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Protein isolation from brown seaweed, *Saccharina latissima*, as part of an integrated bio-refinery

Master's thesis in Biotechnology

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Cover:
[Protein isolate produced from *Saccharina latissima* with the pH-shift process]

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ABSTRACT

Seaweed can be a promising alternative to meet the increasing demands for new sustainable protein sources. A few attempts to isolate proteins from seaweed using e.g. solubilization in water plus ammonium sulphate precipitation or solubilization in alkaline solution followed by isoelectric precipitation (pH-shift processing) have been carried out to date. However, effects of seaweed preservation technique and harvest season on the yield and quality of the protein recovered from seaweed have not earlier been studied. As part of the Swedish Seafarm project, cultivated *Saccharina latissima* harvested in May was here subjected to six different preservation methods; freezing at -20°C/-80°C, oven-drying, sun-drying, freeze-drying and ensilaging where after the pH-shift process with protein solubilization at pH 12 and protein precipitation at pH 2 (+/- freeze-thawing) was applied. Seasonality was studied only for the oven dried samples (harvest in March, April and May). Yield and quality of proteins in terms of nutritional, structural and techno-functional properties were followed.

The freeze-dried biomass gave the highest protein yield, 11.2 %, closely followed by the -20°C frozen and oven-dried biomasses at 11.1 % and 10.0 %, respectively. The ensilaged, sun-dried and -80°C frozen biomasses reached significantly lower yields of 7.6 %, 7.4 % and 6.3 %, respectively. With freeze-thawing-aided precipitation, there was a significant increase in protein yield for all biomasses, except the sun-dried. The freeze-dried, -20°C frozen, oven-dried, ensilaged, sun-dried and -80°C frozen biomasses achieved protein yields of 26.6 %, 19.9 %, 20.3 %, 11.7 %, 13.4 % and 19.8 %, respectively with this technique. Higher protein yield was obtained for the biomass harvested at March (18.7/30.4 % without/with freeze-thawing) compared to April (8.4/24.3 % without/with freeze-thawing) and May (10.0/20.3 % without/with freeze-thawing).

The protein isolate produced from the freeze-dried biomass achieved the highest protein content, 28.0 % on dry matter basis, followed by the oven-dried, -20°C frozen, sun-dried, -80°C frozen and ensilaged biomasses at 24.9 %, 22.0 %, 15.4 %, 15.3 % and 1.3 %, respectively. However, the protein isolates produced with an extra freeze-thawing step during precipitation resulted in significantly different protein contents for the isolates from all biomasses, with isolates from the oven-dried, freeze-dried, -80°C frozen, sun-dried, -20°C frozen and ensilaged biomass containing 40.5 %, 37.6 %, 26.2 %, 20.3 %, 19.0 % and 2.0 % protein/dw, respectively. Compared to the initial biomasses these protein isolates, in most cases, were significantly up-concentrated in protein content, with the concentration factor being from 3 to 5.

The protein isolates achieved a significant increase in essential amino acids (g EAA/ 100 g protein) compared to the initial biomass and met the FAO/WHO adult and infant daily intake recommendations for valine, threonine, isoleucine, leucine, lysine and phenylalanine, in most cases. Preservation method affected the amino acid patterns of the initial biomasses and their respective protein isolates. Total EAA content varied from ~40.2-47.3 g AA/ 100 g protein for the initial biomass, ~49.7-52.6 g AA/ 100 g protein for the

protein isolate produced without a freeze-thawing step and ~47.7-52.6 g AA/ 100 g protein for the protein isolate produced with freeze-thawing-aided precipitation.

Four of the differently preserved biomasses (-20°C frozen, sun-, oven- and freeze-dried) and their respective protein isolates were selected to be investigated further:

As expected, the protein isolates displayed poor solubility in water at pH ~2, however, achieving great solubility at pH 7 (70-80 %) and pH 11 (80-100 %). For the protein isolate produced without freeze-thawing, there was little difference in protein solubility depending on storage treatment of the initial biomass, only the protein isolate produced from the sun-dried biomass had significantly higher protein solubility at pH 2-5. For the protein isolates produced with freeze-thawing, the protein isolates from the sun-dried and -20°C frozen biomasses again differed between pH 2-5, with high protein solubility. However, at higher pH, all isolates displayed a similar pattern.

Seaweed protein isolates showed high emulsion activity index (EAI) (m^2/g protein) at pH 7 and 11, i.e. the range where they were highly soluble. Proteins isolates recovered from sun-dried and -20°C frozen biomasses showed significantly higher EAI compared with isolates from oven-dried and freeze-dried biomasses at all studied pHs. Also, for most protein isolates, the emulsion stability index (min) reached its maximum at pH 11. Polypeptide patterns of the biomasses were affected by storage method. The -20°C frozen, oven- and freeze-dried biomasses displayed bands at 37-75 kDa, 25 kDa, 20 kDa and 15 kDa, however, the sun-dried biomass only showed band at 15 kDa. Protein isolates produced from all the above and seasonal biomasses only displayed two bands at ~15 kDa and ~10 kDa, indicating a fractionation or proteolysis effect of the pH-shift processes, independent of harvest season or storage method. Finally, the protein isolates were subjected to preliminary analysis by Fourier Transform Infrared Spectroscopy (FTIR), where all samples displayed the typical Amide-A, -B, -I, -II and -III bands. Some protein isolates revealed various degrees of peak shifting, possibly indicating changes in their secondary protein structure, i.e. α-helix and/or β-sheet alterations.

There were thus distinct differences in the biomasses and their respective protein isolates depending on storage method and harvest season, also, the use of freeze-thawing-aided precipitation showed to significantly improve the protein yield of the pH-shift process. However, to suggest an optimal harvest season in combination with optimal preservation technique there are several other factors than those investigated in this study that must be determined; available biomass at the different harvest months, energy requirements of the preservation techniques, varying value/possible profit of the seaweed protein isolates depending on area of application etc. Not least, the sensory properties are of importance. There is still a long way to go, but this study proves the possibility of producing protein isolates from differently preserved seaweed biomasses and that these protein isolates, even in this early stage of process development, competes with other vegetable and marine-source protein isolates regarding nutritional value and techno functional properties.

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1 INTRODUCTION

With new trends of vegetarian diets, there is a rise in the demand for vegetable protein sources, which to date mainly composes of legumes, nuts and cereals (1). These terrestrial protein sources require arable land, fresh water and in certain extent fertilizing, however, an alternative source without these demands could be marine macroalgae (seaweed) that grow in the sublittoral zone of the oceans (2). Seaweed, especially brown, grow fast and produces a significant amount of biomass compared to terrestrial plants (2). To date the main usage of seaweed, in Europe, is the production of agar, carrageenan and alginate that are used as thickeners (3, 4). Furthermore, seaweed is also used as feed for livestock (5) and fish (6). Research has been put into producing terrestrial protein isolates, from buckwheat (7), beach pea (8), yellow pea (9), soy bean (10, 11), to possibly enrich the nutritional value or quality of food products. However, little research is done on marine vegetarian protein sources, such as seaweed, where focus has been on green and red algae, leaving the brown fairly unexplored (12-15).

Consumption of whole seaweed plants may have potential health risks since they accumulate heavy metals such as arsenic, mercury and cadmium (3). Also, there can be a potential overconsumption of iodine if large amounts of kelp are consumed (16). Therefore Mæhre et al. (3) suggests that extraction of pure compound fractions like protein or lipids to use as food or feed ingredients might be the way to go with this raw material. Also a study by Horie et al. (17) showed that the abundance of soluble fibers in brown algae decreases the protein accessibility and digestibility, reinforcing the importance of extracting pure compound fractions. Protein content of kelp is relatively low and has been reported to range from 3 to 15% dry weight (DW) (18). Due to the fast growth of seaweed biomass there is still a profit to consider and a possibility to compete with traditional plant protein sources, such as soya beans (40% DW) (19).

To evaluate the nutritional quality of a protein source it is crucial to study the amino acid profile, especially the essential amino acids (EAA) (6). The EAA score, 1.0 for animal origin, of some seaweed species (0.75-1.00) are greater than most terrestrial plants (cereals, legumes and beans 0.4-0.6), implying a higher protein quality and possibly the preferable choice if an adequate protein isolation yield is feasible (3). The most abundant amino acid in most seaweed protein is glutamic acid (15, 18, 20), it can enter the Krebs cycle through conversion to α-ketoglutarate thereby providing an energy supply to the consumer (21). For *S. latissima* the amino acid profile is comparable to that of wheat (15), therefore a protein isolate from this seaweed could potentially serve as a complementary constituent of some fish feed or even as an ingredient in human food (6).

A challenge when considering protein isolation from brown seaweed is its large seasonal variation in biomass quantity, protein content and the amino acid profile (6). A recent study on *S. latissima* by a group in Denmark investigated the amino acid profile and protein content depending on harvest month and location (in conjunction to fish cultivation or not) (6). This group therefor expresses concerns over mismatching harvest seasons considering optimal biomass and optimal protein content. Furthermore, they raise the problem with epiphytic fouling during the summer months and into the fall, which might question if protein isolates from those harvests are food grade. Generally seasonal variation is a crucial parameter when considering a harvest period, as shown by previous work on *S. latissima* (19, 22)

Mass-cultivation of *S. latissima* has previously been explored and studied with respect to optimal harvesting seasons as well as effect on growth when cultivated adjacent to fish farms (6, 19, 22). Vilg et al. (19) studied biochemical composition where total protein content ranged from 30 to 110 mg g^{-1} dry weight (DW), for the months June, August and October, underlining the importance of harvest season. Highest protein levels were found in August, however, during the warmer summer months there is epiphytic growth on the Arctic kelp, which as previously mentioned adds yet another factor when deciding harvest month (6).

1.1 Seaweed Cultivation

Cultivation starts with vegetative seedlings of seaweed being spread on long ropes, that when planted are anchored by chains, marked and held buoyant by buoys resulting in a growth depth of 0.5-1 m below the water surface (23). Mass-cultivation systems of seaweed provide a living environment with high biodiversity, inhabited by invertebrates providing feed for fish who in turn is feed for birds (2). In a study by Sanderson et al. (24) *S. latissima* successfully increased its biomass and uptake of nitrogen when cultivated adjacent to fish farms as a means of reducing eutrophication from the fish feed. Furthermore, a recent LCA-study on *S. latissima* displayed promising results over the energy consumption of offshore seaweed cultivation in Ireland (1.7 MJ_{ex} natural resources consumed per 1 MJ_{ex} produced seaweed) compared to the terrestrial plants sugar beets, maize and potatoes (0.92-3.88 MJ_{ex} MJ_{ex}^{-1}) (23).

1.2 Protein Isolation from Seaweed using pH-shifts

Jordan and Vilter (25) reported signs of an increased solubility of brown seaweed (*L. digitata*) proteins at high (11.8) pH and a decreased solubility at low (6.4) pH, using a two-step aqueous extraction system. They mentioned the potential for this kind of system in large scale processes, since, according to them, this is a fast extraction procedure with low cost of chemicals using simple techniques. Different versions of this kind of "pH-shift process" have also successfully been applied to raw materials as fish (26), yellow pea (9), soy bean (10, 11), krill (27) and tomato seed (28), with protein yields of 59.3% and 57.3%, 20.2%, ~72% and ~60%, 78% and 43.6%, respectively. In the case of Marmon and Undeland (26) a protein isolate from gutted

herring satisfied the FAO/WHO/UNU amino acid profile recommendation, implying the nutritional preservation of their technique. The process itself utilizes the fact that proteins from a homogenized sample has variable solubility in water depending on pH (29). By applying strong alkaline conditions the algal protein gains a negative charge, increasing repulsion between protein molecules promoting the water interaction, i.e. increasing solubilization (30). Furthermore, when drastically lowering the pH of the algae solution the protein loses its charge when the isoelectric point (pI) has been reached, thereby lowering the solubility resulting in precipitation of the protein (30).

To successfully produce a protein isolate from brown seaweed there are a number of limiting barriers, such as; disulfide bonds between the protein and cell wall constituents (31), cell wall anionic polysaccharides increasing viscosity and impairing protein access (25, 32) and phenolic compounds that bind reversibly or even irreversibly, if oxidized to quinones, to the protein (14, 25). Phlorotannins are polyphenolic compounds present in brown seaweed, that can bind to and hinder the extraction of proteins (25). It is believed that this secondary metabolite is excreted to increase plant fitness (33), i.e. a defense mechanism against herbivores, as well as a defense against harmful UV radiation (34). To decrease problematic cellular interactions, it is possible to add reducing agents that prevent and reduce disulfide bonds. However, if applied, the choice reducing agent is important, e.g. N-acetyl-L-cysteine is food grade while β-mercaptoethanol is not (35). Successful protein extraction is thus greatly affected by the chemical content, morphological and structural characteristics of the specific algal species (36).

Protein isolates recovered using the pH-shift process has wide application potential (29). When it comes to pH-shift produced protein isolates from fish, Nolsøe and Undeland described applications like coatings of fried food to decrease the diffusion of frying oil into the food product, marinades that can be injected in meat to improve texture and emulsifiers that will create and maintain stable emulsions. With protein isolates from seaweed displaying great foaming capacity and foam stability, there is also a promising future for using these ingredients in food industry (37).

1.3 pH-SHIFT PROCESSING OF SEAWEED

A previous study on *S. latissima* investigating seasonal effects on compound abundance, showed results of a total protein content of 7.1 ±1.7% on DW (38). pH-shift-like processes (mostly protein extraction, but in some cases also isolation) have previously been applied on several seaweed species, *S. latissima* included; (14, 25, 31, 35-37, 39, 40). Among the obtained protein yields, 5.71-6.48% (on *Enteromorpha*, precipitating with ammonium sulphate) (37) and 7.81% (on *Kappaphycus*, precipitating with ammonium sulphate) (40) has been reported. However, the study by Vilg and Undeland (14) managed to achieve a protein yield of 16.01% for *S. latissima*, whereby their method will be applied in this study.

1.4 SEAWEED PRESERVATION

Part of the vision for future seaweed usage in a bio refinery concept, including protein isolation by for example the simple pH-shift method, is an up-scaled process. For such a process, there would be a demand for large amounts of biomass that must be preserved somehow from the harvest-step, during transport and storage until final usage. The impact of different preservation techniques on the yield of protein isolate from the pH-shift process has not previously been studied. In general, there are few studies made on seaweed regarding the cause and effect of different preservation techniques on content of protein and other valuable compounds (41-43). Wong and Cheung (42) evaluated the different effects of oven- and freeze-drying on three *Sargassum* species, concluding that there was no significant effect on the amount of crude protein (thus based on total N), however, there was a significant effect on the total amount of amino acids, freeze drying being the superior method. In the same study, freeze-dried samples also displayed significantly higher physico-chemical properties, implying a greater potential as a functional ingredient in food. In their following study (31), they showed that protein extractability (via a pH-shift-like protocol) and protein quality (*in vitro* digestibility) is significantly improved when using oven-drying compared to freeze-drying, though with an increased denaturation of the protein thereby affecting its functionality. In the more recent study, Gupta, Cox (43), the effect of different drying temperatures on the phytochemical content of brown seaweed was studied. Results showed that a drying temperature of 25°C reduced the total phenol and flavonoid content by 49% and 51%, respectively, compared to fresh seaweed (43). Black (41) studied the effect of preservation by ensiling the seaweed, results showed that depending on harvest period the effect of fermentation by the added bactericide *L. cloustoni* is varying. If harvested in a period with inorganic nitrogen present and most of the laminarin absent, the result was protein synthesis by *L. cloustoni* on the cost of mannitol (41). If harvested when inorganic nitrogen is absent and laminarin is present, the bactericides utilizes the laminarin also causing a breakdown of the seaweed protein (41). However, when applying the bactericide *A. nodosum* these effects were not observed, instead some non-protein-nitrogen increased at the cost of protein-nitrogen (41). This emphasizes the impact of choosing a bactericide contributing to the aim of your process, if you are not only relying on the endogenous bactericide of your biomass.

1.5 PROTEIN AND AMINO ACIDS

Protein is one of the macro molecules that are the building blocks for life and therefor also present most food stuffs in different amount; peanuts (25.5 %), chicken (20.5 %), cod (17.4 %), egg yolk (16.1 %), soya milk (2.9 %), tofu (8.1 %) (44). Proteins are polymers made up of polypeptides, several linked peptides capable of complex structures, which in turn are made up of monomeric amino acids (AA) combinations link together with peptide bonds. The human body utilizes proteins from the diet by breaking it back down

to amino acids (AA) with hydrolytic enzymes in the gastrointestinal tract, before it synthesizes its own proteins. The structure, function and properties of a protein is all coded by the sequence of amino acids in the polypeptide back bone, which can have a large variation of possible combinations. There are 22 different amino acids defined by their individual functional group. 9 of these 22 are essential amino acids (EAA), i.e. the human body cannot produce them itself and therefore it is crucial that the food we eat cover this need. The essential amino acids are; leucine, isoleucine, valine, lysine, histidine, phenylalanine, methionine, tryptophan and threonine. Even though all these AAs are essential they are needed in different proportions, the WHO recommends a daily intake of AAs for adults according to Table 1 (45).

Table 1. *Recommended daily intake of EAAs for adults, according to WHO.*

Amino acid	mg/kg per day
Leucine	39
Isoleucine	20
Valine	26
Lysine	30
Histidine	10
Phenylalanine	25
Methionine	10
Tryptophan	4
Threonine	15
Total	184

To cover this essential need a varied diet is crucial, Table 2 shows the complete amount of AAs in different food stuffs (44).

Table 2. *AA composition of different food stuffs, calculated as gram of AA per 100 gram protein (44).* ***EAAs*** *are marked in bold font.*

Amino acid	Fresh peas	Wheat flour	Chicken breast	Beef steak	Whole egg	Cow's milk	Human milk	Cod fillet
Glycine	4,3	3,2	5,1	5,6	3	2	2,5	4,6
Alanine	4,5	3,1	6	6,1	5,4	3,6	4,2	6,7
Serine	4,7	5,6	4,1	4,3	7,9	5,2	4,3	4,8
Proline	4,1	1,3	4,3	4,9	3,8	8,5	9,9	4
Valine	5	4,4	5	5,1	7,6	6,6	6,8	5,6
Threonine	4,3	2,7	4,3	4,5	5,1	4,4	4,5	4,7
Isoleucine	4,7	3,9	4,8	4,9	5,6	4,9	5,3	5,2
Leucine	7,5	7	7,8	7,6	8,3	9,1	9,9	8,3
Aspartic acid	11,9	4,4	9,4	9,1	10,7	7,7	9,1	10,2
Lysine	8	1,9	9,3	8,7	6,3	7,4	7,1	9,6
Glutamic acid	17,3	32,9	17,1	16,5	12	20,6	17,4	14,8
Methionine	1	1,6	2,5	2,6	3,2	2,6	1,5	2,8
Histidine	2,4	2,1	3,1	3,5	2,4	2,7	2,5	2,8
Phenylalanine	5	4,8	4,7	4,3	5,1	4,9	3,8	4
Arginine	10	3,6	6,5	6,4	6,1	3,6	3,8	6,2
Tyrosine	3	2,6	3,6	3,7	4	4,1	3	3,4
Cysteine	1,2	2,6	1,3	1,2	1,8	0,8	2	1,1
Tryptophan	1	1,1	1,1	1,2	1,8	1,3	2,3	1,1
TNEAA	61	59.3	57.4	57.8	54.7	56.1	56.2	55.8
TEAA	38.9	29.5	42.6	42.4	45.4	43.9	43.7	44.1

As the nutritional quality of a protein or the protein of a food stuff is generally defined by its AA profile it is important to analyze this, as done in Table 2, usually with liquid chromatography (LC) together with mass spectrometry (MS) (46). However, the true nutritional quality can only be determined with feeding trials since the availability of the protein will vary in food stuffs depending their matrixes (44). One of the larger concerns regarding proteins in food is the possible allergic reactions that people might have against certain types of proteins, in some cases the reactions can even be lethal. The most common proteins sources associated with allergy are: peanuts, tree nuts (almond, cashew, pecan etc.), milk, wheat, egg, fish and shellfish (47). This is yet an incentive to find new reliable protein sources in the nature, where seaweed has the potential to play an important part.

1.6 Amino acid (AA) analysis using LC-MS

Liquid chromatography is a method that uses pressure to separate different components in a sample, depending on their polarity. A mobile phase carries the sample's constituents through a column containing a stationary phase with specific features. There are generally two modes to run a LC; with a polar mobile phase and non-polar stationary phase, called reverse-phase chromatography, or the other way around, called normal-phase chromatography (48). In the case of AAs, the reverse-phase mode is applied (49). Depending on the polarity of the different AAs they will elute, pass through the column, at different time points and to determine what AAs the investigated sample contains a standard is run, that will provide set retention times for the different AAs (49). To quantify the amount of AAs the sample contains the area of the chromatographic peaks for each AA is calculated and compared to the area of the used standard.

However, before the protein or food sample can be run in the LC it needs to have its proteins, polypeptides and peptides hydrolyzed down to their constituent AAs (46). The hydrolysis of the peptide bonds is commonly performed, little changed since the 1954, by adding 6M hydrochloric acid and heating 100-165°C for up to 72 h (46).

1.7 Freeze-precipitation of Proteins

Hernández et al., (50), used freezing as a means of isolating protein from the press juice of the plant alfalfa (a small flower plant used as livestock fodder). The digestibility of the protein isolate was lower than that of soy, however, the biological value was compatible to that of soy and milk protein (50). The act of freezing a protein solution can cause varying degrees of protein denaturation depending on solute, present electrolytes, pH-shifting and freezing rate (51, 52). During freezing ice crystals are formed of the present water, increasing concentration of the proteins, salts, and buffers etc. which can results in increased protein aggregation and/or denaturation (53). Pikal-Cleland et al., (53), reports that by freezing the concentration of sodium chloride in a solution can increase by up to 40 times, destabilizing the protein. They also report that

the freezing of certain components in buffers can cause a drastic change in pH, inducing protein denaturation/precipitation. Both Cao et al., (52), and Chang et al., (51), describes the phenomena of formation of small ice crystals during rapid freezing, increasing the surface area of the ice-liquid interface, also increasing the exposure of the protein to this ice-liquid interface which increases the damage/denaturation to the protein. Chang et al., (51), goes on to strengthen the theory that the denaturation of proteins during freezing is a surface-induced denaturation by proving that Tween 80, a surfactant, protected the protein against denaturation.

1.8 FOOD PROTEIN FUNCTIONALITY

The functionality of a food constituent is the non-nutritional addition of that specific compound, these might be properties that has a beneficial effect on the processing, storage, quality and sensory experience of the product (54). In the case of proteins the main functional properties and examples of their areas of application are; solubility (beverages and yoghurt drinks), water-holding capacity (deli meat and poultry products), gelation (custards and gelatin), emulsification (milk, mayonnaise and gravies) and foaming (sponge cakes and whipped cream) (54). All these functional properties can be affected by the extraction and purification processes of the protein, even as "small" effects as changes in the AA-sequence will affect the secondary, tertiary and quaternary structures, with the latter two being crucial to a proteins overall function (54). In addition to investigating the AA-pattern, analyzes like electro phoresis (SDS-PAGE), protein solubility, emulsion stability and emulsifying activity index can be performed on the protein isolates produced from the pH-shift to provide information about the possible food functionality. The functional properties of vegetable protein isolates has previously been investigated for rice bran (55), wheat (56), pea (57) and cowpea (58).

1.8.1 SDS-PAGE

In short, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique used to separate, for example the proteins/polypeptides of a sample, according to their ability to move through a specific gel mesh with the power of electric current (59). The SDS-molecule will provide a negative charge to the protein/polypeptide, in addition, depending on the protein/polypeptide size the number of binding SDS-molecules will vary, giving all the proteins/polypeptides of the sample the same charge to mass ratio. This feature will cause the proteins/polypeptides to travel through the gel with equal speed and only get separated due to differences in size, therefore experiencing more resistance the further down the sample travels through a gradient mesh gel (59). To visualize the separated proteins/polypeptides a coloring agent like Bromophenol Blue is added to the samples and finally to estimate the sizes of the protein/polypeptides a ladder with known proteins and their sizes is run in an additional gel-lane (59).

By running SDS-PAGE on initial samples and on the final product of at process the effect of protein degradation or separation from the process can be determined. Also, the size of a protein will affect the functionality, smaller peptides will increase the solubility, however, that size might not be optimal for functionalities like emulsion or water-holding capacity (54).

1.8.2 Protein Solubility

For a protein to be useful in any beverage type of product it needs to be soluble to a certain level. However, the solubility of a protein depends on several variables like; pH value, salt type/salt concentration and denaturation of the protein (54). The pH of the protein containing solution will affect the net charge of the protein, either increasing or decreasing the interaction between the protein and the water molecules, in addition, salt ions will aid to enhance this surface charge (54). Denaturation, for example caused by heat or mechanical shear, alters/destroys the original structure of the protein, severely affecting the functions of the protein and an extensive denaturation will render a protein insoluble, greatly affecting its area of application (54).

To measure protein solubility a typical test involves mixing a known amount of protein into a solution, removing insoluble proteins by centrifugation and quantitively measuring the remaining amount of protein in the supernatant (14).

1.8.3 Emulsion Stability Index (ESI) and Emulsifying Activity Index (EAI)

An emulsion is simply a blend of two or several usually unmixable liquids, if unaltered, the liquids of the mix would separate due to a high surface tension between them, e.g. oil and water (54). The surface tension can be modified by adding a surfactant to the mixture, like a protein with hydrophobic and hydrophilic amino acids (54). In foods, the most common emulsion is oil in water (o/w), meaning stable oil droplets in a surrounding continuous water phase, however, the emulsion stability again depends on previously mentioned protein altering factors (54).

To assess the potential of an emulsion the ESI and EAI can be calculated: ESI represents the separation of one or the other liquid from the emulsion over a set time frame, whilst EAI represents the area of stabilized emulsion interface per amount of protein (60). Emulsion properties has previously been investigated on micro algae protein isolate (61)

1.9 Fourier Transform Infrared Spectroscopy (FTIR)

In FTIR-analysis the sample is radiated with light of the infrared (IR) wavelength region, creating a unique IR-spectrum for that sample. For proteins, there are some characteristic spectrum bands called Amide I and Amide II, representing stretching vibrations in the amide C=O bond and bending vibrations in the amide N-H bond, respectively (62). Further, both these amide bonds participate in the hydrogen bonds of the secondary structures of proteins; α-helixes and β-sheets, making it possible to analyze the secondary structure of a sample when comparing with reference FTIR-spectrums in literature (62). However, no detailed FTIR-spectrum analysis will be performed in this study, the spectrums will merely highlight any difference between the protein isolates produced from the different biomasses.

2 MATERIAL AND METHODS

2.1 BIOMASS

S. latissima seeded in the autumn of 2015 at Tjärnö, Sweden (*58° 52' 31.931'' N, 11° 8' 47.434'' E*), was harvested in March, April and May 2016.

The biomass was harvested by manually lifting the cultivation line, pulling the seaweed off it and packing it in mesh bags. A cold room, 4°C, was used for storing the seaweed before transport and to let excess seawater runoff, since some water might get captured in the crevasses of the seaweed surface structure. The seaweed was then packed in plastic bags for the transport to Chalmers, Gothenburg, and stored in a cold room, 4°C, overnight upon arrival. The next day the seaweed was cut in smaller pieces (roughly 3x3 cm) and packaged in Ziploc-bags, prepared for each respective pretreatment. Regarding the March and April biomass, only freezing (-20°C) and oven-drying was applied.

2.1.1 Preservation Techniques

Ensilage: 14 kg biomass (May) was stored in buckets, modified to let excess liquid runoff at the bottom, with 20 ml added acid mix (formic acid/propionic acid, 65%/25% v/v) for 91 days, where after it was packed in Ziploc-bags frozen and stored at -80°C. At day 0 pH was measure to 4.42 and 4.32 at day 91.

Oven-drying: Biomass (March, April, and May) was dried in a heating cabinet at 40°C for 24h, crushed by hand into small flakes, packed in Ziploc-bags and stored in darkness at room temperature.

Sun-drying: Biomass (May) was hung on lines to dry outdoors for 10 days, then stored in one big plastic bag at room temperature.

Freezing: Biomass (March, April, and May) was packed in Ziploc-bags, frozen and stored at -20°C or -80°C.

Freeze-drying: Biomass (May) frozen at -80°C was freeze-dried until constant weight, approximately 4 days of drying, vacuum-packed and stored at -80°C.

2.1.2 Biomass Preparation

Dry biomass (oven-, sun- and freeze-dried) was milled using a coffee grinder (Rubicson 48068, 140W, Sweden). To do so, the dried seaweed was broken into smaller pieces, loaded into the coffee grinder, approximately 4 g per run, and grinded for 2-2.5 min in 5 sec bursts until a powder with a particle size < 0.5 mm was produced. Powder from all runs were pooled in one large container before aliquoted in 15ml Falcon tubes and stored at -80°C until used. Some of the freeze-dried biomass was vacuum-packed and stored at -80°C until used.

Wet biomass (ensilaged, -20°C and -80°C frozen) was minced using a food processer (KitchenAid 5KSM150) with a meat grinder attachment (Jupiter 478100) fitted with a 2 mm hole plate, packed in Ziploc-bags and stored at -80°C until used.

2.1.3 Moisture Content

The moisture content of each individual biomass was measured using an IR-scale (Precisa Moisture Balance HA 300). Approximately 0.5 g of biomass was used in each run and measurements were done in triplicate. The IR-scale was run at 80°C, heat level 8 and stopped automatically when the sample had reached a constant weight.

2.2 THE PH-SHIFT PROCESS

A modified version of the pH-shift method used by Vilg & Undeland, 2016, (14) was performed on all biomasses in duplicate. See Appendix A for a detailed lab manual on how to perform the method, also, see Figure 2 and Figure 3 for overviews of the differently performed pH-shift processes. Below, the settings used in the different steps of the process are described.

2.2.1 Algae-to-Water Ratio and Homogenization

For each separate trial, approximately 3.5 g dry weight (DW) of wet or dry seaweed was weighed and added to a 600 ml beaker. Before homogenization all biomasses were adjusted to a moisture content of 88.5 %, the highest measured moisture content of the wet biomasses, adding cold de-ionized water (DI-water) to the beaker according to Equation I.

$$\frac{Initial\ water\ content\ (g)+X}{Initial\ weight\ of\ raw\ material\ (g)+X} = 88.5 \qquad \text{(I)}$$

The value of X represents the amount of DI-water to add to reach the final moisture content.

The moisture-adjusted biomass was mixed with cold DI-water to a wet weight (WW) ratio of 1:6 (WW algae:water) and homogenized using a polytrone (ULTRA-TURRAX® T18 basic, IKA®) for 2 min at speed 4 (18000 rpm). The seaweed slurry/homogenate was kept on ice at all time, if not stated otherwise.

2.2.2 Alkaline Solubilization

The biomass was osmoshocked in the 6 volumes of DI-water for 15 min while kept on ice, before native pH was then measured under stirring (magnetic stirrer) using a pH-meter (MeterLab® PHM210 STANDARD pH METER). 1M NaOH was added to adjust the slurry to pH 12 and the slurry was then left to incubate under stirring on ice for 20 min. After incubation, samples for measurements of total protein were taken

from the homogenate, one stored overnight in the fridge and one saved as reference sample, stored at -80°C. Lastly, the slurry was centrifuged (Sorvall® RC-5C Plus) at 8500xg, 4°C for 20 min.

2.2.3 Isoelectric Precipitation

Resulting supernatant (S1) was separated from the pellet (P1) using a sieve (~0.5 mm) and weighed. P1 was collected in a 50 ml Falcon tube and stored at -80°C until future use. Samples of S1 were taken for protein measurements before 1M HCl was added to adjust S1 to pH 2 and left to incubate stirring on ice for 20 min. In two types of pH-shift processes (Type I and II, see Figure 2) that were investigated the S1 adjusted to pH 2 was centrifuged directly at 8500g, 4°C for 20 min. Resulting supernatant (S2A) was separated from the pellet (P2A) using a sieve (~0.5 mm) and weighed. P2A was collected in a 5 ml Eppendorf tube and stored at -80°C. In some cases, a small piece of P2 was dissolved in 10 ml 1M NaOH and stored in the fridge overnight until protein measurements. Samples of S2A were taken for protein measurements.

2.2.4 Freeze-thaw-induced Precipitation

In the Type II pH-shift process S2A was frozen overnight at -80°C and thawed the next day under cold running water and centrifuged 8500g, 4°C for 20 min. Samples from the resulting supernatant (S3A) and pellet (P3A) were taken for protein measurements, otherwise the samples were stored at -80°C.

In the third type of pH-shift process (Type III, see Figure 3) the S1 adjusted to pH 2 was frozen overnight at -80°C, thawed the next day under cold running water and centrifuged 8500g, 4°C for 20 min. Samples from the resulting supernatant (S2B) and pellet (P2B) were taken for protein measurements, otherwise the samples were stored at -80°C.

2.2.5 Process Scheme and pH-shift overview

A general process scheme for the project, from raw material to final product, is shown in Figure 1.

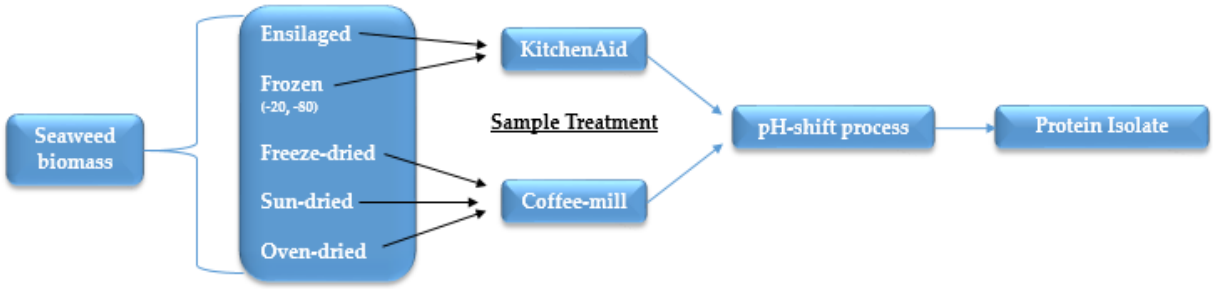

Figure 1. *Six differently preserved biomasses were put through sample treatment: ensilaged and -20°C/-80°C frozen were minced with a KitchenAid grinder, while freeze-, sun- and oven-dried were milled with a coffee-mill. The biomasses were then separately put through the pH-shift process to produce a protein isolate.*

Three different types of the pH-shift process were preformed, where the difference was the use of freeze precipitation of supernatants at different steps of the process:

Type I – pH-shift: "Classical" pH-shift, as described in Section 2.2.1 - 2.2.3 and Figure 2. No freeze precipitation and finished after Step 6 in Figure 2, with the final product of Pellet 2A (P2A).

Type II – pH-shift: The Supernatant 2A (S2A) produced from the classical pH-shift was collected and frozen at -80°C overnight, see Step 7 in Figure 2. The day after it was thawed and centrifuged, producing Supernatant 3A (S3A) and Pellet 3A (P3A). The protein content of P2A and P3A was pooled to calculate the yield of this extended pH-shift process.

Type III – pH-shift: When Supernatant 1 (S1) had been adjusted to pH 2 and incubated for 20 min, stirring on ice, the solution was collected and frozen at -80°C overnight, see Step 5-6 in Figure 3. The day after the solution was thawed and centrifuged, producing Supernatant 2B (S2B) and the final product Pellet 2B (P2B).

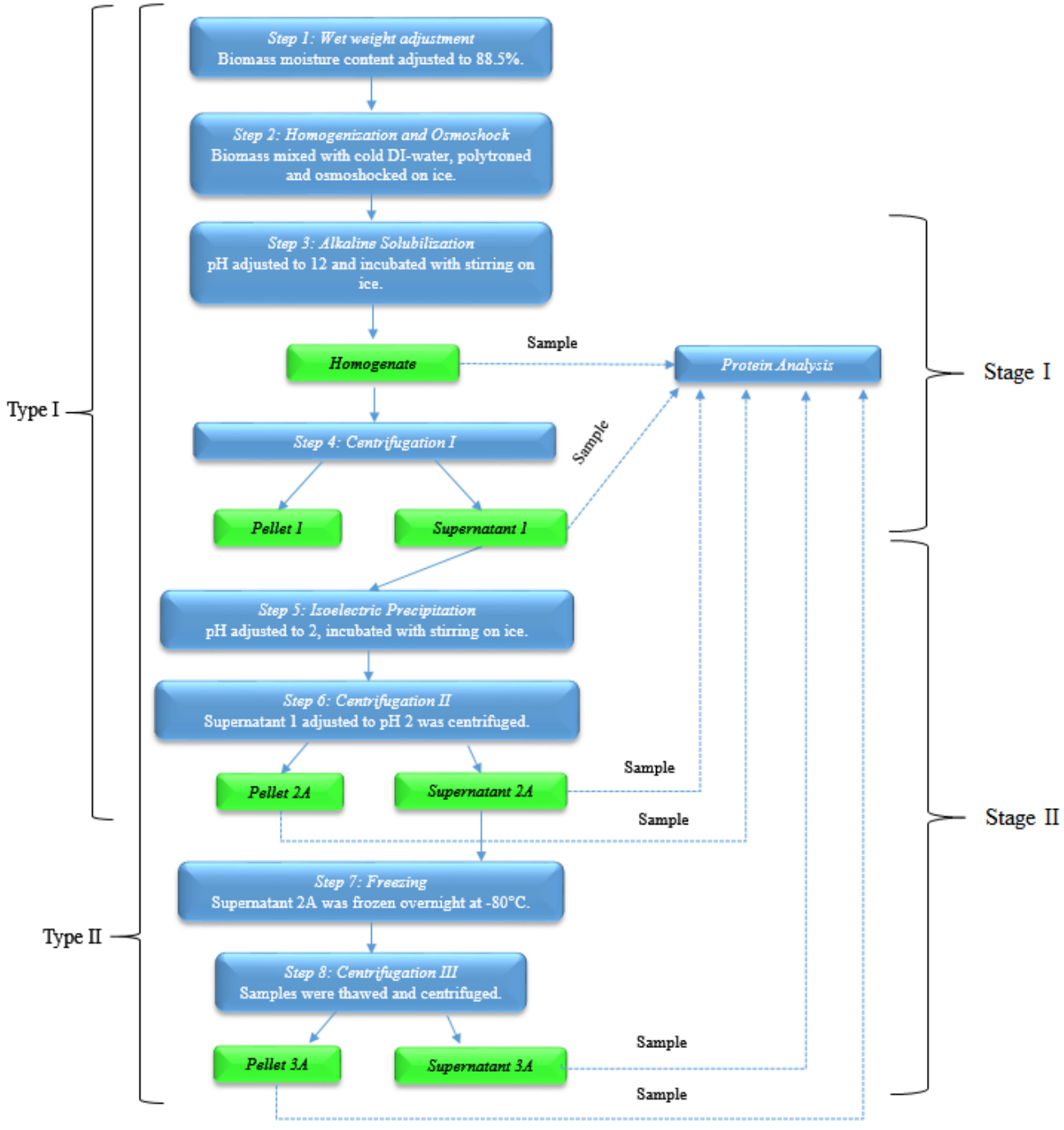

Figure 2. *An overview of the Type I and II pH-shift processes describing the main process steps and some parameters. The final product of the Type I process is Pellet 2A. The final product of the Type II process is a pooled pellet consisting of both Pellet 2A and 3A.*

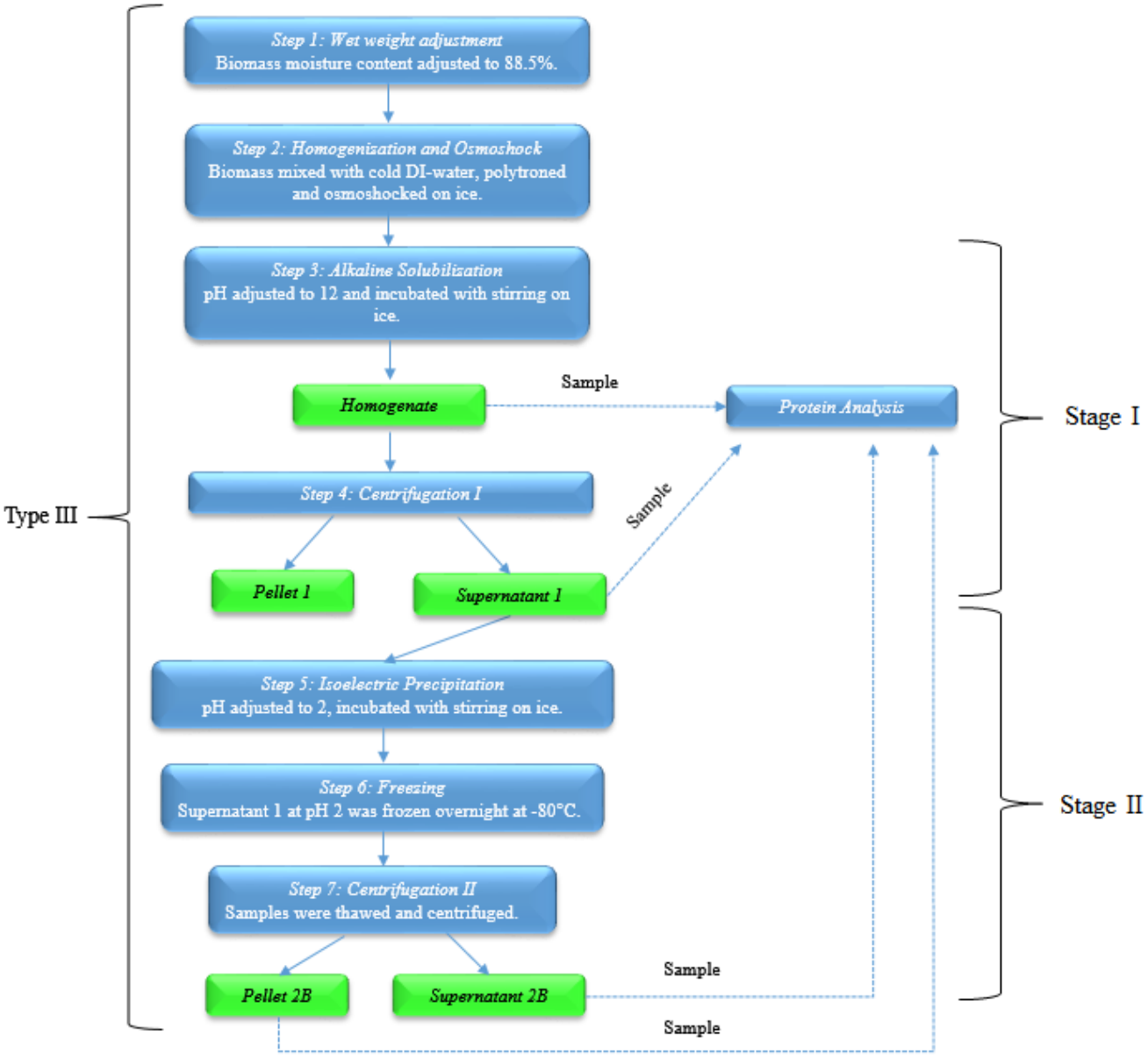

Figure 3. *An overview of the Type III pH-shift processes describing the main process steps and some parameters. The final product of the Type III process is Pellet 2B.*

2.3 PROTEIN ANALYSIS

2.3.1 Investigation of need for sample pre-treatment

Samples of the seaweed homogenate was either diluted, to a protein content of 10-100 μg/ml using 0.1M NaOH, and directly put through the protein analysis method (see Section 2.3.4) or put through a sodium dodecyl sulfate (SDS) boiling procedure, prior to the protein analysis, to investigate the necessity of performing a SDS-boil procedure for measuring total protein. The procedure was performed according to Vilg and Undeland (14), with minor differences:

Seaweed homogenate at pH 12 was prepared as previously described. Half of the homogenate was stored in a fridge until protein analysis, the other half was freeze-dried and ground in a mortar. 50 mg of the produced powder was mixed with 1 ml of extraction liquid (2% SDS, 1 mM dithiothreitol (DTT)), boiled at 100°C for 3 x 5 min with vortexing between. The samples were then centrifuged (Thermo Fisher Scientific, Heraeus Fresco 17) at 4°C, 14000g for 20 min and the supernatant was put through the Lowry protein analysis, see Section 2.3.4.

2.3.2 Standard Curve

A protein standard curve was prepared using bovine serum albumin (BSA), 6-10 points in triplicate, reaching a range within 10-100 μg/ml by diluting a 2 mg/ml water solution with 0.1M NaOH. A new standard curve was made when new chemicals were prepared for the Lowry assay. The standard curve in Figure A1, see Appendix A, was made with six points ranging in protein concentration from 10 to 90 μg/ml, resulting with a R^2 of 0.9942 and Equation II below.

$$y = 0.0035x + 0.0116 \quad \text{(II)}$$

2.3.3 Sample Pre-treatment for Protein Analysis

Samples taken during the pH-shift process were diluted using 0.1M NaOH to reach a protein concentration within the range of the standard curve, i.e. 10-100 μg/ml. Investigated pellets were first mixed with 1M NaOH until completely dissolved before diluted. Dilutions series were made, resulting in a minimum of 4 ml of the final dilution.

2.3.4 Protein Analysis

The protein analysis was performed according to the method of Lowry as modified by Markwell et al., (63), in short;

Prepared chemicals: Reagent A (2.0% Na_2CO_3, 0.40% NaOH, 0.16% Na-tatarate, 1% SDS), Reagent B (4% $CuSO_4$ x $5H_2O$), Reagent C (1 part Reagent B into 100 parts Reagent A, made fresh) and Phenol-reagent (1 part Folin-Ciocalteu phenol into 1 part DI-water, made fresh).

Procedure: 1 ml of adequately diluted sample was mixed with 3 ml of Reagent C, made in triplicate, and vortexed immediately after. Samples were, incubated in darkness at room temperature for 30 min. The phenol reagent was prepared in darkness and 0.3 ml of the phenol reagent was added to each sample and vortexed thoroughly immediately after. Samples were incubated in darkness at room temperature for 45 min. Sample absorption was then measured at 660 nm with a spectrophotometer (Agilent Technologies, Cary 60 UV-Vis) using a quartz cuvette with a 10.00 mm light path.

2.4 Protein Powder pH Dependent Solubility

The pH dependent water solubility of the produced protein powders was analyzed as followed:

20 mg of freeze-dried protein powder was weighed and added to a 50 ml Falcon-tube, where after 25 ml de-ionized water (room temperature) was added and the mixture was thoroughly vortexed. The 25 ml mixture was separated into aliquots of 5 ml in five 15 ml Falcon-tubes. Four of the tubes were adjusted to pH 5, 7, 9 and 11, respectively, using 1M NaOH and controlling with a pH-meter (MeterLab® PHM210 STANDARD pH METER). The fifth tube was left unaltered and referred to as "native pH". All tubes were incubated for 30 min, vortexing every 10 min and finally centrifuged for 10 min, in room temperature at 6000 g. Samples were taken from the resulting supernatants and analyzed for protein content with the Lowry method, see Section 2.3.4.

The sample with the highest measured protein content was considered as having 100 % protein solubility, where after this protein content was used to calculate the protein solubility of the remaining samples, see Equation III in Section 2.9.

2.5 SDS-PAGE

In order to properly display visible bands of protein samples taken during different steps of the pH-shift process, a 10X sample buffer (usually a 2X or 4X is used) was prepared, it was due to very low protein concentrations in general throughout the steps of the pH-shift process. However, samples taken from biomasses had sufficiently high protein content to use a 2X sample buffer. The 10X samples buffer was prepared as follows:

0.606 g of Tris-base and 0.75 g of SDS was added to a 10 ml volumetric flask and dissolved in a glycerol solution (70% glycerol and 30% DI-water). The mixture was kept under agitation with a magnetic stirrer and heated mildly. 2 mg Bromophenol Blue was added and glycerol solution was added to reach a final volume of 10 ml. The 10X sample buffer was stored in room temperature and was heated mildly before use due to the high viscosity of the glycerol solution at room temperature.

Additionally, protein isolate samples were put through dialysis prior to SDS-PAGE analysis. This was done due to destruction of the gel lanes during the first run with non-dialyzed samples. According to the Mini-PROTEAN®-trouble shooting guide, a too high salt content in the sample can cause destruction of the gel. Dialysis was performed as follows:

All protein isolates were freeze-dried and ground into fine powders before mixing with 1M NaOH to create at solution containing ~3 mg protein/ml (1.9 mg/ml protein concentration wanted after dialysis). 3 ml of the protein solutions were respectively loaded into 15 cm long Spectra/Por® Membrane MWCO (Molecular Weight Cut-Off): 3500 Da. The tubes were closed with a tie in one end and after loading the sample the other end was twisted and held closed with dialysis clamps. Clamps were marked with individual numbers, tubes were put in plastic boxes containing ~1 L DI-water and boxes were stored in the fridge for four days, when the dialysis was ended.

The protein content of protein powders, dissolved in 1M NaOH, and biomass samples were measured with the Lowry method and diluted to a final concentration of 2.1 mg protein/ ml. The samples were mixed with the 10X sample buffer in a ratio of 1 (buffer):9 (sample), resulting in a 1X mix with a protein concentration of 1.9 mg protein/ ml. Tubes were then heated in a heating block at 100°C for 7 min, where after the tubes were centrifuged for 5 min at 15000g in room temperature. The final samples were stored in room temperature if they were analyzed the same day, otherwise they were stored in a freezer at -20°C.

The tank buffer was prepared as the kit describes to produce ~800ml 1X tank buffer. The gels (precast mini linear gels 4-20 %, Bio-Rad, USA) and cassettes were assembled as the packaging instructs and the seal of the cassette was checked before any samples were loaded. The ladder used was Bio-Rad's Precision Plus Protein Dual Xtra Standard (2-250 kDa) and 5 µl of ladder sample was loaded in assigned wells. 15 µl of sample was loaded in each designated well, resulting in ~28.5 g protein loaded in each well. Gels were run at 200V until the sample had nearly reached the bottom of the gel, in general 30-35 min.

Gels were stained for 45 min with 0.02 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 7.5 % (v/v) acetic acid, followed by a destaining for 70 min with 50 % (v/v) methanol and 7.5 % (v/v) acetic acid. Pictures of the gels were taken with Bio-Rad's Gel Doc 2000 and light/contrast altered with Adobe Photoshop CC to bring out vague bands.

2.6 Amino Acid Analysis

Protein pellets and biomasses were freeze-dried and ground to fine powders. A mortar was used for the biomasses whilst the protein pellets were ground in their tubes using a spatula. Approximately 30-50 mg of biomass powder and ~10-20 mg of protein powders was weighed into screw cap glass tubes. 4 ml of 6M HCl was added into each tube, air inside the tubes was replaced twice with nitrogen and samples were heated (with caps on) under a fume hood for 24h at 110°C using a heating block. The PPM concentration of each sample was calculated using Equation X, where after samples were diluted to 300 ppm before loading 1 ml in duplicate in glass chromatography vials and sealing with crimping lids. Samples were stored in a fridge until analyze. This sample preparation was not able to recover tryptophan and cysteine.

Samples were loaded, automatically sampled and run in an LC/MS (Agilent 1100 HPLC, Waldbron, Germany). Collected data were then compared against previously run amino acid standards.

2.7 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR-spectras of the produced protein isolates were obtained by placing samples (freeze-dried) of the protein isolates onto the crystal cell of a spectrophotometer (Nicolet 6700, Thermo Scientific, MA, USA). Scanned inverted wavenumbers ranged from 500 cm^{-1} to 4000 cm^{-1}, with data recorded once per 4 cm^{-1}. All spectras were recorded in room temperature (25°C) and 16 times scanning.

2.8 Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI)

At first, 30 mg of protein isolate powder was mixed with 3 ml de-ionized water in a 15ml Falcon-tube, in triplicate. Two mixtures had their pH adjusted to 7 and 11, whilst the third was kept at its native pH. After pH-adjustment all samples were vortexed at full speed for 1 min, where after 1 ml sunflower vegetable oil was added to each sample followed by homogenization with a polytrone (ULTRA-TURRAX® T18 digital, IKA®) for 1 min at a speed of 20,000 rpm. Then, directly after homogenization a 50 µl emulsion sample was taken from the bottom of the tube and mixed with 5 ml of 0.1 % SDS-solution, which was vortex at max speed for 5 s. Directly after vortexing, the absorption at 500 nm was read in a spectrophotometer (Agilent Technologies, Cary 60 UV-Vis) using a quartz cuvette with a 10.00 mm light path. Finally, another 50 µl emulsion sample was taken 10 min after homogenization and put through the same procedure. The EAI and ESI was calculated using Equation XI and Equation XII, respectively.

A small droplet of each emulsion (pH N, 7 and 11 of P2As and P2Bs of each biomass) was investigated by microscopy (Axiostar Plus, Carl Zeiss Microscopy, LLC, USA) using a 10X magnification (A-Plan 10x/0.25 Ph1 Var1, Carl Zeiss Microscopy, LLC, USA). Pictures of the investigated emulsions were taken with a microscope top mounted camera (Canon PowerShot G9, 12.1 Megapixels) with an 6x optical zoom lens.

2.9 CALCULATIONS

2.9.1 Protein Solubility

To calculate protein solubility Equation III and IV was used together with measured and calculated values of protein concentrations in supernatants and homogenate. Equation III was used to calculate the protein solubility of produced protein isolates. C indicates the protein concentration in µg/ml.

$$Solubility\ (\%) = C_{soluble\ proteins\ in\ supernatant} / C_{highest\ recorded\ protein\ amount} \times 100 \quad \text{(III)}$$

$$Solubility\ (\%) = C_{soluble\ proteins\ in\ supernatant} / C_{total\ protein\ in\ homogenate} \times 100 \quad \text{(IV)}$$

2.9.2 Total Protein

To calculate total protein of the biomass in percent of g DW, Equation V was used together with measured and calculated values of protein amounts in the initial homogenate, weight and moisture content of the used biomass. M indicates the amount of protein in mg.

$$Total\ Protein\ (\%) = M_{protein\ in\ homogenate} / \left(\left(1 - \frac{Moist.cont.(\%)}{100} \right) \times M_{Amount\ of\ starting\ biomass} \right) \quad \text{(V)}$$

2.9.3 Protein Yield

To calculate the protein extraction yield over the first stage of the pH-shift process (from the homogenate to S1), Equation VI was used together with measured and calculated values of protein amounts in the homogenate and S1.

$$Yield\ (\%) = M_{total\ protein\ in\ Supernatant\ 1} / M_{total\ protein\ in\ initial\ homogenate} \times 100 \quad \text{(VI)}$$

To calculate the protein precipitation yield over the second stage of the pH-shift process was calculated Equation VII was used. Depending on pH-shift process type, the final pellet is either P2A, P2A + P3A (pooled) or P2B.

$$Yield\ (\%) = M_{total\ protein\ in\ final\ pellet} / M_{total\ protein\ in\ Supernatant\ 1} \times 100 \quad \text{(VII)}$$

To calculate the total protein yield (%) over the whole pH-shift process Equation VIII was used. Depending on pH-shift process type, the final pellet is either P2A, P2A + P3A (pooled) or P2B.

$$Yield\ (\%) = M_{total\ protein\ in\ final\ pellet} / M_{total\ protein\ in\ homogenate} \times 100 \quad \text{(VIII)}$$

To calculate the total protein yield (mg protein/ g DW biomass) over the whole pH-shift process Equation IX was used. Depending on pH-shift process type, the final pellet is either P2A, P2A + P3A (pooled) or P2B.

$$Yield\ (mg\ protein/g\ DW\ biomass) = M_{protein\ in\ final\ pellet\ (mg)}/M_{protein\ in\ homogenate\ (g\ DW)} \times 100 \quad \text{(IX)}$$

2.9.4 Concentration

To calculate the ppm concentration of amino acid samples, Equation X was used.

$$Concentration\ (PPM) = m_{sample\ (mg)}/V_{hydrolysis\ solution\ (L)} \quad \text{(X)}$$

2.9.5 Emulsifying Activity Index (EAI) & Emulsion Stability Index (ESI)

EAI was calculated using Equation XI.

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times DF}{C \times \varphi \times \theta \times 10000} \quad \text{(XI)}$$

A_0 = measured absorbance at 500 nm DF = dilution factor = 200 Θ = cuvette path length = 1cm

C = initial protein concentration (g/ml) φ = volume fraction of oil = 0.25

ESI was calculated using Equation XII.

$$ESI\ (min) = \frac{A_{10} \times \Delta t}{\Delta A} \quad \text{(XII)}$$

A_{10} = measured absorbance at 500 nm after 10 min Δt = elapsed time = 10 min $\Delta A = A_0 - A_{10}$

2.10 Statistical Analysis

To statistically determine significant differences between the resulting data from the six different preservation treatments in the preservation study or between the different harvest months in the seasonal study, one-way ANOVA (ANalysis Of VAriance) was used with a significance level of 5 % ($\alpha = 0.05$) together with Tukey's *post-hoc* test for pairwise comparison. Unpaired Student's t-test was in some cases applied when investigating significance between two specific groups of data.

Error in figures and tables are reported as standard deviation, ±SD. All protein related measurements were run in triplicate, moisture content was measured in triplicate except for the protein isolates which were only measured once and lastly, amino acid analysis and EAI/ESI measurements were performed in duplicate.

3 RESULTS

3.1 SELECTION OF PROTEIN ANALYSIS METHOD TO FOLLOW THE PERFORMANCE OF THE PH-SHIFT METHOD

3.1.1 Results from Method Evaluation

Shown below are the results of some tests evaluating how the protein content of different fractions of the pH-shift process would be determined.

3.1.2 Interference from phlorotannins in the Lowry method

The interference of phlorotannins during the Lowry protein analysis was investigated by running an absorbance scan on the homogenate, S1 and S2A samples when they were in the last step of the Lowry protocol, i.e. ready for spectrophotometric measurement. Resulting scan is displayed in Figure 4. In the Lowry protein analysis, the spectrophotometric measurement was made at 660 nm, looking at Figure 4 there was little interference and difference between the analyzed samples. The Lowry protein analysis was therefore considered suitable for analyzing protein in fractions of the pH-shift process.

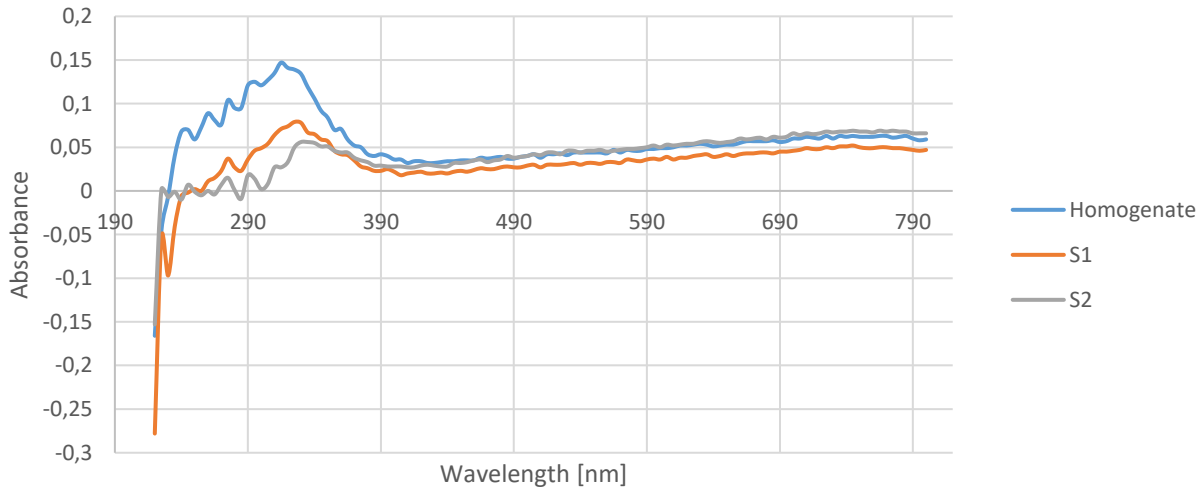

Figure 4. *Absorbance scan of the seaweed homogenate, S1 and S2A fractions, in the last step of the Lowry protocol.*

3.1.3 Sample Pre-treatment

The results of subjecting the initial seaweed homogenates to boiling with SDS or to dilution in 0.1M NaOH was compared based on analysis with the Lowry method. Both SDS-boiling and samples that were simply diluted in 0.1M NaOH resulted in a protein concentration of ~3 mg/ml and there was no significant difference between the treatments.

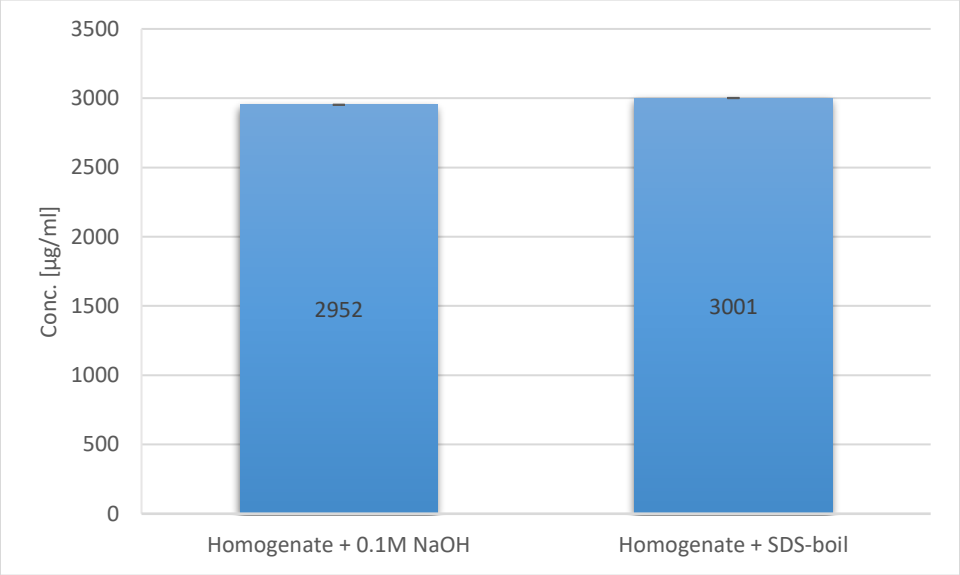

Figure 5. *The concentration of protein in each homogenate sample. The left staple represents the samples that was simply diluted in 0.1M NaOH, whilst the right staple represents the samples that was put through the SDS-boiling procedure prior to the Lowry protein analysis.*

3.2 Results from the Preservation Study

3.2.1 Properties of the Differently Preserved Biomasses

Some basic properties (moisture content, native pH and total protein) of the initial seaweed biomasses were investigated before they were put through the pH-shift process. Results are shown in the sections below.

3.2.1.1 Moisture Content of Biomasses

The results of the moisture content measurements of the biomasses are shown in Figure 6. There was a significant difference between on the one hand the dried biomasses (sun-, oven- and freeze-dried) and on the other the wet biomasses (ensilaged, -20°C and -80°C frozen). However, there was no significant difference between the sun- and oven-dried biomasses, or between the -20°C and -80°C frozen biomasses. The -20°C frozen biomass had the highest measured moisture content of the differently preserved seaweeds, therefore all other biomasses were adjusted to this moisture content prior to the pH-shift process, see Section 2.2.1.

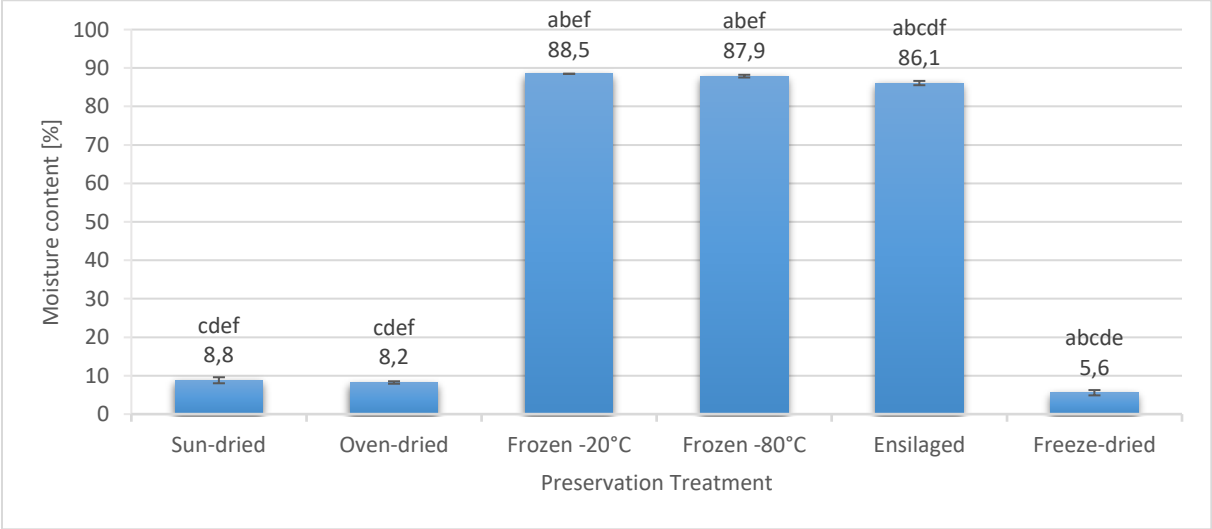

Figure 6. *Moisture content of the differently preserved seaweed biomasses. The numbers adjacent to each column is the moisture content in percent of biomass. Different letters above each data denotes significant differences (α=0.05, a=sun-dried, b=oven-dried, c=-20°C frozen, d=-80°C frozen, e=ensilaged, f=freeze-dried).*

3.2.1.2 Native pH of Biomasses

The native pH of the seaweed biomasses was measured after homogenization in 6 volumes of water, but before the addition of NaOH (Figure 7). There were no significant differences between the oven-, freeze-dried, -20°C and -80°C frozen biomasses, all with a pH close to 7. The sun-dried and ensilaged biomasses however showed significantly lower pH (6.3 and 4.4, respectively) compared to the other biomasses.

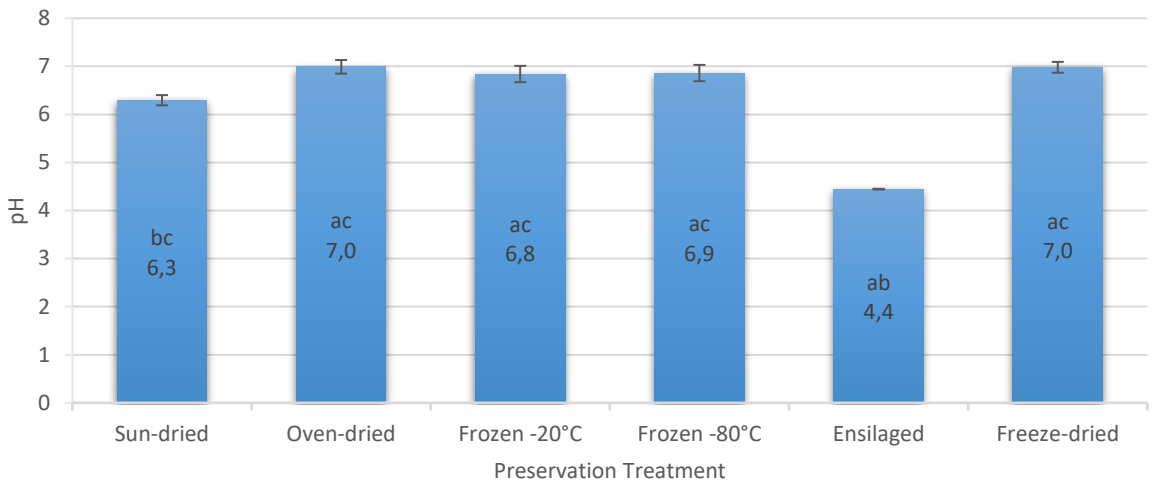

Figure 7. *Native pH of the differently preserved seaweed biomasses after homogenization in 6 volumes of water. The numbers within each staple is the pH of the homogenate. Different letters above each data denotes significant differences (α=0.05, a=sun-dried, b=oven-dried; -20°C frozen; -80°C frozen; freeze-dried, c=ensilaged).*

3.2.1.3 Total Protein of Biomasses

Total protein results are shown in Figure 8. Preservation method significantly affected the protein content of the seaweed biomasses, with minimum protein content in sun-dried biomass (6.0 %) and maximum in freeze-dried biomass (8.2 %).

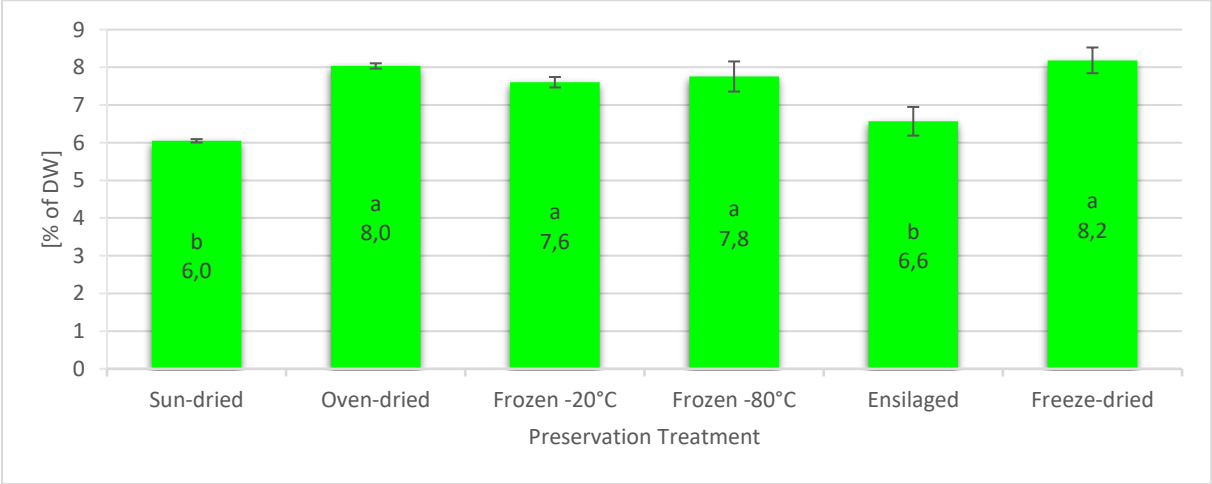

Figure 8. *Total protein amount of each biomass. Numbers adjacent each column represents the percentage of DW biomass that protein accounts for, calculated with Equation V. Different letters above each data denotes significant differences (α=0.05, a=sun-dried; ensilaged, b=oven-dried; -20°C frozen; -80°C frozen; freeze-dried).*

3.3 Outcome of the pH-shift Process – Preservation Study

This section displays the results of the pH-shift processes performed on the differently preserved biomasses, including; protein solubility at alkaline pH, protein extractability at alkaline pH, protein precipitation at pH 2 and total protein yield.

3.3.1 Protein Solubility in the pH-shift Process

Shown below are the results from the protein solubility measurements/calculations for the three different types of pH-shift processes. Overall the choice of storage method influenced protein solubility, protein extraction, protein precipitation and total protein yield.

3.3.1.1 Solubility Results from pH-shift Process - Type I

Protein solubility at pH 12 and at pH 2 of the pH-shift process type I was calculated for each biomass using Equation IV, results are shown in

Figure 9. The oven-dried, freeze-dried, -80°C and -20°C frozen biomasses achieved the highest protein solubilities (83.4 %, 82.5 %, 82.5 % and 80.7 %, respectively) at pH 12. The sun-dried (62.1 %) and ensilaged (31.3 %) biomasses resulted in significantly lower protein solubilities at pH 12. The pattern was similar for regarding solubility at pH 2, with an average decrease in solubility (percentage points) of 11.1 %, compared to at pH 12, over all biomasses. The largest decrease in solubility was achieved with the -20°C frozen biomass with 13.9 % decrease from pH 12 to 2, whilst the smallest decrease in solubility was 8.5 % for the sun-dried biomass.

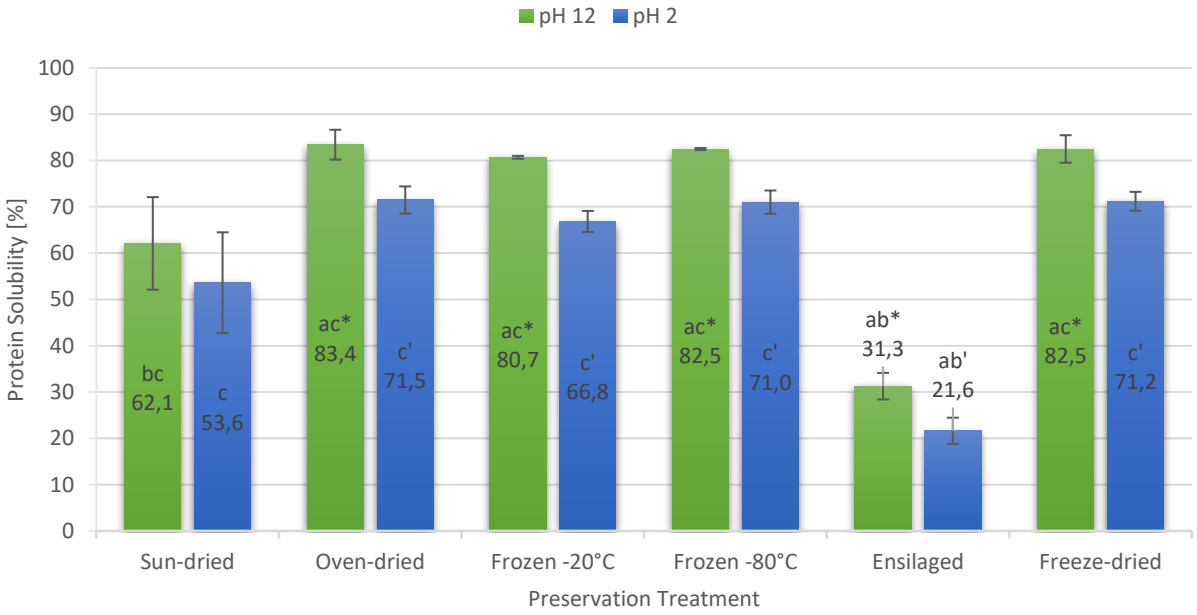

Figure 9. *Protein solubility in the first and second stage of the pH-shift process Type I (i.e. in S1 at pH 12 and S2A at pH 2), for each differently preserved biomass. Numbers within each column represents the protein solubility in percent, calculated with Equation IV. Different letters above each data denotes significant differences between biomasses (a=sun-dried, b=oven-dried; -20°C frozen; -80°C frozen; freeze-dried, c=ensilaged) and symbols denotes differences between supernatants within each biomass (‘ =pH 12, * = pH 2) α=0.05.*

3.3.1.2 Solubility Results from pH-shift Process – Type II

Protein solubility at pH 12, at pH 2 and at pH 2 after freeze-thawing, of the pH-shift process type II was calculated for each biomass using Equation IV, results are shown in

Figure **10**. Resulting protein solubilities at pH 12 and pH 2were the same as during pH-shift type I, see Section 3.3.1.1.

Again, the oven-dried, freeze-dried, -80°C frozen, -20°C frozen and sun-dried biomasses had the highest protein solubilities at pH 12 (63.3 %, 60.0 %, 60.0 %, 60.7 % and 50.7 %, respectively). However, the ensilaged biomass (18.4 %) resulted in significantly lower protein solubilities at pH 2 with freeze-thawing. The average decrease in protein solubility (percentage points) from pH 2 to pH 2 with freeze-thawing was 7.1 %, a significant decrease for the -80°C frozen, oven- and freeze-dried. The freeze-dried biomass had the largest protein solubility decrease, from pH 2 to pH 2 with freeze-thawing, of 11.2 %, whilst the sun-dried biomass had the lowest decrease of 2.9 %.

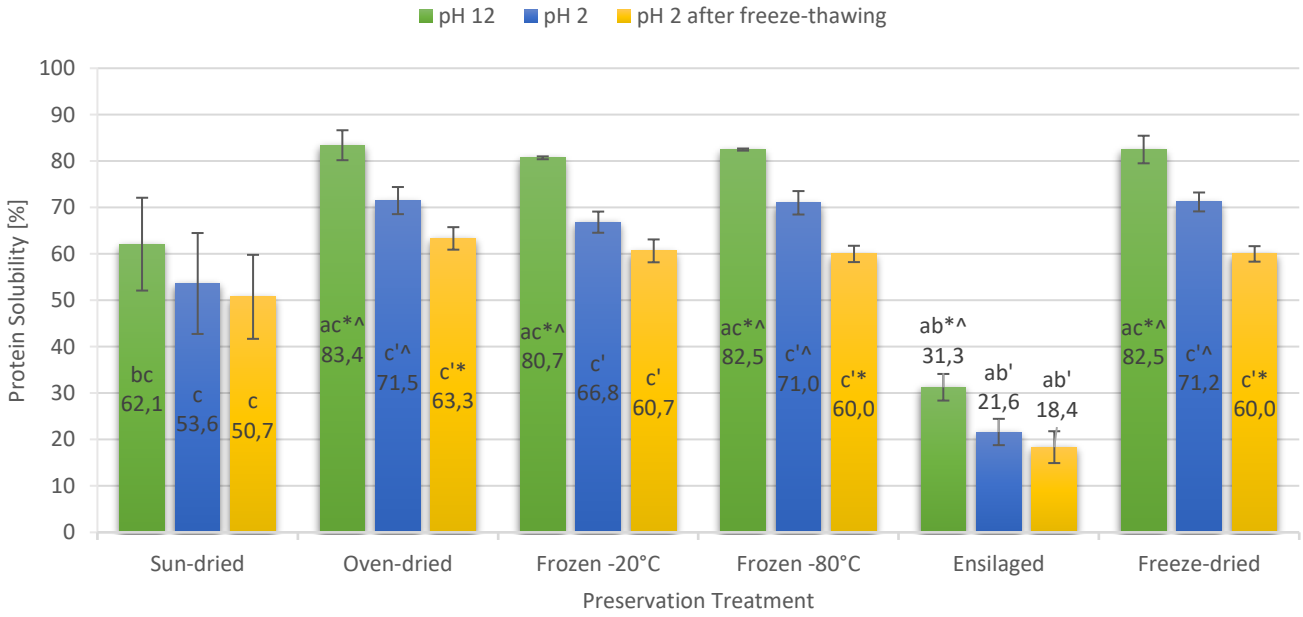

Figure 10. *Protein solubility in the first and second stage of the pH-shift process Type II (i.e. at pH 12 and at pH 2 without and with freeze-thawing), for each differently preserved biomass. Numbers adjacent each column represents the protein solubility in percent, calculated with Equation IV. Different letters above each data denotes significant differences between biomasses (a=sun-dried, b=oven-dried; -20°C frozen; -80°C frozen; freeze-dried, c=ensilaged) and symbols denotes differences between supernatants within each biomass (‘ =pH 12, * = pH 2, ^ = pH 2 after freeze-thawing) α=0.05.*

3.3.1.3 Solubility Results from pH-shift Process – Type III

Protein solubility at pH 12 and at pH 2 with freeze-thawing, of the pH-shift process type III was calculated for each biomass using Equation IV, results are shown in

Figure 11. Again, the resulting solubilities of at pH 12 were the same as for pH-shift type I, see Section 3.3.1.1.

Yet again, the oven-dried, freeze-dried, -80°C frozen, -20°C frozen and sun-dried biomasses had the highest protein solubilities at pH 2 with freeze-thawing (61.6 %, 59.2 %, 60.1 %, 60.1 % and 48.8 %, respectively). As previously, the ensilaged (16.6 %) achieved significantly lower protein solubility at pH 2 with freeze-thawing. The average decrease in protein solubility (percentage points) from pH 12 to pH 2 with freeze-thawing was 19.35 %, a significant decrease for the oven-dried, -20°C frozen, -80°C frozen, ensilaged and freeze-dried biomasses. The freeze-dried biomass had the largest protein solubility decrease of 23.26 %, from pH 12 to pH 2 with freeze-thawing, whilst the sun-dried biomass had the lowest decrease of 13.31 %.

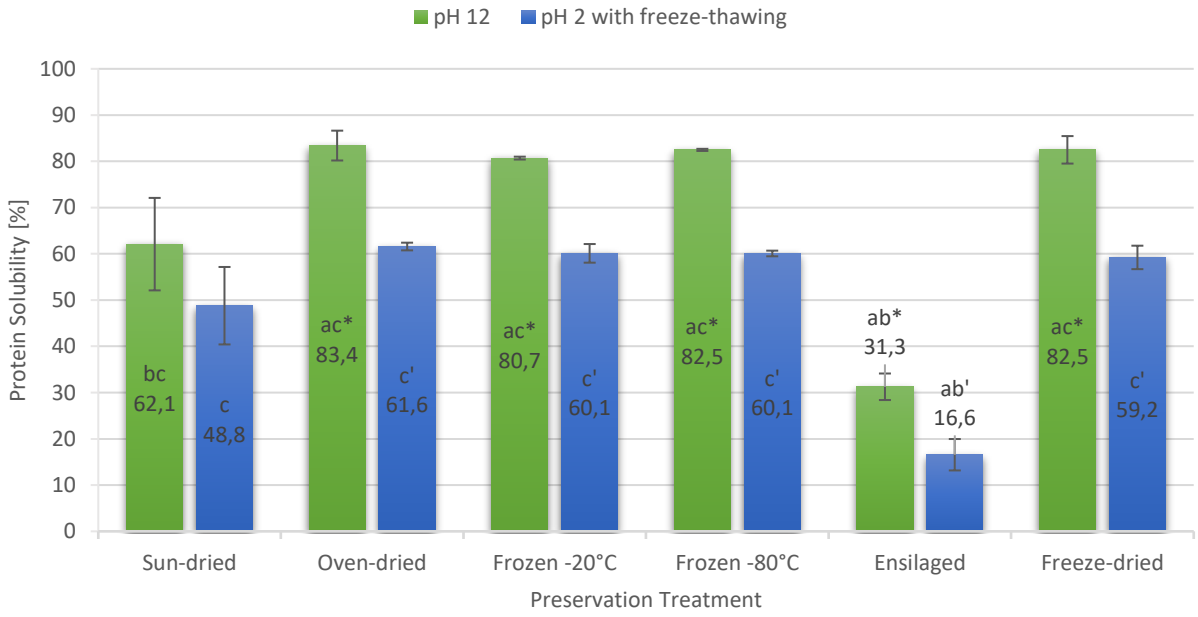

Figure 11. *Protein solubility in the first and second stage of the pH-shift process Type III (i.e. at pH 12 and at pH 2 with freeze-thawing), for each differently preserved biomass. Numbers adjacent each column represents the protein solubility in percent, calculated with Equation IV. Different letters above each data denotes significant differences between biomasses (a=sun-dried, b=oven-dried; -20°C frozen; -80°C frozen; freeze-dried, c=ensilaged) and symbols denotes differences between supernatants within each biomass (' =pH 12, * = pH 2) α=0.05.*

3.3.2 Protein Extraction Yield – First Stage of the pH-shift Process

The total amount of protein that was solubilized from the homogenate and partitioned into S1 during the first stage of the pH-shift process was measured and calculated as protein extraction yield using Equation VI, summarized in

Figure 12.

The freeze-dried biomass exceeded all other biomasses with a protein extraction yield of 90.9 %, followed by the -20°C frozen, oven-dried, -80°C frozen and sun-dried biomasses (79.9 %, 77.2 %, 65.7 % and 64.9 %, respectively). Lastly, the ensilaged biomass reached a significantly lower protein extraction yield (25.4 %) than all the other biomasses. It is important to stress that the yield in the first stage of the pH-shift process depends both on the protein solubility of the seaweed homogenate at pH 12, and on the size of P1, which will still be moist after the centrifugation and thereby retaining some of the solubilized proteins.

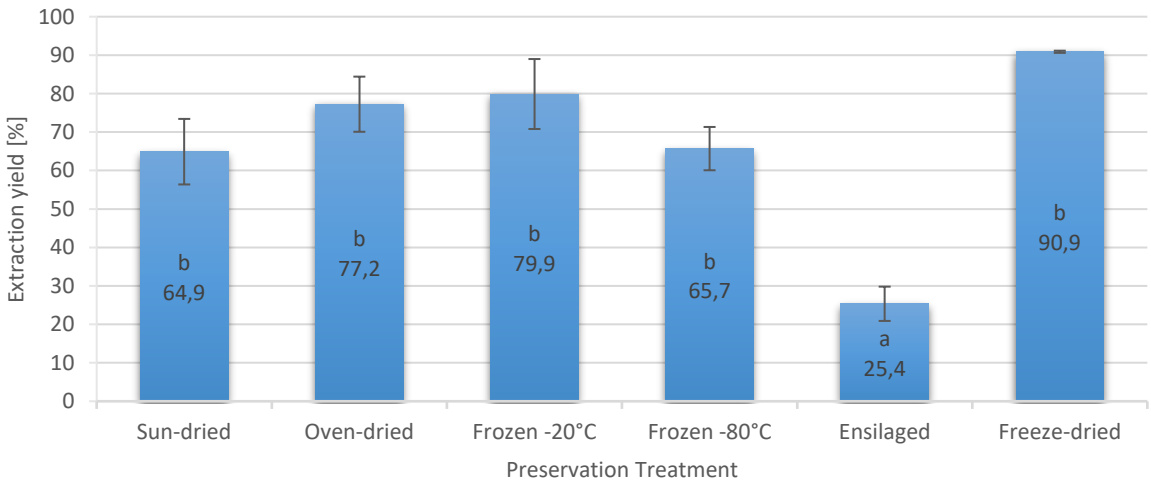

Figure 12. *The protein extraction yield, during the first stage of the pH-shift process, for each different biomass preservation treatment. The yield was calculated as mg of protein in S1 based on the amount of protein in the homogenate, using Equation VI. Different letters above each data denotes significant differences (α=0.05, a=sun-dried; oven-dried; -20°C frozen; -80°C frozen; freeze-dried, b=ensilaged).*

3.3.3 Protein Precipitation Yield – Second Stage of the pH-shift Process

The protein precipitation yields for the different pH-shift processes, i.e. the protein content of the final pellet over the amount of available protein in S1, were calculated using Equation VII and are shown in

Figure 13.

For pH-shift process Type I, the ensilaged biomass achieved the highest protein precipitation yield (30.3 %), followed by the -20°C frozen, oven-, freeze- and sun-dried biomasses (14.0 %, 13.0 %, 12.3% and 11.5 %, respectively). The lowest protein precipitation yield was obtained by the -80°C frozen biomass (9.6 %).

Secondly, for pH-shift process Type II, again the ensilaged biomass obtained the highest protein precipitation yield (42.5 %), followed by freeze-dried, oven-dried, -80°C and -20°C frozen (29.1 %, 26.1 %, 25.3 % and 24.4 %, respectively). The lowest protein precipitation yield was in this case the sun-dried biomass (18.6 %).

Finally, for pH-shift process Type III, yet again the ensilaged biomass gave a significantly greater protein precipitation yield than all other biomasses (47.0 %). The -80°C frozen and freeze-dried biomasses gave similar protein precipitation yields (30.3 % and 29.3 %, respectively). Lastly, the oven-dried, -20°C frozen and sun-dried biomasses gave the lowest protein precipitation yields (26.4 %, 25.1 % and 20.6 %, respectively).

For pH-shift process Type I, II and III, the protein precipitation yield of the ensilaged biomass was significantly higher than for all other biomasses. There was also a significant increase in protein precipitation yield comparing pH-shift process Type I against Type II and Type I against Type III for all biomasses except for when using the ensilaged biomass.

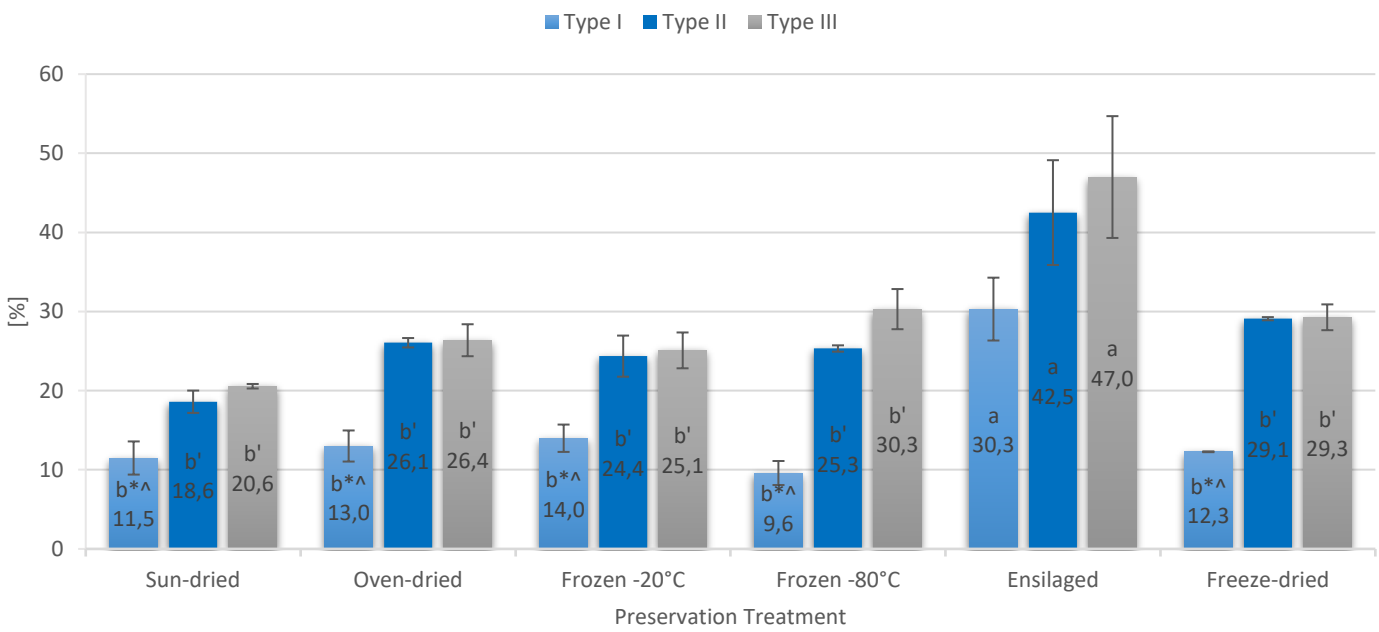

Figure 13. *The protein precipitation yield, i.e. yield in the second stage of the pH-shift process, for each different biomass preservation treatment. The yield was calculated as mg of protein in the final pellet based on the amount of protein in S1, using Equation VII. Different letters above each data denotes significant differences between biomasses (a=sun-dried; oven-dried; -20°C frozen; -80°C frozen; freeze-dried, b=ensilaged) and symbols denotes differences between pH-shift types within each biomass (' = Type I, * = Type II, ^ = Type III), α=0.05.*

3.3.4 Total Protein Yield

The total protein yield for the different pH-shift processes, i.e. the protein content of the final pellet over the amount of available protein in the starting homogenate, were calculated for each differently preserved biomass using Equation VIII and are shown in

Figure 14.

For pH-shift process Type I, the freeze-dried, -20°C frozen and oven-dried biomasses gave rise to the highest total protein yields (11.2 %, 11.1 % and 10.0 %, respectively). The ensilaged, sun-dried and -80°C frozen biomasses reached significantly lower total protein yields (7.6 %, 7.4 % and 6.3 %, respectively).

Secondly, for pH-shift process Type II, the freeze-dried biomass gave rise to a significantly higher total protein yield than all other biomasses (26.4 %), followed by the oven-dried, -20°C and -80°C frozen biomasses (20.1 %, 19.3 % and 16.6 %, respectively). The sun-dried and ensilaged biomasses reached significantly lower total protein yields than the aforementioned biomasses (12.0 % and 10.6 %, respectively).

Lastly, for pH-shift process Type III, a significantly higher total protein yield was again achieved with the freeze-dried biomass (26.6%), again followed by the oven-dried, -20°C and -80°C frozen biomasses (20.3 %, 19.9 % and 19.8 %, respectively). Yet again the sun-dried and ensilaged biomasses reached the lowest total protein yields (13.4 % and 11.7 %, respectively), both being significantly lower than the aforementioned biomasses.

Comparing pH-shift processes, there was a significant increase in total protein yield for all biomasses except for the sun-dried biomass when comparing Type I against Type III. Comparing pH-shift process Type II against Type III there was only a significant difference for the ensilaged biomass.

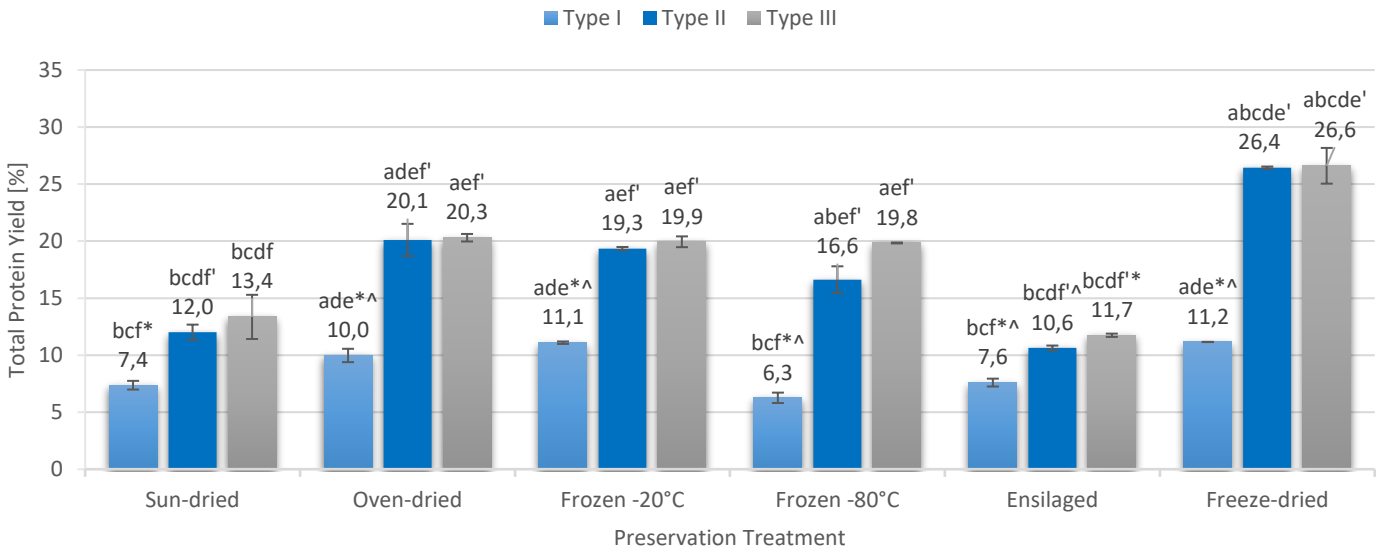

Figure 14. *Total protein yield of the three versions of pH-shift processes, for each different biomass preservation treatment. The yield was calculated as mg of protein in the final pellet based on the amount of protein in the homogenate, using Equation VIII. Different letters above each data denotes significant differences between biomasses (a=sun-dried, b=oven-dried, c=-20°C frozen, d=-80°C frozen, e=ensilaged, f=freeze-dried) and symbols denotes differences between pH-shift types within each biomass (‘ = Type I, * = Type II, ^ = Type III), α=0.05.*

3.3.4.1 Total Protein Yield per Amount Biomass

The actual amount of protein, in mg, produced per g dry of starting material is highlighted in

Figure 15, values were calculated using Equation IX.

For pH-shift process Type I, the freeze-dried biomass gave rise to the highest total protein yield (9.1 mg/g DW), significantly higher than all other biomasses except the -20°C frozen biomass. The -20°C frozen and oven-dried biomasses resulted in total protein yields of 8.4 mg/g DW and 8.0 mg/g DW, respectively, significantly higher than the ensilaged, -80°C frozen and sun-dried biomasses (5.0 mg/g DW, 4.9 mg/g DW and 4.5 mg/g DW, respectively).

Secondly, for pH-shift process Type II, the freeze-dried biomass achieved a significantly higher total protein yield than all other biomasses (21.6 mg/g DW), followed by the oven-dried, -20°C and -80°C frozen biomasses (16.2 mg/g DW, 14.7 mg/g DW and 12.9 mg/g DW, respectively). The sun-dried and ensilaged biomasses gave significantly lower total protein yields than the aforementioned biomasses (7.3 mg/g DW and 7.0 mg/g DW, respectively).

Lastly, for pH-shift process Type III, a significantly higher total protein yield was again achieved by the freeze-dried biomass (21.8 mg/g DW), again followed by the oven-dried, -20°C and -80°C frozen biomasses (16.3 mg/g DW, 15.4 mg/g DW and 15.2 mg/g DW, respectively). Yet again the sun-dried and ensilaged biomasses reached the lowest total protein yields (8.1 mg/g DW and 7.7 mg/g DW, respectively) significantly lower than the aforementioned biomasses.

Comparing pH-shift processes, there was a significant increase in total protein yield for all biomasses except for the sun-dried biomass when comparing Type I against Type III. Comparing pH-shift process Type II against Type III there was only a significant difference for the ensilaged biomass.

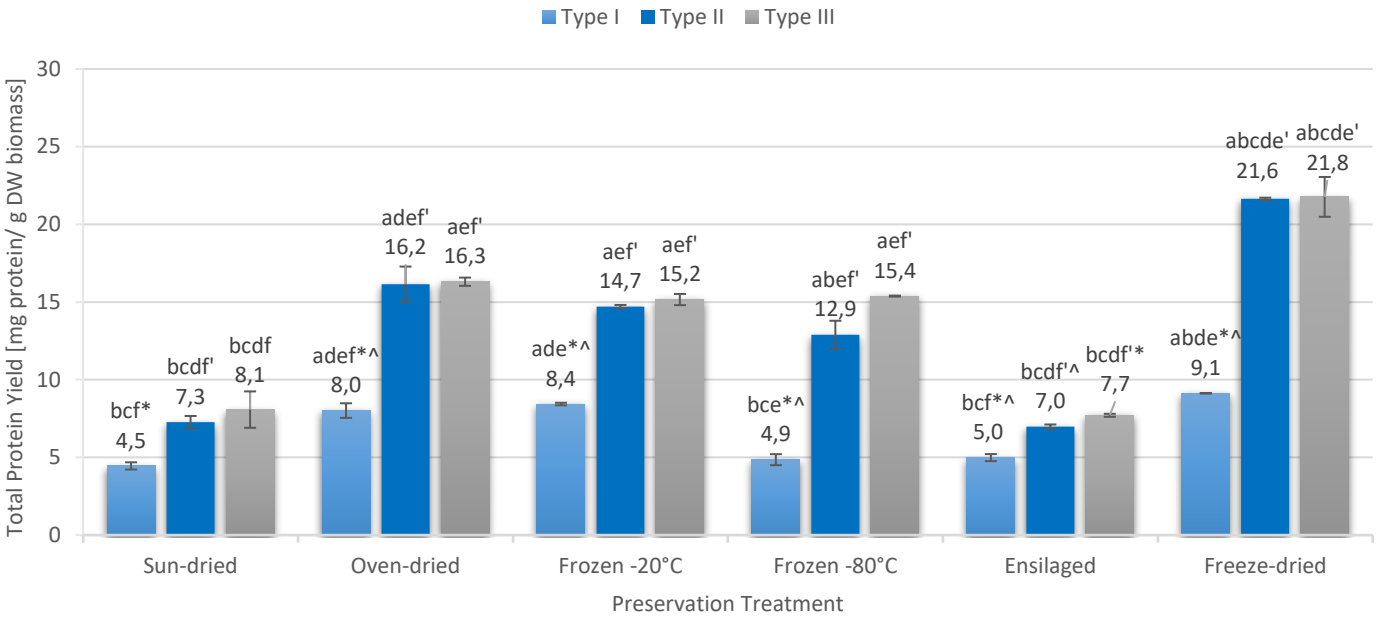

Figure 15. *Total protein yield of the pH-shift processes, for each different biomass preservation treatment. The yield was calculated as mg of protein in the final pellet based on the amount of starting dry biomass, using Equation IX. Different letters above each data denotes significant differences between biomasses (a=sun-dried, b=oven-dried, c=-20°C frozen, d=-80°C frozen, e=ensilaged, f=freeze-dried) and symbols denotes differences between pH-shift types within each biomass (‘ = Type I, * = Type II, ^ = Type III), α=0.05.*

3.4 Up-Concentration of Protein during pH-shift processing as a function of Preservation Method

The moisture content and protein content (percent of freeze-dried biomass (FDB) or freeze-dried protein isolate (FDPI)) for the initial biomasses and their respective produced protein isolates, i.e. P2A and P2B, are reported in Table 3. In almost all cases, the protein content on dry matter basis was doubled or tripled, compared to the starting biomass. There were some exceptions; the protein content of the ensilaged P2A and P2B decreased compared to the initial biomass, however, for both oven- and freeze-dried P2B the protein content increased almost five-fold. Within all treatments there was a significant difference in protein content comparing any combination of the biomass, P2A or P2B. For P2A, cross comparing all preservation methods there was a significant difference in protein content, except when comparing the sun-dried to the -80°C frozen biomass. For P2B, cross all treatments there was a significant difference in protein content, except when comparing the sun-dried to the -20°C frozen biomass.

See Table B3, in Appendix B, for protein content on the wet matter basis of biomasses, P2A and P2B.

Table 3. A*verage moisture content and protein content of each biomass or protein isolate. Protein data are given on a dry matter basis.*

	Biomass		Pellet 2A			Pellet 2B		
Treatment	Moisture Content [%]	Protein Content [% of FDB]	Moisture Content [%] *	Protein Content [% of FDPI]	Conc. Factor	Moisture Content [%] *	Protein Content [% of FDPI]	Conc. Factor
Sun-dried	8,8 ± 0,8	6,1 ± 0,1	94,6	15,4 ± 0,4	2,5	96,3	20,3 ± 0,1	3,3
Oven-dried	8,2 ± 0,3	8,0 ± 0,1	96,8	24,9 ± 0,2	3,1	96,4	40,5 ± 0,5	5,1
Frozen -20°C	88,5 ± 0,0	7,6 ± 0,1	97,2	22,0 ± 0,6	2,9	97,9	19,0 ± 0,8	2,5
Frozen -80°C	87,9 ± 0,4	7,8 ± 0,4	96,2	15,3 ± 0,8	2,0	97,6	26,2 ± 0,6	3,4
Ensilaged	86,1 ± 0,6	6,6 ± 0,4	97,5	1,3 ± 0,1	0,2	94,1	2,0 ± 0,3	0,3
Freeze-dried	5,6 ± 0,7	8,2 ± 0,3	96,7	28,0 ± 0,9	3,4	95,7	37,6 ± 0,7	4,6

** Moisture content was only measured once. FDB: freeze-dried biomass. FDPI: freeze-dried protein isolate.*

3.5 pH Dependent Solubility of Dried Protein Isolates as a Function of Preservation Method

The protein solubility of the produced dried protein isolates after mixing with DI-water and altered to different pH-values were investigated. What is referred to as "native" pH is the pH measured when the protein powder was initially mixed with DI-water. The pH dependent protein solubility of the P2A and P2B protein isolates produced from the freeze-dried, oven-dried, -20°C frozen and sun-dried biomasses are shown in Figure 16 and Figure 17, respectively.

The P2A protein isolates produced from the freeze-dried, oven-dried and -20°C frozen biomasses all showed similar protein solubility as a function of pH (Figure 16); low to moderate solubility at native pH to pH 5 and then increasing to high/maximum protein solubility with more alkaline conditions. The P2A of the sun-dried biomass displayed a different pattern with high protein solubility throughout the pH-range, however, also reaching its maximum at more alkaline conditions.

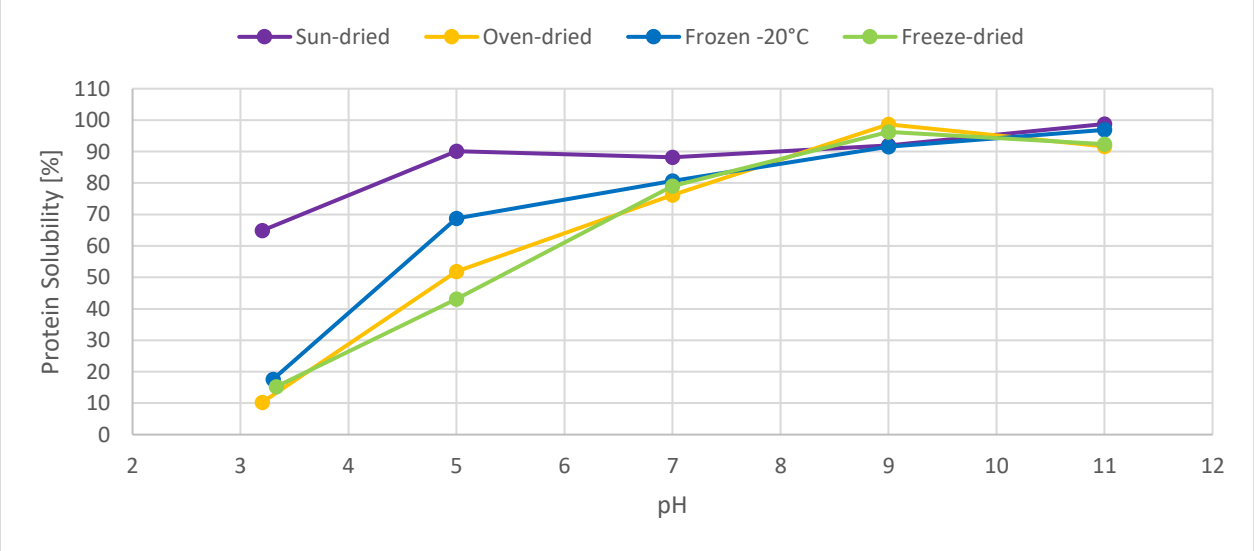

Figure 16. *Protein solubility curve for the P2A protein powders produced from the freeze-dried, oven-dried, -20°C frozen and sun-dried biomasses as a function of pH, ranging from native pH to pH 11. 20 mg powder was mixed with 25 ml water.*

The P2B protein isolates of the oven-dried and freeze-dried biomasses had in general a similar protein solubility pattern to that of their P2A protein isolate, see Figure 17. Both P2B protein isolates of the freeze-dried and oven-dried biomasses, again, displayed low protein solubility in the acid range, however, in the neutral and alkaline range they reached very high/maximum protein solubility. In contrast, the P2B protein isolates of the -20°C frozen and sun-dried biomasses displayed relatively high protein solubilities throughout the whole pH-range. P2B protein isolates of the sun-dried biomass had ~10-20 % less protein

solubility between pH 3 and 9 than its P2A protein isolate. Finally, the P2B protein isolate of the -20°C frozen biomass showed greatly higher protein solubility at native pH and slightly higher protein solubility at pH 5 than its P2A protein isolate.

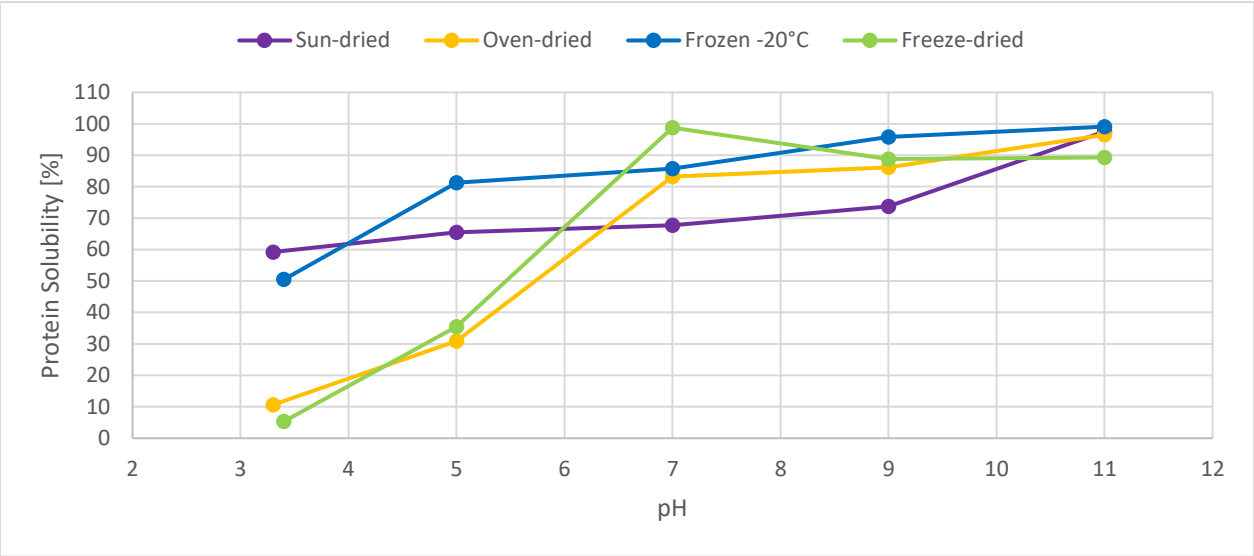

Figure 17. *Protein solubility curve for the dried P2B protein isolates produced from the freeze-dried, oven-dried, -20°C frozen and sun-dried biomasses as a function of pH, ranging from native pH to pH 11.*

3.6 Emulsifying Activity Index & Emulsion Stability Index – Preservation Study

Emulsifying activity index (EAI) was investigated for the P2A and P2B protein isolates produced from the sun-dried, oven-dried, frozen -20°C and freeze-dried biomasses. EAI results are shown as a function of pH in Figure 18 and Figure 19. Due to lack of material the EAI of the P2A protein isolate of the sun-dried biomass was only investigated for pH 7.

All protein isolates displayed an increasing EAI with more alkaline pH. At "native" pH (2.1 - 2.6) the P2A protein isolate of the -20°C frozen biomass reached an EAI of 69 m^2/g, whilst the oven-dried and freeze-dried P2A protein isolates showed a much lower EAI of ~11 m^2/g. Further, at pH 7, P2A protein isolates of the sun-dried and -20°C frozen biomasses reached the highest EAI-values of 115 m^2/g and 97 m^2/g, respectively, again followed by the oven-dried and freeze-dried P2A protein isolates at ~ 70 m^2/g. Lastly, at pH 11, all measured P2A protein isolates reached their maximum EAI at ~90-110 m^2/g.

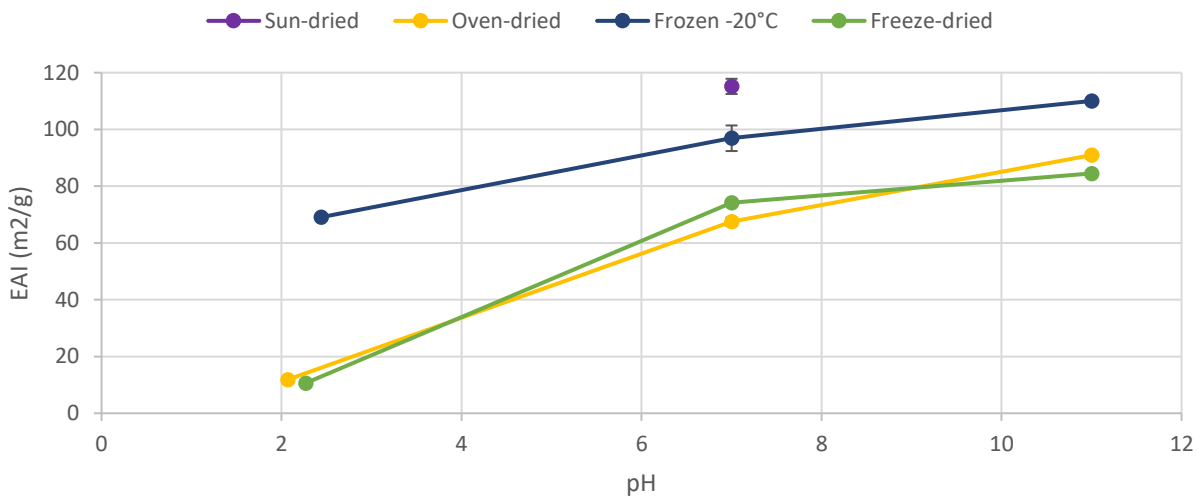

Figure 18. *EAI of the P2A protein isolates produced from the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses, as a function of pH.*

Throughout the whole pH-range there was a great difference in EAI-values between protein isolates from the sun-dried and -20°C frozen biomasses, with the higher EAI-values, compared to the protein isolates from the oven-dried and freeze-dried biomasses. Both P2B protein isolates from the oven-dried and freeze-dried biomasses displayed similar patterns, ranging from minimum EAI of ~20 m^2/g at pH ~2 to maximum EAI of ~60 m^2/g at pH 11. Finally, the P2B protein isolates of the sun-dried and -20°C frozen biomasses ranged from minimum EAI of ~85 m^2/g at pH ~2 to maximum EAI of ~110 m^2/g at pH 11.

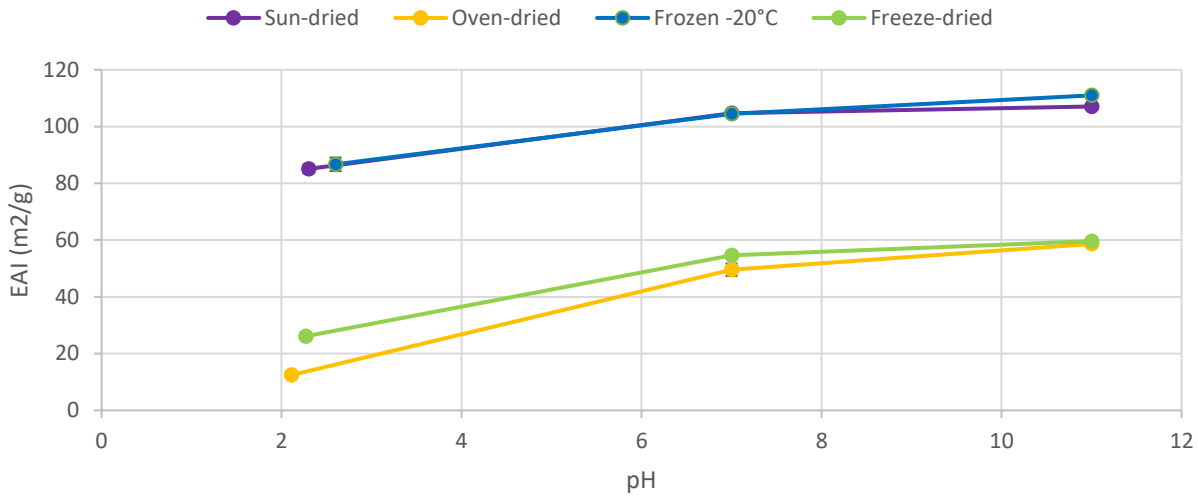

Figure 19. *EAI of the P2B protein isolates produced from the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses, as a function of pH.*

Emulsion stability index (ESI) was investigated for the P2A and P2B protein isolates produced from the sun-dried, oven-dried, frozen -20°C and freeze-dried biomasses. ESI results are shown as a function of pH in Figure 20 and Figure 21. Due to lack of material the ESI of the P2A protein isolate of the sun-dried biomass was only investigated for pH 7.

P2A protein isolates of the oven-dried and freeze-dried biomasses reach their maximum ESI at pH 11 (402 min and 808 min, respectively). The P2A protein isolate of the -20°C frozen biomass reached its maximum ESI of 130 min at pH 7 and showed very low ESI values at pH ~2 and 11 (20 min and 3.8 min, respectively). The P2A protein isolate from the sun-dried biomass was only investigated for pH 7, with a EAI of 190 min.

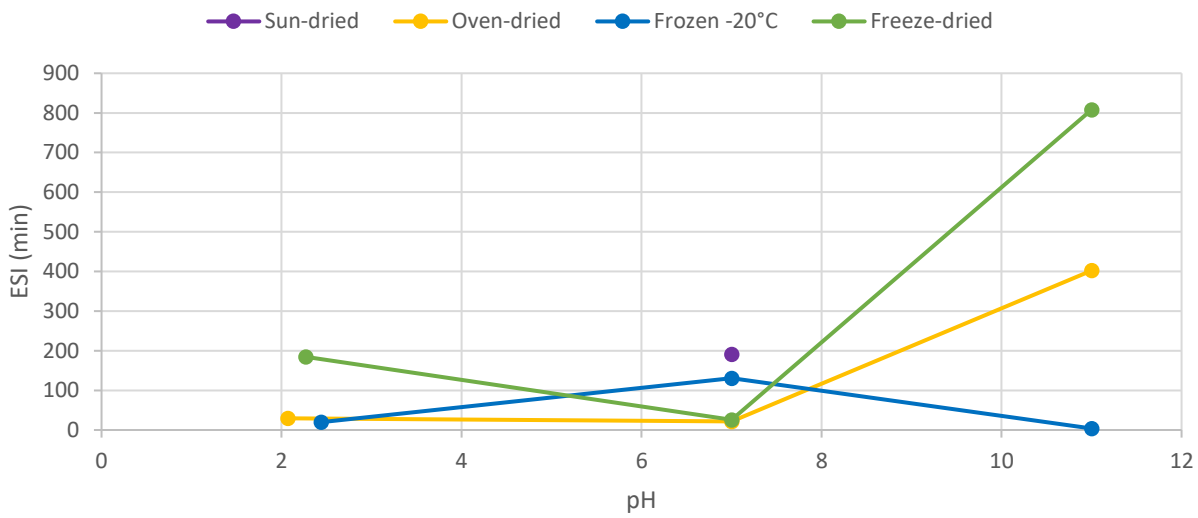

Figure 20. *ESI of the P2A protein isolates produced from the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses, as a function of pH.*

The P2B protein isolates of the -20°C frozen and sun-dried biomasses reached their maximum ESI-values at pH 7 (33 min and 114 min, respectively) and minimum values at pH 11 (4 min and 9 min, respectively). P2B protein isolates of the oven-dried and freeze-dried biomasses reached extremely high ESI-values at pH 11 (3559 min and 3604 min, respectively, outside of the figure range) and minimum ESI-values at pH ~2 (31 min and 95 min, respectively).

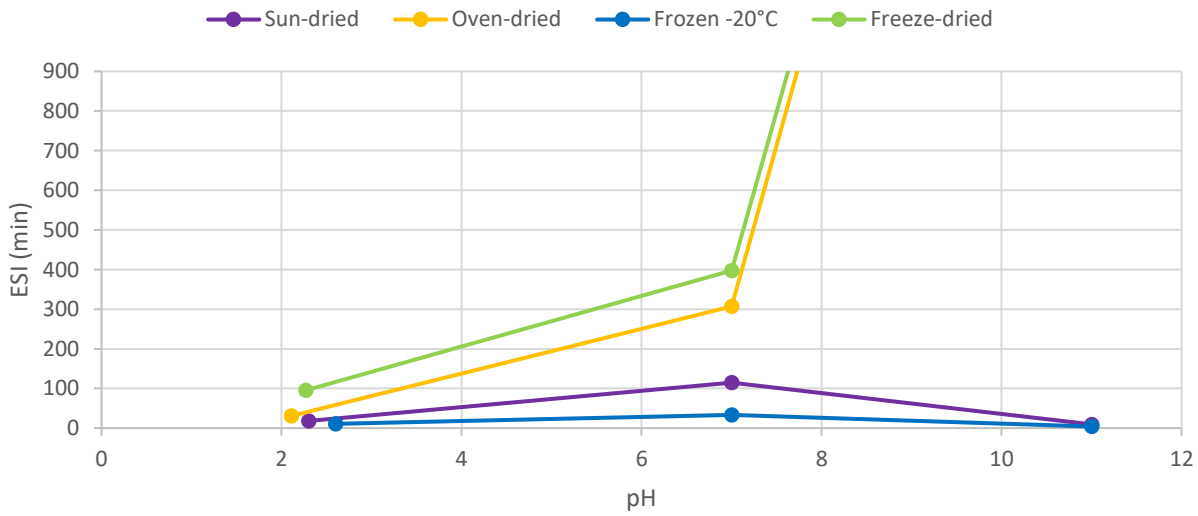

Figure 21. *ESI of the P2B protein isolates produced from the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses, as a function of pH.*

3.7 Microstructure of Protein Isolate Produced Emulsions as a function of Preservation Method

Samples of each produced emulsion, i.e. different protein isolates mixed with water and sunflower oil at different pH, were analyzed with microscopy and pictures are shown below in Figures 22-25. What is referred to as the "native" pH of the emulsion is the pH of the protein isolate mixed with just DI-water.

The P2A protein isolate of the sun-dried biomass was only investigated for pH 7, due to lack of protein isolate material. However, comparing P2A with P2B protein isolates at pH 7, they displayed similar patterns, which can also be seen when comparing these two pictures to the P2B protein isolate at pH 11, see Figure 22. This observation was in agreement with the protein solubility pattern (see Figure 16 and Figure 17) and the EAI-values of both isolates (see Figure 18 and Figure 19). At native pH (2.3) the P2B protein isolates of the sun-dried biomass has fairly high protein solubility, making it possible to still create an emulsion at this low pH (picture 2 in Figure 22).

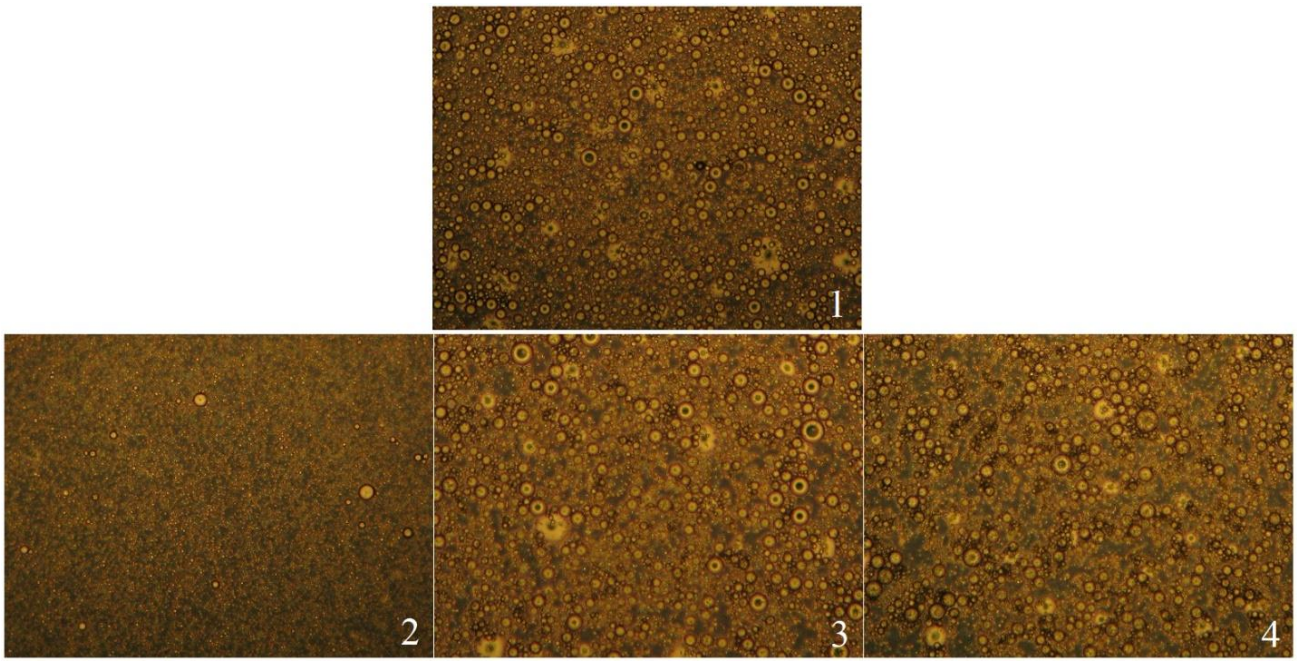

Figure 22. *Pictures of sunflower oil and water emulsions created with P2A and P2B protein isolates of the sun-dried biomass as emulsifiers. What is referred to as the "native" pH of the emulsion is the pH of the protein isolate mixed with just DI-water. Upper row: [1] P2A at pH 7. Lower row: [2] P2B at native pH (2.3), [3] P2B at pH 7, [4] P2B at pH 11.*

The emulsions created with P2A and P2B protein isolates of the oven-dried biomass are shown in Figure 23. Even though the protein solubility of both these isolates was very low at native pH (~10 %, see Figure 16 and Figure 17), a weak emulsion was still created, more visible for P2B protein isolate. For higher pH's (7 and 11) the emulsion-network became tighter and more connected, again in accordance with the protein solubility results and the EAI-values (see Figure 18 and Figure 19) of both protein isolates.

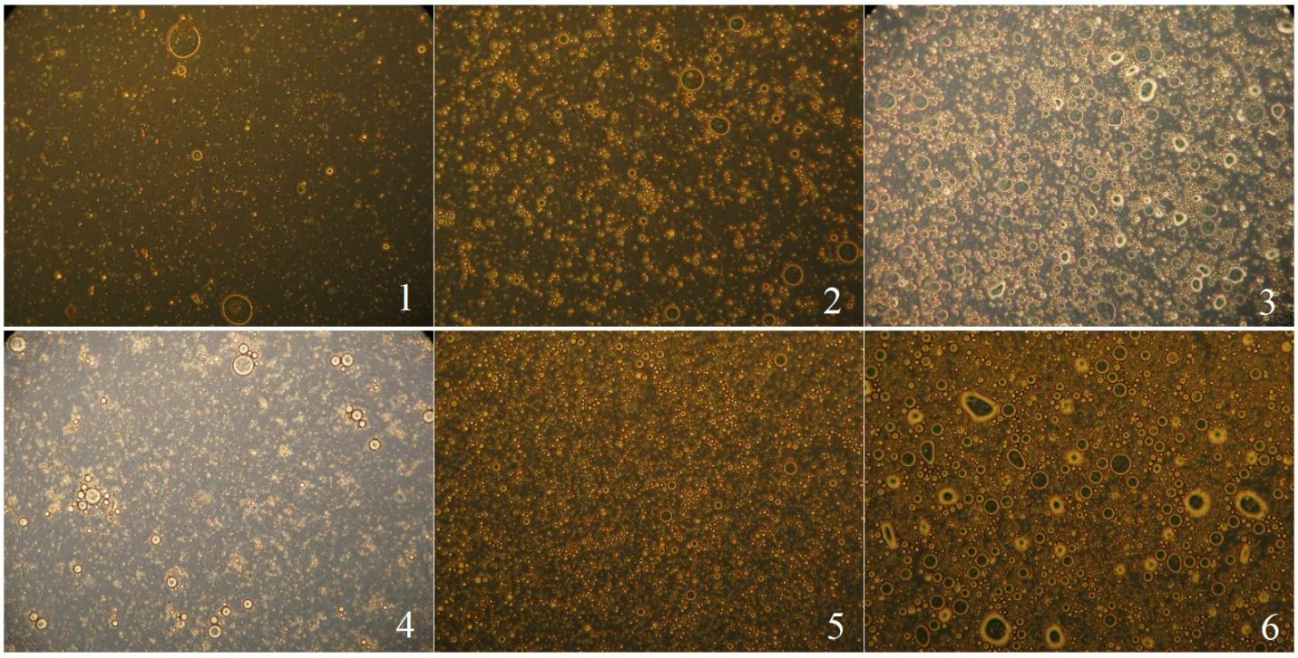

Figure 23. *Pictures of sunflower oil and water emulsions created with P2A and P2B protein isolates of the oven-dried biomass. Upper row: [1] P2A at native pH (2.1), [2] P2A at pH 7, [3] P2A at pH 11. Lower row: [4] P2B at native pH (2.1), [5] P2B at pH 7, [6] P2B at pH 11.*

Further, the emulsions created with P2A and P2B protein isolates of the -20°C frozen biomass are shown in Figure 24. For both protein isolates the previously measured protein solubility (see Figure 16 and Figure 17) was relatively high throughout the whole pH-range, except for the P2A protein isolate at native pH, also in accordance with the EAI-values (see Figure 18 and Figure 19). Even though the difference in protein solubility for P2A and P2B protein isolates at native pH was not that visible in the emulsion pictures (1 and 4, in Figure 24), it was displayed in their EAI-values; 69 m^2/g protein and 87 m^2/g protein, respectively. The emulsion-networks of both protein isolates at pH 7 and 11 were similar (all with EAI-values around 100-110 m^2/g protein).

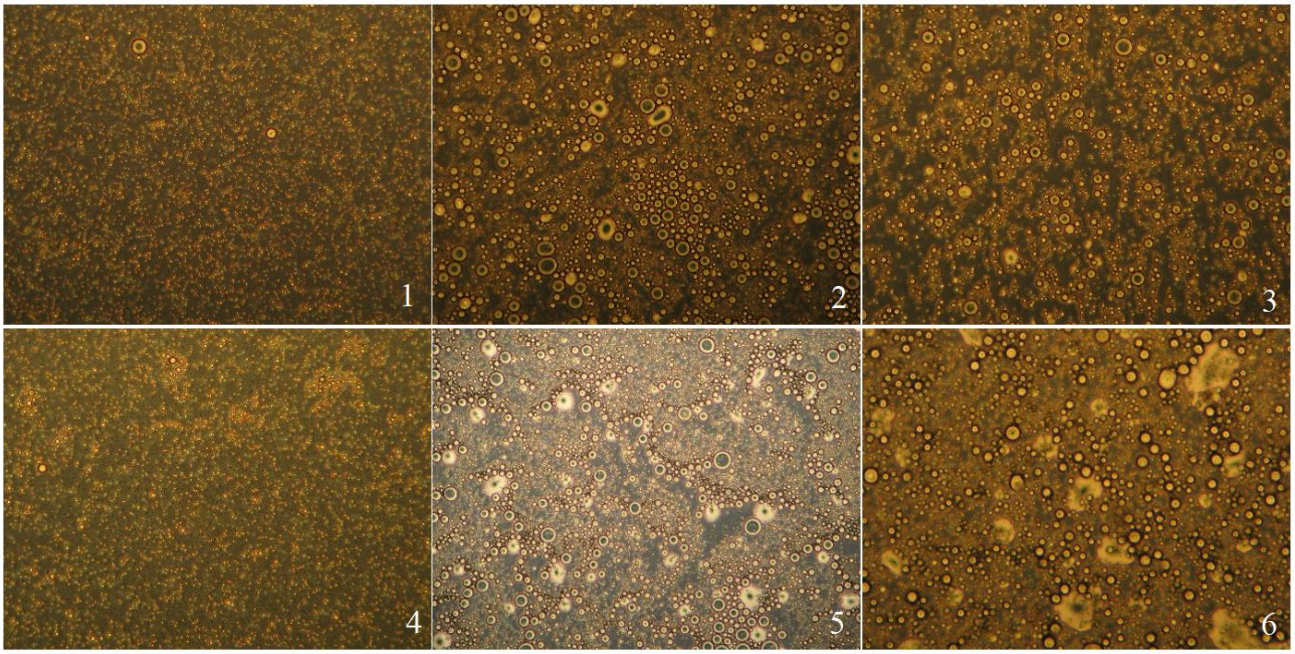

Figure 24. *Pictures of sunflower oil and water emulsions created with P2A and P2B protein isolates of the -20°C frozen biomass. Upper row: [1] P2A at native pH (2.4), [2] P2A at pH 7, [3] P2A at pH 11. Lower row: [4] P2B at native pH (2.6), [5] P2B at pH 7, [6] P2B at pH 11.*

Finally, the emulsions created with P2A and P2B protein isolates of the freeze-dried biomass are shown in Figure 25. Again, the previously measured protein solubility (see Figure 16 and Figure 17) and EAI-values (see Figure 18 and Figure 19) were in accordance with the emulsion-network patterns of Figure 25. The EAI-values of the P2A protein isolates at pH 7 and 11 were slightly higher than those of P2B, which can be explained by the somewhat smaller and more abundant micelles of picture 2 and 3 compared to picture 5 and 6 of Figure 25.

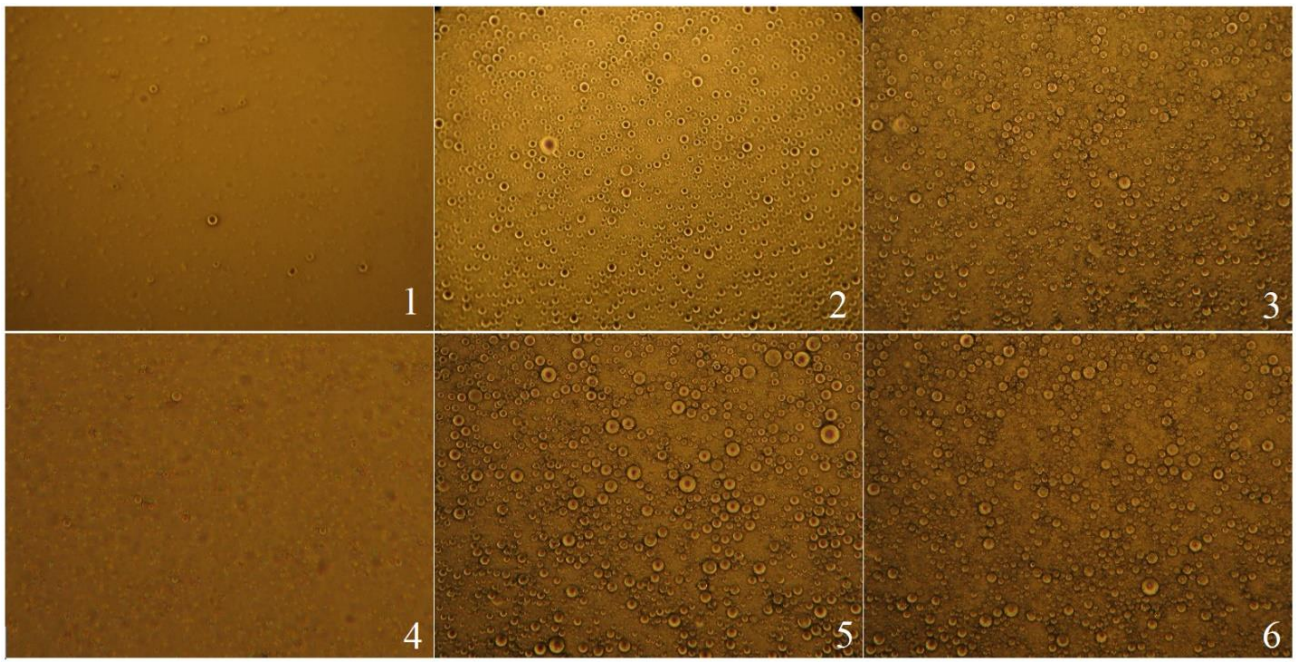

Figure 25. *Pictures of sunflower oil and water emulsions created with P2A and P2B protein isolates of the freeze-dried biomass. Upper row: [1] P2A at native pH (2.2), [2] P2A at pH 7, [3] P2A at pH 11. Lower row: [4] P2B at native pH (2.2), [5] P2B at pH 7, [6] P2B at pH 11.*

3.8 Polypeptide pattern as a function of Preservation Method

SDS-PAGE was performed for four of the differently preserved biomasses (sun-dried, oven-dried, freeze-dried and -20°C frozen) and their produced protein isolates (P2A and P2B), with results shown in Figure 26 and Figure 27, respectively.

For all biomasses shown in Figure 26, except the sun-dried biomass, the polypeptide bands detected were in the range of the 37 – 75 kDa. There was a slightly more intensive band around 50 kDa for the -20°C frozen, freeze-dried and oven-dried biomasses, whilst the sun-dried biomass gave no bands at all in this area. For the -20°C frozen, freeze-dried and oven-dried biomasses it was also possible to see bands at ~75 kDa and somewhat above 37 kDa. Further, the -20°C frozen, freeze-dried and oven-dried biomasses displayed a faint band between 25-37 kDa and at 25 kDa, followed by a more intense band at 20 kDa. Finally, all biomasses showed a sharp band at 15 kDa and except for the sun-dried biomass this was accompanied by a faint band below.

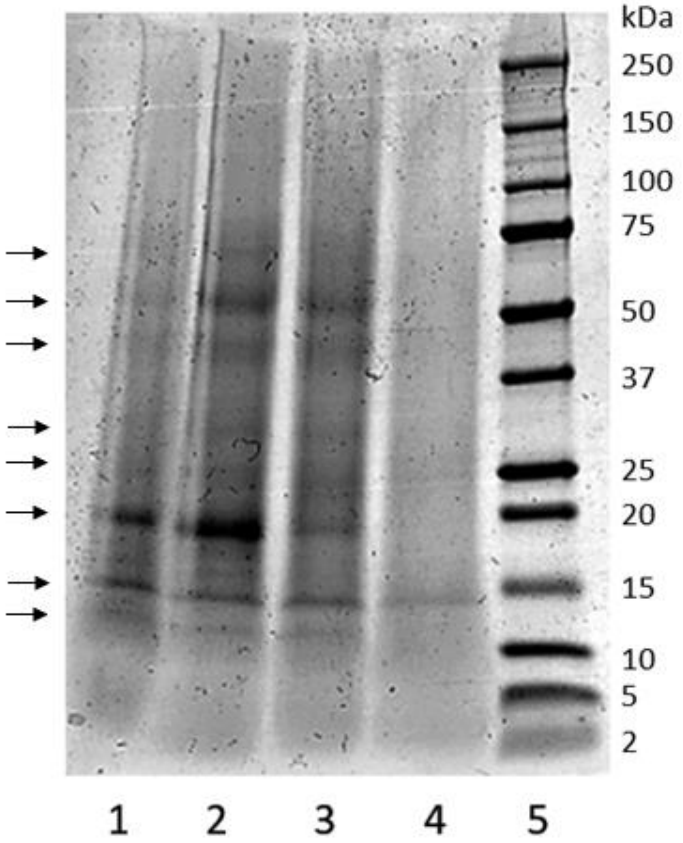

Figure 26. *SDS-PAGE gel of differently preserved seaweed biomasses. 28.5 mg protein was loaded in each well. Lane 1 – -20°C frozen biomass, Lane 2 – Freeze-dried biomass, Lane 3 – Oven-dried biomass, Lane 4 – Sun-dried biomass and Lane 5 – Ladder.*

In Figure 27, the polypeptide-pattern for the P2A and P2B protein isolates of the oven-, sun-, freeze-dried and -20°C frozen biomasses were very similar, with a few weaker bands in the lower molecular weight range. It was possible to see a first faint band at 15-20 kDa, a slightly more visible band showed up somewhat below 15 kDa and finally faint bands were shown just below 10 kDa. There was some smearing in the range of 25-75 kDa, however, it was not possible to distinguish any bands in this range.

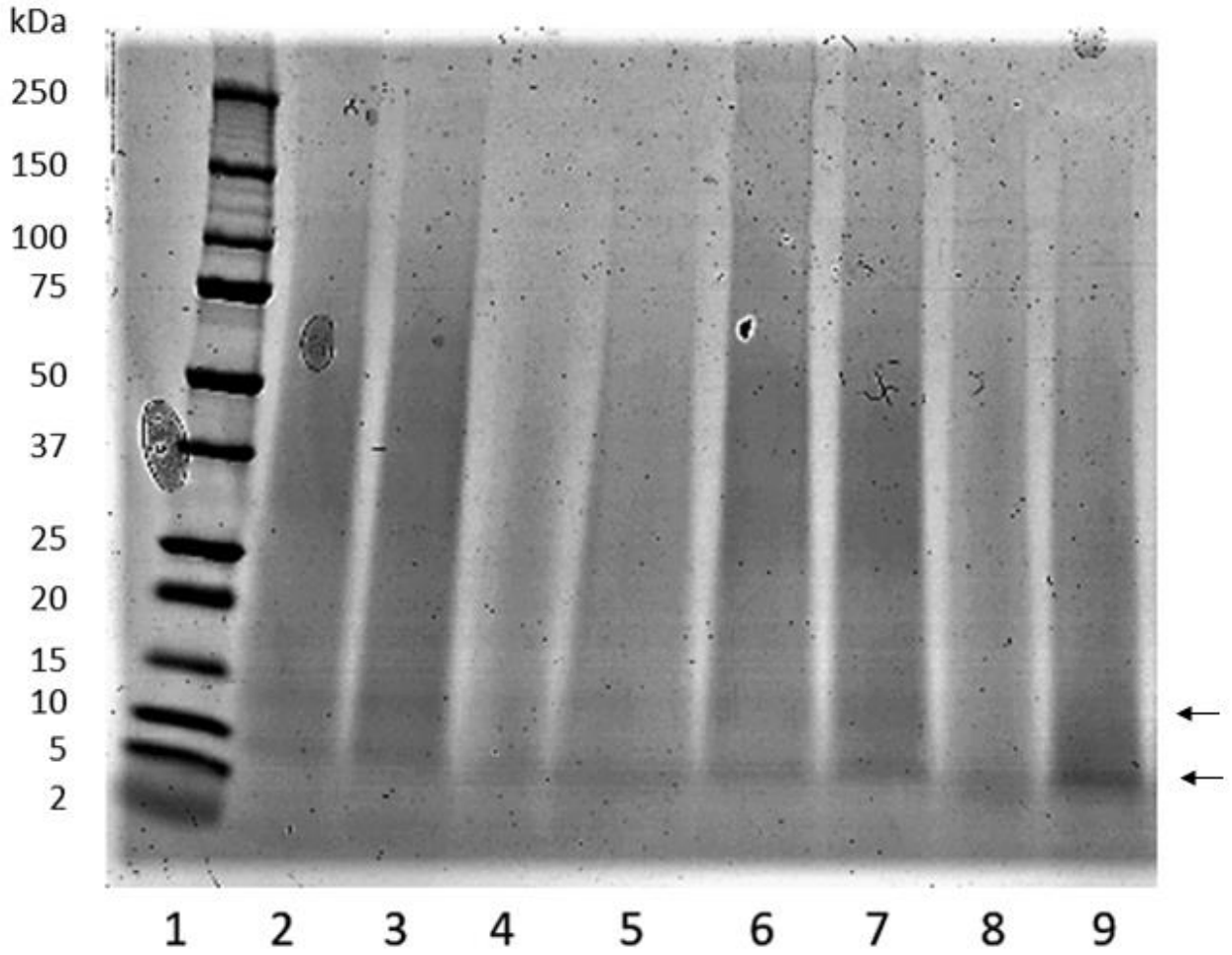

Figure 27. *SDS-PAGE electrophoresis gel of protein isolates. Lane 1 – Ladder, Lane 2 – Oven-dried P2A, Lane 3 – Oven-dried P2B, Lane 4 – Sun-dried P2A, Lane 5 – Sun-dried P2B, Lane 6 – Freeze-dried P2A, Lane 7 – Freeze-dried P2B, Lane 8 – Frozen -20°C P2A, Lane 9 – Frozen -20°C P2B.*

3.9 Fourier Transform Infrared (FTIR) Spectra as a function of Preservation Method

FTIR analysis was performed on the P2A and P2B protein isolates of the sun-, oven-, freeze-dried and -20°C frozen biomasses. An overview of the spectra is shown in Figure 28, ranging in inverted wavelengths of 400 to 4000 cm^{-1}, whilst a closer look on the Amide I-III and Amide A-B bands are shown in Figure 29 and Figure 30, respectively.

Looking at Figure 28, all protein isolates displayed similar peak patterns, however, with varying intensity due to different amount of protein in each sample. All protein isolates displayed bands at 3276-3292 cm^{-1} (Amide A, N-H stretching), 2920-2926 cm^{-1} (Amide B, N-H stretching), 1639-1651 cm^{-1} (Amide I, C=O stretching), 1531-1537 cm^{-1} (Amide II, C-N stretching and N-H bending) and 1217-1227 cm^{-1} (Amide III, C-N stretching and N-H bending) (64, 65).

Exact location of peaks for each protein isolate are shown in Table 4, where some peak shifting can be observed between all samples, however, Amide A and Amide I experienced the most peak shifting.

Table 4. *FTIR peak data for P2A and P2B protein isolates of the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses.*

Sample	**Amide A** *[cm^{-1}]*	**Amide B** *[cm^{-1}]*	**Amide I** *[cm^{-1}]*	**Amide II** *[cm^{-1}]*	**Amide III** *[cm^{-1}]*
Sun-dried P2A	3292	2920	1645	1537	1223
Sun-dried P2B	3279	2922	1645	1533	1221
Oven-dried P2A	3276	2926	1651	1535	1219
Oven-dried P2B	3286	2926	1639	1531	1221
Frozen -20°C P2A	3292	2924	1645	1537	1221
Frozen -20°C P2B	3282	2926	1651	1535	1227
Freeze-dried P2A	3292	2926	1645	1535	1217
Freeze-dried P2B	3284	2924	1641	1533	1219

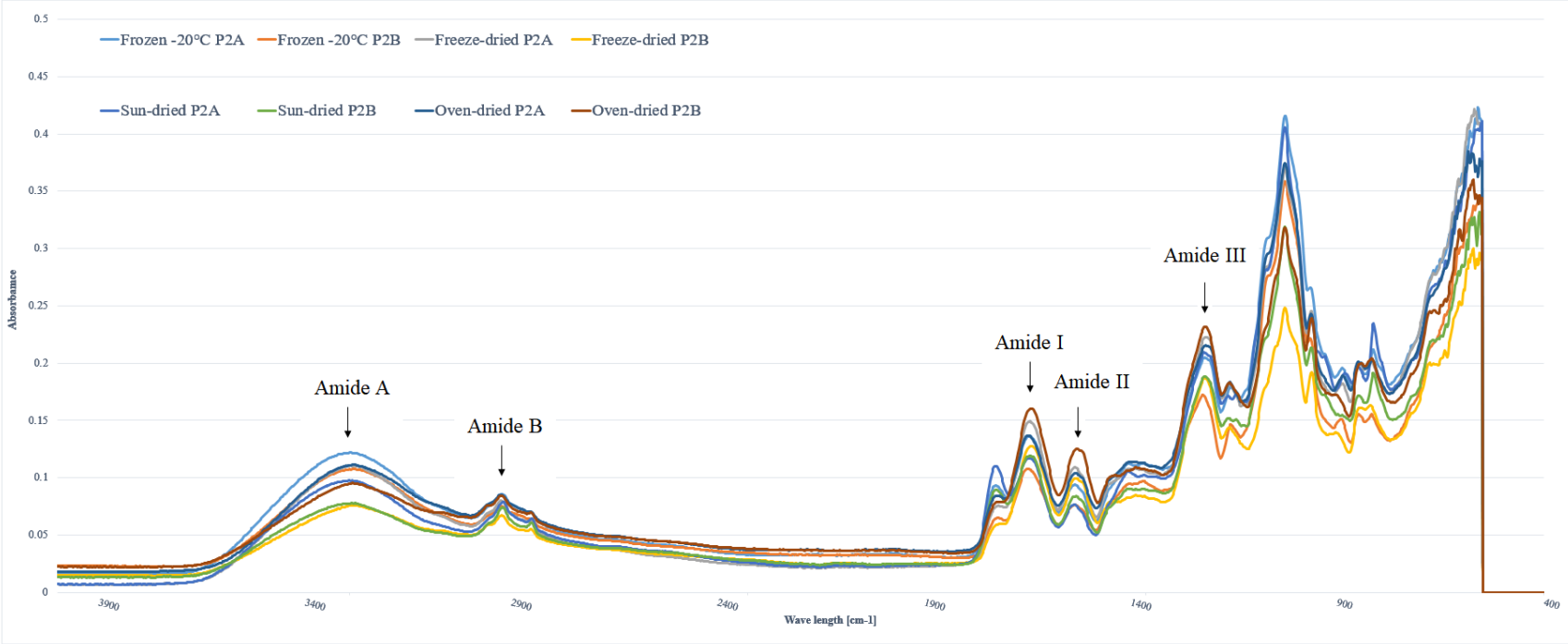

Figure 28. *FTIR-spectra of P2A and P2B protein isolates produced from the sun-, oven-, freeze-dried and -20°C frozen biomasses, in the inverted wavelength range of 400 – 4000 cm^{-1}.*

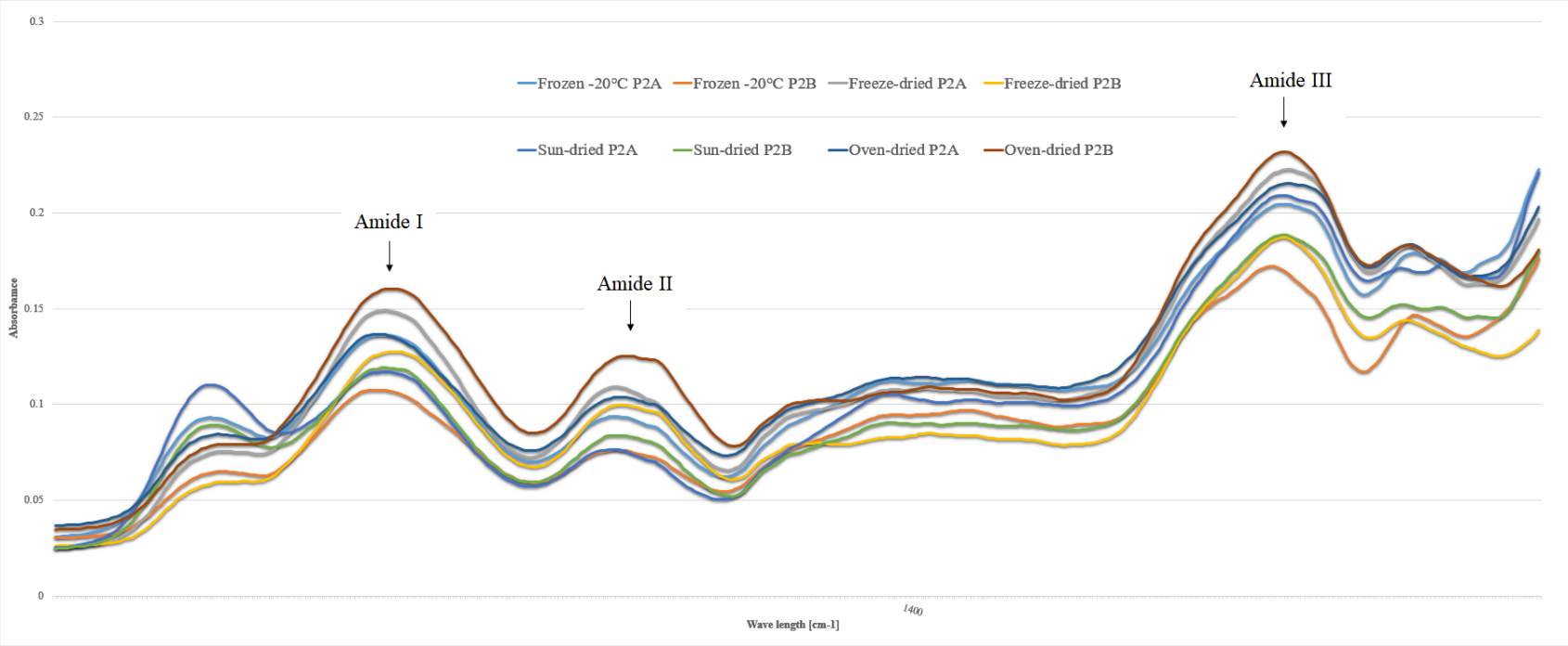

Figure 29. *FTIR-spectra of P2A and P2B protein isolates produced from the sun-, oven-, freeze-dried and -20°C frozen biomasses, in the inverted wavelength range of 1100 – 1800 cm^{-1}.*

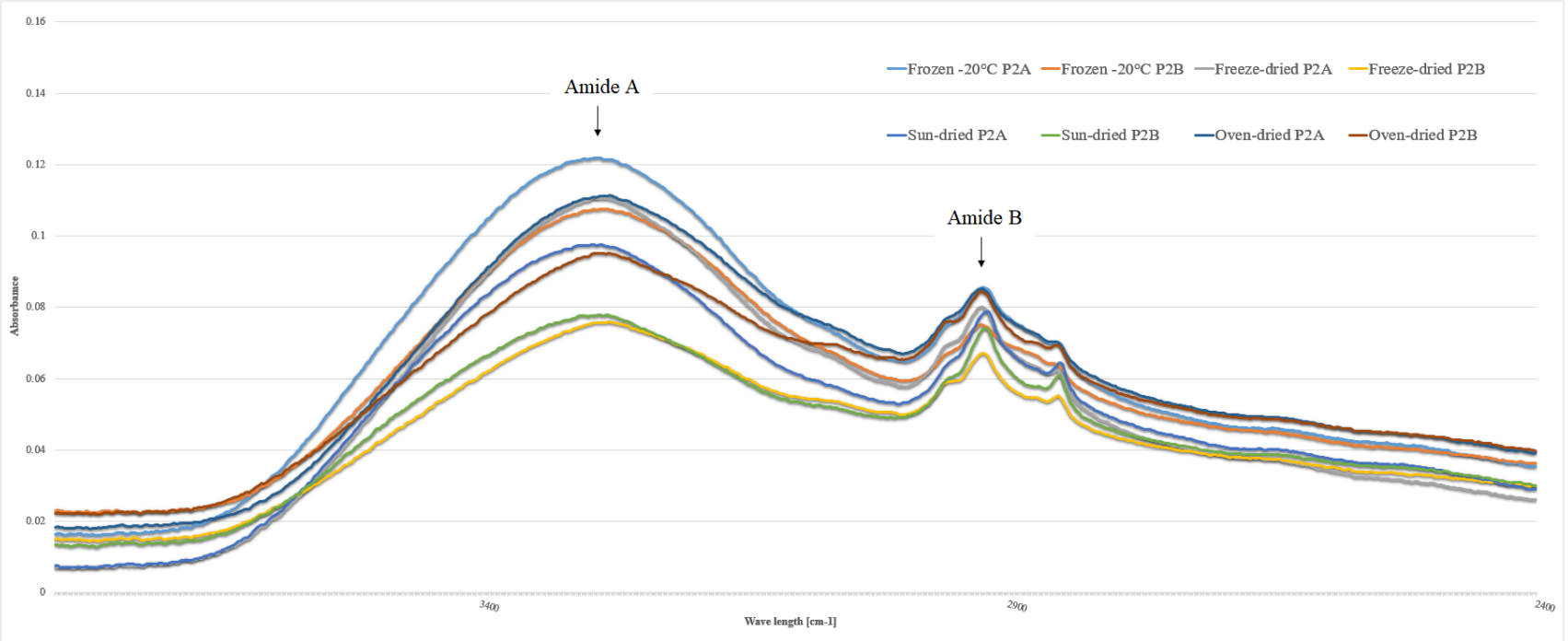

Figure 30. *FTIR-spectra of P2A and P2B protein isolates produced from the sun-, oven-, freeze-dried and -20°C frozen biomasses, in the inverted wavelength range of 2400 – 3800 cm^{-1}.*

3.10 Amino Acid (AA) profile of biomasses and protein isolates as a function of Preservation Method

The amino acid (AA) profile of the initial biomasses, as well as the P2A and P2B protein isolates were analyzed using LC/MS, results are shown in Table 5-7. Cysteine and tryptophan were not reported here due to the destruction of these AAs as an effect of the acid hydrolysis in sample pre-treatment. Preservation method affected the AA-patterns slightly, however, significant differences were not found. The general AA pattern displayed relative high amounts of alanine (~17-19 g AA/ 100g protein), leucine (~10-12 g AA/ 100g protein), glutamic acid (~14-20 g AA/ 100g protein) and valine (~8-10 g AA/ 100g protein). Total essential amino acids (TEAA) accounted for ~40-47 % and total non-essential amino acids (TNEAA) accounted for ~53-60 % of the AA content.

Table 5. *AA profiles of the six differently preserved biomasses reported as g AA/ 100g protein, together with the adult and infant intake recommendations from the FAO/WHO (45). Underlined data represents the highest result when comparing the storage methods.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried	FAO/WHO Adult (Infant)
Glycine	6.43	5.48	5.33	5.30	6.12	5.58	
Alanine	17.03	18.08	18.84	18.39	16.87	19.09	
Serine	3.48	3.10	3.05	2.85	3.24	3.11	
Proline	4.72	3.31	3.33	2.95	3.59	3.05	
Valine	9.76	8.72	8.72	8.36	8.54	8.29	3.9 (4.3)
Threonine	4.87	4.20	4.13	4.08	4.58	4.05	2.3 (3.1)
Isoleucine	6.83	5.83	5.78	5.64	6.09	5.38	3.0 (3.2)
Leucine	12.03	10.30	10.19	9.99	10.72	9.76	5.9 (6.6)
Aspartic acid	3.93	4.44	4.60	4.64	5.19	4.94	
Lysine	3.82	4.19	4.30	4.06	4.06	3.74	4.5 (5.7)
Glutamic acid	13.36	18.74	18.20	19.94	16.70	20.11	
Methionine	3.09	2.89	2.72	2.88	2.76	2.67	1.6 (2.8)
Histidine	0.82	1.18	1.15	1.38	1.26	1.18	1.5 (2.0)
Phenylalanine	6.05	5.60	5.82	5.66	5.94	5.14	3.8 (5.2)
Arginine	2.58	2.61	2.55	2.56	2.92	2.61	
Tyrosine	1.20	1.34	1.28	1.32	1.43	1.31	
Cysteine	-	-	-	-	-	-	
Tryptophan	-	-	-	-	-	-	
TEAA	47.27	42.91	42.81	42.05	43.94	40.21	
TEAA/TAA	0.47	0.43	0.43	0.42	0.44	0.40	

Results for P2A protein isolates (Table 6), were similar to that of the corresponding biomasses, again the preservation method affected the AA-patterns slightly. The most abundant AAs were still alanine (~13-16 g AA/ 100g protein), leucine (~12 g AA/ 100g protein), glutamic acid (~10-11 g AA/ 100g protein) and valine (~9-10 g AA/ 100g protein). TEAA accounted for ~50-52 % and TNEAA accounted for ~47-50 % of the amino acid content.

Table 6. *AA profiles of the P2A protein isolates produced from the six differently preserved biomasses reported as g AA/ 100g protein, together with the adult and infant intake recommendations from the FAO/WHO (45). The arrow adjacent each number represent an increase ▲ or decrease ▼ in abundance of that specific AA compared to the respective initial biomass. Underlined data represents the highest result when comparing the storage methods.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried	FAO/WHO Adult (Infant)
Glycine	7.69 ▲	7.47 ▲	7.23 ▲	7.18 ▲	7.25 ▲	7.42 ▲	
Alanine	14.74 ▼	12.76 ▼	15.05 ▼	13.46 ▼	15.40 ▼	12.44 ▼	
Serine	3.66 ▲	3.79 ▲	3.74 ▲	3.86 ▲	3.84 ▲	3.82 ▲	
Proline	4.55 ▼	3.90 ▲	3.82 ▲	3.66 ▲	4.55 ▲	3.70 ▲	
Valine	9.76 ▼	8.96 ▲	8.98 ▲	8.49 ▲	9.92 ▲	8.63 ▲	3.9 (4.3)
Threonine	5.43 ▲	5.30 ▲	5.46 ▲	5.47 ▲	5.63 ▲	5.38 ▲	2.3 (3.1)
Isoleucine	7.03 ▲	6.47 ▲	6.83 ▲	6.52 ▲	7.22 ▲	6.76 ▲	3.0 (3.2)
Leucine	12.23 ▲	12.10 ▲	12.28 ▲	11.73 ▲	12.20 ▲	12.28 ▲	5.9 (6.6)
Aspartic acid	3.53 ▼	5.31 ▲	3.96 ▼	5.23 ▲	2.71 ▼	5.65 ▲	
Lysine	4.71 ▲	5.73 ▲	5.74 ▲	5.77 ▲	5.25 ▲	5.68 ▲	4.5 (5.7)
Glutamic acid	9.70 ▼	10.51 ▼	10.02 ▼	11.14 ▼	9.29 ▼	10.64 ▼	
Methionine	3.08 ▼	3.17 ▲	3.24 ▲	3.09 ▲	3.41 ▲	3.32 ▲	1.6 (2.8)
Histidine	0.98 ▲	1.47 ▲	1.37 ▲	1.52 ▲	1.39 ▲	1.56 ▲	1.5 (2.0)
Phenylalanine	8.56 ▲	7.26 ▲	7.31 ▲	7.11 ▲	7.57 ▲	6.41 ▲	3.8 (5.2)
Arginine	2.89 ▲	3.70 ▲	3.20 ▲	3.71 ▲	2.98 ▲	4.13 ▲	
Tyrosine	1.45 ▲	2.09 ▲	1.78 ▲	2.07 ▲	1.40 ▼	2.18 ▲	
Cysteine	-	-	-	-	-	-	
Tryptophan	-	-	-	-	-	-	
TEAA	51.78 ▲	50.46 ▲	51.21 ▲	49.70 ▲	52.59 ▲	50.02 ▲	
TEAA/TAA	0.52 ▲	0.50 ▲	0.51 ▲	0.50 ▲	0.53 ▲	0.50 ▲	

For P2B protein isolates (Table 7), the AA patterns were yet again similar to the corresponding biomasses and P2A protein isolates. Preservation method again affected the AA-profiles slightly. The most abundant AAs were yet again alanine (~9-11 g AA/ 100g protein), leucine (~10-11 g AA/ 100g protein), glutamic acid (~11-16 g AA/ 100g protein) and valine (~8-9 g AA/ 100g protein). TEAA accounted for ~48-52 % and TNEAA accounted for ~48-52 % of the amino acid content.

Table 7. *AA profiles of the P2B protein isolates produced from the six differently preserved biomasses reported as g AA/ 100g protein, together with the adult and infant intake recommendations from the FAO/WHO (45). The arrow adjacent each number represent an increase ▲ or decrease ▼ in abundance of that specific AA compared to the respective initial biomass. Underlined data represents the highest result when comparing the storage methods.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried	FAO/WHO Adult (Infant)
Glycine	5.84 ▼	5.54 ▲	5.36 ▲	5.56 ▲	5.75 ▼	5.20 ▼	
Alanine	10.06 ▼	9.70 ▼	10.57 ▼	9.44 ▼	9.30 ▼	8.71 ▼	
Serine	4.30 ▲	4.42 ▲	4.29 ▲	4.65 ▲	4.70 ▲	4.60 ▲	
Proline	4.48 ▼	3.71 ▲	3.61 ▲	3.75 ▲	4.49 ▲	3.79 ▲	
Valine	8.61 ▼	7.75 ▼	7.98 ▼	7.64 ▼	8.54 ▲	7.72 ▼	3.9 (4.3)
Threonine	6.83 ▲	6.50 ▲	6.57 ▲	6.53 ▲	7.11 ▲	6.81 ▲	2.3 (3.1)
Isoleucine	6.22 ▼	5.39 ▼	5.39 ▼	5.43 ▼	6.86 ▲	5.35 ▼	3.0 (3.2)
Leucine	11.24 ▼	10.12 ▼	9.97 ▼	9.82 ▼	11.23 ▲	10.13 ▲	5.9 (6.6)
Aspartic acid	6.75 ▲	8.17 ▲	7.49 ▲	8.05 ▲	6.94 ▲	8.41 ▲	
Lysine	6.71 ▲	8.03 ▲	7.69 ▲	9.02 ▲	6.67 ▲	8.44 ▲	4.5 (5.7)
Glutamic acid	13.04 ▼	14.45 ▼	15.54 ▼	13.85 ▼	11.21 ▼	13.15 ▼	
Methionine	3.13 ▲	3.02 ▲	2.83 ▲	2.87 ▼	3.25 ▲	3.00 ▲	1.6 (2.8)
Histidine	0.71 ▼	1.11 ▼	1.03 ▼	1.23 ▼	1.04 ▼	1.51 ▲	1.5 (2.0)
Phenylalanine	7.45 ▲	6.21 ▲	6.23 ▲	6.14 ▲	7.76 ▲	6.10 ▲	3.8 (5.2)
Arginine	2.71 ▲	3.55 ▲	3.25 ▲	3.66 ▲	3.30 ▲	4.20 ▲	
Tyrosine	1.91 ▲	2.35 ▲	2.20 ▲	2.37 ▲	1.85 ▲	2.88 ▲	
Cysteine	-	-	-	-	-	-	
Tryptophan	-	-	-	-	-	-	
TEAA	50.90 ▲	48.12 ▲	47.70 ▲	48.67 ▲	52.46 ▲	49.06 ▲	
TEAA/TAA	0.51 ▲	0.48 ▲	0.48 ▲	0.49 ▲	0.52 ▲	0.49 ▲	

3.11 Results of the Seasonal Study

3.11.1 Properties of the Seasonal Biomasses

Some basic properties (moisture content, native pH and total protein) of the initial biomasses harvested at different months were investigated before they were put through the pH-shift process. The biomasses investigated for each month were then preserved by oven-drying, no other preservation technique was investigated for the different harvest seasons. Results are shown in the sections below.

3.11.1.1 Moisture Content of Biomasses

Results of moisture content data are shown in

Figure 31. The May- and March-biomasses had significantly higher moisture content (8.2 % and 7.7 %, respectively) than the April-biomass (5.5 %), however, there was no significant difference between the March- and May-biomasses.

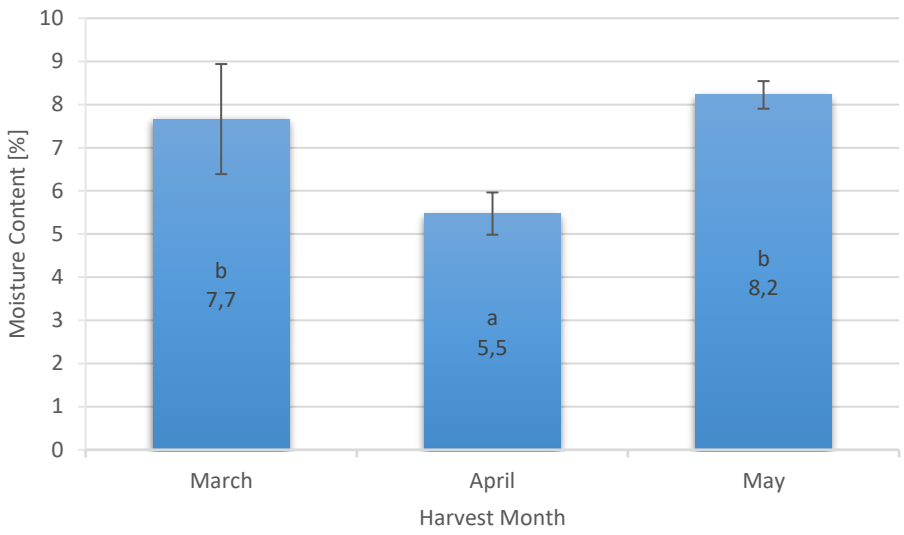

Figure 31. *Moisture content of biomasses harvested at different months. The numbers within each column is the moisture content in percent of biomass. Different letters above each data denotes significant differences between harvest months (α=0.05, a = March; May, b = April).*

3.11.1.2 Native pH of Biomasses

The native pH of the seaweed biomasses was measured after homogenization in water, see Figure 32. The May-biomass had a significantly higher pH (7.0) than the March- and April-biomass (6.7 % and 6.7 %, respectively).

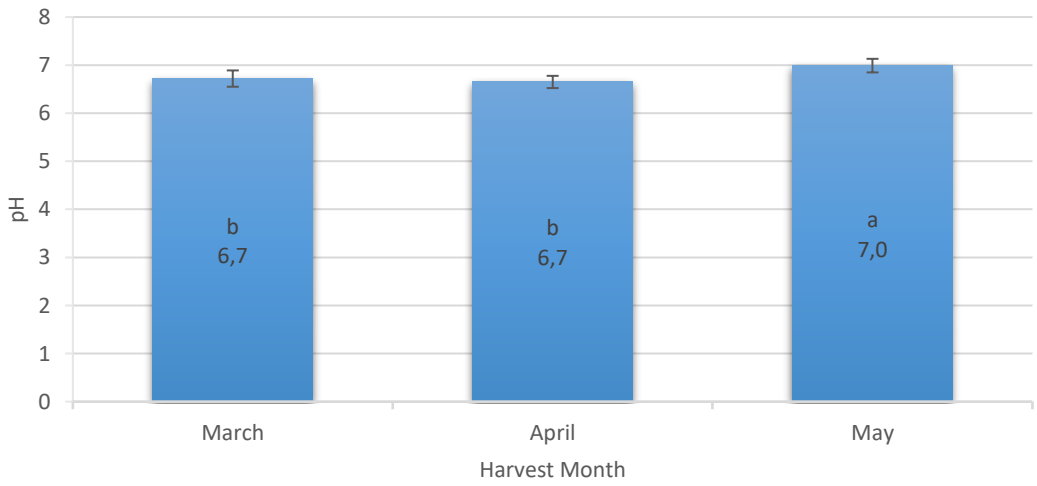

Figure 32. *Native pH of biomasses harvested at different months. The numbers within each staple is the pH of the homogenate. Different letters above each data denotes significant differences between harvest months (α = 0.05, a = March; April, b = May).*

3.11.1.3 Total Protein of Biomasses

As seen in Figure 33, all biomasses had similar total protein content, March-biomass at an average content of 8.2 %, April-biomass at 8.0 % and May-biomass at 8.0 %. There were no significant differences in total protein for the biomasses in any combination of comparison.

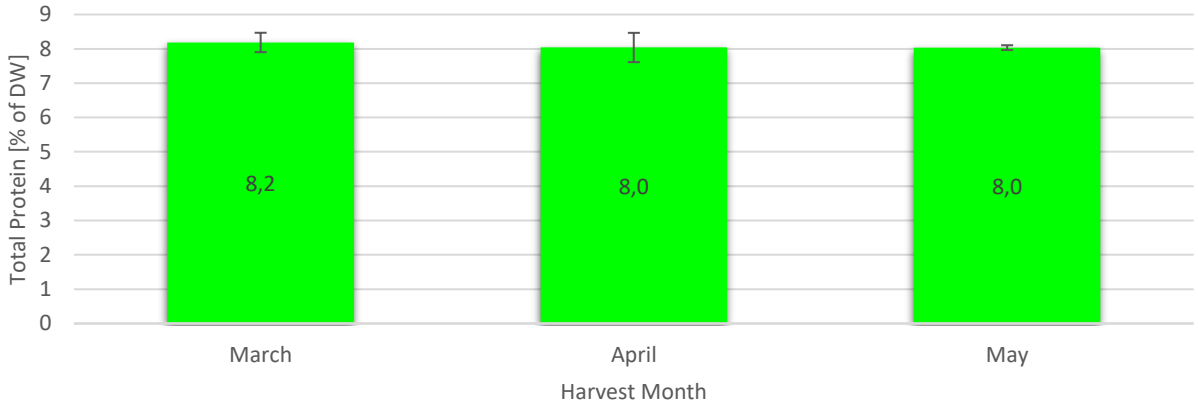

Figure 33. *Total protein amount of each biomass harvested at different months. Numbers within each column represents the percentage of DW biomass that protein accounts for, calculated with Equation V.*

3.12 Outcome of the pH-shift Process as a function of Harvest Season

This section displays the results of the pH-shift processes performed on the biomasses harvested during different months, including; protein solubility, protein extractability, protein precipitation and total protein yield.

3.12.1 Protein Solubility in the pH-shift Process

Shown below are the results from the protein solubility measurements/calculations for the three different types of pH-shift processes performed on the oven-dried biomass harvested at three different months.

3.12.1.1 Solubility Results from pH-shift Process - Type I

Protein solubility at pH 12 and at pH 2, of the pH-shift process type I was calculated for each biomass using Equation IV, results are shown in Figure 34.

The highest protein solubility at pH 12 was reached by the May-biomass (83.4 %), followed by the April-biomass (50.9 %) and March-biomass (36.1 %). The pattern was similar for the S2A protein isolates. The highest decrease in protein solubility (percentage points) between pH 12 and pH 2 was achieved by the March-biomass (19.9 %), followed by the May-biomass (11.9 %) and April-biomass (10.3 %). There was a significant difference in protein solubility comparing any combination of two samples within or between harvest months.

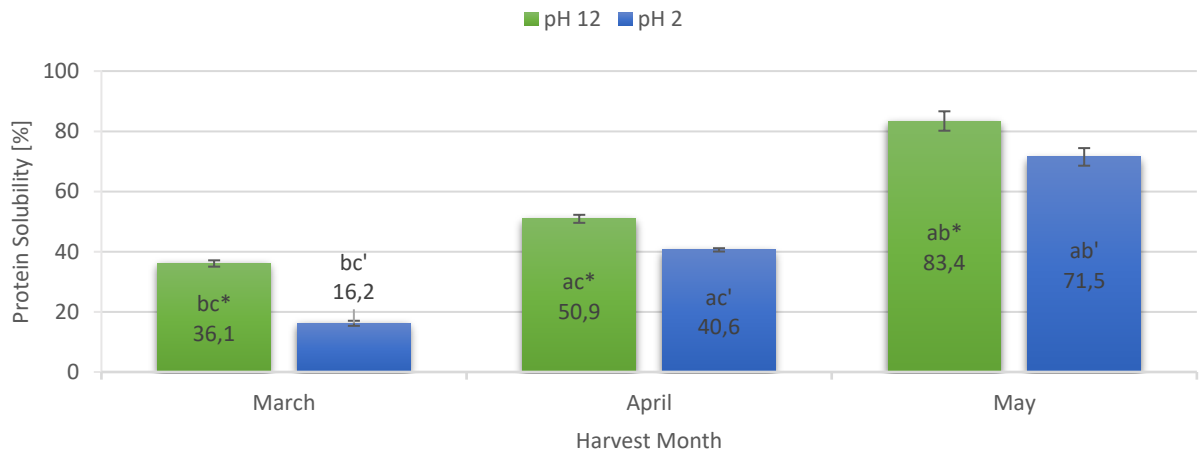

Figure 34. *Protein solubility in the first and second stage of the pH-shift process Type I (i.e. at pH 12 and at pH 2), for each harvest month. Numbers within each column represents the protein solubility in percent, calculated with Equation IV. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between supernatants within each biomass (' = pH 12, * = pH 2), α=0.05.*

3.12.1.2 Solubility Results from pH-shift Process – Type II

Protein solubility at pH 12 and at pH 2 with and without freeze-thawing, of the pH-shift process type II was calculated for each biomass using Equation IV, results are shown in Figure 35.

The resulting protein solubilities at pH 12 and pH 2 were the same as during pH-shift type I, see Section 3.12.1.1. Using freeze-thawing induced precipitation at pH 2, the May-biomass had the highest protein solubility (63.3 %), followed by the April-biomass (25.7 %) and the March-biomass (7.0 %). The largest decrease in protein solubility (percentage points) when applying freeze-thawing was achieved by the April-biomass (14.9 %), followed by the March-biomass (9.2 %) and May-biomass (8.2 %). There was a significant difference in protein solubility when using freeze-thawing induced precipitation comparing any combination of the three harvest months, also, the decrease in protein solubility from pH 12 to pH 2 with a freeze-thawing step and from pH 2 without or with freeze-thawing was significant within each biomass.

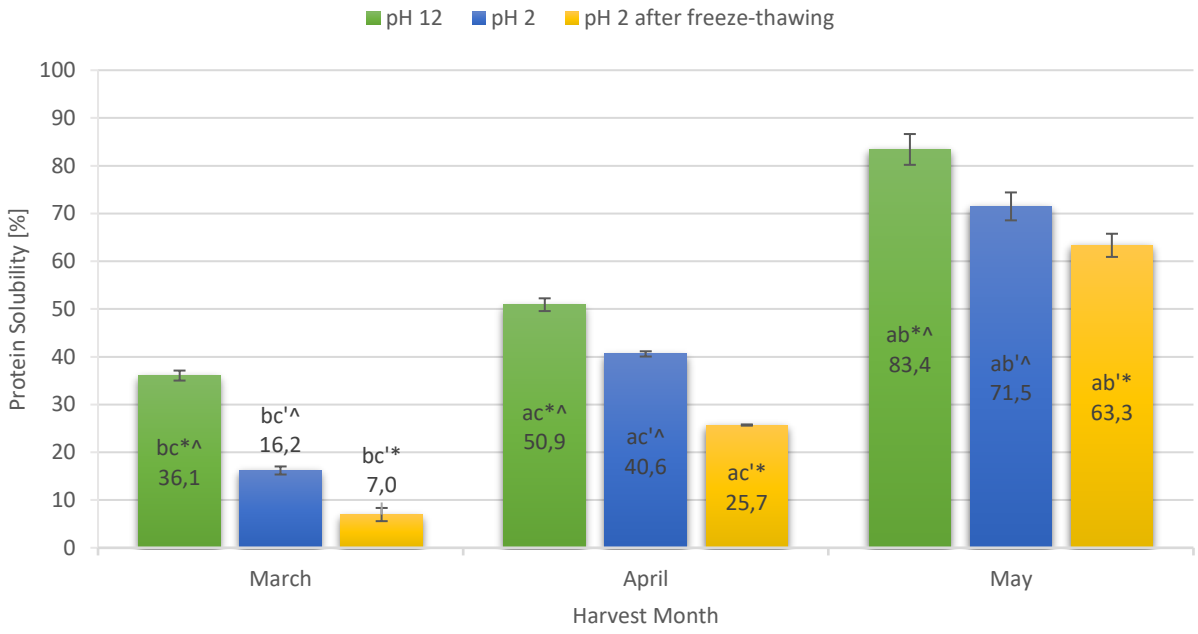

Figure 35. *Protein solubility in the first and second stage of the pH-shift process Type II (i.e. at pH 12 and pH 2 without or with freeze-thawing), for each harvest month. Numbers adjacent each column represents the protein solubility in percent, calculated with Equation IV. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between supernatants within each biomass (‘ = pH 12, * = pH 2), α=0.05.*

3.12.1.3 Solubility Results from pH-shift Process – Type III

Protein solubility at pH 12 and at pH 2 with freeze-thawing, of the pH-shift process type III were calculated for each biomass using Equation IV, results are shown in Figure 36.

Again, the resulting protein solubilities at pH 12 were the same as for pH-shift type I, see Section 3.12.1.1. Moving on to the solubility at pH 2 after freeze-thawing, the May-biomass had the highest protein solubility (61.6 %), followed by the April-biomass (24.4 %) and March-biomass (4.0 %). The largest decrease in protein solubility (percentage points) from pH 12 to pH 2 after freeze-thawing was achieved by the March-biomass (32.1 %), followed by the April-biomass (26.5 %) and the May-biomass (21.8 %). There was a significant difference in protein solubility comparing any combination of two samples within or between harvest months.

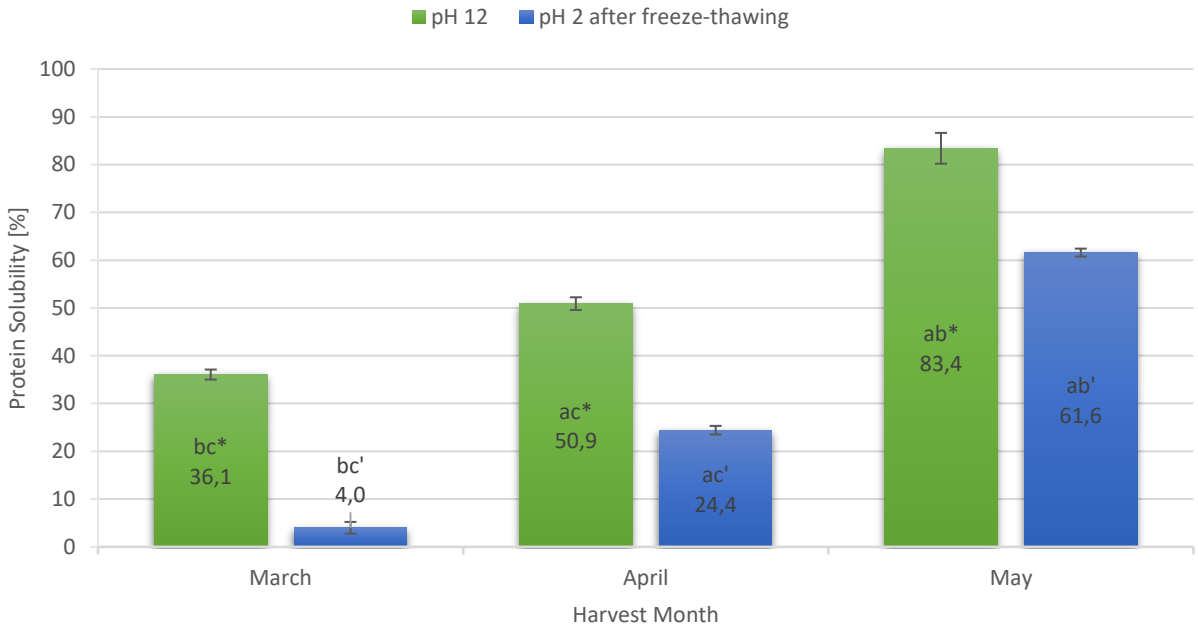

Figure 36. *Protein solubility in the first and second stage of the pH-shift process Type III (i.e. at pH 12 and pH 2 after freeze-thawing), for each harvest month. Numbers adjacent each column represents the protein solubility in percent of total protein concentration in the homogenate, calculated with Equation IV. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between supernatants within each biomass (‘ = pH 12, * = pH 2), α=0.05.*

3.12.2 Protein Extraction Yield – First Stage of the pH-shift Process

The amount of protein that was solubilized at pH 12 during the first stage of the pH-shift process was measured and calculated as extraction yield using Equation VI, summarized in Figure 37.

The May-biomass gave rise to the highest protein extraction yield (77.2 %), followed by the April-biomass (46.8 %) and the March-biomass (34.5 %). There was a significant difference between any combination of harvest month. It is important to stress that the yield in the first stage of the pH-shift process depends both on the protein solubility at pH 12, and on the size of P1, which will still be moist after the centrifugation and thereby retaining some of the solubilized proteins.

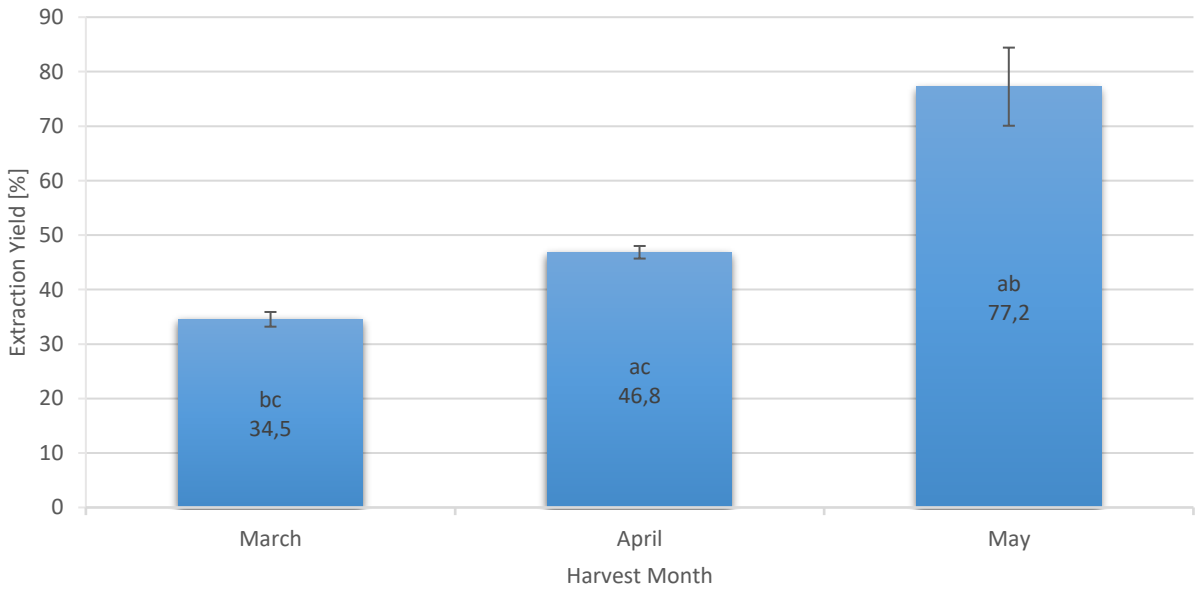

Figure 37. *The protein extraction yield, over the first stage of the pH-shift process, for each harvest month. The yield was calculated as mg of protein in S1 based on the amount of protein in the homogenate, using Equation VI. Different letters above each data denotes significant differences between harvest months (α=0.05, a = March, b = April, c = May).*

3.12.3 Protein Precipitation Yield – Second Stage of the pH-shift Process

The protein precipitation yields for the different pH-shift processes, i.e. the protein content of the final pellet over the amount of available protein in S1, were calculated using Equation VII and are shown in Figure 38.

For the pH-shift process Type I, the March-biomass achieved the highest protein precipitation yield (54.0 %), significantly higher than the April- (18.0 %) and May-biomasses (13.0 %).

Further, for pH-shift process Type II, again the March-biomass obtained the highest protein precipitation yield (80.2 %), followed by the April- (49.1 %) and May-biomasses (26.1 %).

Finally, for pH-shift process Type III, yet again the March-biomass gave the highest protein precipitation yield (88.2 %), followed by the April- (52.0 %) and May-biomass (26.4 %).

For pH-shift process Type II and Type III, there was a significant difference between any combination of the harvest months. Within each harvest month there was a significant difference comparing pH-shift process Type I to Type II and Type I to Type III.

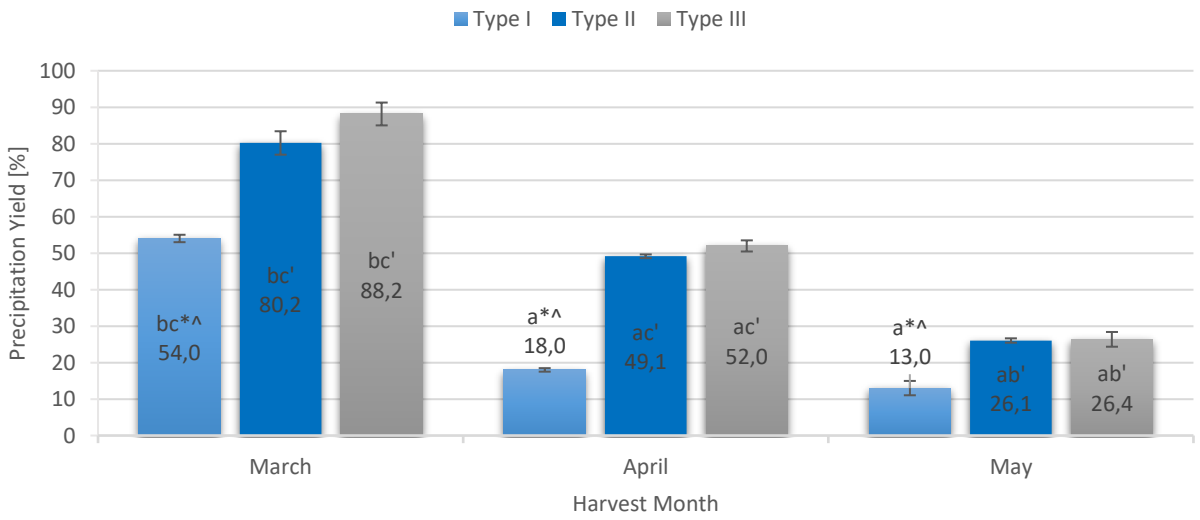

Figure 38. *The protein precipitation yield, over the second stage of the pH-shift process (i.e. at pH 2), for each harvest month. The yield was calculated as mg of protein in the final pellet based on the amount of protein in S1, using Equation VII. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between pH-shift types within each biomass (‘ = Type I, * = Type II, ^ = Type III) , α=0.05.*

3.12.4 Total Protein Yield

The total protein yield for the different pH-shift processes, i.e. the protein content of the final pellet over the amount of available protein in the starting homogenate, were calculated for each harvest month using Equation VIII and are shown in Figure 39.

For pH-shift process Type I, the March-biomass achieved the highest total protein yield (18.7 %), significantly higher than the May- (10.0 %) and April-biomasses (8.4 %).

Further, for pH-shift process Type II, again the March-biomass achieved the highest total protein yield (27.7 %), significantly higher than the April- (23.0 %) and May-biomasses (20.1 %).

Lastly, for pH-shift process Type III, yet again the March-biomass achieved the highest total protein yield (30.4 %), followed by the April- (24.3 %) and May-biomasses (20.3 %). For this process, there was a significant difference between any combination of the harvest months.

Within each harvest month there was a significant difference in total protein yield comparing pH-shift process Type I to Type II and Type I to Type III. For the March-biomass there was also a significant difference comparing pH-shift process Type II to Type III.

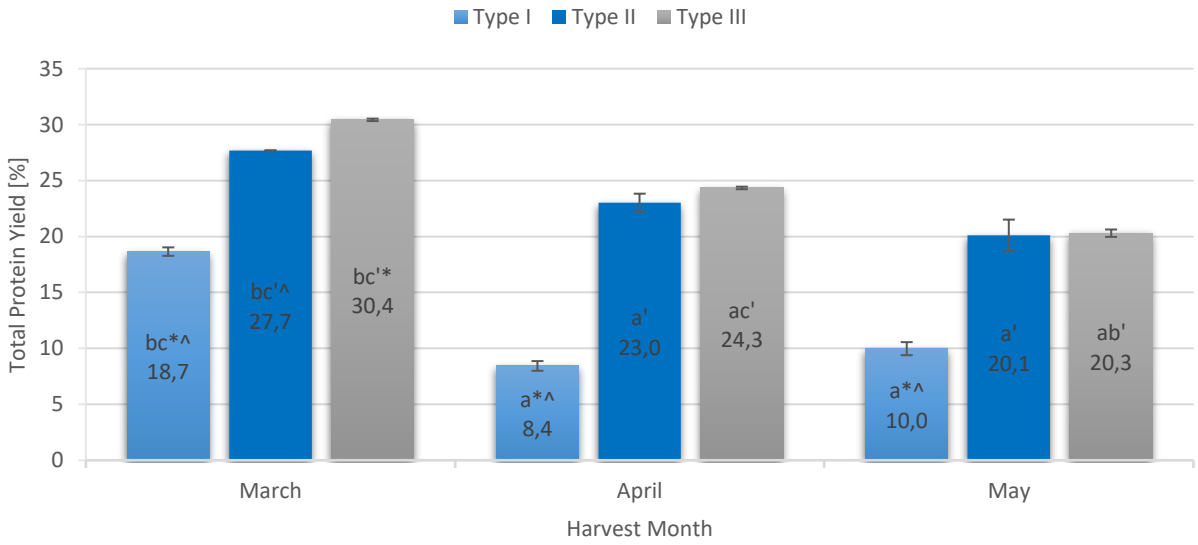

Figure 39. *Total protein yield of the different pH-shift process versions, for each harvest month. The yield was calculated as mg of protein in the final pellet based on the amount of protein in the homogenate, using Equation VIII. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between pH-shift types within each biomass (' = Type I, * = Type II, ^ = Type III) , α=0.05.*

3.12.4.1 Total Protein Yield per Amount Biomass

The actual amount of protein, in mg, produced per g of dried starting material for each harvest month is highlighted in Figure 40, values were calculated using Equation IX.

For pH-shift process Type I, the March-biomass gave rise to the highest total protein yield (15.3 mg protein/g DW biomass), significantly higher than the May- (8.0 mg/g DW) and April-biomasses (6.8 mg/g DW).

Further, for pH-shift process Type II, again the March-biomass achieved the highest total protein yield (22.7 mg/g DW), significantly higher than the April- (18.5 mg/g DW) and May-biomasses (16.2 mg/g DW).

Lastly, for pH-shift process Type III, yet again the March-biomass achieved the highest total protein yield (24.9 mg/g DW), followed by the April- (19.6 mg/g DW) and May-biomasses (16.3 mg/g DW). For this process, there was a significant difference between any combination of the harvest months.

Within each harvest month there was a significant difference in total protein yield on a biomass-basis comparing pH-shift process Type I to Type II, and Type I to Type III. For the March-biomass there was also a significant difference comparing pH-shift process Type II to Type III.

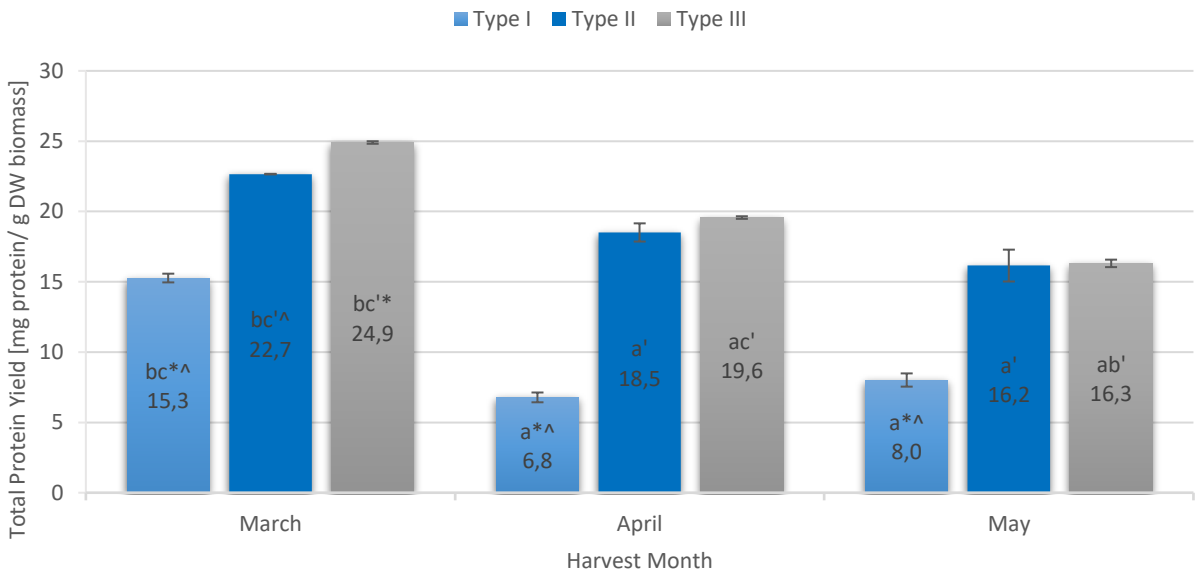

Figure 40. *Total protein yield of the different pH-shift processes, for each harvest month on a biomass-basis. The yield was calculated as mg of protein in the final pellet based on the amount of starting DW biomass, calculated with Equation IX. Different letters above each data denotes significant differences between harvest months (a = March, b = April, c = May) and symbols denotes differences between pH-shift types within each biomass (' = Type I, * = Type II, ^ = Type III) , α=0.05.*

3.13 Up-concentration of Protein during pH-shift processing as a function of Harvest Season

The moisture content and protein content (percent of FDB or FDPI) for the initial biomasses and their respective produced protein isolates, i.e. P2A and P2B, are shown in Table 8 below. In the case of the March- and April-biomass the up-concentration of protein content from the initial biomass to the P2A and P2B protein isolates was about fivefold. For the May-biomass the up-concentration of protein content from the initial biomass to the P2A protein isolate was threefold whilst for the P2B protein isolate it was fivefold. Within all harvest months the there was a significant difference in protein content comparing any the biomasses to the P2A or P2B protein isolates. However, only the March- and May-biomass had a significant difference between their P2A and P2B protein isolates. For P2A, cross comparing all harvest months, there was a significant difference in protein content. However, for P2B, comparing all harvest months, there was only a significant difference in protein content comparing the March-biomass to the April- or May-biomasses.

Table 8. *Average moisture content and protein content of each biomass or protein isolate. Protein data are given on a dry matter basis.*

	Biomass		Pellet 2A			Pellet 2B		
Harvest Month	Moisture Content [%]	Protein Content [% of FDB]	Moisture Content [%] *	Protein Content [% of FDPI]	Conc. Factor	Moisture Content [%] *	Protein Content [% of FDPI]	Conc. Factor
March	7,7 ± 1,3	8,2 ± 0,3	96,5	44,3 ± 1,2	5,4	96,9	46,5 ± 0,3	5,7
April	5,5 ± 0,5	8,0 ± 0,4	97,1	39,3 ± 0,3	4,9	96,7	39,6 ± 1,3	5,0
May	8,2 ± 0,3	8,0 ± 0,1	96,8	24,9 ± 0,2	3,1	96,4	40,5 ± 0,2	5,1

** Moisture content was only measured once. FDB: freeze-dried biomass. FDPI: freeze-dried protein isolate.*

3.14 Polypeptide pattern as a function of Harvest Season

SDS-PAGE was performed on the P2A and P2B protein isolates produced from the biomasses harvested in March, April and May, results shown in Figure 41. The P2A and P2B protein isolates of the March-, April- and May-biomasses displayed similar polypeptide patterns. There were very faint hints of bands for the protein isolates of the March-biomass between 25-75 kDa, however, overall it was not possible to distinguish any clear bands because of smearing in this range. Further, for the protein isolates of the May biomass there were weak bands just below 20 kDa, these bands were not possible to distinguish for the March- or April-biomass. Finally, as for the protein isolates in Figure 27, there were bands visible just below 15 and 10 kDa, however, the intensity was larger for the earlier harvest months; i.e. the ranking was March > April > May.

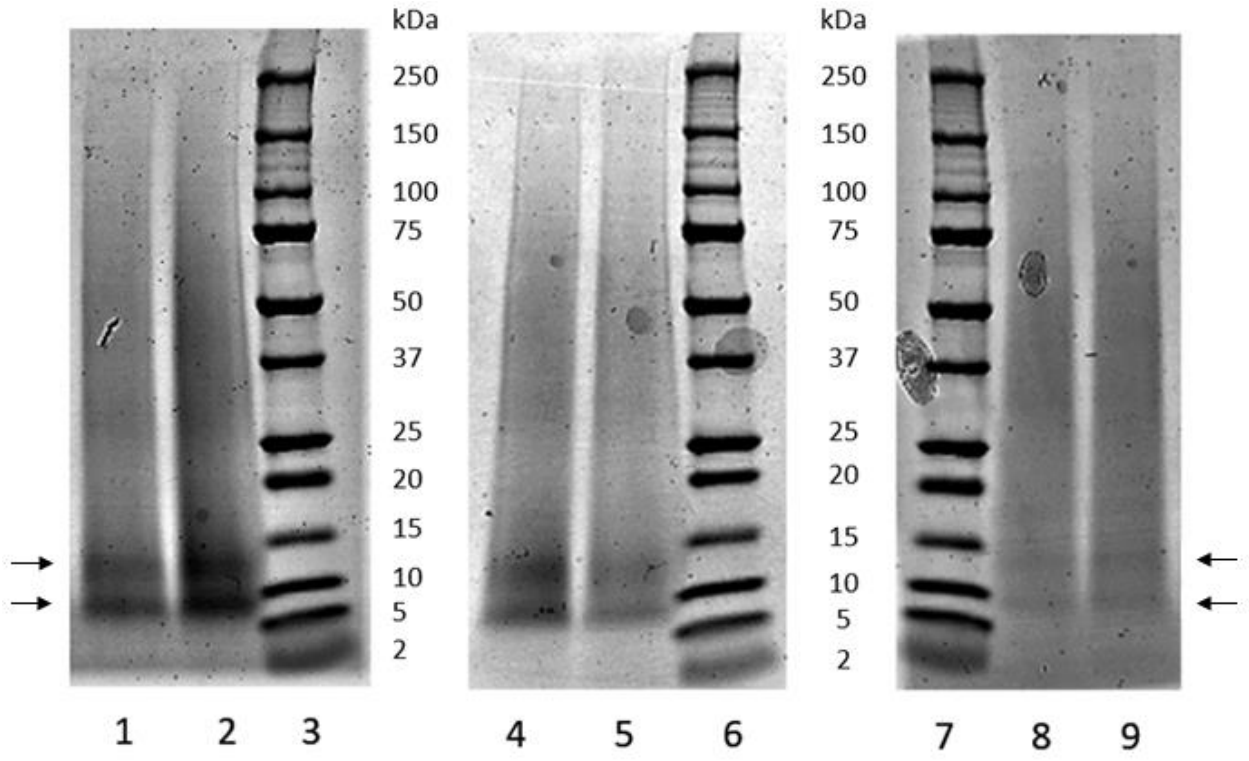

Figure 41. *SDS-PAGE electrophoresis gel of seasonal protein isolates. 28.5 mg protein was loaded in each well. Lane 1 – March P2A, Lane 2 – March P2B, Lane 3 – Ladder, Lane 4 – April P2A, Lane 5 – April P2B, Lane 6 – Ladder, Lane 7 – Ladder, Lane 8 – May P2A, Lane 9 – May P2B.*

4 DISCUSSION

4.1 PRESERVATION STUDY – EFFECT ON THE BIOMASS

4.1.1 Moisture Content of the Biomasses

During the ensilage treatment, there was a runoff of 3 liters of liquid per 45 kg biomass, about a 7 % (v/w) loss. An interesting discovery was then the relatively small difference in moisture content between the ensilaged and frozen biomasses (86.1 % vs 88.5 and 87.9 %), which should not have lost any liquid. This implies that the liquid formed during ensilaging contains solubilized compounds. The run-off liquid contained 6.3 g/L carbohydrates and possibly soluble proteins or peptides, which could explain the similar moisture content in the ensilaged biomass as in the frozen samples, i.e. the ensilaged biomass lost both moisture and macro/micronutrients.

There are several factors that could have affected the moisture content of the sun-dried biomass since it was left to hang on its cultivation lines outdoors for 10 days. It has most likely been affected by the temperature, rain, wind, moisture content of surrounding air, sunlight, insects and birds (66). According to Karam et al. (66), all these impelling factors can affect several quality factors, including moisture content, color, nutrition and hygiene status. Especially the temperature, rain and sun-light will affect the final moisture content of the biomass. In this trial, the end of the drying treatment was not determined by a set final moisture content or water activity, but instead of a set amount of drying days.

The moisture content of the oven-dried biomass was measured after approximately 8 months of storage in Ziploc-plastic bags in darkness. A surprising result, seen in Figure 6, is the small difference (0.59 percentage points (pp)) in moisture between the sun- and oven-dried biomasses. When Chan et al. (67), performed sun-drying (4 days in the sun) and oven-drying (60°C, 15 h) on *Sargassum hemiphyllum* the resulting moisture contents were 12.4 % and 7.60 %, respectively, i.e. a difference of 4.8 pp. A possible explanation for our own data could be the packing in Ziploc-bags, which only provide a plastic seal from the surrounding air and not a completely airtight vacuum seal. Thus, the oven-dried material may have picked up some moisture during the 8 months of storage. Also, the air on the Swedish West Coast may be dryer than in Hong Kong where the study of Chan et al. was performed.

As can be seen in Figure 6 the freeze-dried biomass was not completely dry and still has some residual moisture or moisture that has been picked up during storage. The amount of residual moisture in freeze-dried material is dependent on factors as initial moisture content of the biomass, drying time and the water holding/storage capability of the biomass (68). It is possible that the residual water is vicinal water bound tightly by hydrophilic molecules in the biomass, not being able to sublimate during freeze-drying (44). To sum up, preservation method had an significant effect on biomass moisture content.

4.1.2 Native pH of the Biomasses

Looking at Figure 7, the results implies that some factor of the sun-drying process has affected the biomass pH towards significantly lower values, compared to -20°C/-80°C frozen, oven- and freeze-dried biomasses. One possibility could be rain, which generally has a pH of around 5.6 (69) and would therefore have a slightly acidifying affect. The ensilaged biomass had a native pH significantly lower than all the other biomasses, this was of course due to the ensilage treatment using an acid-mix (see Section 2.1.1) for a duration of 90 days. The other biomasses examined had a pH of around 7 or just below. To conclude, the sun-drying and ensilaging had a significant impact on the native pH of the biomass.

Biomasses with a lower pH will require a larger amount of base to reach pH 12 in the solubilization stage of the pH-shift process and could become an interesting point for considering costs for a scaled-up process. It is possible that a difference in the native pH of the different biomasses could have a slight effect on the protein folding and conformation, therefore also on the protein solubility during the osmoshocking-step (see Section 2.2.2), a higher pH stimulating solubility in the case of *S. latissima* (14).

4.1.3 Protein Analyses of Biomasses

Interference from phlorotannins in the Lowry assay

We suspected there could be interferences from e.g. phlorotannins in the absorbance range where the sample solutions were analyzed in the Lowry-assay (660 nm). Phlorotannins of brown seaweed absorbs light in the range of 280-320 nm (34). As can be seen in Figure 4 there were peaks in the range of 250-360 nm for all samples (homogenate, S1, S2A), but it was however not confirmed by any analysis that these peaks represented phlorotannins, although it is likely. The decrease in absorbance from the homogenate to S1 to S2A was probably due to the partitioning of phlorotannins in to the pellet in each stage. Since the peaks observed were fairly far apart from the area of measurement for the Lowry-assay, they should not interfere with the quantification of protein in the different samples. However, it is known that these molecules interact strongly with proteins (25) and could therefor hinder the solubilization of the seaweed protein under alkaline conditions, reducing the amount of protein possible to extract. Moreover, if protein and/or protein-phlorotannin complexes are extracted into S1, the interaction between the proteins and phlorotannins might still have an impact on how the extracted proteins will fold in later steps of the pH-shift process, affecting the precipitation and total protein yield negatively.

4.1.4 Total Protein content of Biomasses

The Lowry-assay used to quantify protein measures the peptide bonds of the proteins in your sample, compared to the Kjeldahl-assay that measures the amount of nitrogen (70). This means that if peptide bonds are broken during the storage treatment, or sample pre-treatment, this will be reflected in the Lowry-assay. In this study, it seems that the harshest conditions regarding protein degradation were sun-drying and

ensilaging, with the latter being the harshest. Robic et al. (71), performed a study on *Ulva rotundata*, a green seaweed species, including a storage treatment using a chemical mix of acetic acid, citric acid and sodium chloride. Their results from this treatment displayed a protein degradation from 35.5 % protein content of biomass DW (2 weeks), to 31 % (7 weeks), to 19 % (22 weeks). Comparing this with the ensilaged biomass of our study (treated for 13 weeks), using the freeze-dried biomass as a reference, there was a difference in total protein of 1.6 percentage points. This total protein difference would then represent the result of the degradation occurring during the ensiling treatment, since total protein of the non-treated biomass, before any kind of preservation technique was applied, was not measured. Even though the ensilage treatment did not degrade a huge amount of proteins in the biomass, the treatment will come to prove greater effect on protein yield and the protein content of the final protein isolate.

Oven-drying at high temperatures can cause physical degradation of the compounds of a biomass (72), to avoid this, freeze-drying can be applied which minimizes the physical damage and can also avoid losing volatile compounds (42). A study done by Chan et al. (67), found that there was no significant difference of crude protein when the seaweed *Sargassum hemiphyllum*, a brown seaweed species, had been sun-, oven- and freeze-dried separately. Their method for measuring total protein however involved a CHNS/O elemental analyzer and they calculated the protein by multiplying the percent of nitrogen with the nitrogen-to-protein factor 6.25, something which will not detect proteolytic degradation of proteins. Thus, if a protein will degrade during any of the preservation treatments the resulting components might still contain that nitrogen which will be included in the results of an elemental analyzer. In the case of our study there was a significant difference in total protein between sun-dried biomass and both the oven- and freeze-dried biomasses, however, there was no significant difference between the oven- and freeze-dried biomasses. The latter was in accordance with previous reports (42, 67).

Preservation by freezing is today the most common method for food due to its reduction of microbial growth avoiding microbial deterioration and impelling enzymatic and oxidative reactions (73). No previous study has been done on how freezing affects the total protein of seaweed biomass during long time storage. In our study, biomass subjected to two ordinary freezing preservation techniques, at -20°C and -80°C for 6 months, resulted in 7.6 % and 7.8 % total protein on a dry matter basis, respectively. There was no significant difference between the -20°C or -80°C frozen biomasses and the freeze-dried biomass (8.2 %) after this amount of time, possibly longer storage could induce some proteolytic degradation. To further secure good quality during frozen storage, flash freezing can be used since the ice crystals formed during such fast freezing will be smaller than when classic freezing is applied (74).

4.1.5 Protein Solubility During the Different Steps of the pH-shift Processes

It was investigated how the solubility of the seaweed proteins during different steps of the pH-shift processes were affected by the different preservation treatments.

The oven-dried, freeze-dried, -80°C and -20°C frozen biomasses achieved the highest protein solubilities at pH 12, significantly higher than the sun-dried and ensilaged biomasses. Protein solubility is dependent on the interaction between the proteins and the solute.

As described in the introduction; proteins acquire a net negative charge when the pH is altered to a value above its isoelectric point. With this in mind, it is clear that the alkaline nature of the solute must encounter the proteins within the biomass to affects their solubility. However, this will be hindered by the matrix of the biomass, access to proteins by the solute, protein folding and interacting protein-binding compounds such as phenolics. One group of phenolic compounds commonly present in brown seaweed is as previously mentioned phlorotannins, which can bind to proteins and hinder their accessibility to the solute. Even though the content of this compound was not measured in this study, it is possible that this compound is partly responsible for the fact that none of the biomasses reached a protein solubility above ~84 %. Also, it could be possible that the harsh sun-drying and ensiling treatments caused more of these phlorotannin-protein complexes to arise.

The sun-drying process was a relatively undefined treatment since no parameters other than the time the biomass was left to dry was recorded. Therefore, only speculations can be made on what possible elements of this treatment has affected the protein functionality of the seaweed biomass. Young (75) showed that sunlight can denature different kinds of proteins, which could explain why the proteins of the sun-dried biomass in this study only achieved a solubility of ~60 % under alkaline conditions. The denaturation of the proteins would irreversibly affect the folding of the protein, thereby making it difficult solubilize the affected proteins. The treatment of ensilaging in an acid-mix for 91 days also had a strong effect on protein solubility (

Figure 9). The process of ensilage has a proteolytic effect (76), thereby affecting protein functionality, in accordance with the resulting low protein solubilities.

Protein solubility is as mentioned dependent on protein access to solute, that goes hand in hand with the particle size of the biomass homogenate. In this study, it was unavoidable to end up with different particle sizes for the dry and wet biomasses. The smallest available hole-plate for the grinder that was used for mincing the wet biomasses had holes with a diameter of 2 mm, whilst the milling of the dry biomass with a coffee grinder produced a fine powder. Both were homogenized during the pH-shift process using a polytron which only slightly reduced the particle size further of the wet biomasses. However, comparing the protein solubility at pH 12 of the oven-dried, freeze-dried, -20°C and -80°C frozen biomasses they were very similar (

Figure 9). This would then prove that the particle size of the wet biomass was satisfyingly small to achieve as good protein solubility to the same degree as the fine powder of the dry biomasses. Moreover, comparing the sun- and oven-dried biomasses there was a large difference in protein solubility at pH 12 (21.3 %), which is not likely an effect of the particle size since these were milled in the same manner and can only be explained by the effect of some other element of the preservation processes.

When lowering the pH to 2, there was reduction in protein solubility over all biomasses, see

Figure 9. This allowed precipitating the extracted proteins to various degrees for the different biomasses which were then dewatered by centrifugation. The average decrease in protein solubility from pH 12 to 2

was 11.1 pp over all biomasses. The largest decrease in solubility was achieved for the -20°C frozen biomass with 13.9 pp, whilst the smallest decrease in solubility was 8.5 pp for the sun-dried biomass. These results indicate that the refolding of the solubilized proteins was affected differently by the preservation treatment of their initial biomass. Vilg & Undeland (14), reported results on protein solubility for the seaweed *S. latissima*. They showed a decrease in solubility in the range of pH 13 to 2, however, their results indicated that it was not possible to reach an absolute isoelectric point for the proteins of this seaweed species. When the pH-shift process is applied to fish biomass, a more distinct isoelectric point has been found, with increased solubility above and below this point. This type of solubility curve have not been typical for seaweeds (14, 37, 40). Protein precipitation at a lower pH than 2 was not investigated by Vilg & Undeland (14), nor by this study, which leaves concerns if it perhaps is possible to reach an even lower protein solubility below pH 2. However, going extremely low in pH requires very large amounts of acid. Most likely the salt content of the seaweed biomasses and the salt produced during the pH-shift process, by mixing NaOH with HCl, is pushing the isoelectric point of the seaweed proteins down due to interactions with Cl^-.

4.1.6 Changes in Protein Solubility and Protein Precipitation by Freezing

In addition to precipitating proteins by reaching their isoelectric point, as in "classical" pH-shift processing, freeze-precipitation was applied in two additional versions of pH-shift processes (Type II and Type III) in this study. The protein solubility was again analyzed in the resulting supernatants from there processes, as for the pH-shift process Type I discussed above.

In pH-shift process Type II, the final supernatant from pH-shift process Type I (S2A) still contained a lot of proteins (

Figure 13). To recover another fraction of these proteins freezing was applied. During freezing ice crystals are formed from the present water, increasing concentration of the proteins, salts, buffers etc. which can cause varying degrees of protein denaturation and/or protein aggregation depending on solute, electrolytes, pH-shifting and freezing rate (51-53). This way of precipitating proteins in a pH-shift-like process on seaweed has not been performed previously, however, Hernández et al. (50) obtained protein concentrates via freezing of alfalfa (a small flower plant used as livestock fodder) press juice. In our study, we could recover yet some protein by freezing S2A, producing the additional protein isolate P3A, see process scheme in Figure 2. Thus, the protein solubility of the resulting supernatant, S3A, decreased for all biomasses, see

Figure **10**. The freeze-dried, -80°C frozen and oven-dried biomasses gave significant decreases in protein solubility from S2A to S3A (11.2 pp, 11.0 pp and 8.15 pp, respectively), followed by the -20°C frozen, ensilaged and sun-dried biomasses (6.2 pp, 3.3 pp and 2.9 pp, respectively). This proves that the freeze-precipitation of S2A residual proteins was affected by what preservation technique the biomass has been through. Similar findings, but to a lesser extent were also seen when freezing was applied directly after that the pH of S1 was adjusted to 2, such as in pH-shift process Type III (combining both types of protein precipitation techniques) thereby also avoiding one extra centrifugation, saving time and energy, making process Type III the most scalable technique.

4.1.7 pH-shift Protein Yield

The protein yield for various steps of the pH-shift processes were analyzed to investigate how well the proteins from the initial biomass was extracted and then how well these extracted proteins were recovered by isoelectric- and freeze-precipitation. Combining yields in these two steps, provided us with the final total protein yield for the whole pH-shift process.

4.1.7.1 Protein Extraction Yield

The protein extraction yield displayed a very similar pattern to that of the protein solubility at pH 12. Depending on how much S1 is produced from the homogenate (i.e. the volume) the yield will be affected. This means that the water holding capacity of P1 will affect the extraction yield by retaining liquid. Looking at Table B2, in Appendix B, showing the volume of S1 that was produced during the pH-shift process for each biomass, there is a significant difference between the "wet" (-20°C/-80°C frozen and ensilaged) and "dry" (sun-, oven- and freeze-dried) biomasses, with the "dry" biomasses producing larger volumes of S1. This will directly affect the extraction yield. Ways of overcoming the effect of P1 retaining proteins could be to apply a washing of this pellet, to recover trapped proteins.

4.1.7.2 Protein Precipitation Yield

The preservation treatment had little to no effect on the precipitation yield, but overall showed that the use of freeze-precipitation in both pH-shift process Type II and Type III had a significant effect and several cases even doubled the precipitation yield. Whether or not the benefit from this increased yield overcomes the cost of an extra freezing step remains to be proven.

4.1.7.3 Total Protein Yield

Our highest total protein yield, 11.2 %, obtained with the freeze-dried biomass, was lower than the previously reported yield, 16.01 %, achieved by Vilg and Undeland, (14), using a very similar pH-shift process. However, their biomass was harvested in November whilst ours was harvested in May and as previous reports show, there is a crucial seasonal variation in the biomass composition of *S. latissima* affecting the protein content of the biomass and possibly the ability to recover the proteins (6, 19, 22). Our total protein yield was however higher than other previously reported protein yields from pH-shift like processing of seaweed biomasses; 5.71-6.48 % from three green seaweed species (37) and 7.81 % from *K. alvarezii* (40).

The preservation methods used in this study can be theoretically divided into three types including dehydration techniques (sun drying, oven drying and freeze-drying), water solidification technique (freezing at -20°C or -80°C) and wet stabilization (ensilaging). The sun-drying used in this study resulted significantly lower protein solubilization and total yield in the pH-shift process compared to the oven and freeze-drying. Due to economic reasons, sun-drying is usually used for colloid production (agar, carrageenan or alginate), from red and brown seaweeds (71). However, sun-drying is strongly dependent on the weather and the length of the day and it is the most difficult to control drying rate and parameter (67). Similarly, the sun-drying process was the most undefined treatment used in this study since no parameters other than the time the biomass was left to dry was recorded.

Looking at the different preservation treatments and the total protein yields given in pH-shift Type I, we see that the sun-dried and ensilaged seaweed gave two of the lower total protein yields, which was a similar pattern to their protein solubility and protein extraction yield. Also, the -80°C frozen biomass gave a relatively low yield. Preservation by freezing, especially at lower temperatures, is considered to reduce enzymatic reaction rates (73), thereby preventing protein degrading processes caused by e.g. proteases. However, the -20°C frozen biomass (11.1 % protein yield) gave better yield than the -80°C frozen biomass (6.3 %), which means that something else must be the cause of the lower total protein yield. The -80°C frozen biomass had a 14.2 pp lower extraction yield and 4.4 pp lower precipitation yield than the -20°C frozen biomass, implying that the function/folding of the proteins in the -80°C frozen biomass could be different.

Ensilaging is another preservation technique with comparatively low energy input that has been traditionally used for stabilization of wet to partially dry agricultural and fisheries biomasses. Here, seaweeds samples stabilized by acid-aid ensilaging resulted in minimum protein solubility (25.4%) and total yield (7.6%). The harsh treatment of ensilaging the seaweed in an acid-mix for 91 days had a strong effect on protein content of the seaweed biomass as well and reduced it by 37%. The process of ensilaging can prevent microbial deterioration of biomasses with applying low pH (~3-3.5) but has a degrading effect on proteins (52), in that it breaks down proteins into peptides, amino acids and finally non-protein nitrogenous compounds like ammonia and affecting protein functionality, in accordance with the resulting low protein solubilities.

Comparing pH-shift process Type I against Type II and Type I against Type III, there was a significant increase in total protein yield for all biomasses except for the sun-dried biomass in the latter case (Type I against Type III). This proves that freeze-precipitation had a significant impact on the total protein yield of the pH-shift process and since there was no significant difference between the different methods regarding freeze-precipitation, we propose the use of pH-shift process Type III, since it includes one less centrifugation step, saving time and energy, and yielding slightly more protein than Type II. To truly determine the most profitable preservation method one needs to consider the amount of protein that is produced from the processed amount of biomass, i.e. mg protein/g biomass, and not just the total protein yield in percent. This recalculation is shown in

Figure 15. The maximum yield achieved was again achieved by the freeze-dried biomass (21.8 mg protein/ g DW biomass) followed by the oven-dried, -20°C and -80°C frozen biomasses (16.3 mg/g DW, 15.4 mg/g DW and 15.2 mg/g DW, respectively). To further investigate the highest profitability, one then needs to evaluate the quality and functionality of the produced protein isolates from the different biomasses. It could be so that one of the seaweed biomasses might be able to produce a lot of protein isolate but the isolate might not be that favorable, then the use of the isolate will dictate what is the most important, amount or e.g. functionality.

4.2 Preservation Study – Quality Evaluation of Protein Isolates

The protein isolates produced from the differently preserved biomasses were evaluated by several qualitative analyzes. The protein isolate produced from pH-shift process Type II was excluded from these analyzes due to the difficulty to recover the actual protein pellet and since pH-shift process Type III proved to yield even more protein via freeze-precipitation and being a simpler method. Protein content and amino acid profiles were analyzed for all biomasses, whilst protein solubility, emulsion capacity, SDS-PAGE and FTIR were analyzed for isolates from the sun-dried, oven-dried, -20°C frozen and freeze-dried biomasses, since they were more probable preservation treatment scenarios for large scale application.

4.2.1 Up-Concentration of Protein from Biomass to Protein Isolate

The protein content of the produced protein isolates is shown together with the protein content of the initial biomasses in Table 3. The protein content of the protein isolates was significantly increased compared to the initial biomass in all cases, except for the ensilaged biomass where it was significantly lower. The P2B protein isolate had a significantly higher protein content than the P2A protein isolates in all cases, except for the -20°C frozen biomass. This proves that freeze-precipitation significantly increased the protein content of the final protein isolate for all biomasses, except for the -20°C frozen biomass. Also, the choice of preservation method applied to the initial biomass had a significant effect on the protein content of the P2A and P2B protein isolates. The highest protein content was found in the P2B protein isolates made from the freeze-dried and oven-dried biomasses (37.6 % and 40.5 % protein on DW, respectively). Kandasamy et al., (37), using a version of the pH-shift process, produced protein isolates from three green seaweed species containing 33.4 – 60.4 % protein (on DW basis), similarly, Kumar et al., (40), produced a protein isolate from *K. alvarezii* containing 62.3 % (on DW basis). However, these reported protein contents are still lower compared to protein isolates produced from fish or soy, with the pH-shift process, which can contain up to ~80 % and ~90 % protein, respectively (on DW basis) (10, 26).

A full compositional analysis of protein isolates produced with the pH-shift process from the microalgae *N. oculata* were shown to contain ~12 % fatty acids, ~23 % protein, ~25% ash and ~42 % carbohydrates (all on DW basis) (30).

4.2.2 pH Dependent Solubility of Dried Protein Isolates

The solubility of a protein is affected by many factors, like pH and salt content of the protein containing solution, but also denaturation and structural changes to the protein (54). Regarding the pH, it will affect the net charge of the protein, meaning that in acid or alkaline pH, it will increase its interaction with the surrounding water molecules, thereby increasing its solubility (54). The protein solubilization can also be aided or prevented by salt at a given pH, which is an effect called salting-in or salting-out, changing the proteins isoelectric point. The pH-shift process creates salt due to the use of NaOH and HCl, creating NaCl.

Also, the biomass has a native salt content, and the type of storage treatment could affect this content. All P2A protein isolates showed a similar solubility pattern in the neutral and alkaline pH range, however, in the acid range the P2A of the sun-dried biomass displays moderately high solubility whilst the remaining isolates greatly loses protein solubility with decreasing pH down to ~10-20 % at pH ~3.

Oven-drying, -20°C freezing and freeze-drying does not add anything to the biomasses, however, the sun-drying performed in this study could lower salt content due e.g. to rain and dripping. In addition to salt content, the denaturation of a protein can reduce its solubility due to exposure of hydrophobic groups. Further, smaller peptides can have higher solubility than intact proteins (54). As Karam et al., (66), explains, during sun-drying the biomass is be affected by the surrounding temperature, possibly rain, wind, moisture content of surrounding air, sunlight, insects and birds, many of these potentially causing protein degradation. Oven-drying at high temperature can cause physical degradation alterations to the protein (72), however, the drying in this study was carried out at 40°C, which is a relative mild temperature. Freeze-drying is generally considered to minimize the physical damage to the protein (42) and freezing the biomass in its wet state will reduce microbial growth avoiding microbial deterioration and impel possible endogenous enzymatic activity (73).

Also, the P2B isolate of the -20°C frozen biomass followed that of the sun-dried biomass, i.e. had moderately higher protein solubility in the acid range, than the other isolates made with this precipitation technique. P2B isolates of the sun-dried biomass had a ~20 % lower in protein solubility between pH 5 to pH 9, compared to its P2A isolate. This implies that precipitation induced by freeze denaturation may reduce functionality in some types of biomasses. To conclude, the choice of preservation method influenced the solubility of both types of protein isolates and the use of freeze-precipitation alters the solubility of the proteins differently depending on what biomass they were isolated from.

4.2.3 Emulsion Capacity and Microscopic Investigation of Emulsions

Investigation of the emulsion capacity of a protein provides information of how the protein can be applied in emulsion based foods, ex. mayonnaise or dressings. The protein isolates, P2A and P2B, produced from the sun-dried and -20°C frozen biomasses displayed relatively high EAI values (~80-110 m^2/g) throughout the pH-range (pH 2, 7 and 11). However, the same protein isolates produced from the oven-dried and freeze-dried biomasses displayed a relatively low EAI at pH 2 (~10-30 m^2/g), somewhat higher at pH 7 (~50-70 m^2/g) and reaching their maximum at pH 11 (~60-90 m^2/g). To have a good emulsifying effect the protein needs to be soluble at the pH of the aqueous part of the oil/water-mixture to reach the lipid interface and preferably a relative high surface hydrophobicity to interact with the lipid phase (77). The higher EAI values of the protein isolates from the sun-dried and -20°C frozen biomasses could indicate that these isolates were more denatured and experienced some protein unfolding, increasing their surface activity and providing

more of a polar and non-polar end to their structure. Looking at Figure 16 and Figure 17, the protein solubility of the P2A and P2B isolates of the sun-dried and -20°C frozen biomasses were high throughout the pH range 3-11, except for the P2A isolate of the -20°C frozen biomass at pH ~3, which correlated well to their EAI-patterns. The P2A and P2B isolates from oven-dried and freeze-dried biomasses, displayed low protein solubility in the acid range and high/maximum solubility in the neutral/alkaline range. Again, this correlates moderately well to the EAI-patterns of these protein isolates, suggesting less denaturation and unfolding of these protein isolates. However, the surface hydrophobicity needs to be investigated to give the full picture of these correlations. Unfortunately, the SDS-PAGE performed on these protein isolates provide little answer since all isolates shows an identical polypeptide pattern, see Figure 27. Still, all protein isolates tested for EAI in our study outperforms those produced from hake (fish, 2.03 m^2/g), egg white (5.18 m^2/g), soy (7.39 m^2/g) and pea (7.79 m^2/g), all measured at pH 7 (78).

In addition to the EAI measurement of the created emulsions they were investigated microscopically (Figure 22-25). Pictures revealed that P2B isolates from the sun-dried biomass and both isolates of the -20°C frozen biomass could create emulsions at native pH, however, with less complex network than at higher pH. This correlated well to their solubility (Figure 16-17) as well as their EAI-values (Figure 18-19) at this pH. Looking at both isolates from the oven-dried and freeze-dried biomasses (Figure 23 and Figure 25) it was obvious that these produced small to none emulsion-systems at pH 2. However, with increasing pH (7 and 11) all protein isolates created more complex and intense emulsions, correlating well with the protein solubility and EAI patterns. A high protein solubility and EAI around pH 7 means that our proteins could be applicable in a lot of food stuffs requiring emulsification in that pH range. However, more work is needed to optimize the conditions for each protein isolate since the stability (ESI) of the emulsions varied vastly over the whole pH range (Figure 20-21).

4.2.4 Polypeptide Pattern

The polypeptide pattern of the biomasses varied depending on their storage treatment. The freeze-dried and oven-dried biomasses displayed the most bands, three in the range of ~37-75 kDa and four at ~10-15 kDa. The -20°C frozen biomass displayed less intensity for the high molecular weight bands, however, the lower bands were very similar to those of the freeze-dried and oven-dried biomasses. The sun-dried biomass only displayed one band at ~15 kDa. These results show that the choice of storage treatment influences the polypeptide pattern of the initial biomass; with the sun-drying causing a lot of degradation and the -20°C freezing generating some loss of high molecular weight polypeptides.

All analyzed protein isolates displayed the same polypeptide pattern, with two bands at ~10-15 kDa. Garcia-Vaquero et al., (79), performed SDS-PAGE on proteins isolated with a pH-shift like process from the brown seaweed *H. elongate* which displayed several bands in the range of ~37-75 kDa. However, they aided their

protein precipitation using ammonium sulphate possibly that this could aid the recovery of larger peptides otherwise resistant to the isoelectric precipitation method (+/- freezing) used in this study. Another possibility is that there was heavy proteolytic degradation occurring during the process, something that could be prevented by protease inhibitors. Cavonius et al., (30), performed pH-shift processing, very similar to the one used in this study, on the microalgae *N. oculata* and analyzed the polypeptide pattern of the initial biomass, pH-shift fractions and the final protein isolate. Their results display no change in polypeptide pattern, indicating no effect of the pH-shift process itself on polypeptides. However, *N. oculata* is a completely different species from *S. latissima*. Our results show that the choice of preservation method did not affect the polypeptide pattern of the final protein isolates produced from *S. latissima*, whilst the pH-shift process itself did have an effect.

The fact that our protein isolate consisted only of low molecular weight polypeptides is in line with its solubility (54), which was seen around pH 5-6, and could also explain why it could easily create emulsions with relative high EAI-value at pH 7 and 11 by reaching the oil/water interface rapidly. However, low molecular weight polypeptides can cause problems with creating stable emulsion.

What kind of proteins that are present in brown seaweed is not well studied and no previous polypeptide analysis on *S. latissima* biomass has been reported. However, Kim et al., (80) performed proteome analysis of *Saccharina japonica*, a close relative to *S. latissima*. They identified 6 bands, among which two could be the polypeptides we found slightly above 50 kDa and 37 kDa; 57 kDa: 6-phosphogluconate dehydrogenase, 51 kDa: trypanothione reductase and ATP synthase subunit beta (chloroplastic), 41 kDa: Actin-1, 40 kDa: elongation factor Tu, 39 kDa: glyceraldehyde-3-phosphate dehydrogenase, 37 kDa: phosphoglycerate kinase (80). If some of these were present in our biomasses, we know that the preservation methods affect them differently and that, the pH-shift processes do not recover them, since only very low molecular weight protein were present in the final protein isolate.

4.2.5 Fourier Transform Infrared Spectroscopy

The Amide I band is the most sensitive region to discover structural differences in a proteins secondary structure, where the frequencies are closely related to α-helixes, β-sheets, turns and random coils (64). To determine specific contributions from these secondary structure to the overlapping band area of Amide I, mathematic data analysis is needed to resolute the peaks (64), something which was not performed in our study. However, peak shifting, between higher and lower band frequencies, can still provide general information about changes in the protein secondary structure between samples. The frequency bands reported for our protein isolates (Table 4) are the sum of overlapping peaks and can therefore only indicate which secondary protein structure that is dominating in the proteins, and not the distribution of different secondary structures.

Analyzing the resulting bands from our protein isolates revealed that both protein isolates from the sun-dried biomass had a dominating proportion of random coils, that the P2A of the oven-dried biomass was dominated by random coils (1651 cm^{-1}), whilst the P2B isolate contained mostly β-sheets (1639 cm^{-1}). Further, both protein isolates of the -20°C frozen biomass were dominated by random coils and the P2A and P2B isolates of the freeze-dried biomass contained predominantly random coils and β-sheets, respectively. Davenport (81) showed that pH-shift processing of catfish biomass increased the β-shift content at the cost of α-helixes. There was no clear effect of preservation method or pH-shift method on peak shifting towards higher or lower band frequencies, basically, most protein isolates had an Amide I band frequency close to 1645 cm^{-1}.

4.2.6 Amino Acid Analysis

AA analysis was performed to answer if the preservation methods and the version of pH-shift process applied, i.e. the use of freeze-precipitation or not, had an impact on the AA-patterns of the protein isolates.

In general, the EAA-profile of the differently preserved biomasses matched that of previous reports (82) and even slightly higher amounts of EAA were found than was reported for four other brown seaweeds (83). When expressing the amino acids on a protein basis, almost all biomasses met the WHO/FAO adult and infant recommendations for the EAAs; valine, threonine, isoleucine, leucine, methionine and phenylalanine. Both P2A and P2B protein isolates from most biomasses also met the recommendations for these EAAs, and also for lysine, showing an inconcentration from the initial biomasses. Our seaweed isolates were hereby outperforming protein isolates produced from hake (fish) and trout by-products (78, 84). Going from biomass to the P2A isolate, the total EAA increased with ~5-10 g AA/ 100 g protein and from biomass to the P2B isolate the total EAA increased with ~4-9 g AA/ 100 g protein, i.e. both pH-shift process enriched EAA of the proteins. Comparing the EAA-profiles of the P2A and P2B protein isolates to some common foodstuffs, like egg, beef, chicken etc., valine, isoleucine, leucine, methionine, phenylalanine and threonine content is higher for the seaweed protein isolates. Compared to other protein isolates produced with pH-shift like processes, our isolates had higher relative content of valine, threonine, isoleucine, leucine, methionine, phenylalanine and total EAA than that of beach pea protein isolate (8), higher parts of valine, threonine, isoleucine and leucine than krill protein isolate (27), way higher parts of all EAAs than protein isolate from the brown seaweed *H. elongate* (79). To consider using these protein isolates as fodder and feed they need to meet certain standards of EAA-contents. Both P2A and P2B isolates of all biomasses meets the requirements of 45 g lysine/ kg protein and 22 g methionine/ kg proteins (85). The cysteine content was not measured due to limitations in the AA-method.

Using the data of this study one can calculate that an adult with 70 kg body weight needs to consume 191 g, 232 g, 300 g or 160 g of the dry P2A protein isolate produced from the oven-dried, -20°C frozen, -80°C

frozen or freeze-dried biomasses, respectively, to cover the recommended daily intake of EAAs for adults, according to the World Health Organization (WHO) and Food and Agriculture Organization (FAO) data in Table 1. The same calculations performed for the P2B protein isolate shows that an intake of 156 g, 357 g, 218 or 124 g of the isolate produced from the oven-dried or -20°C frozen or -80°C frozen and freeze-dried biomasses, respectively is needed. The isolates produced from the sun-dried and ensilaged biomasses would require even larger intake to cover the recommendations. The seaweed protein isolates can thus only be seen as part of a diet to obtain the needed EAA.

These results show that the choice of preservation treatment has a small impact on the AA-profile of the isolates, however, the choice has a great impact for the daily intake needed to cover the WHO/FAO recommendations, since the protein content of the dry protein isolates vary alot. Also, the use of freeze-precipitation had smaller impact on the AA-profiles, but a greater impact on the daily intake need, requiring less consumption of P2B than P2A protein isolates from the oven-dried, -80°C frozen and freeze-dried biomasses to meet the same limits.

4.3 Seasonal Study – Outcome of the pH-shift Processes

In addition to the biomass harvest in May, used in the preservation study, biomass was also harvest in March and April. The pH-shift processes were applied to the oven-dried samples of these biomasses and some qualitative analyses were performed on the protein isolates produced.

4.3.1 Moisture Content of the Biomasses

The April-biomass had significantly lower moisture content than the March- and May-biomasses. Previous studies analyzing the moisture content of fresh *S. latissima* revealed very little difference within the March to May months (6, 38). It should be mentioned that the March- and April-biomasses were vacuum packed after oven-drying, whilst the May-biomass was stored in Ziploc-bags, which could explain the differences between the harvest months.

4.3.2 Native pH of the Biomasses

The May harvested biomass had a significantly higher native pH (7.0) than the March- and April-biomasses (6.7 and 6.7, respectively). No previous studies have measured the pH of *S. latissima* biomass harvested at different months.

4.3.3 Total Protein of Biomasses

There was no significant difference in total protein between any combination of the seasonal biomasses. Marinho et al., (6), also investigated the seasonal compositional variations of *S. latissima*, they found no significant difference in total protein between March, April or May on a DW basis. However, Schiener et al., (38), and Manns et al., (82), found a 2.3 pp and ~5 pp difference in total protein between March and May, respectively, with May containing the least amount of protein. Differences in protein content can be explained by factors like water temperature, nutrition availability, underwater irradiance, cultivations systems etc. (86). These factors were all varying for the studies mentioned above, for example the biomass studied by Manns et al., (82), and Marinho et al., (6), were harvested in Danish waters whilst Schiener et al., (38), worked with biomass from Scotland, creating obvious variations in the environmental factors. In terms of actual growth of seaweed biomass, Peteiro et al., (86), declared that April or May are the optimal months for harvest, however, in terms of the most protein profit from the pH-shift methods used in this study one needs to asses a combination of optimal harvest month for bulk biomass and which biomass preservation technique is yielding the most protein.

Schiener et al., (38), revealed that there was a large difference micronutrient composition between *S. latissima* biomasses harvested in March and May, the latter generally containing more Al, Cr, Mn, Fe, Cu. However, their March biomass contained more iodine. To further investigate the applicability of our isolates these kinds of analyzes need to be performed on the biomass in food products.

4.3.4 Protein Solubility During the Different Steps of the pH-shift Processes

For pH-shift process Type I, Type II and Type III, there was a significant difference in protein solubility between biomasses from each harvest month and within each harvest month (comparing supernatants). May biomass gave the highest protein solubilities for each pH, followed by April and then March, i.e. the older the seaweed plant was the higher protein solubility it had. Which is good for the extraction part, but a disadvantage for the protein precipitation. No previous studies have investigated the seaweed protein solubility depending on harvest season. However, Marinho et al., (6), and Manns et al., (82), who investigated the seasonal AA-patterns of the biomass, found that biomass harvested during earlier months of the year contained more hydrophobic AAs than biomasses harvested during later months. Further, Schiener et al., (38), reported seasonal variations in the content of polyphenols, where phlorotannins are included, however, specific polyphenols were not measured. In summary, a combination of the AA-profile, polyphenol content and the biomass matrix could explain the seasonality in protein solubility seen in our results.

Again, as for the differently preserved biomasses, freeze-thawing had highly significant impact on protein precipitation. For pH-shift Type II, the March biomass gave a 5.2 times higher protein solubility and the April biomass gave a 2-fold reduction in solubility. For pH-shift process Type III, the March biomass gave a 9-fold reduction in protein solubility by applying freeze-thawing and the April biomass gave a 2.1-fold reduction. This again proves that the simple act of freezing the protein rich supernatant S1 together with pH adjustment to the pI will lower the protein solubility greatly.

4.3.5 Protein Yield during pH-shift Processing

As for the preservation study, the protein yield for different steps of the pH-shift processes were calculated to evaluate which biomass that yielded the most protein. This is a question that no previous studies have focused on.

4.3.5.1 Protein Extraction Yield

The seasonal difference in protein extraction at pH 12 was vast, and the protein yield increased significantly with later months, from 34.5 % in March to 46.8 % in April to 77.2 % in May. Protein extraction yield goes hand in hand with protein solubility at pH 12 and size of the first sediment. In this case, solubility at pH 12 was highest for the May biomass. As discussed in 4.3.4, factors like AA-pattern, phlorotannin content and biomass matrix can affect these features.

4.3.5.2 Protein Precipitation Yield

Again, there was a great seasonal difference between the biomasses. The protein precipitation yield was highest with earlier harvest months with up to 3.3 times higher precipitation yield in March than May, with pH-shift Type III.

4.3.5.3 Total Protein Yield

As a result of the higher extractability the total protein yield was higher with earlier harvest months, except for pH-shift process Type II where May had higher total protein yield than April. The choice between April and May biomass in terms of total protein yield only matters if one applies pH-shift process Type III, which then yielded significantly higher amount for these months. However, to determine the optimal harvest month for seaweed to be used in protein extraction one needs to look at more than the highest total protein yield, e.g. amount of biomass per plant, the quality and functionality of the protein extract etc.

With our simplest pH-shift process, Type I, the March biomass yielded 18.7 % protein, which was higher than any previous reported pH-shift like process protein yield for *S. latissima*; 16.01 % was seen by Vilg et al., (14), and for three green seaweed species; 5.71-6.48 % (37) and finally for *K. alvarezii* 7.81 % (40). With the addition of freeze-precipitation, pH-shift process Type III, we managed to reach a total protein yield of 30.4 % for the March biomass, greatly exceeding previous reported yields in pH-shift like processing of seaweed and exceeding the protein yield from yellow pea, 20.2 % (9). This again proves that the simple method of freeze-precipitation increases total protein yield significantly and that harvest season is a crucial factor affecting outcome of the pH-shift processes investigated in this study.

Considering the protein yield in on a biomass basis (DW) the pattern was identical to that discussed above. The March biomass achieved the highest total protein yield of 24.9 mg protein/ g biomass when pH-shift process Type III was applied.

4.4 Seasonal Study – Quality Evaluation of Protein Isolates

4.4.1 Concentration of Protein in the Protein Isolate

There was up to 5.7 times inconcentration of protein during the pH-shift process, however, the choice of harvest month had a significant impact on the protein content of the final protein isolate. The only exception was for when applying pH-shift process Type III on April and May biomasses. Also, applying freeze-precipitation increases the protein content of the final protein isolate, independent of the choice of harvest month.

The P2B isolate of the March biomass contained the most protein per dried protein isolate powder, 46.5 %. This is in the mid-range of what Kandasamy et al., (37), found for protein isolates produced from three green seaweed species (33.4 – 60.4 % on DW basis). However, ammonium sulphate was then used to precipitate the proteins leading to the need of dialysis of these protein isolates, complicating potential large scale processing and consuming more time. Kumar et al., (40), also produced a protein isolates with a pH-shift like process obtaining isolates with 62.3 % protein on DW basis. However, also here, the protein was precipitated using ammonium sulphate.

Again, as for the protein isolates produced in the preservation study, the protein content was still much lower than what is achieved with pH-shift processing of soy (~90 % on DW basis) (10). To get full picture of what our protein isolates contains a full compositional analysis has to be performed.

4.4.2 Polypeptide Pattern

All isolates showed similar patterns, with two thin bands slightly below 15 kDa and 10 kDa. This is the same pattern as all the isolates analyzed in the preservation study. This indicates that the pH-shift process has isolated specific proteins, regardless of the preservation treatment or harvest season of the biomass. As discussed earlier, the small size polypeptides could also indicate severe proteolysis during the process. The intensity of the bands increased with earlier harvest months, i.e. the amount of protein in the intense bands is higher, even if the same amount of protein was loaded in each well. It is possible that the isolates produced from the April and May biomasses contained peptides so small, that they would flush out of the gel, or that parts of these proteins are “smeared” out in the 25-75 kDa area.

5 CONCLUSION

Choice of preservation technique greatly affected total protein and polypeptide patterns of the seaweed biomasses, as well as protein solubility at extreme pH's and final total protein yield from the pH-shift processes performed on the seaweed biomass. Two-step (Type II) and one-step (Type III) freeze-precipitation both increased the protein yield of the pH-shift process significantly, for most biomasses. One-step freeze-precipitation gave the highest protein yields, up to 30.4 %, and was a simpler and faster method than the two-step version. Among the large scale applicable preservation techniques, i.e. when excluding freeze-drying, the oven-dried and -20°C frozen biomasses achieved the highest total protein yield when applying classic pH-shift processing with added freeze-precipitation.

Protein content, protein solubility at low pH and emulsion capacity of the protein isolates created with the classic pH-shift method and with the added one-step freeze-precipitation varied significantly depending on the biomass used. However, the protein solubility at pH > neutral and AA-patterns of these isolates were relatively constant over all biomasses. Polypeptide patterns of the biomasses differed depending on preservation treatment, whilst all produced protein isolates displayed similar polypeptide pattern indicating that choice of preservation treatment does not affect the size of the peptides in the final product. The relatively high protein solubility in the neutral pH range, and the good emulsion activity index makes our protein isolates applicable in a wide range of food stuffs. Also, protein isolates of most biomasses met the WHO/FAO adult and infant recommendations for the EAA; valine, threonine, isoleucine, leucine, lysine, methionine and phenylalanine, and had a total EAA content of ~50 g/ 100 g protein. The oven-dried biomass gave rise to the protein isolate with the highest protein content 40.5 % (on DW basis), when one-step freeze-precipitation was applied. A person with a body weight of 70 kg thus needs to consume only 156 g of this protein powder to meet the WHO/FAO requirements for daily EAA intake.

Oven-dried *S. latissima* harvested in March, April or May did not differ significantly in total protein content, however, protein solubility and total protein yield during the pH-shift processes varied significantly. Biomass harvested in March achieved a total protein yield of 30.4 % and the final product from the pH-shift process with one-step freeze-precipitation applied had a protein content of 46.5 %. However, further qualitative analysis of these protein isolates is needed to determine what the rest of the isolate consists of. Initial ionic strength analyses revealed that a large fraction is salt. In terms of optimal harvest season, one needs to consider the bulk amount of biomass available to harvest for each month, e.g. each seaweed plant becomes larger with later harvest months. Thus, a combination of biomass availability, protein yield from the pH-shift processes and the quality of the produced protein isolates will determine the optimal harvest season.

With increasing demands for novel vegetable protein sources and an emerging area of utilizing seaweed biomass to produce food products in the western world, we have shown that is it possible to produce a high-quality protein isolate from the seaweed *S. latissima* with simpler than previously applied methods, the pH-shift process.

5.1 Future Approaches

To improve the pH-shift processes applied in this study, a screening of optimal protein solubilization pH and precipitation pH is needed. Even though Vilg et al., (14), found that pH 12 and 2 was optimal for protein solubilization and protein precipitation, respectively, their biomass was harvested in November whilst the biomass in this study was harvested during spring months, and was subjected to various preservation treatments that, in hindsight, clearly affected the proteins. The use of freeze-precipitation proved to significantly increase the yield from the pH-shift process, however, this methodology also needs an optimization e.g. regarding freezing temperature. Another factor needing investigation is the salt content of the initial biomasses and final protein isolates. A high salt content in the initial biomass and during the pH-shift process stages will obstruct the recovery of proteins, due to the salting in/out effect of salt. Also, a too high salt content in the final product will complicate its applicability in foodstuffs. Potentially a desalting step of the biomass should be applied prior to processing.

A few pH-shift process runs were performed in larger scale with ~340 g starting amount of wet biomass instead of ~30 g. This was performed on all differently preserved biomasses except the ensilaged. In this scale, the -20°C and -80°C frozen biomasses retained a jelly layer on top of the first pellet and finally produced only a small amount of protein isolate, relative to what was achieved in smaller scale processing. The "dry" biomasses; sun-dried, oven-dried and freeze-dried, did not display any jelly layer and produced an expected amount of protein isolate. Future studies should imply compositional analysis on this jelly layer and test ways of recovering it in case the protein content is high. These results awaken speculations about how well the biomasses will performed in even larger scale and is an important point for future research in this area.

To truly compare preservation techniques, harvest seasons and pH-shift process types towards each other in different combinations, comprehensive life cycle analyses are needed. That information would prove if an extensive cost, like frozen storage of biomasses, might be cancelled out by a more valuable protein isolate being produced from that biomass. However, another factor to consider when it comes to freezing is that the transport of wet biomass will be less efficient than transport of dried biomass which might make a dry biomass more profitable. In terms of harvest months, the amount of available biomass is an important factor together with the protein yield from a specific harvest month. Thus, even if May yielded the least protein the seaweed plants are larger in this month which will provide more raw material. Without full calculations on amount protein per hectare ocean, it is difficult to exclude any of the biomasses.

From the pH-shift processes applied in this study there are two types of by-products produced. Firstly, P1 that is created after the alkaline solubilization and centrifugation of the homogenized biomass, which contains biomass debris and to various degree, proteins, depending on preservations treatment and harvest

month of the biomass. Secondly, residual protein containing supernatants are produced during various steps of the pH-shift processes. P1 is currently passed on to the biogas producing part of the bio-refinery in the Seafarm project. To recover proteins from the residual liquid fractions, i.e. supernatants, the water needs to be removed, which can hypothetically be done by e.g. evaporation or spray drying. Evaporation could be done by lowering the air-pressure, i.e. creating a vacuum, whereby lowering the boiling point of the liquid which would potentially cause less damage to the proteins. Spray drying, could produce a protein powder in much faster time protecting the proteins from vast denaturation, however, this process is expensive and must be evaluated compared to the possible profit of the protein powder produced.

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Appendix A – Standard Curve and Process Manual

BSA Protein Standard Curve

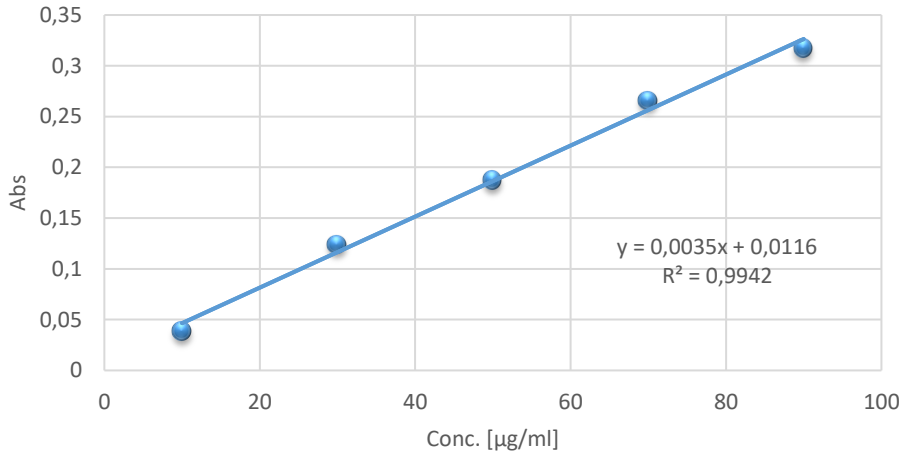

Figure A1. *BSA standard curve used in the Lowry protein analysis. Absorbance is plotted against the known protein concentration of each sample.*

pH-shift Process Protocol

Start the pre-cooling of the centrifuge and rotor.

Type of biomass:_____

Moisture content:_____ [%]. Seaweed to DI-water ratio (wet weight basis):_____

Note: Use cold DI-water and keep the algae-water slurry on ice at all time.

Amount of seaweed:_____[g]. Adjusted to:_____[g].
(_____[g DW].) To achieve moist. Cont.:_____[%].

Amount of water:_____[g]. (Save 6 ml (g) to rinse polytrone shaft)

Total seaweed + water amount:_____[g] ([ml])

(Use for tot protein/dilution calc.)

Polytrone for 2 min at speed 4. Rinse the polytrone shaft using the saved 6 ml DI-water. Use a rubber spatula to scrape down algae from the sides of the beaker into the mix.

Osmoshock for 15 min on ice.

Add a magnetic bead (not too small) and place on a magnetic stirrer.

Weigh the canister containing the 1M NaOH:

Weight before NaOH addition:_____ [g]. Measure native pH:_____.

Adjust to pH 12 using 1M NaOH and weight the beaker when complete.

Weight after NaOH addition:_____[g]. NaOH density: 1.0477 g/ml.

Amount of 1M NaOH added:_____[g] ~ _____[ml].

□ Take 2 x 1ml samples of the Homogenate.

Incubate for 20 min on ice still stirring with the magnetic bead.

Weigh two centrifuge tubes **without seal and lid**: (check that the seals and lids weigh the same)

Centrifuge tube 1:_____[g].

Centrifuge tube 2:_____[g].

Centrifuge tube 3:_____[g].

Centrifuge tube 4:_____[g].

Weigh the tubes again (containing the algae) **without seal and lid**:

Centrifuge tube 1 + slurry:_____[g]. Algae slurry:_____[g].

Centrifuge tube 2 + slurry:_____[g]. Algae slurry:_____[g].

Centrifuge tube 3 + slurry:_____[g]. Algae slurry:_____[g].

Centrifuge tube 4 + slurry:_____[g]. Algae slurry:_____[g].

Load the tubes into the centrifuge and run for 20 min, 8500g, 4°C.

Separate Supernatant 1 (S1) from the tubes and add to a beaker. **Keep on ice**!

Weight of S1:_____[g]. Weight of Pellet 1 (P1):_____ [g].

□ Take 2 x 1ml samples of S1.

Total volume of S1:_____[ml]. (Use for protein calc.)

Volume of S1 – sample (2ml):_____[ml]. (Use for dilution calc.)

Weigh the canister containing 1M HCl:_____[g].

Adjust pH to 2 using 1M HCl.

Weight after HCl addition:_____[g]. HCl density: 1.0231 g/ml.

Amount of HCl added:_____[g] ~ _____[ml].

Incubate on ice, with stirring, for 20 min.

Freeze half of "S1 pH 2" and centrifuge the day after.

Pre-weigh one (possibly 2) centrifuge tube **without seal and lid**: (check the weights of seals and lids)

Centrifuge tube 5:_____[g].

Centrifuge tube 6:_____[g].

Centrifuge tube 7:_____[g].

Centrifuge tube 8:_____[g].

Add S1 to the tube(s) evenly and weigh:

Centrifuge tube 5 + S1:_____[g]. Amount of S1:_____[g].

Centrifuge tube 6 + S1:_____[g]. Amount of S1:_____[g].

Centrifuge tube 7 + S1:_____[g]. Amount of S1:_____[g].

Centrifuge tube 8 + S1:_____[g]. Amount of S1:_____[g].

Load the tubes into the centrifuge and run for 20 min, 8500g, 4°C.

Separate Supernatant 2 (S2) from the pellet using a sieve and add to a 100 ml beaker.

Weight of S2:_____[g]. Weight of P2 (wet):_____[g].

Moisture content:_____ [%].

□ Take 2 x 1 ml samples of S2.

Total volume of S2:_____[ml]. (Use for protein calculations below).

Save P2 in at least two (maybe three) separate containers, mark and place in -80°C freezer.

Dilution due to NaOH and HCl addition

Volume of homogenate before NaOH addition:_____[ml].

NaOH added: _____[ml].

Resulting dilution: (NaOH added)/(Homogenate volume) + 1 = _____.

(Use this for exact calculations of Lowry measurements for Homogenate and S1.)

Volume of S1 before HCl addition:_____[ml].

HCl added: _____[ml].

Resulting dilution: (HCl added)/(S1 volume) + 1 = _____.

Total dilution after HCl addition:_____ x _____ = _____.

(Use this "total dilution" for exact calculations of Lowry measurements for S2.)

The three samples taken (homogenate, S1 and S2) will then be diluted further and put through the Lowry protein assay.

Appendix B – Additional Data and Results

The pH-value of cold DI-water was measured using a pH-meter (MeterLab® PHM210 STANDARD pH METER), results are shown in Table B1.

Table B1. pH measurements of cold DI-water.

Replicate	*pH*	*Mean*	*S.D.*
1	5,1		
2	5,1	5,1	0,1
3	5,2		

All fractions created during the pH-shift processes were weighed, in Table B2 the volumes of S1 are shown (assuming a density of ~1 g/ml).

Table B2. Volume of S1 [ml], produced during the pH-shift process.

Biomass	*Replicate 1* *[ml]*	*Replicate 2* *[ml]*	*Mean* *[ml]*
Sun-dried	191.7	190.4	191.0
Oven-dried	190.3	191.3	190.8
Frozen -20	182.6	184.4	183.5
Frozen -80	185.0	185.2	185.1
Ensilaged	179.7	179.0	179.3
Freeze-dried	191.6	194.8	193.2

Up-concentration of Protein

The moisture content and protein content (percent of WW) for the initial biomasses and their respective produced protein isolates, i.e. P2A and P2B, were measured/calculated, see Table B3. The moisture content of the P2A and P2B protein isolates were very high (94.1 - 97.9 %) automatically causing the protein content on WW-basis to be very low (0.03 – 1.6 % of WW). For the oven-dried, -80°C frozen, ensilaged and freeze-dried biomasses the protein content on a WW-basis was higher in P2B than P2A, however, for the sun-dried and -20°C frozen biomasses the protein content was higher in P2A.

Table B3. M*oisture content and protein content of each biomass or protein isolate in percent of WW.*

	Biomass		Pellet 2A		Pellet 2B	
Treatment	Moisture Content [%]	Protein Content [% of WW]	Moisture Content [%] *	Protein Content [% of WW]	Moisture Content [%] *	Protein Content [% of WW]
Sun-dried	8,8 ± 0,8	5,52 ± 0,04	94,6	0,84 ± 0,02	96,3	0,75 ± 0,00
Oven-dried	8,2 ± 0,3	7,37 ± 0,06	96,8	0,79 ± 0,01	96,4	1,45 ± 0,02
Frozen -20°C	88,5 ± 0,0	0,88 ± 0,02	97,2	0,61 ± 0,02	97,9	0,39 ± 0,02
Frozen -80°C	87,9 ± 0,4	0,94 ± 0,05	96,2	0,58 ± 0,03	97,6	0,63 ± 0,01
Ensilaged	86,1 ± 0,6	0,91 ± 0,05	97,5	0,03 ± 0,00	94,1	0,51 ± 0,02
Freeze-dried	5,6 ± 0,7	7,73 ± 0,32	96,7	0,93 ± 0,03	95,7	1,60 ± 0,03

** Moisture content was only measured once. WW: wet-weight.*

Additional Amino Acid Calculations

For all the biomasses and their respective produced isolates P2A and P2B the AA profile was also calculated in units of mg AA/ g FDBP (freeze-dried biomass) and mg AA/ g FDPI (freeze-dried protein isolate). These AA profiles for the biomass, P2A and P2B are shown in Table B4, Table B5 and Table B6, respectively.

Table B4. *AA profiles of the six differently preserved biomasses reported as mg AA/ g FDBP.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried
Glycine	3.86	4.38	4.05	4.14	4.04	4.58
Alanine	10.22	14.46	14.32	14.34	11.14	15.65
Serine	2.09	2.48	2.32	2.22	2.14	2.55
Proline	2.83	2.64	2.53	2.30	2.37	2.50
Valine	5.86	6.98	6.63	6.52	5.63	6.80
Threonine	2.92	3.36	3.14	3.18	3.02	3.32
Isoleucine	4.10	4.66	4.39	4.40	4.02	4.41
Leucine	7.22	8.24	7.74	7.79	7.08	8.00
Aspartic acid	2.36	3.55	3.50	3.62	3.42	4.05
Lysine	2.29	3.35	3.27	3.17	2.68	3.07
Glutamic acid	8.02	14.99	13.83	15.55	11.02	16.49
Methionine	1.85	2.31	2.07	2.25	1.82	2.19
Histidine	0.49	0.95	0.88	1.07	0.83	0.97
Phenylalanine	3.63	4.48	4.42	4.42	3.92	4.21
Arginine	1.55	2.09	1.94	2.00	1.93	2.14
Tyrosine	0.72	1.07	0.97	1.03	0.95	1.07
Cysteine	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-
TAA	60.00	80.00	76.00	78.00	66.00	82.00
TEAA	28.36	34.32	32.53	32.80	29.00	32.97
TEAA/TAA	0.47	0.43	0.43	0.42	0.44	0.40

Table B5. *AA profiles of the P2A protein isolates produced from the six differently preserved biomasses reported as mg AA/ g FDPI.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried
Glycine	11.82	18.55	15.87	10.99	1.39	20.80
Alanine	22.65	31.72	33.06	20.61	2.96	34.89
Serine	5.62	9.42	8.21	5.91	0.74	10.71
Proline	6.98	9.69	8.38	5.60	0.87	10.37
Valine	14.99	22.26	19.73	13.00	1.90	24.20
Threonine	8.35	13.18	11.99	8.37	1.08	15.08
Isoleucine	10.79	16.09	14.99	9.98	1.39	18.95
Leucine	18.79	30.08	26.96	17.96	2.34	34.45
Aspartic acid	5.43	13.20	8.69	8.01	0.52	15.85
Lysine	7.23	14.23	12.59	8.83	1.01	15.93
Glutamic acid	14.91	26.11	22.00	17.05	1.78	29.84
Methionine	4.73	7.87	7.11	4.73	0.66	9.30
Histidine	1.51	3.66	3.02	2.33	0.27	4.37
Phenylalanine	13.15	18.03	16.06	10.88	1.45	17.97
Arginine	4.44	9.20	7.02	5.67	0.57	11.57
Tyrosine	2.23	5.19	3.92	3.16	0.27	6.11
Cysteine	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-
TAA	153.60	248.50	219.60	153.10	19.20	280.40
TEAA	79.53	125.40	112.45	76.08	10.10	140.25
TEAA/TAA	0.52	0.50	0.51	0.50	0.53	0.50

Table B6. *AA profiles of the P2B protein isolates produced from the six differently preserved biomasses reported as mg AA/ g FDPI.*

Amino acid	Sun-dried	Oven-dried	Frozen -20°C	Frozen -80°C	Ensilaged	Freeze-dried
Glycine	11.85	22.44	10.18	14.57	4.88	19.53
Alanine	20.41	39.27	20.08	24.74	7.89	32.73
Serine	8.73	17.89	8.15	12.18	3.99	17.31
Proline	9.10	15.02	6.87	9.81	3.81	14.27
Valine	17.48	31.39	15.16	20.03	7.25	29.04
Threonine	13.86	26.34	12.49	17.12	6.04	25.62
Isoleucine	12.62	21.82	10.24	14.22	5.83	20.12
Leucine	22.82	40.97	18.95	25.73	9.53	38.10
Aspartic acid	13.70	33.07	14.22	21.10	5.89	31.63
Lysine	13.62	32.52	14.61	23.62	5.66	31.72
Glutamic acid	26.48	58.54	29.53	36.29	9.52	49.45
Methionine	6.35	12.24	5.37	7.52	2.76	11.28
Histidine	1.45	4.48	1.96	3.21	0.88	5.66
Phenylalanine	15.12	25.13	11.84	16.08	6.59	22.92
Arginine	5.51	14.38	6.17	9.58	2.80	15.79
Tyrosine	3.87	9.50	4.18	6.20	1.57	10.84
Cysteine	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-
TAA	203.00	405.00	190.00	262.00	84.90	376.01
TEAA	103.33	194.89	90.62	127.53	44.54	184.47
TEAA/TAA	0.51	0.48	0.48	0.49	0.52	0.49