead to a higher water concentration in the algae extracts in the end. In addition, the lower chelating ability for FD samples can be due to loss of water under freeze-drying, and therefore cause destabilization of bioactive molecules. The

cellular components may be attached to each other in the absence of water, and further lead to difficulties during extraction with different solvents, and hence reduce the antioxidant activity of the extracts (Li et al. (2006), referred to by Amorim et al. (2020)).

By the research done for the present study no earlier studies were found that was using the same drying methods, extraction solvents, the same two brown macroalgae and calculation of results, a direct comparison of results are therefore not possible, which is the same as for DPPH and ABTS radical scavenging activity.

4.2.4 Ferric reducing antioxidant power (FRAP)

The results from FRAP assays shows a similar trend as for TPC, especially for the wet samples, with high reducing capacity for *A. esculenta*. The difference was that 70% ethanol exhibit a better antioxidant potential than 70% acetone. The reducing capacity is indicating how able extracts are to donate electrons, and further donate electrons to free radicals and convert them to stable compounds (Dorman et al., 2003). In the literature, Chan et al. (2015) observed higher reducing capacity for the red algae, *G. changii* extracted in ethanol compared to water and acetone, with the following trend; 80% ethanol > 80% acetone > water. This agrees with the findings in the present study, with only small differences between extracts for the FD and OD samples. The variations in extraction solvents indicate that ethanol extracts are more able to reduce Fe^{3+} to Fe^{2+}. In addition, Pérez-Jiménez and Saura-Calixto (2006) performed FRAP analysis on catechin;gallic acid in water and ethanol solution, and observed no significant differences between the solvents for reducing capacity. The same observation was done by Mashkor (2014), investigating Fenugreek Seeds Extract, and small differences in reducing capacity were observed for water, 70% acetone and 70% ethanol. An increase in polarity of a solvent by mixing solvents up to 50% water is reported to enhance the solubility of antioxidant compounds. Extraction efficiency is dependent upon solvents polarity and the cell matrix of the material (Alcântara et al., 2019). Earlier studies observed a delicate distinction between the mixing of water and ethanol or acetone, and demonstrated a decrease in the ferric reducing antioxidant power for acetone extracts between 60-80% aqueous solution, and between 80-100% for ethanol aqueous solution (Meneses et al., 2013). A perfect balance of water and ethanol or acetone for finding the optimum concentration to obtain the highest reducing capacity is therefore important. It must be mentioned that some of these studies are performed on terrestrial plants, and solvents can influence the extractions differently because of differences in cell matrix, and compounds in the cell wall, but generally it is the same mechanism.

Generally for wet, FD and OD, *A. esculenta* demonstrated a higher antioxidant activity compared to *S. latissima*. This observation can be explained by *A. esculenta* being a high-phenolic species as described in chapter 4.2.1. As mentioned, the results from FRAP assay and TPC followed the same trend with high antioxidant potential for *A. esculenta*, indicating that polyphenols might contribute to the antioxidant activity.

The OD samples demonstrated a statistical significantly lower antioxidant activity compared to wet and FD. In contrast to Sappati et al. (2019), which found highest reducing capacity for wet *S. latissima*, followed by oven-dried, and lastly freeze-dried for *S. latissima* harvested early May. On the other hand, they observed higher reducing capacity for FD compared to OD for algae harvested late June. The FRAP value used in Sappati et al. (2019) is given in μmolFSE/g dry solid, and is therefore not direct comparable with the results in the present study. On the contrary, Dang et al. (2017) reported a higher reducing capacity for freeze-dried brown algae, *H. banskii*, compared to oven-dried at 40°C, and this is consistent with the present results. As mentioned, studies have suggested that reducing capacity is related to phenolic content (Neoh et al., 2016), this trend; low phenolic content followed by low reducing capacity for especially the oven-dried samples, is observed in the present study. However, a strong correlation between TPC and FRAP might not be obtained because of small differences for the algae and extraction solvents, but a general lower TPC and reducing capacity is easy to observe for the OD samples.

4.3 Correlation between TPC and antioxidant activity

Correlations were found between total phenolic content and ABTS radical scavenging activity for FD and wet samples, with r = 0.91 for both. In addition, a correlation was found for wet samples of TPC and DPPH radical scavenging activity with r = 0.95, and for N = 6 the probability for the measurements to be uncorrelated are 1.4% (p = 0.014), which indicate highly significant correlated measurements. It must be noted, that the correlation between FD TPC and ABTS (Fig. 3.11) has one point in the upper right corner, and without this point there would have been no correlation. It could probably been done more experiments to make the correlation graph more reliable. The correlation indicate that polyphenols is a major contributor to antioxidant activity for the *S. latissima* and *A. esculenta* extracts, and then especially as a potent radical scavenger. Although *S. latissima* and *A. esculenta* may contain other antioxidant molecules, such as fucoxanthin, ascorbic acid and carotenoids, they will in this context contribute less to the antioxidant activity

(Jiménez-Escrig et al., 2001). These findings agrees with different studies, where a high correlation were found between TPC and radical scavenging activity of seaweeds (Wang et al. (2009), Zakaria et al. (2011), Jo et al. (2005)).

Interestingly, no clear correlation was found for the oven-dried samples. The correlation coefficient between TPC against DPPH radical scavenging was determined to r = 0.66, this could be considered as a relatively high correlation, but with N=6 the probability for the measurements to be uncorrelated is above 5%, and therefore it is concluded that there is no correlation. For the oven-dried samples it might be other bioactive compounds present in the extracts which can contribute to the scavenging activity (Zakaria et al., 2011), and the reported role of polyphenols as the main contributor to radical scavenging activity may be overestimated (Wang et al., 2009).

Correlations coefficients between total phenolic content and metal ion-chelating ability for wet, freeze-dried and oven-dried were close to zero, and indicates no correlation. The non-existent correlation agrees with the findings in Rice-Evans et al. (1996) which may indicate that phenolic compounds is not the main chelator of metal ions, and other metal chelators might contribute more.

Average and low positive correlation coefficients were found between TPC and FRAP, indicating that polyphenols are not the major contributor for the reducing capcity of *S. latissima* and *A. esculenta*, and other antioxidant compounds such as fucoxanthin and carotenoids might affect the reducing capacity more. However, similarly trends between the TPC and FRAP results were observed and this might be an indication that phenolic compounds plays a minor role for the reducing capacity. Both Dang et al. (2017) and Sappati et al. (2019) reported high positive correlations between TPC and FRAP for brown-algae, and indicated that polyphenols are the major contributors for reducing capacity.

4.4 Effects of dry processing

The effect of drying processes has been well studied in the literature, and there is conflicting reports of which drying methods that is the most efficient for extraction of polyphenols and antioxidant activity in seaweed or terrestrial plants. Ling et al. (2015) compared seven different drying techniques for a red seaweed, where oven-dried at 40°C and freeze-dried

were two of them. Oven-dried samples at 40°C exhibited the highest total phenolic content and a better DPPH and ABTS radical scavenging activity than the freeze-dried samples. On the other hand, Amorim et al. (2020) and Badmus et al. (2019) observed a higher TPC and higher antioxidant potential for freeze-dried seaweeds compared to OD at 40°C.

For the present study, there were significant differences in antioxidant activity between the wet, FD and OD samples for the five antioxidant assays. The variations between the results from the antioxidant activity assays indicate that there are various contributors to total antioxidant potential for the different methods (Cao and Prior, 1998). In general, the antioxidant potential had this order; freeze-dried > wet > oven-dried, with of course some deviation between algae and extraction solvents. The different antioxidant behavior in the wet, FD and OD samples for each antioxidant activity assay can be explained by the different chemical systems and/or reactions of the assay (Uribe et al., 2019). For instance, Folin-Ciocalteu, ABTS, FRAP and DPPH assays is based upon single electron transfer mechanism (SET), and measures the activity of primary antioxidants. Conversely, the metal ion-chelating ability assay is based upon inhibition of the Fenton reaction and chelating agents are effective as secondary antioxidants (Kumar et al., 2008) (Gordon, 1990).

The disadvantages of freeze-drying is mainly high production costs and high energy consumption. The only thing from the literature which may contribute to a decrease in bioactive substances is the destabilization of the native conformation of molecules and the absence of water, which influence extraction of compounds (Franks, 1998). However, studies have reported a positive effect of ice-crystals formed in the cell matrix under freeze-drying. The ice-crystals can rupture the cell structure and allow access of solvent and exit of cellular components and consequently increase the efficiency of extraction, In addition, heat-sensitive compounds are being preserved and the process prevent degradative enzymes to function (Zhang et al., 2009), (Bernard et al., 2014).

The degradation of polyphenols in seaweeds which further leads to a decrease in antioxidant activity when it is exposed to high temperature, is well discussed in the earlier sections. However, literature have reported that high radical scavenging activity, reducing capacity, and phenolic content, on oven-dried seaweeds and/or plants at 40°C (Ling et al. (2015), Bernard et al. (2014)), can be due to rapid inactivation of enzymes (Lim and Murtijaya, 2007). In addition, the most aggressive degradation of phytochemcials will occur at higher temperatures than 40°C (Ling et al., 2015). Lower antioxidant activity for the wet samples might be due to breakage of the cell wall and cell membrane during freeze-

and oven-drying, which makes the extraction of antioxidant compounds more efficient (Amorim et al., 2020). The drying process time might play a role in degradation of phytochemicals, the wet algae was only thawed for 24 h, while freeze-drying took 48 h and oven-dried 76 h. It is reported that a longer drying time leads to more degradation of phytochemicals (Ling et al., 2015).

However, the content of polyphenols and antioxidant activity in *S. latissima* and *A. esculenta*, as discussed, can be affected by high temperatures, long drying time and dehydration during drying process. Lastly, because of differences in cell walls and physiology for all species it can be concluded that no single drying methods can be considered as the most optimal (Cox et al., 2012).

Chapter 5

Conclusion

The results from the five different antioxidant activity assays varied both between *S. latissima* and *A. esculenta*, different drying methods, and different extraction solvents. It can therefore be concluded that more than one antioxidant activity assay is important for a full analysis of the macroalgae's antioxidant potential. *A. esculenta* demonstrated in general the highest antioxidant activity, and is probably a better choice as a functional food ingredient compared to *S. latissima*. High correlations were found between wet TPC and ABTS and DPPH radical scavenging activity, and a correlation was found for freeze-dried TPC and ABTS, concluding that polyphenols contribute to radical scavenging activity. No correlations were found for metal chelating ability and TPC, indicating that other compounds contribute to the metal chelating part of the antioxidant activity. Correlation coefficients between TPC and FRAP were low with $p > 0.05$ indicating no significant correlation, but the results demonstrated similar trends. The overall results indicate that polyphenols are playing a minor role as an antioxidant activity contributor.

Different extraction solvents affected the amount of phenolic compounds, radical scavenging activity, metal chelating ability and the reducing capacity differently. Where 70% acetone extracted out more polyphenols and demonstrated a better inhibition of free radicals, but water extracts demonstrated a higher ability to chelate transition metals. Ethanol exhibit the highest reducing capacity. In conclusion a mix of water and a less polar solvent, ethanol or acetone, would be the most suitable extraction solvent for achieving the highest antioxidant activity.

The drying methods demonstrated different effects on the antioxidant activity assays, but oven-dried samples exhibited highest metal chelating ability compared to freeze-dried. Freeze-dried is overall the best drying process alternative for *S. latissima* and *A. esculenta* for preserving phenolic compounds and displaying high antioxidant activity. In addition, freeze-dried samples even demonstrated a higher radical scavenging potential than wet control samples. The choice of drying methods depends upon preserving nutritional content, but also on production costs.

Chapter 6

Further work

Since there was no correlation between total phenolic content and the two radical scavenging activity assays (DPPH and ABTS) for the oven-dried samples, it could be interesting to investigate if fucoxanthin may contribute to the radical scavenging activity as well. In addition, no correlations were found between metal ion-chelating ability and total phenolic content, and it could therefore be interesting to find out if polysaccharides, proteins and peptides are capable to chelate metal ions as Saiga et al. (2003) suggested and mentioned in the discussion.

It can also be useful to investigate oven-dried *S. latissima* and *A. esculenta* at lower temperature, around 25°C - 30°C, because of the generally lower phenolic content and antioxidant activity observed for the oven-dried samples. Other extraction solvents, such as methanol, and a lower ethanol and acetone concentrations around 50-60% would also be interesting to study and compare those results with the 70% acetone and 70% ethanol extracts.

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Appendix

Statistical analysis

Standard deviation

Standard deviation for all measurements were determined by the built in Excel function STDAV.S with following formula;

$$\sigma = \sqrt{\frac{\sum (x_i - \bar{x})^2}{N-1}} \tag{6.1}$$

Significance analysis

Statistic significance was determined using the built in Excel function T.TEST and finds the p-value, $p < 0.05$ indicates significant differences between two data sets.

Calculation of total phenolic content

The total phenolic content was determined by:

$$TPC = \frac{cV}{m} \tag{6.2}$$

Where c is the concentration obtained by the standard curve, the concentration was further converted from [mM] to [mg/ml] with the molar mass of PG, 212.2 g/mol. V [ml] is the volume of solvent used in extraction and m [g] is the mass of algae. For wet and oven-dried samples the dry matter multiplied with the algae mass, and the dry matter percentage is shown in table 3.1 and 3.2). TPC given in mgPGE/g dried algae.

Standard curves for TPC by Folin-Ciocalteu assay
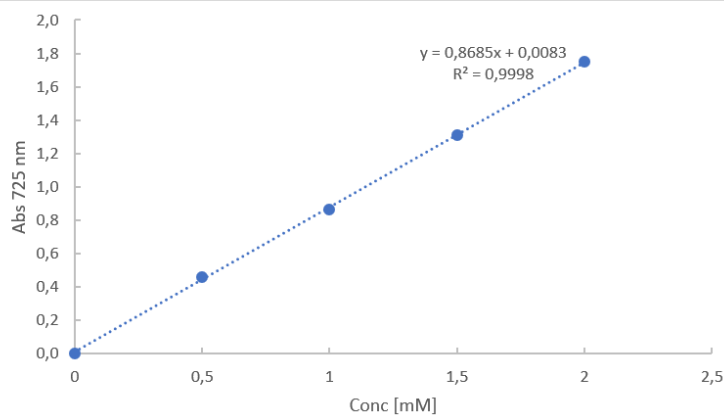
Figure 6.1: Standard curve of dilution series of propyl gallate for calculations of TPC for freeze-dried samples.
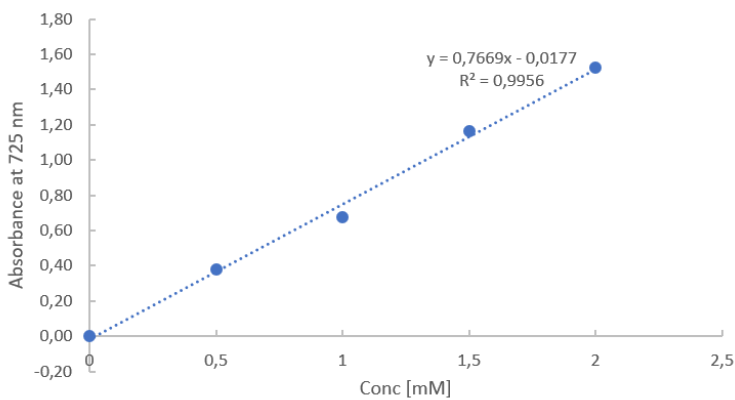
Figure 6.2: Standard curve of dilution series of propyl gallate for calculations of TPC for oven-dried samples.
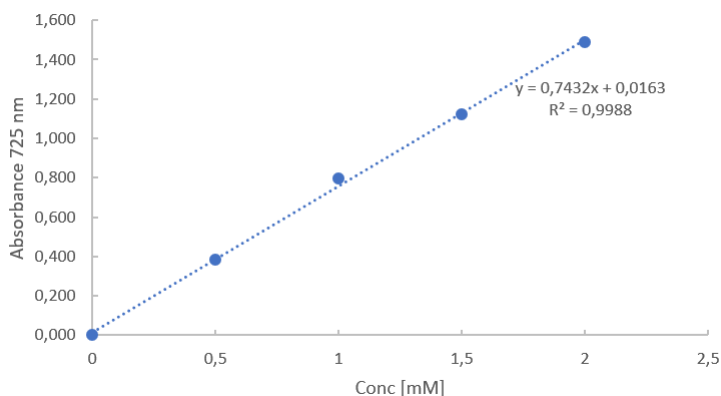
Figure 6.3: Standard curve of dilution series of propyl gallate for calculations of TPC for wet samples.

Absorbance measurements and TPC by the Folin-Ciacalteu assay, 725 nm

Freeze-dried

Extracts	Dilution factor	Abs 1	Abs 2	Abs 3	Avg.abs
S.L water		0,6	0,635	0,625	0,620
A.E water		1,157	1,22	1,171	1,183
S.L acetone	2	0,514	0,521	0,513	0,516
A.E acetone	3	1,174	1,23	1,161	1,188
S.L ethanol	3	0,155	0,15	0,146	0,150
A.E ethanol	3	0,313	0,322	0,316	0,317

Figure 6.4: Absorbance measurements for freeze-dried samples for TPC calculations.

Extracts	Conc [mM]	Conc [mg/ml]*DF	TPC [mg PGE/g dried algae]
S.L water	0,70	0,15	2,99
A.E water	1,35	0,29	5,67
S.L acetone	0,58	0,25	5,51
A.E acetone	1,36	0,86	17,30
S.L ethanol	0,16	0,10	2,24
A.E ethanol	0,36	0,23	4,45

Figure 6.5: Concentration and TPC determination for freeze-dried samples.

Oven-dried

Extracts	Abs 1	Abs 2	Abs 3	Avg.abs
S.L water	0,152	0,16	0,153	0,155
A.E water	0,245	0,23	0,253	0,243
S.L acetone	0,206	0,189	0,19	0,195
A.E acetone	0,159	0,157	0,144	0,153
S.L ethanol	0,163	0,145	0,141	0,150
A.E ethanol	0,138	0,135	0,13	0,134

Figure 6.6: Absorbance measurements for oven-dired samples for TPC calculations.

Extracts	Conc [mM] avg	Conc [mg/ml] avg	TPC [mg PGE/g dried algae] avg
S.L water	0,225	0,048	2,40
A.E water	0,340	0,072	5,67
S.L acetone	0,277	0,059	2,97
A.E acetone	0,223	0,047	3,60
S.L ethanol	0,218	0,046	2,32
A.E ethanol	0,198	0,042	3,30

Figure 6.7: Concentration and TPC determination for oven-dried samples.

Wet

Extracts	Abs 1	Abs 2	Abs 3	Avg.abs
S.L water	0,074	0,075	0,072	0,074
A.E water	0,25	0,247	0,245	0,247
S.L acetone	0,113	0,106	0,108	0,109
A.E acetone	0,417	0,412	0,42	0,416
S.L ethanol	0,075	0,069	0,071	0,072
A.E ethanol	0,344	0,342	0,339	0,342

Figure 6.8: Absorbance measurements for wet samples for TPC calculations.

Extracts	Conc [mM] avg	Conc [mg/ml] avg	TPC [mg PGE/g dried algae] avg
S.L water	0,077	0,016	2,81
A.E water	0,311	0,066	13,66
S.L acetone	0,125	0,026	4,74
A.E acetone	0,538	0,114	23,67
S.L ethanol	0,074	0,016	2,85
A.E ethanol	0,438	0,093	19,11

Figure 6.9: Concentration and TPC determination for wet samples.

Calculation of radical scavenging activity

The radical scavenging activity measured in percentage inhibition, for both DPPH and ABTS assay, were calculated in Excel with the formula below:

$$\text{Radical scavenging activity}[\%] = (1 - \frac{A_{sample}}{A_{blank}}) * 100\%$$

(6.3)

Where A_{sample} was the absorbance of the sample, and A_{blank} was the absorbance of the blank.

Absorbance and RSA [%] for DPPH assay, 517 nm

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,829	0,888	0,89	0,869	
S.L water	0,351	0,319	0,301	0,324	62,75
A.E water	0,595	0,579	0,289	0,587	32,45
S.L acetone	0,432	0,465	0,417	0,438	49,60
A.E acetone	0,272	0,275	0,272	0,273	68,58
S.L ethanol	0,643	0,612	0,607	0,621	28,58
A.E ethanol	0,482	0,622	0,523	0,542	37,59

Figure 6.10: Absorbance measurements and RSA [%] for freeze-dried samples, DPPH assay, value in red is neglected because of large deviation.

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,86	0,852	0,856	0,856	
S.L water	0,644	0,672	0,682	0,666	22,20
A.E water	0,5234	0,616	0,6	0,580	32,27
S.L acetone	0,788	0,794	0,597	0,791	7,59
A.E acetone	0,801	0,796	0,764	0,787	8,06
S.L ethanol	0,826	0,804	0,778	0,803	6,23
A.E ethanol	0,804	0,813	0,792	0,803	6,19

Figure 6.11: Absorbance measurements and RSA [%] for oven-dried samples, DPPH assay, value in red are neglected because of large deviation.

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,8	0,795	0,793	0,796	
S.L water	0,826	0,755	0,753	0,754	5,28
A.E water	0,691	0,59	0,676	0,652	18,05
S.L acetone	0,677	0,658	0,649	0,661	16,92
A.E acetone	0,326	0,342	0,323	0,330	58,50
S.L ethanol	0,762	0,725	0,754	0,747	6,16
A.E ethanol	0,5	0,481	0,438	0,473	40,58

Figure 6.12: Absorbance measurements and RSA [%] for wet samples, DPPH assay, value in red are neglected because of large deviation.

Absorbance and RSA [%] for ABTS assay, 734 nm

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,647	0,646	0,643	0,645	
S.L water	0,501	0,498	0,485	0,495	23,35
A.E water	0,517	0,516	0,508	0,514	20,40
S.L acetone	0,364	0,332	0,334	0,343	46,80
A.E acetone	0,167	0,065	0,081	0,104	83,83
S.L ethanol	0,491	0,472	0,472	0,478	25,88
A.E ethanol	0,414	0,384	0,349	0,382	40,75

Figure 6.13: Absorbance measurements and RSA [%] for freeze-dried samples, ABTS assay.

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,659	0,655	0,65	0,655	
S.L water	0,58	0,564	0,568	0,571	12,83
A.E water	0,633	0,615	0,611	0,620	5,35
S.L acetone	0,567	0,572	0,578	0,572	12,58
A.E acetone	0,609	0,617	0,62	0,615	6,01
S.L ethanol	0,607	0,562	0,596	0,588	10,13
A.E ethanol	0,638	0,629	0,605	0,624	4,68

Figure 6.14: Absorbance measurements and RSA [%] for oven-dried samples, ABTS assay.

Extracts	Abs 1	Abs 2	Abs 3	Abs. avg	RSA [%]
Blank	0,644	0,641	0,639	0,641	
S.L water	0,624	0,62	0,613	0,619	3,48
A.E water	0,614	0,606	0,61	0,610	4,89
S.L acetone	0,615	0,6	0,6	0,605	5,67
A.E acetone	0,532	0,508	0,512	0,517	19,33
S.L ethanol	0,623	0,615	0,615	0,618	3,69
A.E ethanol	0,572	0,55	0,544	0,555	13,41

Figure 6.15: Absorbance measurements and RSA [%] for wet samples, ABTS assay.

Absorbance and calculation of metal ion-chelating ability, 562 nm

The concentration of the extracts were 5 mg/ml and diluted 1:5, and the metal ion-chelating ability was calculated by the equation below, in Excel:

$$\text{Chelating activity } [\%] = (1 - \frac{A_0 - A_1}{A_2}) * 100\% \qquad (6.4)$$

Where A_0 was the absorbance of the extract (protein), A_1 was the absorbance of the sample (called blank in the materials and methods part) and A_2 was the absorbance of the control. Figures below show the metal chelating ability for different extracts, the values in the yellow marks are averages and the one used in the present study, red color of absorbance measurements is neglected for calculations because of large deviations.

S.L water	Abs 1	Abs 2	Abs 3	Abs.avg	A.E acetone	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,906	0,909	0,909	0,908	Protein	1,198	1,2	1,164	1,187
Sample	0,029	0,029	0,029	0,029	Sample	0,151	0,147	0,167	0,155
Control	1,115	1,117	1,111	1,114	Control	1,158	1,161	1,163	1,161
Chelating ability %	21,35	21,22	20,79	21,12	Chelating ability %	9,59	9,30	14,27	11,06

A.E water	Abs 1	Abs 2	Abs 3	Abs.avg	S.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,648	0,714	0,672	0,678	Protein	1,008	0,995	1,071	1,025
Sample	0,089	0,09	0,089	0,089	Sample	0,167	0,176	0,215	0,186
Control	1,112	1,107	1,126	1,115	Control	1,152	1,121	1,116	1,130
Chelating ability %	49,73	43,63	48,22	47,20	Chelating ability %	27,00	26,94	23,30	25,76

S.L acetone	Abs 1	Abs 2	Abs 3	Abs.avg	A.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	1,218	1,236	1,173	1,209	Protein	1,214	1,166	1,183	1,188
Sample	0,181	0,16	0,165	0,169	Sample	0,229	0,223	0,23	0,227
Control	1,156	1,159	1,157	1,157	Control	1,157	1,154	1,157	1,156
Chelating ability %	10,29	7,16	12,88	10,11	Chelating ability %	14,87	18,28	17,63	16,93

Figure 6.16: Absorbance measurements and calculation for metal chelating ability for freeze-dried samples.

S.L water	Abs 1	Abs 2	Abs 3	Abs.avg	A.E acetone	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,863	1,081	0,849	0,856	Protein	0,079	0,105	0,091	0,092
Sample	0,09	0,085	0,088	0,088	Sample	0,025	0,025	0,025	0,025
Control	1,113	1,079	1,11	1,101	Control	1,159	1,142	1,131	1,144
Chelating ability %	30,55		31,44	30,19	Chelating ability %	95,34	92,99	94,16	94,17

A.E water	Abs 1	Abs 2	Abs 3	Abs.avg	S.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,19	0,18	0,196	0,18866667	Protein	0,236	0,27	0,258	0,255
Sample	0,086	0,089	0,089	0,088	Sample	0,017	0,017	0,024	0,019
Control	1,078	1,113	1,114	1,10166667	Control	1,12	1,126	1,127	1,124
Chelating ability %	90,35	91,82	90,39	90,86	Chelating ability %	80,45	77,53	79,24	79,07

S.L acetone	Abs 1	Abs 2	Abs 3	Abs.avg	A.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,9	0,882	0,878	0,88666667	Protein	0,079	0,101	0,097	0,092
Sample	0,022	0,021	0,022	0,022	Sample	0,025	0,025	0,021	0,024
Control	1,097	1,106	1,113	1,105	Control	1,126	1,113	1,11	1,116
Chelating ability %	19,96	22,15	23,09	21,74	Chelating ability %	95,20	93,17	93,15	93,85

Figure 6.17: Absorbance measurements and calculation for metal chelating ability for oven-dried samples.

S.L water	Abs 1	Abs 2	Abs 3	Abs.avg	A.E acetone	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,664	0,824	0,51	0,587	Protein	0,931	0,919	0,944	0,931
Sample	0,063	0,066	0,067	0,065	Sample	0,205	0,207	0,269	0,227
Control	1,101	1,104	1,099	1,101	Control	1,117	1,121	1,118	1,119
Chelating ability %	45,41		59,69	52,63	Chelating ability %	35,00	36,49	39,62	37,04

A.E water	Abs 1	Abs 2	Abs 3	Abs.avg	S.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,245	0,296	0,425	0,322	Protein	0,868	0,854	0,822	0,848
Sample	0,239	0,228	0,265	0,244	Sample	0,074	0,075	0,077	0,075
Control	1,114	1,086	1,104	1,10133333	Control	1,102	1,077	1,121	1,100
Chelating ability %	99,46	93,74	85,51	92,92	Chelating ability %	27,95	27,67	33,54	29,76

S.L acetone	Abs 1	Abs 2	Abs 3	Abs.avg	A.L ethanol	Abs 1	Abs 2	Abs 3	Abs.avg
Protein	0,663	0,65	0,639	0,65066667	Protein	1	0,982	0,971	0,984
Sample	0,07	0,07	0,072	0,071	Sample	0,255	0,264	0,286	0,268
Control	1,077	1,09	1,08	1,083	Control	1,09	1,114	1,098	1,101
Chelating ability %	44,94	46,79	47,65	46,46	Chelating ability %	31,65	35,55	37,61	34,95

Figure 6.18: Absorbance measurements and calculation for metal chelating ability for wet samples.

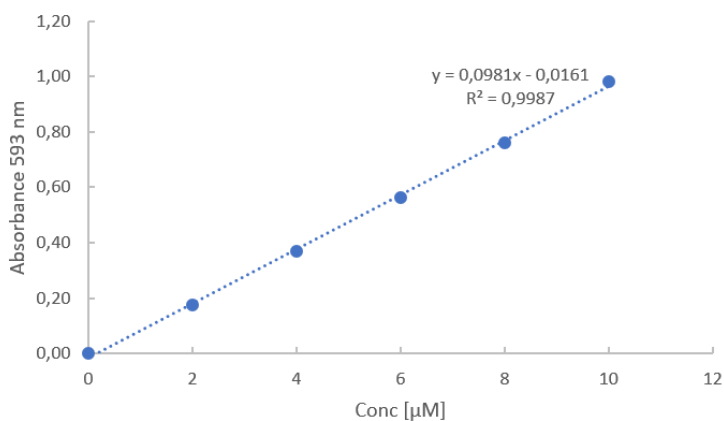# Standard curves of propyl gallate for FRAP assay

Figure 6.19: Standard curve for propyl gallate dilution series, used for the freeze-dried algae samples.
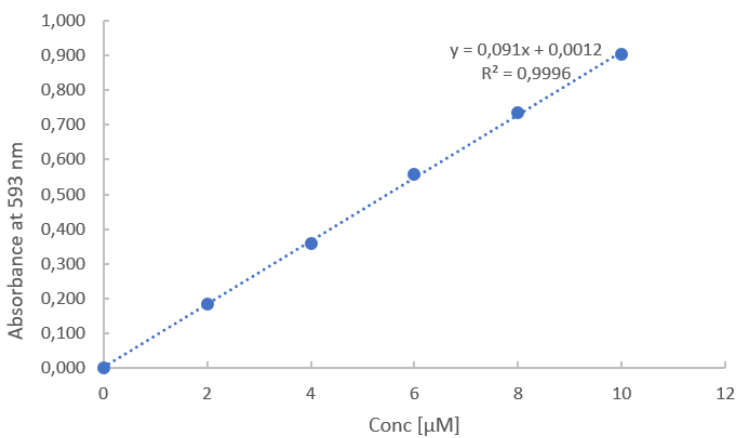

Figure 6.20: Standard curve for propyl gallate dilution series, used for the oven-dried algae samples.
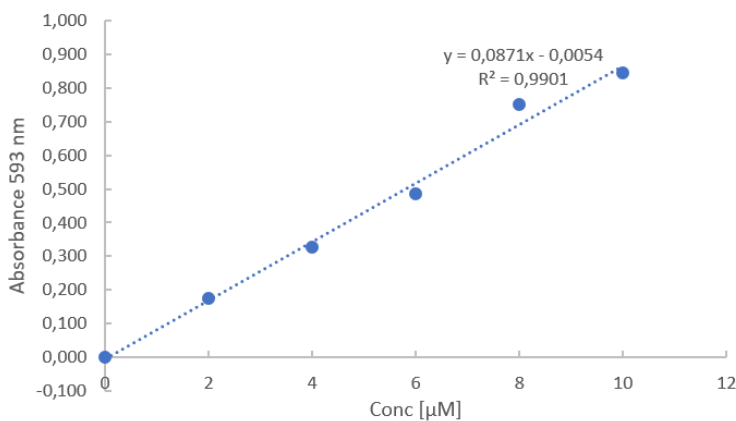
Figure 6.21: Standard curve for propyl gallate dilution series, used for the wet algae samples.

Absorbance measurements for FRAP assay, 593 nm and calculation of reducing capacity

Reducing capacity was measured in mgPGE/g dry extracts. Concentrations in μM were calculated from standard curves of PG and further converted to mgPGE/ml by PG molar mass, 212,2 g/mol. Lastly the PGE concentrations were divided with sample concentration 0.025 g/ml, obtaining mgPGE/g dry extracts. Here the dry mass are freeze-dried algae extracts powder.

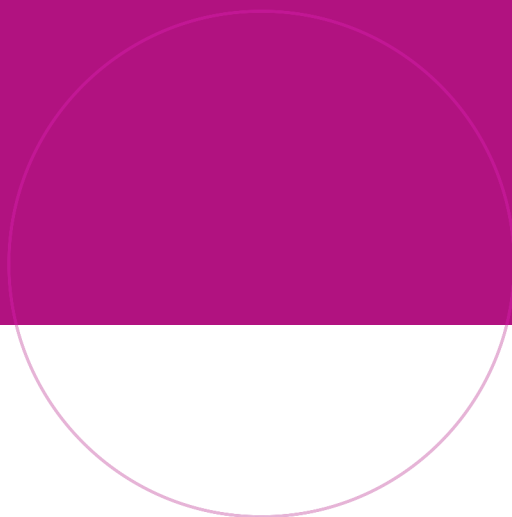| FREEZE-DRIED						
Extracts	DF	Abs 1	Abs 2	Abs 3	Abs avg	
Blank		0,035	0,042	0,045	0,041	
S.L water		0,54	0,677	0,677	0,631	
A.E water		0,611	0,674	0,706	0,664	
S.L acetone	5	0,2	0,216	0,229	0,215	
A.E acetone	5	0,248	0,279	0,295	0,274	
S.L ethanol	5	0,217	0,238	0,246	0,234	
A.E ethanol	5	0,263	0,278	0,294	0,278	
		Conc [µM]	Conc [µM] *5	Conc [mg/ml]	mg PGE/g dw	
Blank		0				
S.L water		6,60		0,001400462	0,056	
A.E water		6,93		0,001470403	0,059	
S.L acetone		2,36	11,78	0,002499461	0,100	
A.E acetone		2,96	14,79	0,003137575	0,126	
S.L ethanol		2,55	12,73	0,00270135	0,108	
A.E ethanol		3,00	15,01	0,003184442	0,127	

Figure 6.22: Absorbance measurements at 593 nm and calculation of FRAP-values for freeze-dried samples.

OVEN-DRIED	Abs 1	Abs 2	Abs 3	Abs avg	
Blank	0,021	0,022	0,023	0,022	
S.L water	0,308	0,336	0,344	0,329	
A.E water	0,413	0,494	0,496	0,468	
S.L acetone	0,286	0,273	0,297	0,285	
A.E acetone	0,471	0,506	0,504	0,494	
S.L ethanol	0,266	0,285	0,303	0,285	
A.E ethanol	0,278	0,296	0,293	0,289	
	Conc [µM]	Conc [mg/ml	mg PGE/g dw		
Blank	0,000				
S.L water	3,606	0,00076516	0,0306		
A.E water	5,126	0,00108774	0,0435		
S.L acetone	3,122	0,00066256	0,0265		
A.E acetone	5,412	0,00114837	0,0459		
S.L ethanol	3,115	0,00066101	0,0264		
A.E ethanol	3,163	0,00067111	0,0268		

Figure 6.23: Absorbance measurements at 593 nm and calculation of FRAP-values for oven-dried samples.

WET	DF	Abs 1	Abs 2	Abs 3	Abs avg
Blank		0,026	0,024	0,024	0,025
S.L water		0,315	0,391	0,457	0,388
A.E water	3	0,413	0,494	0,496	0,468
S.L acetone	3	0,3	0,341	0,361	0,334
A.E acetone	5	0,423	0,455	0,492	0,457
S.L ethanol	5	0,208	0,218	0,221	0,216
A.E ethanol	5	0,735	0,803	0,849	0,796
		Conc [µM]	Conc [µM]*DF	Conc [mg/ml]	mg PGE/g dw
Blank		0			
S.L water		4,5		0,000957621	0,038
A.E water		5,4	16,29	0,003457569	0,138
S.L acetone		3,9	11,69	0,00248062	0,099
A.E acetone		5,3	26,53	0,005628619	0,225
S.L ethanol		2,5	12,69	0,002692902	0,108
A.E ethanol		9,2	45,99	0,009758114	0,390

Figure 6.24: Absorbance measurements at 593 nm and calculation of FRAP-values for wet samples.