Master's thesis

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The effect of maturation process on chemical and sensory properties of Palmaria palmata

Master's thesis in Biotechnology
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May 2019

NTNU
Kunnskap for en bedre verden

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Science and Technology

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Submission date: May 2019

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Abstract

The high demand for sustainable and healthy food is caused by the rapid growth of the world population. The ocean has an enormous potential to produce food for humans and feed for animals. However, this potential has not been fully exploited despite the high potential ocean food-producing. Macroalgae, both cultivated and wild harvested are popular in Asian countries, while it is mainly through wild harvesting for Western countries. Macroalgae as food for humans and feed for animals has not much been utilized in the West especially in Europe.

This thesis is a part of the PROMAC project which has worked on three macroalgae species *Alaria esculenta, Sacchina latissima* and *Palmaria palmata*. The objective of this master thesis is to evaluate how the storage conditions with different moisture content in the *P. palmata* (Dulse) samples affect chemical and sensory characteristics. The effective storage conditions should not only maintain the soluble protein content but also improve the flavor of the seaweed.

The red seaweed *P. palmata* harvested in France in November 2017 was the study target. The moisture content (MC) and water activity (a_w) of air-dried samples were around 6% and 0.28 respectively. While in matured samples, MC was 20% with an a_w approximately 0.62.

For the total soluble protein content, the commonly used Lowry method was applied. According to the obtained data, the air-dried control sample D-126 showed the highest level of total soluble protein content with 30.33 mg/g DW. After 126 storage days, there was an increase by 8mg/g DW from 22.17 mg/g DW in D-0 sample to D-126 sample. The remaining samples in the same testing group did not show any changes in total soluble protein level after storage. However, the FD control group resulted in a significantly lower value with only around 11 mg/g DW. The total soluble protein contents in all matured samples were around 21 mg/g DW.

The total soluble free amino acids (FAA) content of all samples was evaluated by HPLC. The total soluble FAA level decreased by 50% in both control groups. The freeze-drying step after storage did not affect the total soluble protein level since no difference was

detected between D-126 and D-126 NFD samples. Only the total soluble protein level in the semi-hydrated samples stayed the same regarding different storage days.

The FAA profile from HPLC results showed that glutamic acid was the most abundant, followed by alanine and aspartic acid. The content of glutamic acid and aspartic acid decreased after 126 days of storage in both FD and D control groups. However, the level of alanine declined during the maturation time only in the FD control group to half the value compared to this level the FD-0 sample. While the three semi-hydrated M-12, M-61 and M-126 samples showed the stable amount of these 3 FAA regardless of the storage days.

From the sensory analysis, the control sample D-126 and the early matured samples M-12 had stronger marine taste including seaweed and fish skin aroma and dried-fish in flavor. These two samples also were more tough and crunchy in texture. Whereas, the marine taste faded, and the texture was softer in longer matured time sample. The two samples M-61 and M-126 had sweeter taste. M-61 had the highest score in hay aroma and the least score in a bitter taste.

According to the outcome of the presented analysis, M-61 samples showed a stable amount of total soluble protein and FAA. Moreover, M-61 not only had a less marine and bitter taste with softer texture but also richer in sweet taste and flavor richness as umami taste. This is the most optimal sample among the 4 sensory testing samples.

Although 20% in moisture content showed a stable amount of total soluble protein and free amino acids content, further studies are needed to find out the optimal moisture content and time of maturation to obtain higher value in protein and a richer flavor.

Acknowledgments

From the bottom of my heart, I would like to thank Professor Turid Rustad who has guided me from the very first step. She is always available and helpful, the most dedicated and kindest teacher I have ever met.

Special thanks to Oskar Speilberg and Veronica Hammer Hjellnes who has instructed me through all the lab methods and analysis.

To Pierrick Stévant, my co-supervisor, for patiently helping me with all the questions I had during my thesis and always responded quickly.

To my friend, study mate and lab partner Monika Katarzyna Kopczyk from whom I have learned a lot from doing my lab work with her. I would not have felt that much of fun in studying if we have not been friends.

To Siri Stavrum for being in charge of a big part of my thesis by running HPLC samples.

To my boyfriend Ronny Andre Værnes for always supporting, staying by my side and lifting my mood up.

And the last is my family, my dearest people, for encouraging and basically always be there for me when I need. Without them, I would never have had a chance to study at NTNU.

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1 Introduction

The world population has steadily increased and is expected to reach 9.6 billion by the year 2050 (1). The increase in population results in high demand for food. Today hunger kills more people than AIDS, malaria, and tuberculosis combined amounting to approximately 9 million people every year (2). While the ocean is responsible for 50% of the earth's biological production, only 2% of the average calorie intake from food originates from the ocean (3). The available land for agriculture is limited and the potential to utilize the ocean for food production should be exploited.

This thesis is a part of a project which studies seaweed as food for human and animal feed applications. The project investigated 3 specific types of seaweed *Alaria esculenta, Sacchina latissima,* and *Palmaria palmata.* The project duration was from 2015 to 2018. Several organizations as research partners in the project including Møreforsking, SINTEF, NIBIO, NTNU, NMBU, Matís, Ceva and Slu. The benefits, cost, nutrient value, quality and different primary processes combined with several extraction methods have evaluated the benefits of the 3 algae types for Norway commercial potential. The project was funded by the Research Council of Norway, Sustainable Innovation in Food and Bio-based Industries Program (4). With the new knowledge from the results of this project, macroalgae will have a chance to become an alternative high-quality food for humans and feed for animals.

1.1. Macroalgae and *P. palmata*

1.1.1 Macroalgae

Macroalgae, common name seaweed, are multi-cellular and macroscopic organisms. They are taxonomically divided into green (phylum Chlorophyta), red (phylum Rhodophyta) and brown (phylum Phaeophyta) groups (5). They can perform photosynthesis as the other marine plants, but the roots, leafy shoots, flowers and vascular tissues are missing. The different colors in

seaweed depend on the pigment, the absorbed wavelength in water and the location where they locate. The main pigments have been found in algae are carotenoids, chlorophylls, and phycobiliproteins (6). Among that, chlorophylls present in all groups of macroalgae with varying amounts depending on the types, the extraction buffer and the quantitative methods (7).

The green seaweed needs good levels of light which explains their shallow and open water location at the ocean. This type only uses the chlorophyll pigments from both chlorophylls a and b to absorb red and blue light respectively and reflect green color. While the red seaweed locates at deepest depth among the three types due to the low intensity and light level in their life. Red seaweed uses phycoerythrin and phycocyanin to absorb blue light and reflect red light resulting in the red color. The final in the three types of seaweed is brown seaweed which has the most pigments. The branching structure with a high number of tiny floats maximizes the capacity in light absorbing and makes it buoyant. Brown seaweed is found in deeper water compares to the green seaweed due to the lower requirement in light intensity. Chlorophyll a, b and d can be found in this type of seaweed. And the brown color is the result of the reflection of blue, green and yellow light to brown (8).

Macroalgae have several ecological functions such as photosynthesis, reef framework, and habitats for many species. Macroalgae were recorded to be responsible from 50% up to 70% oxygen production and 25% of total carbon monoxide uptake in the world (9).

The chemical compounds in seaweed have caught the interest and curiosity of scientists for a long time. The composition of seaweed varies greatly among different groups (10). The content of polysaccharides ranges from 4% to 75% of seaweed dried weight and protein varies from 10% to 30%. In addition, it was also found with low-fat content 1% to 5%, phenolics compounds and rich of minerals such as Ca, Mg, Na and P, but extremely low in Zn, I and Mn (11). Moreover, seaweed has been well known not only for low-calorie content but also for a high amount of nutrients such as vitamins, minerals, antioxidants, proteins and dietary fiber in some species (12). Furthermore, it has been shown that other polysaccharide compounds actively aid to fight obesity and diabetes. Seaweed is a rich source of antioxidant carotenoids (13), anticoagulants and antibacterial (14)(15)… All these compounds contribute to the potential as a promising commercial and sustainable food for humans and feed for the animals (16).

Besides food, seaweed has been used for a wide variety of purposes. In the industry, some compounds have been extracted including agar, alginate and carrageenan for gel or thickening, pigments and antioxidants such as chlorophyll and carotenoids…(17) Among those compounds, alginate extraction mainly from brown seaweed *L. hyperborean (*kelp) has been used as an important seaweed extract substance. Besides the common usage as food thickening or emulsified substance, alginate multi-function in the biopharmaceutical industries as drug delivery, tissue engineering and wound healing due to its vast availability, low price, great biocompatibility and easy to modify (18). The applications of alginate are important in Norway leading by the company FMC Biopolymer. The company produces ultra-pure alginate from *L. hyperborea* for pharmaceutical, industrials and research products which had 1.5 billion NOK avenue in 2016 (19). FMC has used kelp as a sustainable and renewable resource for alginate with an annual production of 150 000 – 160 000 tons (20). As a result, other countries have tried to copy Norway for this sustainable production.

In agriculture, seaweed extracts adding to the field for growing plants has positive effects on phytochemical compounds, growth, and yield. In 2013, Theodoura and her colleagues found that 3 seaweed extracts stimulated the content of flavonoids and other phenolics compounds in cabbages with different levels (21). Another study showed that by the addition of seaweed extract to the soil, the betaines compound in the extract enhanced the chlorophyll level in tomato leaf (22). Furthermore, different ways of how seaweed extract benefit plants have been reported comprising leaf development, susceptible improvement of weather and disease tolerance (23)(24)(25).

1.1.2 Seaweed usage and production around the world

In Asia, particularly China, Japan, and South Korea, seaweed has been an important and familiar food for centuries. It has been written that the Chinese were the earliest to use seaweed for food. This has a history that is more 2000 years long. Seaweed was prepared in a wide variety of different ways. It was cooked as a soup, stir-fried, steam pan fry to be as filling in dumplings (26). In Japan, it has been universally known for “nori” as sushi wrap from the red algae family *P. yezoensis* and *P. tenera* (figure 1). The same species are used for kimbap in Korea. Kimbap is a Korean wrapping dish which has a similar form as sushi. The difference between kimbap and sushi is the rice and filling. Kimbap is made from rice seasoned with

sesame oil instead of vinegar added in sushi rice. The filling is from plentiful premade materials including tuna, fried egg, vegetables, pork cutlets, cooked beef, cheese…

In Europe, only a few countries have used seaweed as food. Historical documents record the usage in Ireland, France, Iceland, and Norway. Uses of seaweed as a dish was more common during World War II, after that time the consumption of seaweed dropped sharply. However recently, with the high demand for healthy and sustainable food, using seaweed as food has become more popular in Western countries. In Norway and Ireland, it was mainly *P. palmata* that was used. Ireland has a long tradition of using Irish moss (*Chondrus crispus*) as a part of milk dishes. Seaweed products are packed in plastic bags can be found in the supermarkets in some countries such as Ireland, Spain, Iceland, Norway, Denmark, Germany, Italy… Among European countries, France was reported as the most outstanding country to import seaweed for human consumption in 2016 with more than 9 million $ in value (27).

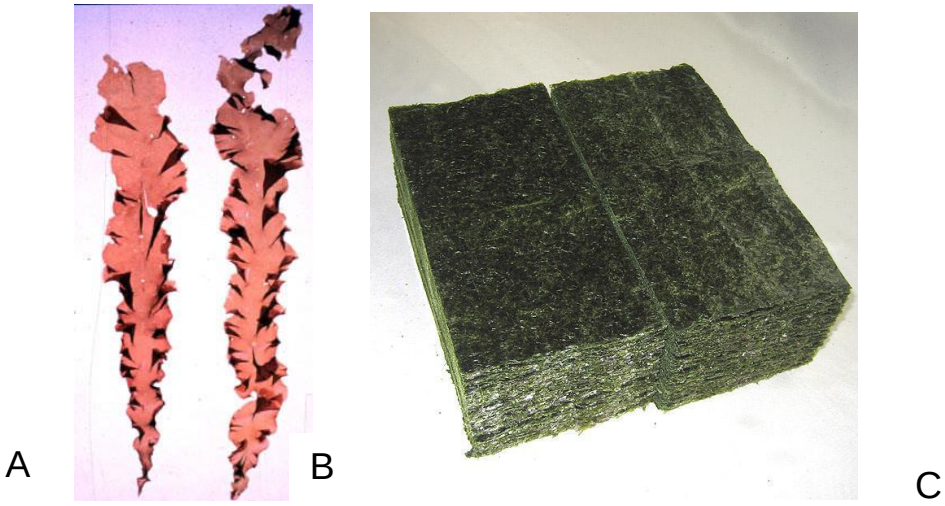

Figure 1 Picture of the red algae used for nori in Japan

A. P. yezoensis raw

B. P. tenera raw

C. Processed nori

The pictures were taken from http://www.seaweed.ie/aquaculture/noricultivation.php and https://alicegordenker.wordpress.com/2013/02/19/how-is-nori-made/

Overall, it was recorded that China and Indonesia were the leading countries in seaweed production with more than 10 million tonnes followed by the Philippines and South Korea with 1 million ton in 2016 (28). The large scale seaweed farming cultivation has been applied in

other Asian countries such as Japan and Malaysia, while for Europe, Canada and Latin America the industries it is mainly wild harvesting (29).

1.1.3 Seaweed applications in Norway

Norway has several advantages in seafood production with long coastlines extend to over 100,000 km and cold Northeast Atlantic water. This has contributed to Norway's position as the second largest seafood exporting country in the world. Norway has a long tradition of using seaweed. It has been used as fertilizer and to produce ash. Both as used in the production of glass. Later, seaweed was used as an iodine source. The period was from the end of the 17th century to the year 1930.

There has been ongoing research to utilize seaweed for industrial applications mainly with wild harvested seaweed. *Laminaria hyperborean* (forest kelp) and *Ascophyllum nodosum* have been the main species for biomass production in Norway. Since 2005, studies on seaweed cultivation (*Saccharina latissimi*) techniques at the sea has been carried out. This started because of the success in seaweed farming in France and Germany. The demand to find the optimal cultivation technique and upscale the production of biomass has risen. Therefore, several research units such as SINTEF collaborating with private companies to upscale the biomass production of *Saccharina latissimi* (sugar kelp) to industrial scale. There has been researching and pilot productions but the first permission for commercial seaweed cultivation came in 2014. This was licensed by Integrated Multi-Trophic Aquaculture system for large scale cultivation. Since that time, the number of companies that applied seaweed large scale cultivation technique increased from 6 to 83 (2014 to 2018) and the seaweed cultivation production skyrocketed from 51 tons in 2015 to 149 tons in 2017 (30).

The mentioned rapid development of seaweed cultivation is caused by the demand for both human food and animal feed. There are several reasons as well as proofs for the increased interest in seaweed as food. For human consumption in the last 10 years, sushi has been introduced to Norway from Japan and has become increasingly popular. For now, it is not difficult to find a sushi store in big cities in Norway, although from approximate 40 years ago, it was quite rare that a Norwegian knew what sushi was. Another reason is getting inspired by the cooperation with Korea, some restaurants and students in Frøya high school organized the

first series in a workshop that focuses on seaweed as food (31). As one of the leading countries that support a sustainable lifestyle, Norwegian prefers an alternative and healthy food type. Seaweed is a promising candidate for that. Several restaurants have caught that trend and included it in their menu including Smalhan in Oslo and Credo in Trondheim. Recently, Zoe Chritiansen and her co-workers published a book name "Tang og tare, et hav av mat" (Seaweed, an ocean of food) which introduced a wide range way of seaweed cooking along with cooking classes.

In animal and fish feed, it is also worth to mention that seaweed has potential as a new protein source. Norway is one of the leading countries in exporting salmon and trout. This leads to an enormous demand for fish feeding. It was estimated that by 2050 the need for salmon feed could be 6 million which are 6 times higher than the need today (32). Seaweed has varying protein content but can be up to 40% dry weight is a potential alternative source for animal feed in the future.

Seaweed has been also applied for non-food purpose, European countries including Norway has shown interest in using kelp as a source to produce biofuel due to the high level of carbohydrate in the dry weight. Two studies in 2012 and 2014 described a successful protocol using brown algae as alginate feedstocks for genetically modified yeast to produce ethanol for biogas (33)(34). Belonging to one of the biofuel products, biogas from macroalgae as feedstock was another application (35). Due to the low level of cellulose and lignin, it can be more efficiently hydrolyzed than conventional material wood. Tedesco and his collaborates described an optimal protocol of the mechanical treatment in 10 minutes at 53^{o}C of *Laminariaceae* yielded 52% extra biogas as well as 53% extra methane than the unprocessed samples (36).

1.2. The background for this study

This thesis is one step in the PROMAC project to find a novel raw material for human food and animal feed. There were 3 macroalgae as the target in this project *Alaria esculenta, Sacchina latissima,* and *Palmaria palmata*. The project included several different stages from evaluating the composition and quality of seaweed harvested from wild and cultured resources. Also developing an optimal primary process and extracted methods to assess the nutritional values of macroalgae for both human and animal. This thesis belongs to the 2nd stage

developing an optimal primary maturation process which has not only high nutritional values but also improving the taste of *P. palmata*.

The primary aim of the work done in this thesis has been to understand how maturation process including partial moisturization and storage for different times affect the moisture content (MC), water activity, sensory properties and phytochemical properties characterization of *P. palmata*. Among those parameters, the sensory property is the most important because of the limited information and the importance of product perception.

Several factors influence people when they buy food including taste, cost, nutritional value, convenience, and weight control but the taste has been reported as the most important factor (37). The taste of the food has a critical role in people's decision which food they will eat. One of the most common unpleasant feelings that were found in seaweed taste in a study was "fishy, marine taste and tough texture" (38). To overcome this barrier, this study was set up to improve the taste of seaweed and find out the optimal maturation condition to retain the high nutritional compound in the red seaweed *P. palmata.*

1.2.1 What do we know about P. palmata?

The red macroalgae *Palmaria palmata (P. palmata),* also known as Dulse, is the target species of this study. It was considered as a tasty but unexploited food material especially in the Western world by Mouritsen 2012 (39). Cold Atlantic water in the North and North-East is the habitat of this species. Macroalgae have not been known as a familiar ingredient in Europe, but the red algae are an exception. It belongs to one of the few algae that have been used as human food for centuries or even millennia. In the wild, the color purple or brownish-red is the most common, however under drying and bleaching by the sun it can show lighter color as pinky-red.

In Europe, the idea of eating seaweed probably began from Ireland because the old written records reported that Dulse was used as a spice with bread, butter, and milk in the 5^{th} century. Irish people have used Dulse in various ways such as snack, soup ingredients, dried salted seaweed, eaten raw as the outer wrap around baked fish… This is described in the book "Irish seaweed kitchen" of Dr. Prannie Rhatigan (40). It was believed that the trend in eating Dulse migrated to England as "cow weed" to Iceland and then to Norway as "horse seaweed". Into the modern household kitchen, Dulse can be eaten together with bread, fish or ingredients in

vegetable soups. Another tasty way to prepare it is adding a little oil and roasting on the pan which serves as a perfect snack with beer. It is interesting that young and fresh Dulse can be eaten as a raw salad after being soaked in water. Drying seaweed before being packaged into plastic bags has been applied in many places to prevent the microbial spoilage and reduced the weight in the transporting process. Dulse has a high content of umami flavor because it releases more sweet amino acid comparing to the dashi from Japan (41). With the high demand for sustainable and healthy food as a replacement, this species possesses a promising opportunity to appear in New Nordic Cuisine.

1.2.2 Chemical composition of *P. palmata*

Like other macroalgae, Dulse has high moisture content from 73% to 89% (wet weight), the amount of protein and carbohydrate level is 8%-35% and 38%-74% respectively; while ash occupies 12-37% of dry weight and only 0.2-3.8% in lipid (42). In the same study, *P.palmata* was found to have a wide range of mineral elements particularly high in chlorine, potassium, and sodium, while iron, aluminum and zinc are dominant among the trace elements. In addition, the variation in composition mainly depends on the season, drying method and source of harvesting, geology difference and extracting methods (43)(44)(45). Heat treatment also contributes to the bioaccessibility leading to an increase in nutrient value (46). In addition, there are some pigment compounds in Dulse including chlorophyll α (47), allophycocyanin, R-phycoerythrin and β-phycoerythrin (48)…

Table 1 Amino acid composition of *Palmaria palmata*

Amino acidds	Schilichting & Purdom, 1969 (49) mg/g DW	Munda Gubensek, 1976 (50) mg/g DW	Mai K et al., 1994 (51) %/ total AA	Anne-Valéria et al., 1999 (52) %/ total AA
Asp	2.05	1.84	18.5	9.3
Glu	2.36	1.00	9.9	13
Asn	-	-	-	-
His	0.29	0.26	0.5	2.1
Ser	1.21	0.67	6.3	4.6
Gly	1.30	1.02	13.3	7.2
Arg	1.37	0.87	5.1	6.2
Thr	1.04	0.87	3.6	3.6
Ala	1.64	1.32	6.7	7.5
Tyr	-	-	3.4	4.5
Aba	-	-	-	-
Met	2.0	0.39	2.7	1.9
Val	1.37	1.05	6.9	7.3
Phe	1.44	0.71	5.1	5.2
Ile	1.06	1.02	3.7	5.3
Leu	1.84	1.05	7.1	7.8
Lys	1.63	0.83	3.3	8.2

The amino acid composition has been characterized by several researchers. The results of four of these are given in table 1. The results of Schilich 1969 showed that glutamic acid, aspartic acid, and methionine were the most abundant (49). Later, Munda et al 1976 had the same conclusion about aspartic acid and glutamic acid, but valine and leucine accounted for the 3rd and 4th highest level (50). Data from both studies were expressed in mg/g drey weight (DW) unit. The other studies gave the chemical composition of *P. palmata* in % of total amino acid. In 1994, Mai K et al presented that aspartic acid occupied the highest content, followed by glycine and glutamic acid. In 1999, Anne-Valéria's group studied Dulse from France monthly except for August. And the result confirmed that aspartic acid had the highest content followed by glycine and glutamic acid as reported in the study of Mai K et al. It was interesting that the protein content in this study varied according to the season and was 21.9 ± 3.5 % in winter-spring period, while in the summer and early fall it was only 11.9 ± 2.0% (52). The most recent study about this species was *P. palmata* collected May and December 2015 in Norway. P.

palmata harvested in December 2015 total protein content was analyzed by acid hydrolysis method and was 11.6 ± 0.3 % dry weight. The study also presented the most abundant free amino acids (FAA) were glutamic acid, followed by aspartic acid and alanine by HPLC method for *P. palmata* collected in May 2015. The study did not evaluate the FAA content from *P. palmata* harvested in December 2015 (53).

1.2.3 The related parameters and applied method

1.2.3.1 Moisture content (MC) and water activity (a_w)

Water plays a key role and is an essential part of all living organisms. It is involved in many biological processes from biological reactions to the transport process. In food, water is usually considered in the term of moisture content (water content) or water activity a_w. Moisture content (MC) in food is defined as the percentage of the mass of water in the food compared to the total mass of the food sample given as the formula below (54).

$$\%\, moisture = \frac{Mwater}{Msample}\ x\ 100$$

MC can affect the food sensory though its effects on the food texture. The sensory of food texture is highly dependent on the food structure. When food is handled or eaten, food structure is broken and deformed. Any processes affect this change in structure will have a strong impact on the food texture. Packing, storage conditions, processing method…, all these can change the structure of the food. MC together with fat content plays a key role in the perception of food structure. The lower MC in the food, the more saliva it requires to cover the surface of food and moisturize it. While in the fresh solid food with high MC, the rapid release of high moisture content leads to higher pleasing for mouthfeel. As a result, higher MC is one of the reasons for the satisfying feeling in the mouth.

Another important factor that discrepancies in MC causes is the difference in the breaking size of food after being chewed compared to the original size. The newly formed particles have a massive impact on human eating experience and strategy. It is because food particles need to be chewed until reaching a thresh hold size and then mix with saliva and to be swallowed (55). The particles forming after being chewed can be measured by a breakage function parameter (56). This is a quantitative parameter giving the measurement of how solid food is broken and

fractured. The breakage function can be quantified by the ratio between the weight of newly broken food particles which are below half of the original food size. Dry solid food is highly fragmented during the chewing process due to the crispiness, crunchiness, and crumbliness in low MC leading to high breakage function. While in wet solid food, it requires more chewing in order to break the food down to the same size as in dry solid food. Wet solid food has low breakage-function. Therefore, those listed factors caused by variety level of MC can cause major differences in food texture both in wet and dry solid foods (57).

Water activity (a_w) is known through the following equation where aw is water activity, pw is the partial water vapor pressure of water above the testing food sample and pow is the water vapor pressure of pure water. Both are measured at equilibrium in the same temperature (58).

$$aw = \frac{pw}{pow}$$

The a_w reflects the thermodynamic activity of water in the food. This is a measure of the water which is available for microbial and chemical reactions. By controlling this factor, the food industry can limit microbial spoilage and improve food stability (59). These two terms are positively correlated to each other non-linearly following sigmoidal curve presented in figure 2 and also are two of the testing parameters in this study.

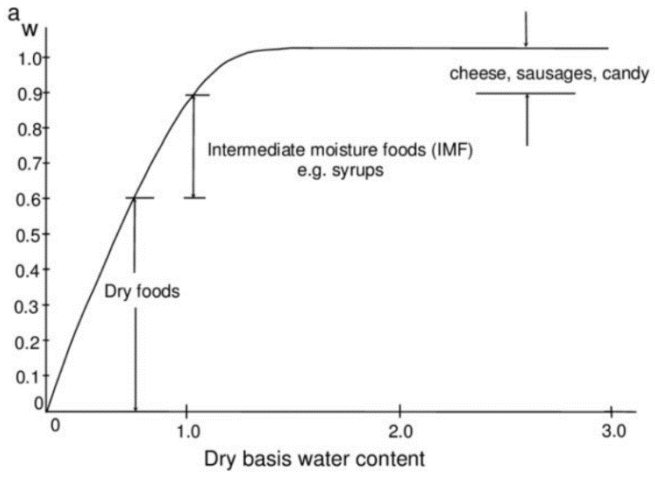

Figure 2 Water activity versus moisture content plot for different food types

Available at https://www.researchgate.net/publication/318456369_Mass_transport/figures

1.2.3.2 Sensory analysis and Principle component analysis (PCA)

Sensory quantity is defined as the aspects of texture, taste or flavor, aroma and visual. And sensory analysis is one method to assess the sensory quantity of food. Sensory analysis is defined as a scientific discipline need to evoke, measure, analyze and interpret to a product perception using 5 basic senses of the human being including vision, smell, touch, taste, and hearing (60). The sensory analysis used as a scientific method and when it is carried out with appropriate and qualified panel, it can give accurate and sensitive results.

There are 3 main basics testing methods: difference, affective and descriptive testings. The first test can be considered as the simplest form indicating if there are any perceptible differences between two kinds of product. The typical examples of these tests are the triangle, duo-trio and paired comparison tests (61). While the second one, known as hedonic testing, is ranking. It is not only for like/ dislike attitude but also for the color or texture of a product. To investigate people's thinking to a specific type of food, the common grading is 9 point-scale from the extreme like to extreme dislike (62). A group of 75 to 150 frequent customers will be asked for their ideas on the new product. This ranking test is not only used with customers but also with a panel. The most advanced form of sensory analysis is using the descriptive method. This requires the use of trained panels. A change in ingredients, storing condition, packing or processing can be pointed out by a special panel consisting of 10 to 12 people. This panel has a critical role in the outcome of descriptive testing because they have been trained to perform the assessment scales in a similar manner not only minimizing variance error but also keep the accuracy and sensitivity of the test despite a low number of samples (63). In this study, the descriptive test was chosen to examine the development of seaweed flavor after maturation.

The outcome of the sensory analysis was analyzed by Principal Component Analysis (PCA). PCA is a multivariant analytical method that was first developed since 1891. In 1933, Hotelling (64) modified it by turning complex statistical variances into the principal analysis. Nowadays, this method is useful and one of the most popular solution analysis of datasets that deals with large and complex data (60). PCA is efficient in processing complicated research data by reducing dimensional presented data interpretably and only keeps the most important information. The differences between testing samples will be converted into new variances that

linearly correlated with the original data. In this way, the variance value will be maximized, and the discrepancies revealed by logarithm calculation eigenvectors(65). PCA is a suitable method for food sensory analysis because food flavor is usually the result of many different components. In this thesis, PCA was used to evaluate the 12 sensory attributes of seaweed after maturation. In those characteristics, marine flavor (seaweed and dried fish skin aroma), sweetness, bitterness, crunchiness, and toughness were the most important which are closely related to dislike responses from participants in a taste trial test (66).

Within the food industry, PCA has been applied and proven to be effective for a variety of different types of food. An example was for the fermented food with appearance, texture, flavor, acid activity and mouthfeel (67). In addition, PCA was chosen to analyze the different food patterns and concluded that high fast food intake as the cause of a fourth overweighed and obesity Nepalese (68). Furthermore, PCA revealed the key attributes such as sweetness, texture, rancidity, and firmness which helped in the development and marketing of an Indian traditional milk product (69). It was also used to evaluate the phenolic, mineral, and phenol for red and white wine coming from a different place in Greece (70). By selecting optimal testing conditions and method, PCA is a powerful and reliable tool for sensory analysis (71).

1.2.3.3 High performance liquid chromatography (HPLC)

The purpose of chromatography is to separate the compounds in a mixture of a sample based on the difference in molecular structure and molecular composition. There are two phases involved in the process, one is static and called the stationary phase, while the other can move along and is named as the mobile phase. The mobile phase will carry the mixture of compounds across the stationary phase. The speed and the time for each compound depending on how strong these substances interact with the stationary phase. In the end, this data will be analyzed through a computer via program detector (54). There are several types of chromatography with the different polarity of both phases such as adsorption, reverse-phase, affinity or size-exclusion chromatography. The conventional chromatography uses the column but in a low pressure, the mobile phase movement created by only gravity or the addition of low air pressure.

HPLC is based on the same principle but is improved from the conventional method by replacing gravity movement by pressure up to 400 atmospheres which accelerates the process.

A typical HPLC is presented in figure 3, the HPLC solvent is commonly a mixture of both polar and non-polar liquid with varying concentrations. Next HPLC pump draws out the solvent and forces it to go through injector where the sample is injected. With the pressure from the pump, the solvent carries loaded sample through HPLC column and detector. The process ends when the waste is collected, and data obtained through a computer program.

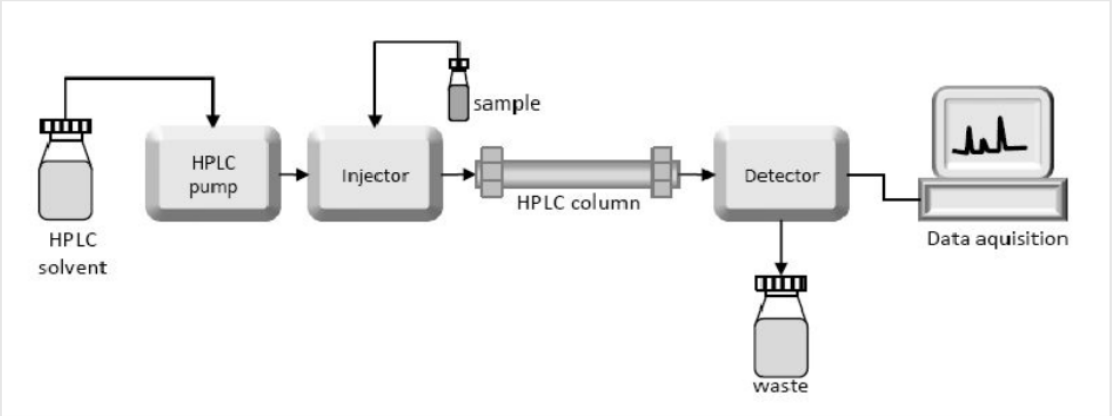

Figure 3 HPLC instrument

The picture was taken from https://laboratoryinfo.com/hplc/

HPLC has been considered as a rapid and reliable method for a variety of purposes including clinical laboratory (72)(73), pharmaceutical field (74)(75), forensic major (76)(77), food analysis (78)(79). For applications of fish and algae, HPLC has been efficiently used for fish free amino acids (FAA) (80), fish identification (81), microalgae FAA (*Chlorella pyrenoidosa* and *Chlamydomonas rinhardtii*) (82), analyzing FAA in 5 macroalgae from France (83), D-aspartate and D-alanine in 8 Japan algae (84), protein content and FAA profile of New Zealand *Undaria pinnatifida*… HPLC was chosen in this study as well based on the effective results from previous research.

1.2.3.4 Lowry Method for protein content determination

The Lowry method is one of several methods used to determine the concentration of soluble protein in a sample. It was developed from 1951 by Oliver H.Lowry (85). The protocol was modified in 1972 to give a clearer color yield of the standard solution bovine serum albumin

(BSA) and other pure proteins (86). The principle of this method is the reaction of nitrogen in the peptide backbone with Cu^{2+} under alkaline condition resulting in the formation of Cu^{+}. These newly formed molecules will oxidize Folin-Ciocalteu reagent to heteropolymolybdenum with blue color. The blue color intensity is correlated to the number of aromatic amino acids tyrosine and tryptophan. Lowry is sensitive down to concentrations of 0.01 mg protein in 1 ml (87). This method is probably the most widely used to analyze total soluble protein due to its simplicity and that it is time efficient. The result of this method is affected by the presence of other substances such as divalent cation (88), some amino acid derivatives, types of the buffet, some macromolecular (lipids, sugar, salt) (89)…except for amino acid analysis. Lowry has been used to determine algae protein level *Chaetoceros gracilis, Tetraselmis tetrathele* and *Pavlova* from Japan (90) *lutheri Porphyridium cruentum, Scenedesmus almeriensis,* and *Muriellopsis sp* (91), microalgae biomass (92), chlorophyta (93)…

2 Methodology

2.2 Experimental design

Wild *P. palmata* was collected at Roscoff (France) in November 2017. The epiphytes were removed, and samples were maintained at 32^{o}C on a shelf-dryer for approximately 24 hours. All samples were transferred to the laboratory reception. They were divided into 2 groups: the control and testing groups. The control samples were either kept untreated (D-126, D-126 NFD) or freeze-dried (FD-0 and FD-126), while D-0 control sample was sent immediately to a -40^{o}C storage room. The testing samples (M-12, M-61 and M-126) were partially rehydrated by spraying unfiltered tap water on the surface until a moisture content (MC) of 20% was achieved. All samples were marked with the storage and air-dried or freeze-dried treatment in PE bags. Each sample contained 3 replicates.

Samples from both groups were transferred to 12^{o}C in a dark room. The control group was divided into 2 categories, one group including D-126, FD-126 and D-126 NFD underwent 126 maturation days, while the other was kept freeze-dried at -40^{o}C (in a freezing room). Samples D-126 and FD-126 after maturation time were also kept in the freezing room. However, this -40^{o}C storage was omitted for D-126 NFD sample which was used as a backup control if the

freeze-dried step before analyzing results has impacts on the protein level. After being partially rehydrated, testing samples were stored and matured at the mentioned condition for 12, 61 and 126 days labeled as M-12, M-61 and M-126 respectively. All samples were stored at -40^{o}C in the dark throughout the experiment until being analyzed. The following analyses were performed with freeze-dried samples to avoid the biased outcome between two groups of samples with different moisture content. Figure 4 below gives an overview of the experimental design

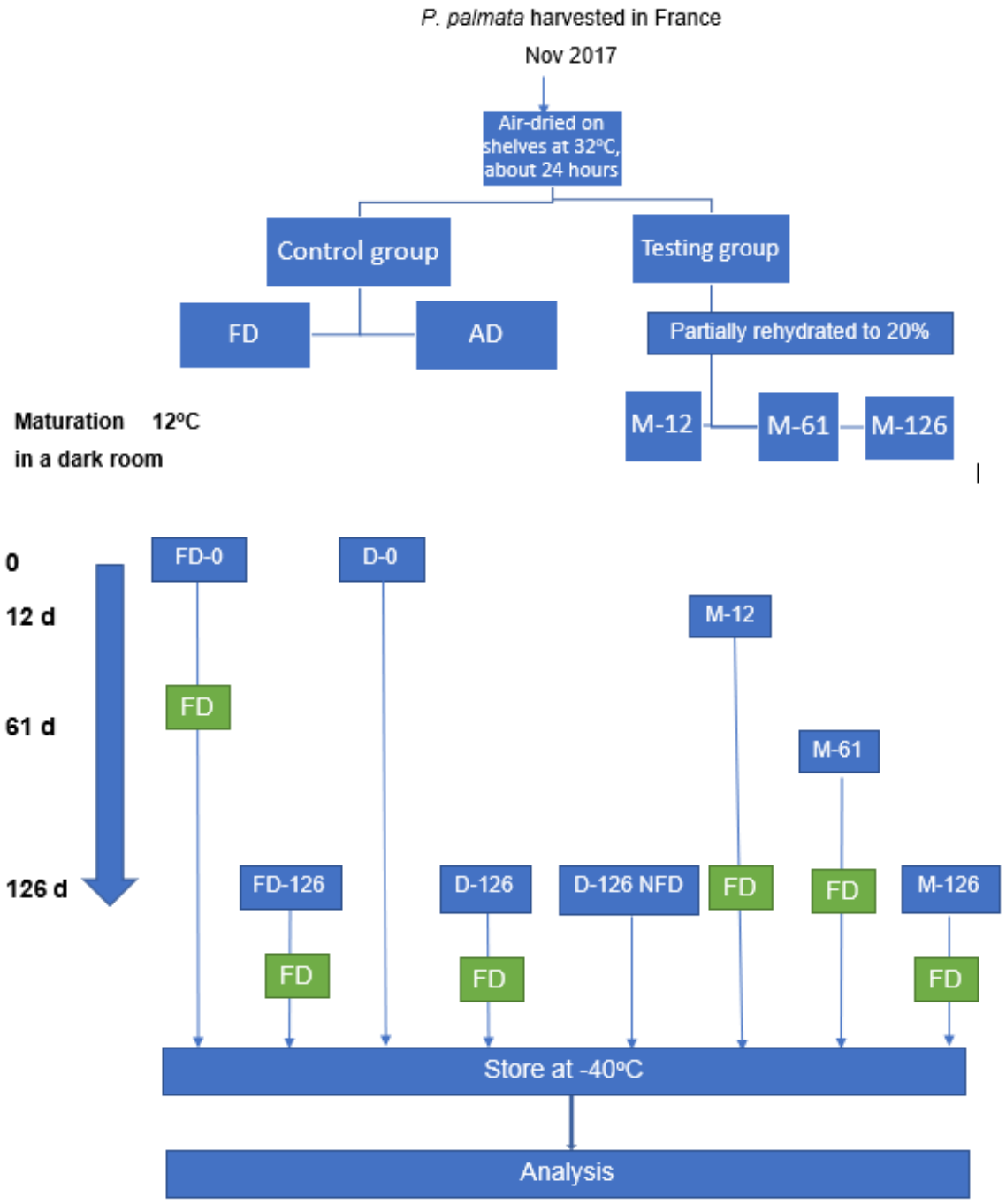

Figure 4 The overview experimental design to study the impact of storage conditions on the protein concentration and water activity of *P. palmata.* The freeze drying (FD) process indicates

that samples were kept overtime in a dark freeze room under -40°C to avoid microbial spoilage and enzyme activities

2.3 Protein concentration determination

2.2.1 Protein extraction

Samples were taken from the freezer room at -40°C. The first step was using a food homogenizer Braun (Germany) at the highest speed for 2 mins followed by grinding with mortar and pestle until 2 – 3 mm of seaweed pieces were obtained. This homogenizing process was evaluated as an effective step for macroalgae protein extraction (94). Three extracts were made from each sample. This was done by mixing 0.8 g of dried grinded seaweed with 12ml distilled water. The solution was stirred at room temperature in 1 hour by a magnetic stirrer. For each suspension, the pH value was recorded before centrifugation at 12000 x g for 20 mins at 4°C. The supernatants were used for Lowry and HPLC analysis.

2.2.2 Lowry method

The Lowry method estimates the total protein concentration in a solution based on the chemical reaction of Cu (II) interacting with peptide in the protein. Cupper molecules will be reduced into Cu (I) under the alkaline condition with the aromatic groups of tyrosine, tryptophan and cysteine amino acids reacts with Folin-Ciocalteu reagents forming molybdenum/tungsten blue product (95) (85).

The concentration of samples corresponds to the intensity of the blue-purple color and is calculated based on the reduced Folin-Ciocalteu reagents. The concentration of proteins can be calculated based on the standard curve. In this experiment, Bovine Serum Albumin (BSA) was used as standard curve due to its high effectiveness for the Lowry method (94).

The detailed protocol and the standard curves can be found in Appendix A and B respectively.

2.2.3 High performance liquid chromatography (HPLC) method

Free amino acid (FAA) profile has played an important role in food science (96). The fact that different chemical substances react differently to the stationary and the mobile phase in HPLC system depends on the affinity of the interesting compound to those phases (97). HPLC was

chosen to identify the free amino acids in each testing sample. The concentration of free amino acid was determined by the time and area on the detector computer.

Procedure: The experiment was started by preparing setting 3 parallels per sample. In each parallel, 1 ml of extracted seaweed was added in a 1.5 ml Eppendorf. Then 0.25 mL of 10% sulphosalicylic acid was mixed thoroughly in the eppendorf tube. Sulfosalicylic acid causes protein precipitation which aids in protein elimination out of testing sample (98). Cold room storage at 4^{o}C for at least 30 mins to all the parallels facilitated protein precipitation. Then, the eppendorfs were transferred to be centrifuged at 10000 rounds/ minute (rpm) speed for 10 mins. One out of the three parallel tubes was chosen to perform the same described procedure above until the centrifuge process. At this step, there were two cases obtained which either showed the precipitate or only supernatant in the new repeat Eppendorf. If the former was observed, the other two parallels were precipitated with the same procedure as the 1st parallel. If no precipitate was formed, the others 2 parallels were used directly for the next step. After all the protein was precipitated, a dilution step with ratio 1:50 was applied for all parallels followed by filtration with a 0.2 µm filter. According to technical guidance, this pore-sized minimizes the system errors and maximizes the performance was recommended (99). Before performing HPLC analysis, 0.205 ml of filtrated samples were poured to HPLC tubes and was kept at -20^{o}C. Figure 5 illustrates an overview of separated steps in HPLC protocol.

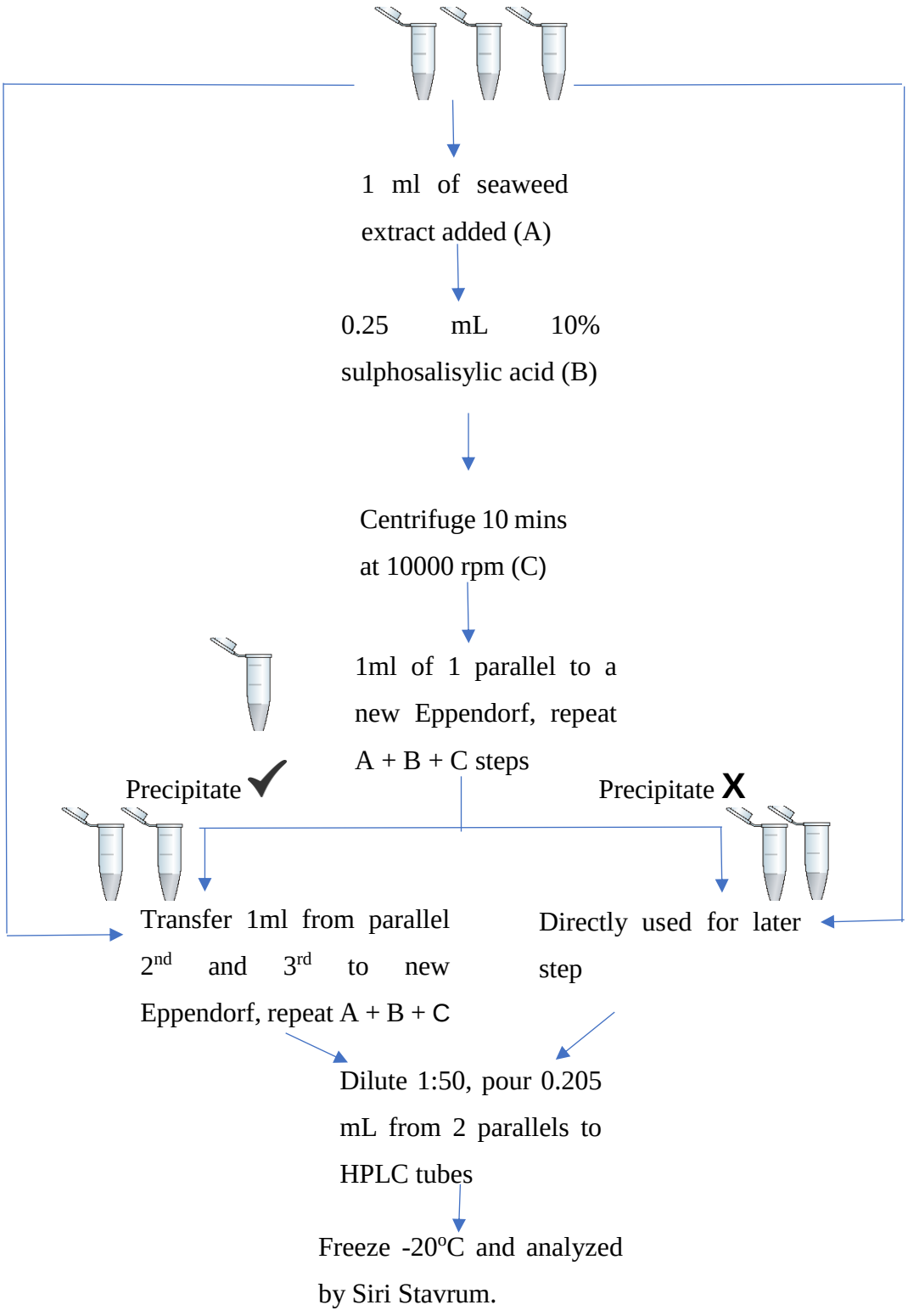

Figure 5 Overview step for HPLC analysis protocol

2.4 Moisture content and water activity analysis

The moisture content (MC) in all samples was assessed gravimetrically by drying at 105^{o}C till a stable weight was obtained after approximately 24 hours. The obtained results from chemical analyses were presented as part of samples dry weight (DW). The water activity (a_w) was carried out by LabMaster-aw (Novasina AG, Lachen, Switzerland) at NTNU Kalvskinnet campus.

2.5 Sensory analysis

The profile of all semi-hydrated samples (M12, M61, M126) and D-126 were built from a panel of 9 trained judges following standard ISO 8586:2014 (100). After maturation, samples were stored in a freezing room (-80^{o}C) before undergoing sensory analysis (section 2.1). Each sample was triplicated in a separate vacuum PE bag.

The general descriptive analysis (GDA) was chosen to examine *P. palmata* samples. The intensity of the 12 selected attributes of aroma, flavor and texture was measured to describe the sensory characteristics. These attributes were evaluated at Matís (Iceland) in June 2018.

There were 3 training phases before the sensory measurement. In the first two phases, a scale based on the previous assessments of seaweed was established. In the final phase, the 9 panelists were defined more with the attribute vocabularies describing the 3 typical sensory properties sample odor, flavor and texture listed in table 2. After that, the established scale and vocabularies for sensory attributes were synchronized by the panels. During analysis, the intensity of the 12 attributes was assessed by 15 cm unstructured scale. From that scale, all the obtained results were transcribed into ranging grade from 0 to 100 numbers for the statistical test.

Before analysis step, all samples were gently squeezed into small pieces Due to the difference in moisture content from the testing M samples to the control D sample which may lead a bias in the result, they were all rehydrated to 20% MC. The rehydration process was performed by the addition of water by pipetting. After that, all samples were held in a closed container for 2 hours until being analyzed.

During the analysis process, each sample consisted of 2.5g to 3g semi-hydrated seaweed and they were set up in a 160ml white drinking glass with lid at 25^{o}C. The panel was assigned to different booths to minimize the bias in judgments. The differences between samples appearance were masked by the red lights in the booths. There were 3 rounds of analyzing and each round of measurement involved one in the triplicate from each sample. Each sample was coded with three-digit numbers and consisted of triplicates in the analysis. The program FIZZ (2.5B, Biosystemes, France) was chosen as the data collector, while the accessors performance and the team member performance were determined by program Panel check (V1.4.0, Nofima, Norway).

Table 2: Sensory attributes and their definitions, associated with the *P. palmata* samples

Sensory attribute	Label	Scale anchors	Definition
Aroma			
Seaweed	O-Seaweed	none || much	Odour of seaweed or sea shore
Sweet	O-Sweet	none || much	Sweet odour that reminds of sweet soy sauce
Hay	O-Hay	none || much	Odour of dry hay
Fish skin	O-Fish skin	none || much	Odour of dried fish skin or dried fish heads
Flavor			
Salty	F-Salty	none || much	Salty taste
Seaweed	F-Seaweed	none || much	Flavor of seaweed or sea shore
Flavor richness	F-Richness	none || much	Flavor richness, stock, umami
Processing	F-Processing	none || much	Processed seaweed, heavy and complicated flavor, green tea, honey
Dried fish	F-Dried fish	none || much	Skin of dried fish
Bitter	F-Bitter	none || much	Bitter aftertaste
Texture			
Crunchy	T-Crunchy	none || much	Crunchiness at the beginning of chewing
Tough	T-Tough	tender || tough	When chewing. Tough: takes long time to disintegrate

2.6 Statistical test

All the parallel results from HPLC, Lowry and sensory analysis were calculated in average with the correlated standard deviation. ANOVA and Tukey Posthoc test were performed by SPSS Statistics (version 25) from NTNU accessed software ($p<0.05$).

3 Results and discussion

3.2 Moisture content (MC) and water activity (a_w)

Table 3: Water activity (a_w) of dried and semi-dried samples at different storage days. Values include mean and standard error with n =3.

	D-0	D-126	M-12	M-61	M-126
MC	5.5 ± 0.2	6.2 ± 0.3	18.5 ± 0.3	21.5 ± 0.9	18.9 ± 0.9
a_w	0.28 ± 0.01	0.29 ± 0.01	0.62 ± 0.00	0.65 ± 0.01	0.60 ± 0.02

Table 3 illustrates the measurement of MC and a_w of dried-control samples D-0, D-126 and the 3 matured samples. The air-dried (D) samples had a low MC than the matured samples. In D-0 and D-126, MC was only 5.5 and 6.2 % respectively. The aim was that the matured samples should have a MC of 20%. As expected, the MC of the control samples was approximately 6%, while M-12, M-61 and M-126 had a MC of 18.5%, 21.5 % and 18.9% respectively. The MC in all 3 matured samples showed close MC to 20%. Because the relation between MC and a_w follows the sigmoidal curve, the high MC in food results in higher a_w. The obtained data from table 3 matched with this theory.

3.3 Content of soluble protein

The contents of soluble proteins in the control and testing samples were determined by the Lowry method. The results are given as the average between three parallels with standard deviations. As can be seen in figure 6, the sample D-126 had the highest concentration of soluble protein 30.33 mg /g DW. The content increased from 22.17 mg/g DW in D-0 sample to 30.33 mg/g DW in D-126 sample. The air-dried control samples were the only group showed an increase in soluble protein content. The remaining samples which were in the same groups showed no change in the level of soluble proteins during 126 storage days. It was noticeable that there was only 11.11 mg and 11.27 mg protein detected in FD-0 and FD-126. The results for the control D-0 and all matured samples were approximately 21 to 22 mg protein / g DW.

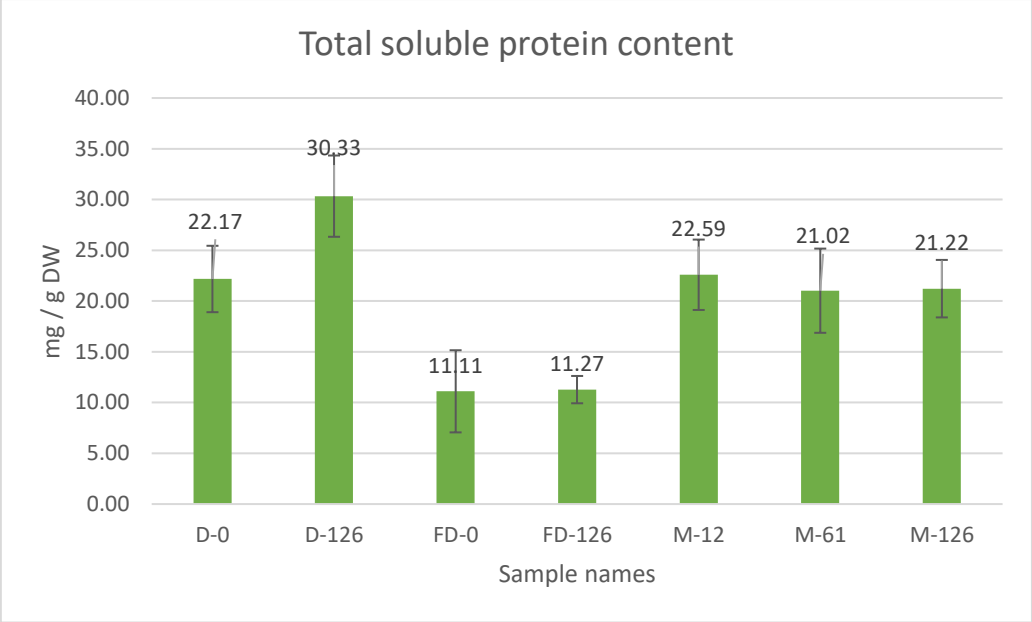

Figure 6 Total soluble protein content of control and testing samples by Lowry method

A homogenous Tukey test was performed to compare the differences in total soluble protein between all samples. And the results indicated that the samples could be divided into 3 groups. The first group included only the D-126 sample which had an outstanding high content, followed by D-0 and all matured samples. The final group was the freeze-dried control group with only about 11 mg/ g DW. There was a significant difference between the D-126 sample and the remaining samples; however, there was no statistical difference between D-0 and all moisturized samples. The moisturized samples M-12, M-61 and M-126 showed no significant difference in the total soluble protein content.

3.2.1 Protein level comparison with other studies

Apart from the unexpected low protein level in the FD-0 and FD-126 freeze-dried samples, the amount of protein in the remaining samples matched with previous studies on *P. palmata*. It was 21.9 ± 3.5 % dry weight of the same species collected in France on the Brittany coast during the winter time 1999 (52). Furthermore, all the obtained soluble protein levels also fell within the range from 8% to 35% of the values found in a study by Morgan 1980 (42).

The low values of both FD-0 and FD-126 were unexpected. The obtained total soluble protein content was opposed to an unpublished project results from a project studying the total soluble protein content of *P. palmata* in the same maturation condition but with bicinchoninic acid (BCA) protein reagent assay. In that study, there was a slight decrease in the total soluble protein level from D-0 to D-126 samples, but a dramatical fall from M-12 to M-61 and M-126 from 18.6 mg/g DW to only 11.6 and 10.5 mg/g DW respectively. In contrast, there was an increase of 8 mg/g DW from D-0 to D-126 samples and stable soluble protein content between matured samples in this study.

Compared to the Lowry assay, the BCA assay is based on the same principle by the reaction of tyrosine, cysteine and histidine with the reagent in the assay. However, the BCA assay consists of two steps, the first is the biuret reaction which reduces Cu2+ to Cu+, then the cuprous ion from the first reaction will form complex with BCA by the enhancement of those AAs. This method depends greatly on the contribution of the peptide backbones, for example, di- and tri-peptides. Moreover, the BCA-Cu^{+} can remove the weakly binding chelated peptides from the biuret reaction which allows those peptide groups to rebind with other Cu+ ions. The large amount of BCA and Cu ions in the will prolong the reaction. This results in that BCA is not as effective as Lowry for an end-point assay (101). The difference in the method for protein quantification can lead to variation between the results of this thesis and the other study.

In 1997, Chan & Cheung investigated the effect of different drying methods including freeze-drying and sun-drying for brown seaweed. The sun-drying could be considered to take place as 32^{o}C on an average because the temperature for shade and sun-drying used in other study was from 30-40^{o}C (102). Therefore, sun-drying and air-drying methods have approximately the same temperature. In the Chan & Cheung study, there was not a significant difference in the crude protein between the two groups (103). However, the brown seaweed was treated differently in the study of Chan & Cheung for the freeze-dried samples which were sent to a room at -70^{o}C and frozen immediately after the washing step. Unlike in this thesis, all samples were dried at 32^{o}C in 24 hours, this could be the reason for the dissimilar results. Another research on red seaweed in Malaysia 2016 also showed the same results for crude protein as Chan & Cheung in the soluble protein level (104). Therefore, the air-dried initial step at the

beginning of the experiment could have had an impact on the low protein level obtained from FD samples.

Although there were changes in the D control samples and a noticeable low amount of protein in the FD controls, the level of soluble protein in all matured samples almost remained unchanged. The results of total soluble protein from D controls and matured M samples were expected to decrease slightly. This was the result of an unpublished project of *P. palmata*. However, the total soluble protein remained almost unchanged among matured samples. The difference in the moisture content between can be one of the reasons for this variation. With higher MC in the matured samples, the amount of water-soluble protein and polysaccharide released could result in higher protein extraction. The proteins bound tightly to other polysaccharide compounds in *P. palmata* such as sulfated polysaccharides etc through hydrogen and ionic bonding (105)(106). In addition, the polysaccharides may have changed in the structure and revealed more binding sites to soluble proteins. These tight bindings limited the decrease of total soluble protein level in the 3 matured samples.

The MC was not determined in the FD samples, but other studies showed that freeze-dried samples possessed lower MC than sun-dried sample by approximately 3% (103). Therefore, with even less MC than the air-dried samples, the total soluble protein level from FD samples was less than all testing samples.

3.4 Free amino acids (FAA) profile analysis

3.3.1 Total soluble FAA content

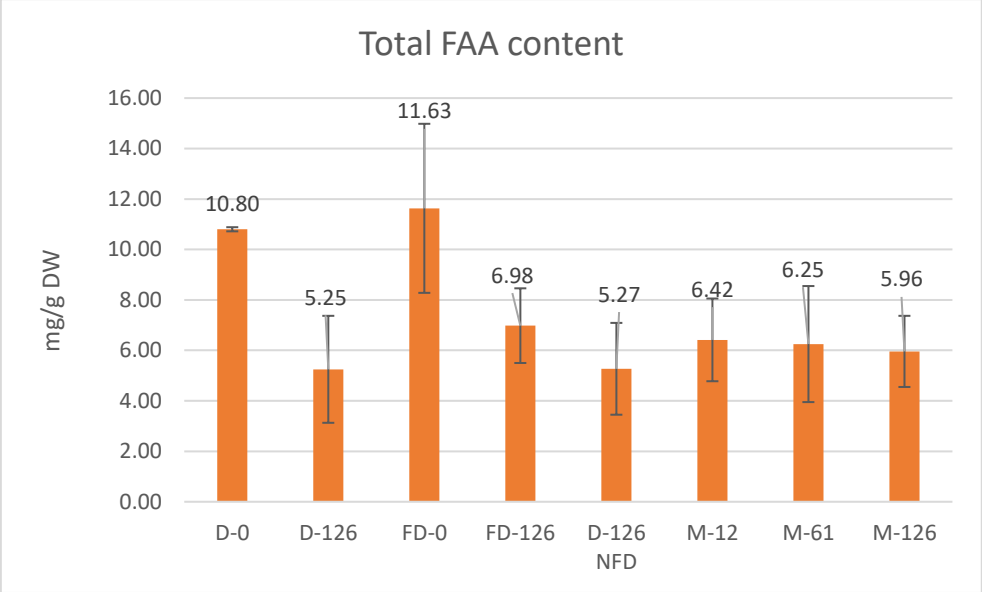

Figure 7 Total FAA content of control and matured sample. Values expressed as an average number from the HPLC method and in mg/g DW unit. Each triplicate from a sample was measured twice.

The protein extracts were made as described in section 2.2 and the supernatant was used for HPLC analysis. Each sample consisted of 3 replicates and each replicate was measured twice by HPLC. The average value for the total soluble FAA of all samples is presented in figure 7**.** It was noticeable that the FAA content decreased after 126 maturation days in all samples except for the matured samples. The highest FAA levels were found in FD-0 control and D-0 control values of approximately 11.63 and 10.80 mg/g DW respectively. For control air-dried samples, it was reduced by nearly half from approximately 10.80 mg/g DW of D-0 compared to the amount in the D-126 sample. The same trend was observed in FD control, a decrease of 5 mg/g DW of FAA content was obtained during the storage period. There was no significant difference in the total level of FAA between D-126 NFD and D-126, this showed that the freeze-drying process did not affect the FAA content. As a result, D-126 NFD sample was excluded from the sensory analysis.

The FAA contents for the M-12, M-61 and M-126 samples were not different according to statistical testing. It was noticeable that the soluble FAA level of the two initial control samples D-0 and FD-0 were higher than the early matured M-12 sample, approximately twice as high. After 61 and 126 storage days, the level of FAA in all control samples D-126, FD-126 and D-126 NFD was almost equal to the level in M-61 and M-126 samples.

One noticeable outcome was from FD-0, where the total soluble protein content was lower than the total FAA level. This unexpected result could be due to the use of the Lowry method because the amount level of the FAA should be lower than the total soluble protein in a sample. Lowry has been proved to have high sensitivity even with small amount of input (1mg), but it is easily interfered by other chemical compounds in the lab such as buffers, salts, sugar…(107). Moreover, the efficiency in aromatic amino acids extraction can interfere with the final results by changing the intensity of color interaction and absorbance from the spectrophotometry (108). The samples with higher content aromatic free amino acids might have higher absorbance value. Another reason is the incubation time for color development, there was a significant difference in the absorbance between incubation times for 30 mins and 60 mins. A study found that the 60 mins incubated samples showed higher absorbance than 30 mins. This could be a reason for FD low total soluble protein content. Since the experiment was done with many samples at a time, the difference in the Lowry's incubation time is difficult to avoid (109).

3.3.2 FAA profile

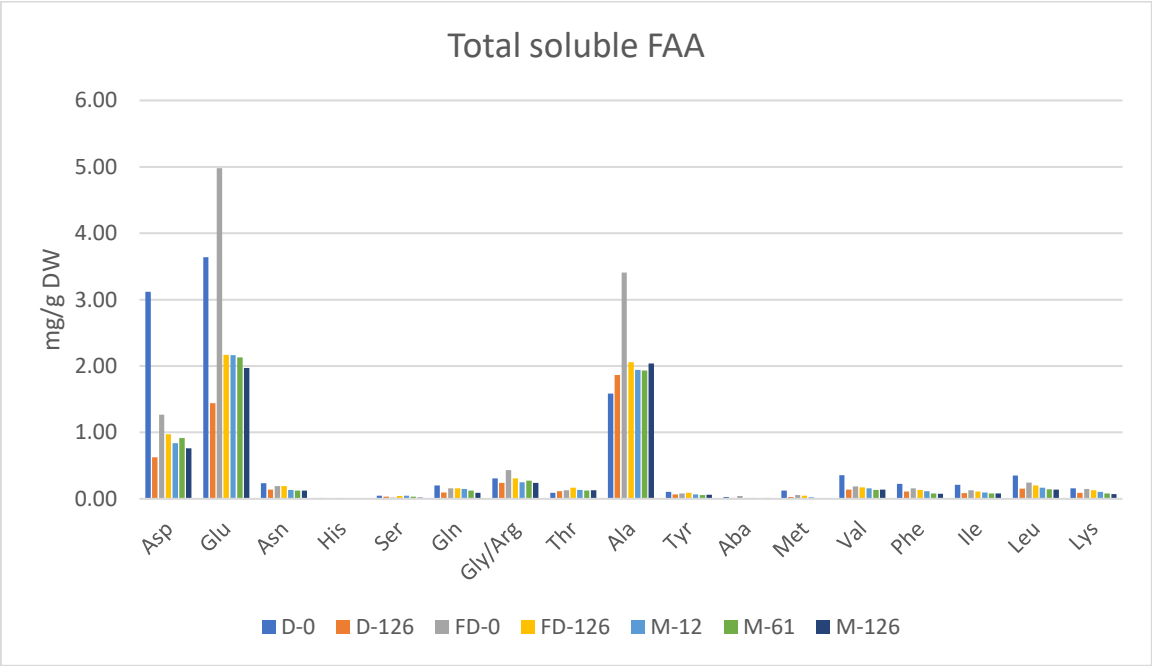

Figure 8 FAA profile of air-dried (D), freeze-dried (FD) and matured (M) samples of P. palmata during 126 storage days

Figure 8 presents an overview of the level of 17 FAA in all samples. The average values of the triplicates from each sample was analyzed by HPLC and was used in this figure. The three amino acids glutamic acid, alanine and aspartic acid are dominant among FAA. The 14 remaining FAA accounted for a small portion compared to the 3 most abundant FAA. This outcome shows similarities with previous studies for FAA profile of *P. palmata* (52)(53)(110).

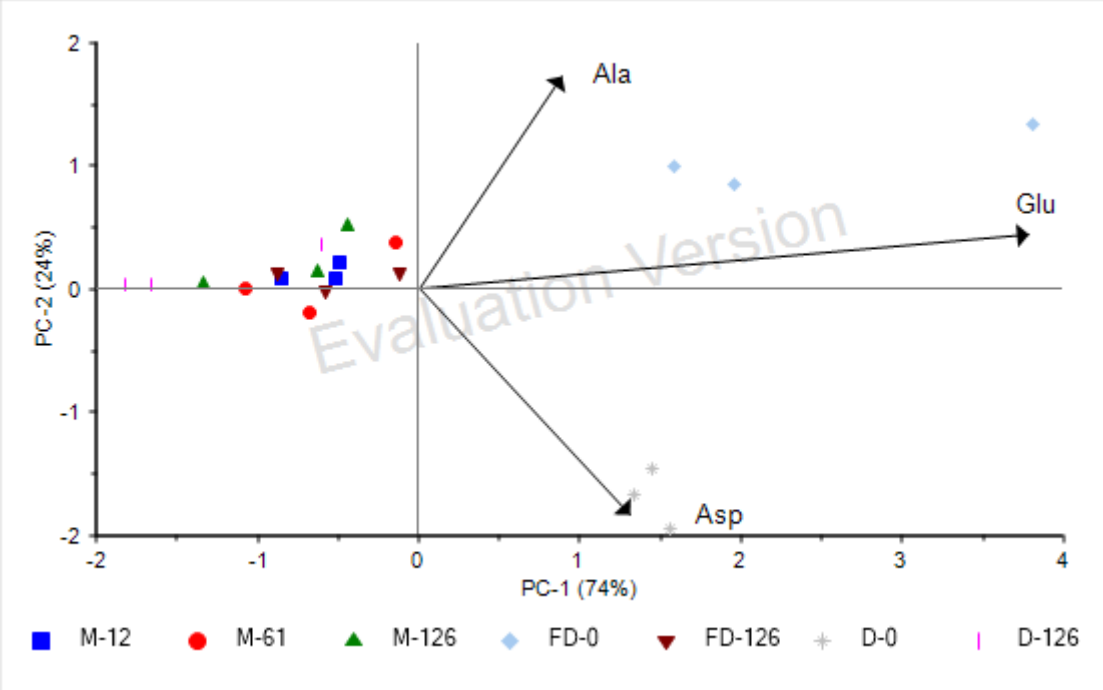

Figure 9 PCA plot (1st and 2nd principal component axes) obtained from HPLC analysis of P. palmata samples in both air-dried (D), freeze-dried (FD) and matured (M) samples. Average values from the triplicates of each sample were used for the PCA. The vectors indicate the specific amino acids that cause the variation.

Figure 9 shows the result of the PCA analysis and shows the causes in the differences in the level of FAA between all samples. PCA of the mean values for each FAA explains 98% of the difference in the data (74% for PC1 and 24% for PC2, figure 9). Variation in Glu is explained by PC1 while ala is explained more by PC2.

The high content of Glu and some Ala causes FD-0 samples to group together, while D-0 samples group together with high Asp level. The matured and stored samples are grouped together.

In detail, the two initial controls D-0 and FD-0 samples grouped on the right side of the figure and were close indicating a high content in the three amino acids. This showed that these two groups were more related to each other than the rest of the samples. For FD-0 samples, the amount of glutamic acid was the main reason for the variation by the long vector of Glu along the horizontal axis. The FD-0 samples lied oppositely with M-12, M-61, M-126, FD-126 and

FD-0 samples. The level of FD-0 alanine presented the same trend but the difference in alanine content in FD-0 to the remaining samples was not as high as in glutamic acid level. While for D-0 samples, the aspartic acid level was particularly higher than the other samples.

The other samples including M-12, M-61, M-126, FD-126 and D-126 were located near each other and on the opposite side of the glutamic level. It showed that these samples had the related FAA content, but D-126 values were slightly lower in glutamic level than in the groups located on the left side in the figure.

3.3.3 Difference in Glu, Ala and Asp content

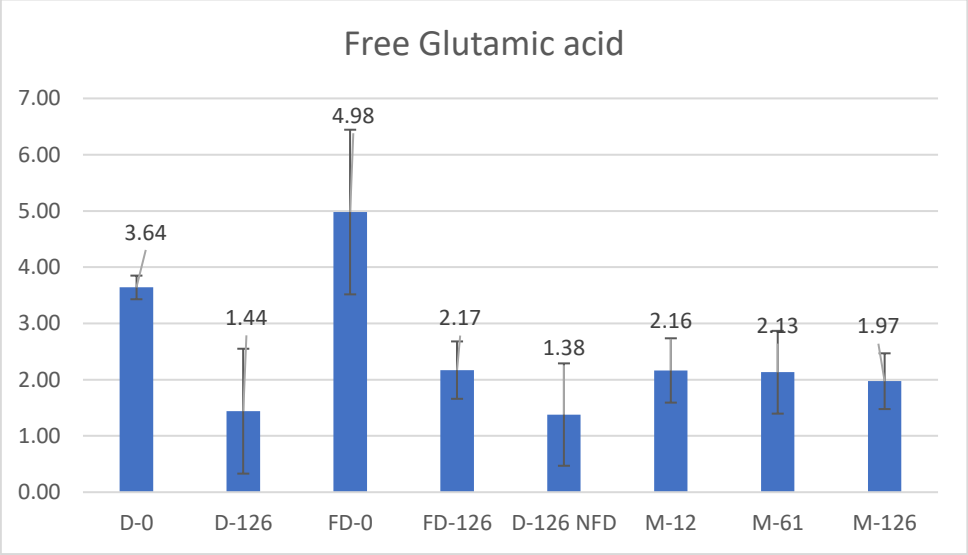

Figure 10 Glutamic acid level between control and matured samples. The value expressed as an average number from the HPLC method and in mg/g DW unit. Each triplicate from a sample was measured twice.

According to the PCA analysis described above, the three FAA contents which caused the difference between the testing samples are clarified in figure 10,11 and 12. The most dominant amino acid was glutamic acid, followed by alanine and aspartic acid. The content of these 3 amino acids in all control samples declined after 126 maturation days. The glutamic acid level in both control samples was reduced to 50% of the original value after the storage period. The level of glutamic acid between 3 matured sample groups was not statistically different. Sample FD-0 was the highest among the rest of the samples with approximate 5 mg/g DW.

There is no statistical difference between the amount of glutamic acid between D-126 and D-126 NFD. The D-0 and FD-0 control samples had a higher content of glutamic acid compared to the M-12 and M-61 samples. However, after 126 days of storage, the glutamic acid content showed no statistical difference between D-126, FD-126, D-126 NFD and M-126 samples.

Compared to the other studies on *P. palmata*, the glutamic acid content in this study generally showed lower results. In a study by Morgan 1980, the glutamic acid level in the crude protein of *P. palmata* was around 7 % DW (42). But in a study by Anne-Valéria in 1999, the average glutamic acid level throughout the year was 9.9 ± 8.5 % DW; however, the level of glutamic acid was not able to be detected during the winter months November, December and January(52). In 2002 Ole G. Mouritsen combined several studied of FAA composition of *P. palmata* which showed a wide range variety of average glutamic acid levels which were 9.9, 13, 40 ± 10 (Denmark) and 10 ± 5 (Iceland) % DW respectively. And the most recent study by Line Hovdenak Lyng in 2016 presented glutamic acid content of *P. palmata* harvested in Norway May 2015 as 8.7 ± 1 mg/g DW. Therefore, the amount of glutamic acid varied from different studies depending on the place and the time of harvest. The samples from this study were collected in a winter month November which probably a reason for the low content of free glutamic acid.

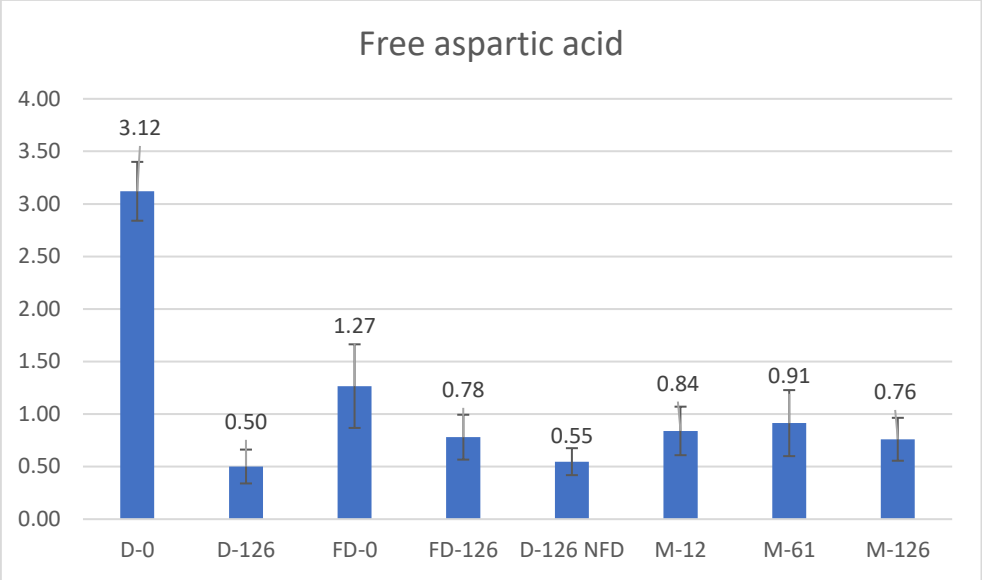

Figure 11 Free aspartic acid level between control and matured samples. The value expressed as an average number from the HPLC method and in mg/g DW unit. Each triplicate from a sample was measured twice.

The same trend was recorded for aspartic acid level in figure 11. There were large reductions in both control groups D-0 to only a sixth of the original level in D-126 and in FD-0 only half the level in FD-126 after the storage period. It is worth to mention that D-0 had the highest aspartic acid level with 3.12 mg/g DW and is significantly outstanding compared to the remaining samples. The level of aspartic acid between D-126 and D-126 NFD samples was not statistically different. The FD-0 samples with 1.27 mg/g DW resulted in the second highest value. For the matured sample, there was no statistical difference between three samples M-12, M-61 and M-126. The same result was obtained between D-126, FD-126, D-126 NFD and M-126

The aspartic acid contents in this study were also lower compared to other results from studies investigating *P. palmata*. Morgan and his team in 1980 presented that aspartic acid level in *P. palmata* crude protein was approximately 8.5 % DW (42). The average level of this FAA in a study by Anne-Valéria during 1999 was 18.5 ± 6 % DW. During November, December and January, the aspartic acid levels were recorded as the highest with around 26 mg/g DW compared to the other months (52). In 2002 Ole G. Mouritsen combined several studied of FAA composition of *P. palmata* and showed the average glutamic acid levels were 9.9, 13, 40

± 10 (Denmark) and 10 ± 5 % (Iceland) DW respectively. The aspartic acid content in Line's study of *P. palmata* harvested in Norway May 2015 study was 3.9 ± 0.5mg/g DW. This study used *P. palmata* from France which was harvested during wintertime. The amount of aspartic acid was different between studies depending on both place and time harvest. These differences could lead to lower results in the aspartic acid content between the results of this study and other research.

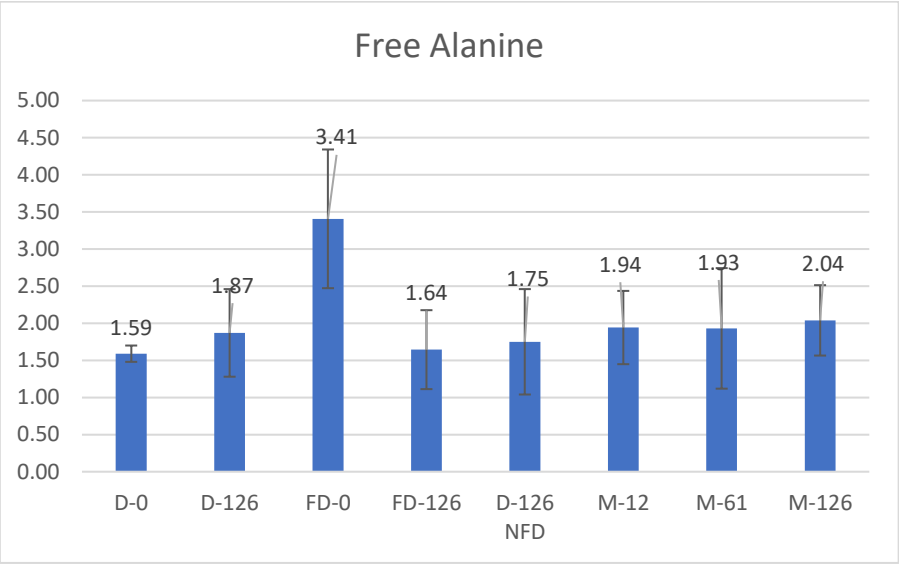

Figure 12 Free alanine level between control and matured samples. The value expressed as an average number from the HPLC method and in mg/g DW unit. Each triplicate from a sample was measured twice

Unlike glutamic acid and aspartic acid content, figure 12 illustrates that FD-0 sample had the highest alanine level with 3.41 mg/g DW. There was a decrease from 3.41 mg/g DW to only half of this value in FD-126 samples. The alanine level of air-dried control samples showed no statistical difference between the D-0 and D-126 samples. The same outcome was obtained between all maturation samples. There was a slight increase in alanine content in M-126 compared the two shorter times matured samples M-12 and M-61, but this increase was not significant. All samples both matured samples and D-126, FD-126 and M-126 had the same level of alanine.

The levels of alanine in this study were also lower compared to other research. In 1980, Morgan et al found that the alanine level in crude protein of P. *palmata* was approximately 6.5 mg/g DW (42). This level was the same as the average value to study by Anne-Valéria during 1999 with 6.7 ± 5.6 % DW. The study in 1999 showed a highly varied amount of alanine during the year, *P. palmata* harvested in November, December and January, the alanine levels were very low which could not be detected like in glutamic acid's level (96). The samples in this thesis were harvested in November which probably explained the reason for low alanine amount. In 2002 Ole G. Mouritsen combined several studied of FAA composition of *P. palmata* and also obtained a wide range variety of average alanine levels with the value 6.7, 7.5, 25 ± 6 (Dulse harvested in Denmark) and 10 ± 5 (Dulse harvested Iceland) % DW. The alanine content in Line's study of *P. palmata* harvested in Norway May 2015 study was 2.11 ± 0.07 mg/g DW (53) which was closer to the obtained number in this thesis.

3.3.4 General values found from FAA analysis

From the outcome of the HPLC for total soluble FAA content and the FAA profile, FD-0 and D-0 had the highest total content of soluble protein. After the maturation period, there were decreases in both FD-126, D-126 and D-126 NFD in the protein level by 50% compared to the level in the original samples.

However, the total soluble FAA content remained unchanged in the three matured samples regardless of the storage time. The same trend was found for the 3 most abundant FAA glutamic acid, aspartic acid and alanine content. Although there were increases in alanine content and reductions in glutamic acid and aspartic acid between the initial and after 126 days of storage for air-dried or freeze-dried controls. The levels of those amino acids in matured samples remained almost unchanged.

The outcome from the HPLC analysis for total soluble FAA content supports the results of total soluble protein content. Because HPLC presented the stable content of FAA regardless of the days of storage similar to total soluble protein content from the Lowry method outcome. Overall, the protein content in both control samples D and FD was reduced after 126 maturation days, only semi-hydrated samples showed no significant differences. Two out of three of the most dominant amino acids including glutamic acid and aspartic acids showed the same results. The level of these two FAA dropped dramatically in both control samples but stayed almost

unchanged in all matured samples M. While alanine content showed a drop from FD-0 to FD-126, the remaining testing samples were not statistically different. Between M-12, M-61 and M-126, the level of alanine presented negligible discrepancies.

There was a common outcome that for the matured samples regardless of the storage time, from the total protein content to the total soluble FAA and even the 3 dominant FAA, all the obtained data show no significant changes between them.

The control D-126 NFD was used as an extra control and to investigate whether the freeze drying at the final step before being stored at -40^{o}C has any impact on the Lowry and HPLC analysis processes. It turned out that this step did not show any effect on the HPLC analysis by no significant changes in total protein, soluble FAA level, and the 3 dominant AA.

Regarding other studies of Dulse, the outcome of the FAA from the 3 most abundant amino acids was lower. The differences in the harvesting place including France, Norway, Denmark or Iceland, the time (winter or summer) and the analysis method can be the reasons for this variation.

3.3.5 The reduction in the content of free amino acids

The decrease in total FAA in general, as well as the three most abundant amino acids in both control groups, were unexpected. It is hard to explain the exact mechanism or reasons leading to such an outcome due to limited studies investigating *P. palmata* under the same conditions. It was expected that these levels would increase. One reason is the effect of the proteolytic activity in the macroalgae. During storage, the proteases in macroalgae would be expected to cut the soluble proteins leading to more FAA yield. However, this enzymatic activity is highly variable and not fully understood. In a study by J. Lucas Pérez-Lloréns who characterized proteases in macroalgae and based on his results, it was thought that this variation was caused by the differences in the environment and laboratory experimental conditions (111). Another reason for the expected rise in the level of FAA in both control samples is the activity of polysaccharide degrading enzymes. These enzymes could be expected to degrade the polysaccharide network releasing entrapped proteins. However, the decline in the level of FAA in D control group after maturation time matched with an unpublished study of *P. palmata* under the same storage condition. Furthermore, the FAA can be oxidized and degraded during long time storage by reactions with the fatty acids in the tissue (112).

In contrast, the total FAA in general and glutamic acid, aspartic acid and alanine levels showed stable content regardless of storage for 12, 61 or 126 days. The difference in the MC content of these samples could be a reason for this stable outcome. Higher MC can lead to higher efficiency in soluble protein extracts and free amino acids in the extract. In higher water content, the chance of crossing-linking binding between sulfhydryls proteins will be reduced (113). The reduction in cross-linking bindings between sulfhydryls proteins could lead to the stable FAA contents in the matured sample. Another explanation for this unchanged FAA level might be from the more availability of polysaccharides in the extract leading to the interaction between the FAA and these compounds. FAA-polysaccharides complexes are formed through strong H-bonding, hydrophobic and steric interactions (114). These tight bondings could also be a reason that limited the FAA lost during the long storage time of matured samples.

3.5 Sensory analysis

Table 4 The mean values of points given by a sensory panel of 9 judges. Samples were analyzed in triplicate. The 12 attributes belong to aroma (A), flavor (F) and texture (T) sensory characteristics. The attributes were examined and compared using two-way ANOVA, $p < 0.05$. Bold p-value showed significant differences in the means between them.

Attributes	**D-126**	**M-12**	**M-61**	**M-126**	***p*-value**
Aroma					
Seaweed	40.63	38.15	31.93	31.11	**0.039**
Sweet	30.22	29.74	38.89	40.22	**0.001**
Hay	31.00	35.89	47.15	17.34	**0.005**
Fish skin	29.07	22.81	11.41	10.52	**0.001**
Flavor					
Salty	60.70	59.74	58.30	63.15	0.455
Seaweed	42.41	39.00	33.85	34.85	**0.016**
Flavor richness	39.52	43.78	47.04	43.81	0.155
Processing	32.37	35.74	43.00	41.37	**0.033**
Dried fish	22.41	18.41	12.30	14.59	**0.012**
Bitter	8.52	8.56	7.63	10.85	0.319
Texture					
Crunchy	57.33	51.59	44.48	44.41	**0.003**
Tough	60.93	58.96	47.78	39.22	**0.001**

Significant differences (bold numbers of *p*-value in table 4) were detected between the majority of the 12 examined sensory attributes except for salty, flavor richness and bitter flavor. Table 4 describes the average value for the intensity of each attribute from the panel. The control D-126 and short time matured samples M-12 showed almost similar values compared to M-61 and M-126 samples. For the seafood flavor such as seaweed and fish skin in the aroma as well as dried fish and seaweed flavor, D-126 and M-12 resulted in a higher score than M-61 and M-126. The same trend was also observed in texture characteristic, M-61 and M-126 had softer and less crunchy texture compared to D-126 and M-12 samples. While, for the sweet and hay in aroma and the processing in flavor attributes, the longer matured samples M-61 and M-126 came out with higher score than then dried control D-126 and short time matured M-12 samples.

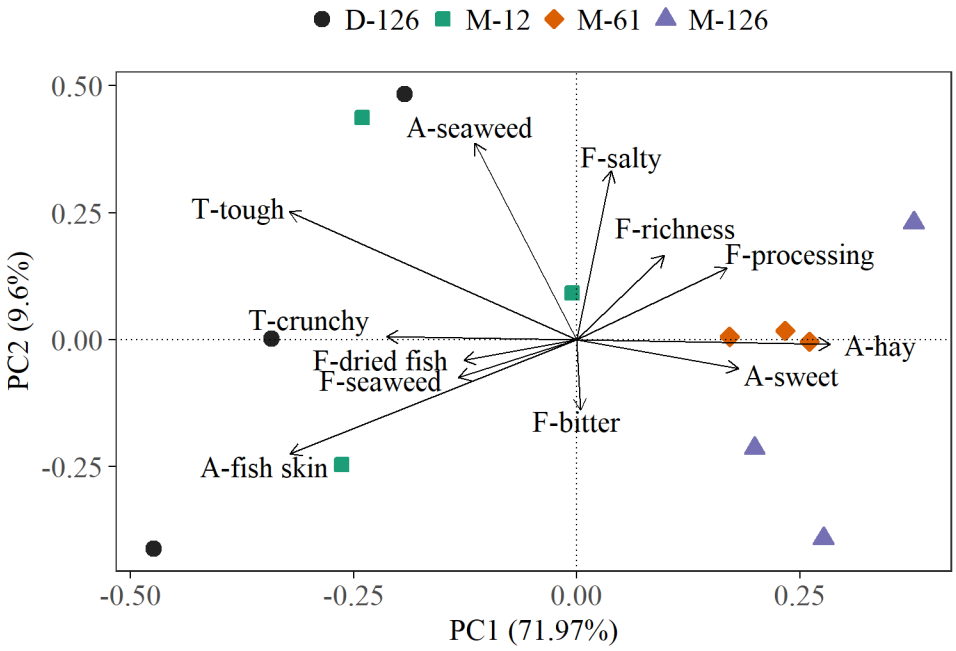

Figure 13 PCA biplot recorded from the descriptive sensory analysis of D-126 and all 3 matures sample in 126 storage period. The mean scores were used for PCA analysis from 9 panelists. The 12 vectors indicate the difference in the intensity for the 12 sensory attributes consist of aroma (A), flavor (F) and texture (T).

Figure 13 presents PCA which clarifies how the testing attributes were correlated to each other and to the samples. PC1 and PC2 from figure 10 explain 71.97% and 9.6% respectively of the variations resulting in approximately 81% of the variations in the sensory data numbers. Hay aroma and crunchy texture attributes contributed most to PC1, while salty and bitter flavors were responsible for PC2. The control D-126 and short-time matured M-12 samples located on the left side, while, the medium- and long-time matured M-61 and M-126 were on the right side of PCA biplot.

The most noticeable sample was M-61 showing significant higher value in hay and sweet flavor compared to D-126 and M-12 samples. The three M-61 spots lie almost on the horizontal axis and completely opposite to the control and early matured samples. It is worth to mention that M-61 spots were close to the sweet and processing flavor than the remaining samples. M-126 dots were spread out more than M-61 on the graph. Although there were no significant differences, M-61 possessed not only the notably higher value of flavor richness but also lower in salty and bitter flavor compared to the remaining samples.

3.4.1 Relate the sensory analysis results with other sensory studies

From the sensory analysis in table 3 and PCA analysis in figure 12, it was concluded that the marine-related attributes including fish skin, dried fish and seaweed aroma and flavor became milder in the maturated samples M-61 and M-126. This result matched with the observation in *konbu* maturation in Japan. Konbu is dried under the sun after harvesting from one up to ten years, but the most common is two years. The marine flavor after maturation is less strong together with milder sea smell (115). A study in 2017 examined the sensory analysis of P. palmata which were dried at 46^{o}C to 48^{o}C and stored less than 15 days. The results also showed strong fish, marine and seaweed odor (116). In addition, control sample D-126 and early matured sample M-12 also resulted in higher crunchy and tough attributes in texture.

In contrast, the samples that underwent longer maturation time M-61 and M-126 had milder marine attributes but were richer in sweet, hay aroma and processing flavor. Two samples also were softer and less crunchy in texture. Although all samples were rehydrated to 20% MC, the D-126 sample had the highest score in a crunchy and tough texture. This is probably due to the extreme low MC (6.2 ± 0.3%) before rehydration which changes the structure of that sample and the sample remained harder in the texture compared to matured samples.

In a study by Henry Richard Prager (NTNU), it was indicated that the reason why seaweed is still not a common dish in the western countries was the strong marine flavor including fish-smell and fish taste, uncommon taste and the hard pieces when it is eaten (38). The M-61 showed lower value in bitter and salty flavor, also sweeter in the aroma. The outcome from M-61 overcome those undesirable characteristics and was the best among the 4 testing samples in sensory testing.

3.4.2 Combine FAA profile and sensory attributes.

Although FAA are not the only factor that contributes to the food taste, they have an important role in human perception for food flavor. The role of FAA for food taste was first discovered by Dr. Ikeda in 1908 for the umami flavor. However, it took almost 100 years for umami to be scientifically and globally published and recognized as the 5th taste in the basic food flavors (117). The umami taste is built mainly from glutamate amino acid and 5'-ribonucleotides such

as *inosinate* and *guanylate*. It has been proven that almost every FAA possesses a certain level of sourness, sweetness, bitterness and umami (118). FAA contribute to the characteristic of food and is especially important in many kinds of seafood (119).

The variation in the results of the sensory analysis between the D-126 control and the early matured M-12 samples with longer matured samples M-61 and M-126 can be caused by the difference in FAA content. D-126 has slightly lower levels of total FAA levels compared to all matured samples. Moreover, the glutamic acid content of D-126 was lowest among all 4 samples. From figure 10 and figure 11, it can be seen that D-126 and M-12 had less free aspartic acid and alanine compared to M-61 and M-126 samples. Although all listed data was not statistically different, these discrepancies may contribute to the sensory analysis outcome. Alanine was found to express a strong sweet taste due to its attachment with sweet substance receptors (119). This might be one of the reasons for the sweeter taste of longer matured samples compared to D-126 and M-12 samples.

3.4.3 The contribution of P. palmata in food and health benefits.

The high nutritional value with a high protein content (can be up to 35%) of *P. palmata* and the potential as a food flavor enhancer has caught the interest of both scientists and chiefs. A study of several Irish seaweed including Dulse was conducted to study the flavor and taste potential of seaweed. It found out that *P. palmata* has the true potential in improving the round flavor of chicken broth after being added in. The addition of Dulse can make the soup base with more rounded taste without extra seasoning added in.

P. palmata can overcome the problem for people who do not have enough iodine intake or suffer from hypothyroidism. Dulse has a wide range variety of mineral compounds and especially rich in iodine level. In a study by Ole G. Mouritsen 2013, the average level of daily iodine intake could be achieved by consuming 30g dry weight of Dulse every day (110).

The food industry has been searching for a natural salty flavor that can be used instead of the table salt. Another advantage of Dulse is that it can be used as a salt replacement in soups and other dishes. In 1g spoon of table salt (NaCl), the content of sodium (Na) is 0.4g. In 1g of Irish Dulse, it was estimated that the Na content was which means it requires 16g of Irish Dulse to

reach the amount of Na in 1g table salt. The Irish are developing more cooking recipes that use Dulse dried powder instead of adding salt. In one of the recipes, adding 5 g of the dried Dulse powder in one product equals to add 0.125 g of Na. While in a product without the addition of Dulse, 2g or 3g of salt will be added which means 0.8 or 1.2 g Na added. This replacement can help to keep the limit of 2g Na intake daily for people, especially in Europe. The average salt intake of a European is 7 – 12g /day which is far more than 2.5 g of salt intake that is recommended (120). Adding seaweed to daily food can be a solution for the over-consumption of salt.

4. Conclusion

The purpose of this study was to find out how different storage conditions and moisture content would influence the level of soluble protein and free amino acid profile as well as the sensory characteristics. The two control samples group had lower MC and aw compared to the matured samples.

For the total soluble protein content, the air-dried control D-126 showed the highest level around 30 mg/g DW, while D-0 and the three matured samples had no significant differences with a level of approximately 21 mg/g DW. The two freeze-dried controls had unexpected low values with only 11 mg/g DW.

The total FAA content of all samples was examined by HPLC. The three matured samples M12, M-61 and M-126 had no change in the level of FAA. However, both control sample groups experienced a significant decrease by 50% after 126 days of storage. The freeze-drying step before storage at -40^{o}C had no impact on the FAA level.

Glutamic acid was the most dominant FAA, followed by alanine and aspartic acid in all samples. Regarding glutamic acid content, freeze-dried FD-0 had the highest level with around 5 mg/g DW, followed by D-0 control. The level of glutamic acid decreased in both control samples and remained unchanged for matured samples. The same trend was observed for the aspartic acid content, but D-0 had the highest level. For the alanine level, there was a drop from FD-0 to FD-126. The remaining samples showed no significant differences.

Sensory analysis was performed on 4 samples: D-126, M-12, M-61 and M-126. The marine flavor and odor such as dried-fish skin and seaweed characteristics faded after long maturation time. In addition, longer maturation caused softer and less crunchy texture. D-126 and M-12 shared several similarities in the 12 sensory attributes, while M-61 and M-126 were almost the same in those attributes. Among the 4 samples, M-61 had the 2nd highest score for the sweet aroma and the least score in bitter taste. Therefore, the sample M-61 was the best sample in sensory analysis among all tested samples.

Although samples with a moisture content of 20% showed a stable amount of total soluble protein and free amino acid content, further studies are needed to find the optimal moisture content and time of maturation to obtain higher value in protein and a richer flavor.

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6 Appendices

Appendix A Detailed protocol for the Lowry method and calculation

A1. Detailed Lowry protocol

Detailed protocol: Solution A was made by dissolving 20g Na_2CO_3 in 1l 0.1 M NaOH while dissolving 1g of CuSO4 x 5H2O in 100 ml water for solution B. Solution C requires 2g of potassium sodium tartrate in 100 ml distilled water. A mixture with the 3 listed substances with a ratio 1:1:1000 of B:C:A contributes to solution D which only was made on the performing day. Solution E includes 2N Folin – reagents stirring with distilled water ratio 1:2 respectively which was made daily. Finally, solution F is the standard 1000 µl/ml BSA.

The experiment started with preparing all the mentioned solutions. Then, 7 standard solutions with different concentrations were prepared as 125 µL, 250 µL, 500 µL, 1000 µL, 1500 µL, 2000 µL and 3000 µL per ml. For the testing samples, 0.5 ml of each 1: 10 dilution with 1ml of seaweed extraction into 9 ml distilled water was made and poured into a test tube. Adding 2.5 mL solution D came after with mixing thoroughly and those mixtures were left at room temperature in 10 minutes precisely. The final step before measuring spectrophotometer absorbance was adding 0.25 mL solution E with mixing through. The absorbance was evaluated from spectrophotometer at OD 750nm.

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

Lowry method was used to determine the total soluble protein content in testing samples by relating the absorbance level of samples to a stock solution with a known concentration.

To achieve that, a standard curve based on the stock solution concentration was built. A linear standard curve is constructed by the horizontal axis is the stock solution BSA concentration (µg/ml) and absorbance (at OD 750nm). Data from the Lowry experiments were recorded in Excel 2016 generating the standard curve as the formula: y = ax + b, a is the intercept of x-axis and b is the slope.

The absorbance at OD 750nm of the testing samples will be measured three times. In each parallel, the soluble protein concentration c is evaluated as in the equation A1 below:

$$c = \frac{A - b}{a} \qquad \text{(A1)}$$

In the equation A1, c (µg/ml) is the soluble protein level of the testing sample and A is the absorbance value obtained from the standard curve.

The average values (c average) of the three parallel in each sample were calculated and applied to find the actual protein content (c actual) of the testing sample by the equation A2:

$$\text{c actual} = \frac{\text{c average}}{m} \frac{d}{10\text{^}6} \times 100\% \qquad \text{(A2)}$$

In the equation A2, the c actual is the protein content in % of dry weight *P .palmata* extract, d is the dilution factor and m is the weight of *P. palmata* used in the extract. The number 10^6 is the conversion from µg/ml to g/ml.

Appendix B

Standard curve built from protocol in appendix A

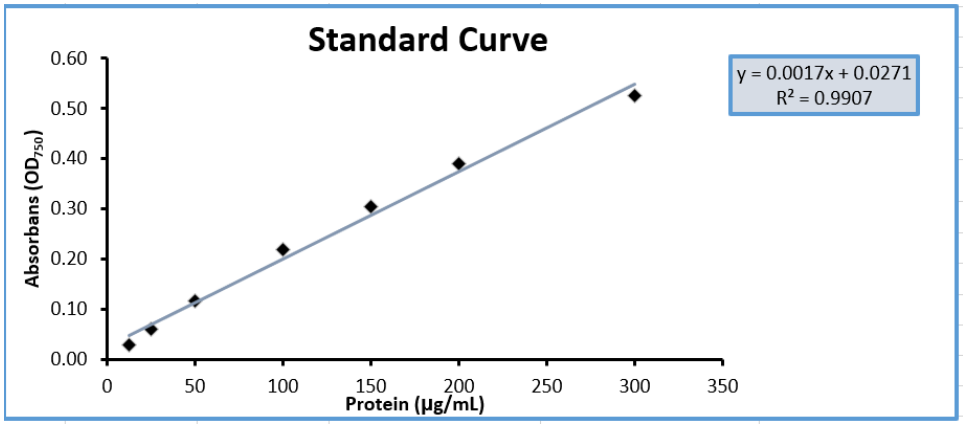

Figure B1 One of the standard curves prepared for the determination of total soluble protein content of *P. palmata*. In this figure, the total soluble protein content of samples M-12 and M-126 were determined. Values are recorded as average, n = 3.

Appendix C Tables and numbers

Table C.1: The total soluble protein content from all testing samples. The values were obtained from the Lowry method following the procedure described in Appendix A. Data was expressed as average values ± standard deviations, n=3

Samples	Total soluble protein contents (mg/g DW)
D-0	22.17± 3.27
D-126	30.33 ± 4.00
FD-0	11.11 ± 4.05
FD-126	11.27 ± 1.35
M-12	22.59 ± 3.47
M-61	21.02 ± 4.15
M-126	21.22 ± 2.83

Table C.2 The free amino acids contents and profile from all testing samples. The data was evaluated by HPLC method and expressed as mg/g DW material. All values were recorded as average values ± standard deviations, n = 3.

	M-12	M-61	M-126	FD-0	FD-126	D-0	D-126	D-126 NFD
Asp	0.84 ± 0.23	0.91 ± 0.31	0.76 ± 0.20	1.27 ± 0.40	0.78 ± 0.21	3.12 ± 0.28	0.50 ± 0.16	0.55 ± 0.13
Glu	2.16 ± 0.57	2.13 ± 0.74	1.97 ± 0.50	4.98 ± 1.46	2.17 ± 0.51	3.64 ± 0.21	1.44 ± 1.11	1.38 ± 0.97
Asn	0.14 ± 0.05	0.12 ± 0.06	0.12 ± 0.04	0.19 ± 0.06	0.19 ± 0.05	0.24 ± 0.03	0.14 ± 0.04	0.14 ± 0.03
His	0.01	-	-	-	0.01	0.01	0.01	0.01
Ser	0.05 ± 0.04	-	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.05 ± 0.04	0.03 ± 0.02	0.02 ± 0.01
Gln	0.15 ± 0.04	0.12 ± 0.06	0.09 ± 0.04	0.16 ± 0.05	0.16 ± 0.05	0.20 ± 0.05	0.10 ± 0.04	0.11 ± 0.02
Gly/Arg	0.25 ± 0.06	0.27 ± 0.07	0.24 ± 0.06	0.43 ± 0.12	0.31 ± 0.09	0.31 ± 0.03	0.24 ± 0.08	0.23 ± 0.09
Thr	0.13 ± 0.04	0.12 ± 0.06	0.13 ± 0.3	0.13 ± 0.05	0.17 ± 0.04	0.09 ± 0.01	0.11 ± 0.04	0.13 ± 0.03
Ala	1.94 ± 0.49	1.93 ± 0.81	2.04 ± 0.47	3.41 ± 0.93	2.06 ± 0.66	1.59 ± 0.11	1.87 ± 0.59	1.75 ± 0.71
Tyr	0.07 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.08 ± 0.03	0.09 ± 0.02	0.11 ± 0.01	0.07 ± 0.02	0.19 ± 0.04
Aba	-	-	-	0.04 ± 0.01	-	0.03 ± 0.01	-	-
Met	0.02 ± 0.01	-	-	0.06 ± 0.03	0.05 ± 0.02	0.12 ±0.02	0.03 ± 0.02	0.04 ± 0.02
Val	0.16 ± 0.05	0.13 ± 0.06	0.14 ± 0.04	0.19 ± 0.06	0.17 ± 0.05	0.35 ± 0.03	0.14 ± 0.04	0.14 ± 0.04
Phe	0.11 ± 0.04	0.08 ± 0.04	0.08 ± 0.03	0.16 ± 0.07	0.14 ± 0.03	0.23 ± 0.03	0.11 ± 0.04	0.10 ± 0.04
Ile	0.10 ± 0.03	0.08 ± 0.03	0.08 ± 0.02	0.13 ± 0.04	0.11 ± 0.03	0.21 ± 0.02	0.08 ± 0.02	0.08 ± 0.03
Leu	0.17 ± 0.05	0.14 ± 0.04	0.14 ± 0.04	0.25 ± 0.07	0.20 ± 0.05	0.35 ± 0.04	0.15 ± 0.05	0.15 ± 0.05
Lys	0.11 ± 0.03	0.08 ± 0.04	0.07 ± 0.02	0.15 ± 0.05	0.13 ± 0.03	0.16 ± 0.02	0.09 ± 0.03	0.10 ± 0.03
Total:	6.42 ± 1.64	6.25 ± 2.30	5.96 ± 1.41	11.63 ± 3.35	6.98 ± 1.48	10.80 ± 0.08	5.25 ± 2.12	5.27 ± 1.82

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