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Increased utilization of marine rest raw material

Extraction of gelatin and optimization of the extraction procedure from salmon (Salmo salar) backbone

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Preface and acknowledgements

This master thesis is the final delivery of a 5-year program (MTKJ) at NTNU, the Norwegian University of Science and Technology. The thesis was performed in co-operation with Sintef Ocean as a part of the DAFIA project, which aims to increase the utilization of marine rest raw materials. The lab work was performed at the food science laboratory at NTNU Gløshaugen during the spring of 2018.

First and foremost, I want to thank my main supervisor, Professor Turid Rustad, and my co-supervisor Dr. Rasa Slizyte at Sintef Ocean, for all their help and guidance. Their knowledge and expertise have been invaluable for the work performed, and have helped motivate me. In addition, I want to thank the technical staff of the food science laboratory, Veronica Hjellnes and Siri Stavrum for tips and help during the work. Thanks to the other students in the lab for participating to the creation of a positive environment at the lab, this made it much easier to wake up early and do the work.

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Lastly, I want to thank all the people that have proofread and supplied input for the thesis. Thank you all!

Abstract

Every year large amounts of marine rest raw materials are produced worldwide, including Norway. In 2015 the total marine rest raw material produced in Norway was 676 000 tonnes. This amount is expected to increase every year with the increase in production from the marine industry. The majority of this rest raw material is used for production of low value products such as silage or bioenergy. Currently only small amounts are utilized for production of high value products, like oil and fat for human consumption. The increasing global population creates a demand for a higher food production. There are several options to meet this demand to increase the supply of food, either by increasing the production, better utilizing the raw material, or changing people's diet. Due to this there has been an increased interest in utilization of rest raw materials.

The aim of this project has been to study the possibility of utilizing marine rest raw materials from Atlantic salmon backbone (*Salmo salar*) for production of gelatin, a high value product for human consumption. This project built on results from a project performed fall 2017, which was focused on running trials on an extraction method by Arnesen & Gildberg (Arnesen & Gildberg, 2007), modified by Sintef Ocean. The project was performed in co-operation with Sintef Ocean and NTNU under the DAFIA project.

The pre-treatment consisted of a seven-step acidic procedure, followed by a two-step extraction. The extraction of gelatin was performed thermally in water. The goal was to study how varying the parameters of the pre-treatment and extraction influenced the yield, purity and physiochemical properties of the extracted gelatin.

The different variations of the extraction procedure did not significantly influence the yield or purity of the extracted gelatin. Gelatin yield was found to vary between 25.9% and 34.2%, with a purity of 75.5% to 83.3%. The procedure that consisted of 10 minutes shorter pre-treatment steps was found to give the highest yield (34.2±5.75%). This sample also had the second highest purity, which was found to be 81.6±10.3% of freeze dried sample. The basic method was found to give a yield of 29.5±1.6% and a purity of 80.5±5.8%. All samples had a total amino acid content at around 1000 mg AA/g sample, which further reinforce the

assumption of a high purity. No statistical difference ($p < 0.05$) was found between the yield and purity of the samples based on the extraction and pre-treatment parameters.

The dry matter content of all extracts was found to lie between 1.5% and 2.5%. Results of SDS-page indicates that the gelatin contain considerable amounts of peptide fragments with a size of 669000 Mw, and between 440000 Mw and 232000 Mw. The high molecular weight distribution was reflected in the gel forming properties for the gelatin, which formed strong gels at low temperatures (4 °C). In addition, the gels were found to have a low melting point (~15 °C). The physiochemical properties were shown to be good, and followed the expected properties of cold water fish gelatin. Based on these result, gelatin extracted from salmon backbone by this method produces gelatin with high purity and good yield and is likely to have areas of application in food, pharma and cosmetics.

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Introduction

The worlds food supply

With an ever-increasing world population there will be a greater need for increased food production. Even though the population growth is generally slowing down, it is estimated by the UN that there will be 9.8 billion people by 2050 and 11.2 billion people by 2100. The demand for food is projected to increase by 50 percent by 2050 because of the population increase combined with an increase in the overall wealth of the poor nations, more people living in cities and the influence of food habits from the western world on the poorer countries. Because the diet of the people living in the developed countries consists of a higher amount of high value or animal proteins compared to the diet of the less developed regions, the demand for animal protein and fats will increase (FAO.a, 2013).

The current food supply chains are not capable of meeting this demand, and will need to increase their production drastically if everyone is going to adopt a western diet. FAO estimates that to feed the world population in 2050 the agriculture will need to produce almost 50% more feed, biofuel and food than the current amount (FAO.b, 2017). This required production increase will be most difficult for the agriculture in sub-Saharan Africa and South-Asia, which will have to more than double their current production, while Europe and North-America will only need to increase their production by a third. This can be achievable, as larger increases in agricultural production have been achieved over the same amount of time, however not for high value proteins and meat (Erisman & MA Sutton, 2008).

There are several ways of meeting the increased demand, such as increasing the production, adjusting the diet to accommodate a lower consumption of high value proteins, and increasing the reducing the food waste. Increasing the production of meat and fish with the current methods will probably be highly taxing on land area and on the environment, and such a large increase will not be sustainable. The most viable option for increasing the production would be increasing the production per square meter. There have been several agricultural revolutions where the yield per square meter have increased drastically,

especially with the discovery of industrial fertilizers which were estimated to be necessary for one third of the current production of food and being required for feeding half the people of earth (Stewart, Dibb, Johnston, & Smyth, 2005. Vol 1). To drastically change the diet of the developed regions to consist of a lower amount of animal proteins and distributing it better, there needs to be a willingness from the population to change it, which will undoubtedly be difficult. There are several studies concerning the required daily intake (RDI) of protein for an average human. According to numbers from the Organization for Economic Co-operation and Development (OECD), an average American consumes 90 kg of meat a year, which translates to approximately 250 grams per day. Globally this number is 35 kg year (OECD, 2018). This implies a large difference between the diet of the more developed countries and the less developed countries. The recommended daily intake (RDI) of proteins is suggested to be 0.75 grams/kg bodyweight, for a diet based at 2000 kcal (British Nutrition Foundation, 2012). The potential to reduce the intake is there, but will be probably be challenging. Lastly, to meet the coming demand for a higher animal protein production there has been an increasing interest in rest raw material utilization and lower food waste.

The increasing interest for rest raw material utilization gives rise to many opportunities and several new jobs. By using the rest raw materials that is discarded, the amount of food supplied to the consumers could be increased without taxing the environment more. In addition, the amount of waste that needs to be handled by the producers will decrease, thereby increasing their profits.

Currently there is a large waste of viable and good sources of raw materials at several points of the food supply chain, also after it reaches the consumers. Food loss and waste is defined by the FAO as a decrease in quantity or quality of food. This can mean that the nutritional value, the economic value or the availability is decreased, either because of physical displacement or food safety. According to FAO roughly one third of the food produced for human consumption gets lost or is wasted (FAO.c, 2017). This equates to approximately 1.3 billion tonnes every year. In developing countries approximately 40% of the loss is from producer to retailer, due to poor infrastructure, lack of knowledge and lack of technology for harvesting and post processing. In industrialized countries approximately 40% of the loss happens at retailer and consumer levels, in parts due to an over-emphasis on appearance and sensory aspects. This shows a large discrepancy between how the rich countries utilize the resources that are present, and some areas where there are large possibilities for

improvement. Not only does the food loss result in a reduced ability to feed a larger population, it also results in an economic loss for farmers and other workers in the food supply chains, which in turn makes food more expensive for the consumers. All wasted food is also an indirect waste of land areas used for that production, waste of energy used to produce it, and is therefore indirectly responsible for millions of tonnes of greenhouse gasses. With global warming becoming a more prominent and obvious threat, reducing the emission footprint from food production will be extremely beneficial for reducing the threat posed to food production, especially in food insecure areas (FAO.c, 2017).

When talking about rest raw materials there is no unified single definition that is used. However, it is usually defined as everything that remains after the main product has been extracted from the raw material (Rustad, Storrø, & Slizyte, 2011).This includes everything edible and inedible, such as bones, skin, viscera, head, cut-offs and blood. For salmon and most other fish, the main product is the filet. The rest raw materials from different processes can make up to 70% of the fish (Olsen, Toppe, & Karunasagar, 2014). According to the FAO the amount of rest raw materials produced from the fish industry represents between 50 and 70 percent of the initial weight of the fish (FAO.d, 2017). This is a very large amount compared to bovine where the rest raw material can be as low as 20 percent of the initial weight. For cows the utilization of the byproducts is higher so the relative rest raw material is lower. The total production of Atlantic salmon in Norway in 2017 is estimated to be 1.2 million tonnes and the amount is increasing each year (SSB, 2017). The total rest raw material produced in Norway each year is also steadily increasing, as can be expected with a higher production in the marine industry. In 2015 the total marine rest raw material produced in Norway was 676 000 tonnes, which was a 7 percent increase from 2014 (Richardsen & Nystøl, 2015).

The Norwegian fish industry utilizes almost 91 percent of the raw material from salmon. The only part not used is blood, which is hard to keep sanitary and is therefore discarded. In Norway the rest raw material is used for three main products: silage, products for human consumption and biogas. Silage products are by far the most important, and form largest industry of the three as 79 percent of the total amount of rest raw material produced in Norway is used for this purpose (Richardsen & Nystøl, 2015). Silage is a way to utilize low value raw material for animal feed. Manufacture of silage is done by mixing the raw material with an acid, usually formic acid. The raw material is completely degraded and turn in to a

liquid. This process requires fairly simple equipment, such as a grinder, a container with agitation and an acid dispenser. Because of the simplicity of the required equipment the procedure can be done on site, and is therefore an easy and simple way of using the rest raw material in field even though it is low value.

One product that can be obtained by better utilizing rest raw material is collagen and gelatin. These compounds have several areas of application in the food industry, as well as in cosmetics and pharmaceuticals.

Collagen and Gelatin

Market, value and production

With an estimated production of 460 000 metric tonnes of gelatin in 2018, gelatin is one of the most commercially produced biopolymers today. It is one of the most used gelling agents in food industry, and has hundreds of other applications in several industries, due to its versatility, ranging from pharmaceutical to scientific research to technical (Haug, Draget, & Smidsrød, 2004). In 2011 the global market was worth approximately $1.79 billion, $2.79 billion in 2018 and is estimated to reach $4.08 billion in 2024 (Grand View Research, 2016) (Transparency Market Research, 2012).

Gelatin extracted from fish represents roughly 1.5 percent of the total global gelatin production today (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011), while the rest is extracted from either bovine skin, pork skin or ossein, each accounting for 29.4%, 46.0% and 23.1% of the production respectively (Karim & Bhat, 2009). With such a large part of the gelatin produced from non-halal and kosher sources there are some challenges for Muslims and Jews who, according to their faith, should not eat products where gelatin from pork is used (Shafie & Othman, September 28-30. 2006). This is also an issue for Hindus who consider cows sacred. With the outbreak of bovine spongiform encephalopathy (BSE), there has also been an increasing demand for gelatin from sources other than cow.

Physio-chemical properties

Gelatin is extracted by partial hydrolysis of collagen. Collagen is the main structural protein in the body, and the most prevalent, totaling between 25 and 35 percent of the total protein mass in mammals. It is the main component of the connective tissue and in ligaments, and is present in bones. Small amounts are found in the muscle tissue, roughly 2 percent of the fresh weight.

Collagen is a triple helix that usually consists of two identical chains of amino acids and an additional chain which differs slightly (Szpak, 2011). The four most common amino acids in the collagen protein are, in decreasing order, glycine, proline, alanine and hydroxyproline. The high hydroxyproline content is atypical for proteins, an attribute that can be used for identification and quantification. There are 28 different types of collagen that have been identified, which are divided by the structure they form (fibillar and non-fibillar). The most common are types 1 to 5, and the collagen extracted is mostly type 1, which is present in bone, skin and tendons.

The collagen monomer is approximately 300 nm long and 1.5 nm in diameter. It is composed of three left-handed α-helixes, containing approximately 1050 amino acids each, which are wound together to form a right-handed super-helix. This helix is stabilized by intramolecular hydrogen bonds and hydroxyproline and proline, which gives the triple helix increased structural strength because of their rigid R-groups that limits the rotation of the polypeptide backbone (Francis, 2000). Collagen is insoluble in water due to inter- and intra-molecular covalent cross-linkages. These cross-linkages organize the collagen into a quarter staggered array which is how it is found in connective tissue. The solubility is also highly influenced by the age of the source tissue because of the trivalent cross-links that form from the divalent links with increasing age (Haug & Draget, 2009).

Gelatin is a pure protein made by thermally degrading collagen into shorter polypeptides. There are two main types of gelatin, Type A and Type B. The type of gelatin extracted in a process is mainly dependent on the pre-treatment conditions. These are separated by their isoionic points, which are 7-9 and 4.8-5.2 respectively. These isoionic points occur depending on the pre-treatment: Type A is a result of acidic pretreatment, and Type B from alkali pretreatment. Because gelatin and collagen are the only protein in animals with a substantial

amount of hydroxyproline, it is used to determine collagen or gelatin content. The extracted gelatin is a mixture of polypeptide chains with different molecular weights. The most important factor for the molecular weight distribution (*MWD)* is the resulting length of the polypeptide chain, but it is also dependent on the amount of inter- and intra-molecular covalent bonds in the raw material and the amount of these that were not hydrolyzed. The covalent bonds between the α-chains are strong enough for some of these to survive through the treatment and extraction, resulting in free α-chains, some β-chains and some γ-chains, and in some cases chains with even higher *MW.* A β-chain consists of two covalently linked α-chains and a γ-chain consists of three α-chains linked together, as shown in *Figure 1* where the red dots are the covalent linkages (Francis, 2000).

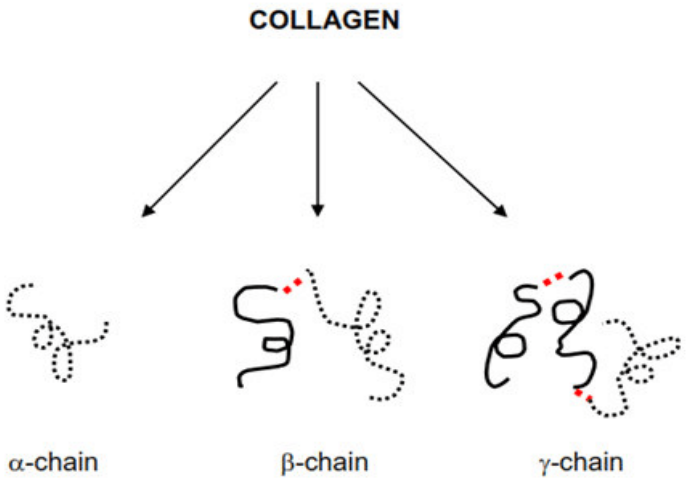

Figure 1: Pure gelatin is a polydisperse protein which consists of several strands of α-, β-, and γ-chains and some chains with higher and lower molecular weight than the α-chain. The red dots are covalent bonds. (Mahmood, et al., 2016)

The hydroxyproline and proline content in collagen varies from species to species and animal to animal because the physiochemical properties changes with the amount. In cold water fish the proline and hydroxyproline content is lower compared to warm water fish and mammals. Since the ambient temperature of the environment the fish lives in increases the rigidity of the collagen a high hydroxyproline content would make it too stiff. Water does provide some external support, so the need for the mechanical strength that hydroxyproline and proline provides is not as necessary for fish as for mammals. This is reflected in the higher hydroxyproline and proline content for mammals (Szpak, 2011). In *Table 1* the average amino acid content for skin and bones in mammals, bony fish and cartilaginous fish is shown.

Table 1: The average amino acid composition of fish and mammalian skin and bones. (Szpak, 2011)

Raw mat	**Bone**			**Skin**		
Source	**Mammalia**	**Bony fish**	**Cartilaginous fish**	**Mammalia**	**Bony fish**	**Cartilaginous fish**
Gly	**330**	**343**	**339**	**329**	**339**	**332**
Pro	**119**	**105**	**97**	**126**	**108**	**109**
Ala	115	119	117	109	114	114
Hyp	**95**	**72**	**77**	**92**	**67**	**74**
Glu	75	73	79	74	76	77
Arg	49	50	50	49	52	50
Asp	48	46	42	47	47	45
Ser	35	42	44	36	46	46
Leu	26	22	21	24	23	23
Lys	26	27	27	29	26	25
Val	23	18	23	22	21	24
Thr	19	26	30	19	26	24
Phe	14	14	14	13	14	12
Ile	11	10	15	11	11	17
Hyl	6	9	7	6	8	6
His	5	7	7	5	7	8
Met	4	13	11	6	13	13
Tyr	3	3	3	3	3	3

The physical properties and temperature characteristics of collagen reflect the habitat poikilotherm animals live in, but this is not the case for homeotherm animals which regulate their own temperature. This can be observed when looking at the amount of hydroxyproline and proline in fish living in cold water and fish living in warmer water, shown in

Table 2. Cold-water fish do not have as high hydroxyproline and proline content as fish living in warmer water as a high hydroxyproline and proline content is not beneficial, since it will result in less flexible collagen strands. Collagen extracted from cold-water fish raw material will give gels with lower gel strength and melting point, while collagen extracted from mammalian or warm-water fish gelatin will have higher gel strength and the gel will keep its integrity at higher temperatures. Because the collagen extracted from warm-water fish closely

resembles the mechanical properties of gelatin from mammals it can be used as an alternative to pork gelatin in products where pork gelatin is used. Gelatin from cold-water fish cannot be used to replace mammalian gelatin directly. Some studies show that the addition of some cofactors can mitigate the lacking physiochemical properties of cold water gelatin (Fernández-Díaz, Montero, & Gómez-Guillén, 2001).

Table 2: Mean amino acid composition in cold- and warm-water fish, sorted from highest to lowest content for warm-water fish. (AA residues/1000 total AA residues) (Szpak, 2011)

Amino Acid	Cold-water fish	Warm-water fish
Gly	**340**	**337**
Ala	109	119
Pro	**105**	**110**
Hyp	**59**	**76**
Glu	77	75
Arg	52	51
Asp	50	44
Ser	55	38
Thr	25	26
Lys	27	26
Leu	24	22
Val	21	21
Met	14	13
Phe	14	13
Ile	12	11
Hyl	6	7
His	9	6
Tyr	3	3

The amino acid composition will also differ based on the gelatin type, not only its raw material source. The different types of gelatin, have quite different amino acid content, as shown in *Table 3.* These give Type A and Type B gelatin different properties. Most commercial fish gelatin is Type A.

Table 3: Mean amino acid composition of Type A and B gelatin from mammalian source. The different types of gelatin have different amino acid content. T*he Glu and Asp for Type B gelatin also includes glutamine and asparagine (Haug & Draget, 2009).*

Amino Acid	Type A Gelatin	Type B Gelatin
Gly	330	335
Ala	112	117
Pro	132	124
Hyp	91	93
Glu	25	72*
Arg	49	48
Asp	16	46*
Ser	35	33
Thr	18	18
Lys	27	28
Leu	24	24
Val	26	22
Met	4	4
Phe	14	14
Ile	10	11
Hyl	6	4
His	4	4
Tyr	3	1

Applications

Because of the aforementioned physio-chemical properties of cold-water fish gelatin, the commercial interest in production of cold water fish gelatin has been relatively low. This is reflected in the amount produced each year, less than 1.5 percent (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Cold-water fish gelatin shows good emulsifying properties and because of this the main uses are currently the embedding of oil-based vitamins for stabilization, such as vitamin A (Schrieber & Gareis, 2007). There are several other uses that do not require a high melting point in both food and pharma, as well as in cosmetics. In

addition, it can be used as a foam stabilizer, such as in marshmallows. Gelatin can also function as a protective colloid, e.g. water with low concentration of gelatin forms individual discrete crystals as the gelatin stops the formation of a solid ice block. This effect is highly desirable when making ice lollies and when making other ice creams to make sure the lactose does not precipitate into granules. It can be used to help precipitation of impurities in juice, and as a stabilizer in yoghurt (Francis, 2000). In pharma, gelatin can be used in soft and hard gel capsules, in tablets, or as an emergency blood substitute.

Gel strength and formation

The gel formation of gelatin with water is attributed to the partial recovery of the triple helical structure in collagen and intertwining of the strands. When gelatin is dissolved in water using heat, the helices expand and intermolecular linkages are broken. When the temperature decreases the intermolecular hydrogen bonds reform and the helices contract slightly, which leads to gel formation. The regions with a high Gly-Pro-Hyp concentration is of crucial importance for the initiation of the intermolecular bond formation, strength and stabilization of the triple helices which partially reforms when cooling.

To determine the gel strength of a gelatin gel, the most important measurement is the Bloom value, which is defined as the weight required for a specific plunger with a diameter of 12.7 mm to depress the surface of a 6.67% (w/w) gelatin gel, that has matured for 16-18 hours at 10 °C, by 4 mm without breaking the gel (Phillips & Williams, 2000).

The extraction conditions highly influence the strength of the gel. Gelatin extracted at mild conditions will usually produce higher strength gel than gelatin extracted with more extreme methods, such as high temperature. This is because prolonged exposure to heat will result in thermal hydrolysis of the polypeptide chains.

One of the most important factors for a gel is its melting point, since it determines its possible applications. A typical gel made from pork or bovine sourced gelatin typically have a melting point at around 35 °C which is just below human body temperature, which is an important aspect for the mouthfeel and stability. Fish gelatin does not generally reach a melting point close to the melting point of mammalian gelatin, this is especially the case for cold-water fish gelatin, which have a melting point of between 15-20 °C. Warm water fish gelatin can

however reach temperatures as high as 34 °C which is close to mammalian gelatin (Pranoto, et al., 2016).

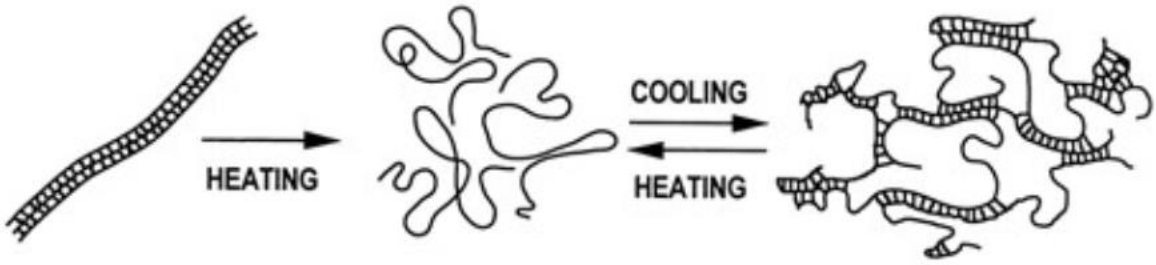

Figure 2: Collagen is partially hydrolyzed and converted to gelatin by heat. The gelatin can then form a gel by being cooling below its melting temperature. The degeneration of collagen is non-reversible, but the gelatin gel formation is reversible and can be done several times (Lee, 2018).

Gelatin extraction process

All gelatin extraction methods consist of three main stages: pre-treatment, extraction and purification. Depending on the pretreatment method, the gelatin will either be Type A gelatin or Type B. If the pre-treatment is alkali, the resulting gelatin will be Type B, and if it is acidic, it will be Type A. For fish skin, the acidic pretreatment is the most appropriate since the collagen has low amounts of covalent linkages which makes the alkali method unnecessarily harsh (Mahmood, et al., 2016) (Schmidt, et al., 2016).

Alkaline pre-treatment

The alkaline pre-treatment is usually used on raw material where the collagen is highly cross-linked. This can be raw material from old animals, cattle hide or ossein, which is a porous material made by soaking bovine bones. This method usually involves either a short intensive treatment where the raw material is soaked in NaOH for a few days at ambient temperature, or a less aggressive treatment where it is soaked in supersaturated lime for 8-12 weeks at 14-20 °C. The lime solution gives a pure gelatin since the non-collagenous proteins dissolve in the solution.

Acidic pre-treatment

The acidic pre-treatment is the most common method used for fish raw material, as it requires much shorter time compared with the lime process. The raw material is usually soaked in a low concentration inorganic acid (2-5%), such as sulfuric acid or hydrochloric acid. The pH is adjusted to 4 when the solution is fully acidified or there is no more swelling. Alkaline extraction differs from the acidic process with the use of strong alkali treatment, which leads to a different isoelectric point compared to acid extraction and results in different functional properties.

Enzymatic pre-treatment

Another pre-treatment method is the enzymatic process. This method is more expensive and has been found to have a lower yield. It does however give gelatin with higher gel strength (Mahmood, et al., 2016). The enzymatic process is based on using a hydrolyzing enzyme, such as pepsin, to prepare the gelatin for extraction. There are several benefits with the

enzymatic pre-treatment - the process has been observed to be shorter than liming or the chemical process, and gives the ability to be highly specific in which bonds that are hydrolyzed. Because hydrolysis can continue after the gelatin have been extracted, an intermediate enzyme deactivation step is important before extracting the gelatin. If this is not performed the active enzymes in the extract will give in gelatin with poor functional properties.

Extraction

When extracting gelatin from fish, the conditions are usually very mild compared to extraction from mammalian sources, since it is well known that harsher conditions influence the properties of the product (Jonhard, Haug, Elharfaoui, Djabourov, & Draget, 2009). The most common extraction mediums are water or mild acids. When extracting from fish the temperature is usually lower than when extracting from pork or bovine sources. For pork and bovine raw materials there are usually several steps which ends up at between 90-100 °C. Extraction from fish usually takes place at temperatures between 50 °C and 70 °C. If the extraction is performed at higher temperatures, the polypeptide can be too hydrolyzed, which will result in little to no gel-forming capabilities and reduced foaming and emulsifying properties. When the raw material is bone, it is common to add more steps like demineralization since bones have much higher ash content.

Raw material source

When looking at rest raw materials as a source for production of gelatin and other high value products, there are several challenges that need to be solved. Since rest raw materials are highly perishable the logistics of transportation is a problem. Transporting the rest raw material and keeping it fresh will be challenging without large energy, due to keeping, and space investments. This will be an especially large challenge when it comes to the difference between land based and coastal fish industry, such as salmon farming and coastal fishing, and offshore industry, such as large fishing boats. The offshore fishing boats stay out for several days to weeks and they would need to dedicate large storage facilities for storage of rest raw material. Currently, rest raw materials from land based sources is almost completely utilized, although mostly for low value products like silage (Richardsen & Nystøl, 2015).

Since the farming of Atlantic salmon is an all year industry in Norway, the rest raw material supply is stable and sustainable. The salmon farming industry produces large quantities of salmon products and a large quantity of rest raw material each year (Thorkelsson, Slizyte, Gildberg, & Kristinsson, 2009). A stable supply of rest raw material will also decrease the need for storage, resulting in products made from fresh raw materials and a purer and higher quality product.

Goals for the project

One of the most important aspects for a product to be successfully applied in industry is that the earnings outweigh the production costs, so that the profit is substantial enough to make it worthwhile. Sintef Ocean AS joined the DAFIA project which aims to find new and improved ways to utilize municipal solid waste and marine rest raw material (DAFIA, 2018). Under the DAFIA project Sintef Ocean focuses on utilization of marine rest raw material, such as salmon rest raw material to produce high value products. This thesis had gelatin as the main product. The method used in this project was tested on salmon back bone earlier in the project and the results showed that the gelatin extracted was of high purity and good yield (Speilberg, 2018). The aim for this thesis is to further expand on this procedure, in an effort decrease the cost of production by decreasing the time and energy requirement of the method with the hope of reducing. The raw material used in this project was salmon backbone, and this was chosen because of its large supply in Norway due to the salmon farming industry.

Optimization of the chosen method have to be based on the purity, yield and physiochemical properties of the extracted gelatin. This due to that these are the most important aspects of the gelatin and its extraction process. The purity, yield and physio-chemical properties were examined using HPLC to investigate the amino acid composition. Hydroxyproline content was determined to analyze the purity and SDS-Page was done to determine the molecular weight distribution. Dry-matter analysis and sensory analysis were performed to determine the dry-matter content and product odor. The optimization was done by varying the time for each pre-treatment step of the raw material or by varying the temperature and/or time of the extraction.

Materials and methods

All reagents used for analysis and experiments were of analytical grade.

Raw material and preparation

The raw material used for this experiment was salmon backbone from a commercial Atlantic salmon (*Salmo salar*) filleting plant (Nutrimar). After delivery the backbones were stored at -20 °C until processing. To separate the excess meat and fat from the backbone a purely mechanical method using spoon, knives and hands was used. To make the separation easier the fish was slightly frozen when starting the separation, as the meat kept its structure better and separated from the bones and cartilage easier and with less damage to it. This made for a cleaner product and less raw material. After the separation most of the raw material that contained little collagen was removed. The bones were then ground while slightly frozen, using a large grinder (Hobarth AE200) with a 10 mm mesh hole size.

Extraction and analytical methods

Manufacture

The original extraction method used was a method by Arnesen and Gilberg (Arnesen & Gildberg, 2007) that was modified by SINTEF Ocean. To look at possible optimizations of the method, several different changes in processing parameters were tested one at a time. The original modified method is described below, and each variation is shown in *Table 4*.

The process is divided into three main parts: pre-treatment, extraction, and cleaning and drying. The washing solutions was made the day before the extraction and stored at below 4 °C until right before they were used. Extraction was performed on raw material that had been stored at -20 °C, then thawed for 45 minutes at ambient temperature. Two parallels of 150 gram raw material was weighed out, rinsed in cold tap water for approximately two minutes using a fine mesh sieve, then transferred to separate plastic beakers. To remove unwanted

fats, proteins, minerals and meat a pre-treatment was performed on the raw material, split into 7 separate steps. After the raw material was rinsed, it was transferred back into the beakers and mixed with 0.04M NaOH in a 1:3 (w/v) ratio of raw material and washing liquid. The beaker was put on a stirring table (Orbital Shaker, PSU-10i, Grant Bio) with gentle stirring for half an hour, after which the raw material was separated from the NaOH using a fine mesh sieve and the NaOH discarded. The raw material was briefly rinsed in cold tap water and reintroduced to the beaker and mixed with a new round of NaOH and stirred for 30 minutes before being separated and rinsed again to remove the last NaOH. The same washing procedure was done twice with 0.12M H_2SO_4, as the washing liquid with the same time and ratio. After washing with H_2SO_4 the raw material was washed with 0.005M citric acid for another 30 minutes to neutralize the H_2SO_4. The final two steps of the pre-treatment were washing with 10% ethanol for 35 minutes each to remove fat and oil. The pre-treatment was done at ambient temperature (~22 °C). To monitor the temperature-rise during each washing steps, the temperature was measured before and after each step. The temperature was not observed to rise above 12 °C during any of the steps.

Figure 4: The raw material after all pre-treatment steps. In this form most of the low collagenous material is removed, like meat, fat and oil.

Figure 3: The raw material before any pre-treatment, the content of low collagen material is much higher.

After the pre-treatment, the gelatin was extracted in two 2-hour long steps. The extraction liquid was distilled water, this was mixed with the raw material in a 1:1 (w/v) ratio of initial raw material weight and distilled water. After the raw material and distilled water was mixed, the beaker was put into a water bath at 56 °C and left for two hours and stirred with a small metal spatula every 15 minutes. Afterwards the beakers were moved from the water bath that held 56 °C to another bath that held 65 °C for another two hours. The mixture was stirred every 15 minutes.

Figure 5:The remaining raw material after it was separated from the extract.

After the extraction was completed, the remaining raw material was separated from the extraction liquid and frozen. The extraction liquid was filtered through a double tea strainer to remove the larger particles and some oil residues, and weighed. A small amount was then put aside for dry matter analysis and the rest was frozen before being freeze-dried.

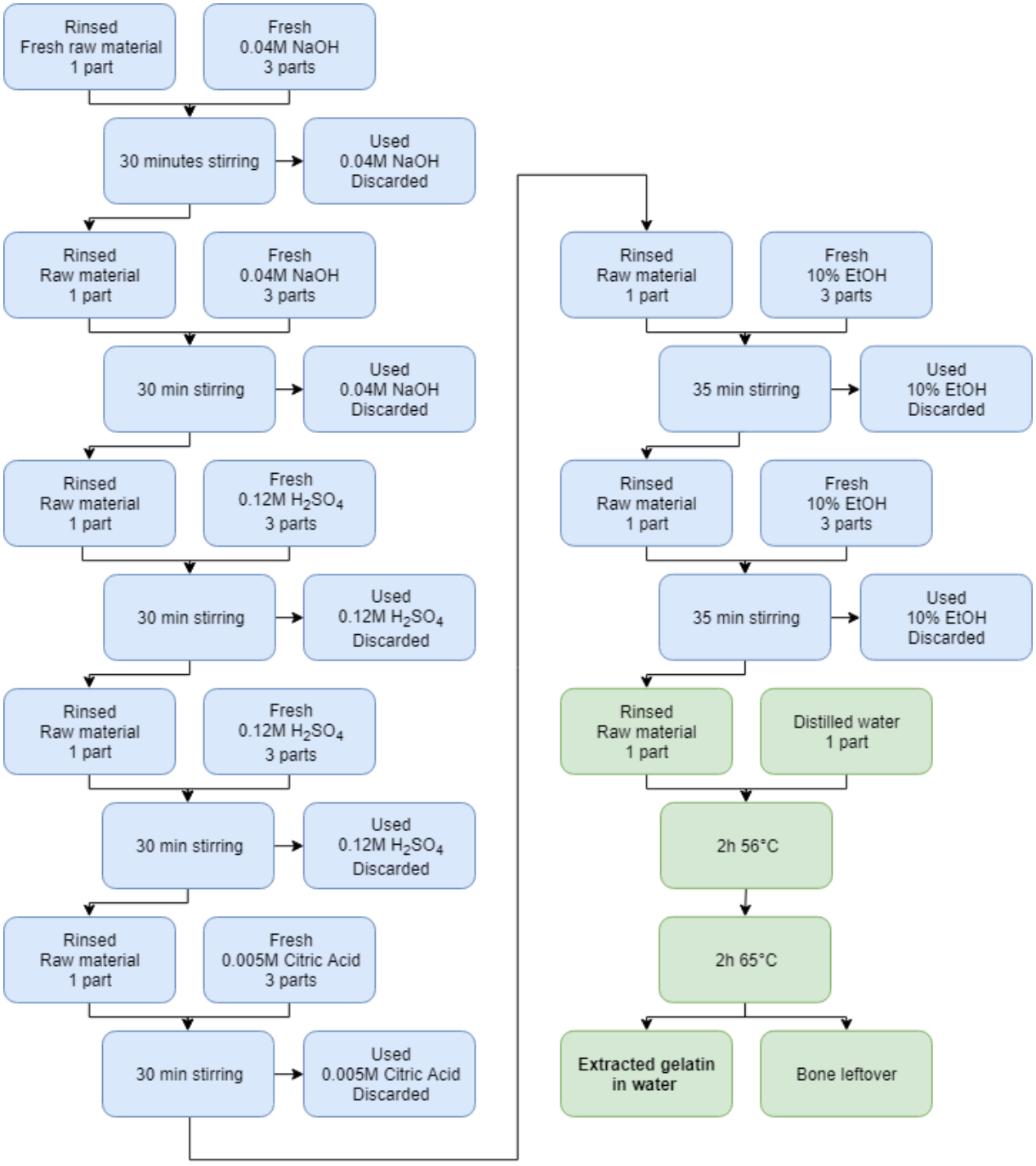

Figure 6: A flowchart of the modified method from Arnensen and Gildberg (Arnesen & Gildberg, 2007). This extraction procedure is shown without modifications. The pre-treatment steps are colored blue and the extraction steps green. The fresh raw material is 150 grams of ground salmon backbone. The pre-treatment steps were all done in the same beaker, which was rinsed between each step, and with a 1:3 ration of raw material and washing solution. Between each step the raw material was rinsed in cold tap water after being separated from the washing liquid, after rinsing the raw material was reintroduced to the empty beaker and then fresh washing solution was added. The extraction was done in one beaker with a 1:1 ration of distilled water and initial raw material weight.

Table 4: Each variation of the extraction procedure. For samples B through E the time of each of the pre-treatment steps were reduced by the given time. For samples F through M the extraction was done over the given time with stirring every 15 min without removing from the water bath.

Sample	Parallel	Treatment	Abbreviation
A	I	Basic procedure	Basic
	II		
B	I	10 min reduction for each pre-treatment step	PT-10
	II		
C	III	10 min reduction for each pre-treatment step	
	IV		
D	I	15 min reduction for each pre-treatment step	PT-15
	II		
E	III	15 min reduction for each pre-treatment step	
	IV		
F	I	4h extraction at 56 °C	4h-56°
	II		
G	III	4h extraction at 56 °C	
	IV		
H	I	4h extraction at 65 °C	4h-65°
	II		
I	III	4h extraction at 65 °C	
	IV		
J	I	2h extraction at 56 °C	2h-56°
	II		
K	III	2h extraction at 56 °C	
	IV		
L	I	2h extraction at 65 °C	2h-65°
	II		
M	III	2h extraction at 65 °C	
	IV		

Dry matter

The dry matter content was analyzed gravimetrically after the AOAC method by accurately weighing out approximately 4 grams of the extraction liquid in a pre-weighed crucible. The crucible was then put into a heating cabinet at 105 °C for 24 hours. After 24 hours the crucibles were removed from the heating cabinet and stored in an exicator until they reached room temperature. The crucibles were then weighed, to see the difference before and after the heating cabinet and the dry matter content calculated.

Gel electrophoresis

Gel electrophoresis was used to determine the molecular weight distribution (MWD) of the extracted gelatin. Gel electrophoresis is a method that can identify the MWD of a mixture of proteins or other particles, like DNA or RNA, by separation on a gel the time used to travel through a gel. To analyze the *MWD* of the manufactured gelatin, a high molecular weight standard (Amersham™, HMW calibration kit) was used as the standard of choice, the buffer was a SDS run buffer (ClearPAGE™, SDS Run Buffer, 20x 500 ml, C.B.S Scientific), the gel was an 4-20% acrylamide gel (ClearPAGE™ SDS Gel, 4-20%, 12-well) and PS300B power supply (AA Hoefer). 20 mg of gelatin was diluted in 10 ml of distilled water, and 0.5 ml taken out and mixed in a 1:1 ratio with the buffer, the sample was then placed in a water bath at 70 °C for 10 minutes. After being taken out of the water bath, 0.1 ml of glycerol was added to the sample and mixed. 10 wells were loaded with 10 µl sample and two were loaded with the standard. To the pre-made buffer, 2% DTT was added and thoroughly mixed. The running buffer was prepared by mixing 40 ml of the buffer with 760 ml distilled water. This was added to the running chamber after the gels were placed and the inner and outer chamber was separated. The power supply turned on, with a voltage of 180 V and left on until the dye front of the standard reached the steel thread that symbolizes the bottom of the gel.

After the gel was done, it was removed from the cassette, rinsed in distilled water, and dyed with a protein stain (*InstantBlue Protein Stain™*, Expedon*)* overnight. The following day, the gels were rinsed, photographed, and stored in distilled water.

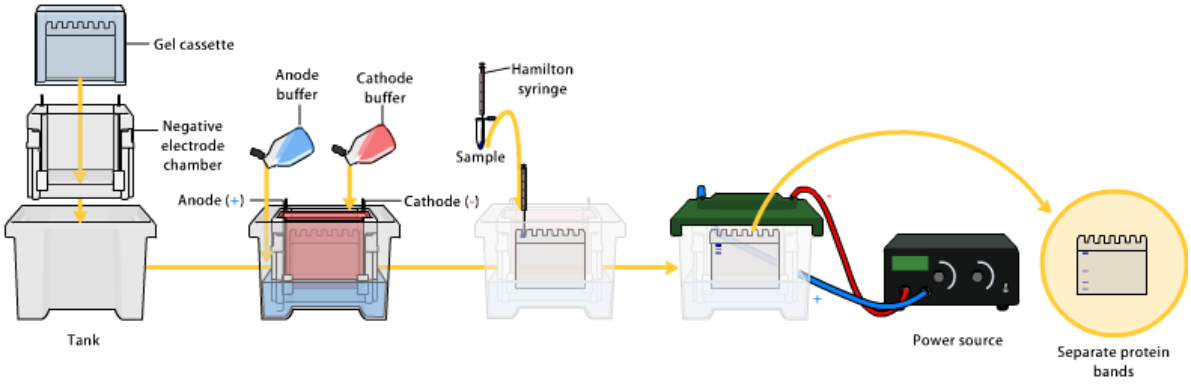

Figure 7: Typical setup of a gel electrophoresis. In this experiment the anode and cathode buffer is the same buffer solution, and up to two gels can be run at the same time. (Bensaaccount, 2009).

Total amino acid composition

Total amino acid composition was analyzed by an Ultra High-Performance Liquid Chromatography (UHPLC) (Dionex UltiMate® 3000 UHPLC+ focused, Dionex UltiMate® 3000 Autosampler, Dionex RF Fluorescence Detector, Thermo Scientific, USA) and Nova-Pak column (Nova-Pak C18 4 µm, 3.9·150 mm).

The UHPLC analysis is based on difference in the interaction of different molecules in the sample with an absorbent material that is present in the column. The sample is passed through the column using pumps. The different particles will have different flow rates through the column because of the different interactions and can be identified.

The preparation for the HPLC was done following the method described by S. Blackburn (Blackburn, 1978). For each gelatin extraction procedure two parallels were run. 50 mg of freeze dried sample was completely hydrolyzed by adding 1 ml of 6M HCl in a small sample glass. After weighing out the gelatin in the sample glass they were put in a heating cabinet at 105 °C for 22 hours. Half an hour after being put into the heating cabinet the lids were retightened. After 22 hours had passed the samples were removed from the heating cabinet and the lids loosened immediately.

The sample were then neutralized to approximately pH 7 using NaOH and HCl. After the pH was within the accepted area, 6.5 to 7.5, the samples were filtrated through a glass microfiber filter (Whatman, GF/F, CAT No. 1825-025) using vacuum and the filtrate was transferred to a 10 ml volumetric flask and doubly distilled water was added to 10 ml. The samples were prepared for the HPLC by thinning it 1:2000 with distilled water and filtering it through a syringe filter (0.2 µm, Whatman, F30/0.2 CA-S) and 0.205 ml were transferred to HPLC sample glasses.

The UHPLC procedures was performed by Siri Stavrum at the Department of Biotechnology and Food science at NTNU.

Figure 8: The sample glasses used for HPLC analysis loaded with 205 µl sample. After preparation the samples were stored at -20 °C until they were analyzed.

Sensory analysis

A simple sensory analysis was conducted by smelling the freeze-dried gelatin at room temperature and by looking at the freeze-dried gelatin under sunlight and under LED lights installed in the lab.

Hydroxyproline content

Hydroxyproline content was analyzed according to the method described by *A. A. Leach 1960* (Leach, 1960), which is a modification of a method from Neuman and Logan (Neuman & Logan, 1949). This method was performed with specific conditions for fish.

A standard stock solution containing 100 µg/ml hydroxyproline was prepared by dissolving hydroxy-L-proline in distilled water.

To a test tube containing either 0.5 ml standard, sample or blank, 0.5 ml 0.05M $CuSO_4$ and 0.5 ml 2.5M NaOH were added and the tubes shaken, the samples turned blue. The tubes were then placed into a 50 °C water bath for 10 minutes, removed and 0.5 ml 6% H_2O_2 were added turning the samples green. The tubes were immediately shaken and placed back into the 50 °C water bath for 10 minutes. The samples were removed from the water bath and cooled under running water until they reached room temperature and 2 ml 1.5M H_2SO_4 and 1 ml 5%-*p*-dimethylaminobenzaldehyde in 1-propanol was added to each tube while in a fume hood and immediately shaken. The tubes were covered using marbles and put into a 70 °C water bath for 16 minutes. The oxidation product will react in the presence of *p*-dimethylaminobenzaldehyde and heat, and produce a red color which will be compared with the standard curve. After 16 minutes the samples were cooled to room temperature, vortexed and left for 2 minutes. Then the OD were measured at 555 nm using a spectrophotometer.

The standard curve was plotted and the slope and intercept found for the standard curve. This was used to calculate the amount of hydroxyproline in undiluted sample, which was used to find the percentage content of HyP. The percentage HyP in freeze dried sample was multiplied with a factor of 11.42 for salmon to get the collagen content, which is also the gelatin content (Eckhoff, Aidos, Hemre, & Lie, 1998).

Results

Dry matter analysis

To evaluate the efficiency of a given extraction method based on gelatin yield, determination of the dry matter content becomes necessary. *Table 5* shows the average dry matter content in the extraction liquid as percentage of wet weight of the extract. For the basic method, two parallels were analyzed and for the rest four parallels were analyzed, each sample's different treatment can be seen in *Table 4*. The average dry matter content in the extraction liquid are quite low. The extract from the basic method had 2.48 $\frac{g}{100\ g}$, and the extract from the *4h-56°* had 1.78 $\frac{g}{100\ g}$. There was no large outlier in the samples which is expected, previous analysis on the raw material shows that the ash content is quite low (Speilberg, 2018). The H_2SO_4 pre-treatment step will demineralize the raw material to a point where there is little contribution of minerals to the dry matter fraction. Filtration of the extract also reduces the dry matter content. Based on these assumptions, the main contributor apart from the gelatin is residual oil and fat. The low dry matter content is due to the high amount of water used for the extraction of the gelatin. The process uses a large water to raw material ratio to ensure that the extraction solution does not become saturated during the extraction. If the extraction solution becomes saturated it would prevent the extraction of more gelatin from the raw material.

Table 5: Average percentage dry weight in wet weigh gelatin extract. The dry matter content is reasonable considering the large amount of water. The sample abbreviations are shown in Table 4 (Average DM ± SD) (For Basic: n=2, for the rest: n=4).

Sample	$\frac{\mathbf{g\ DM}}{\left(\frac{\mathbf{100\ WW}}{\mathbf{extract}}\right)}$
Basic	2.48 ± 0.03
PT-10	2.29 ± 0.13
PT-15	2.39 ± 0.14
4h-56°	2.09 ± 0.06
4h-65°	2.32 ± 0.14
2h-56°	1.78 ± 0.19
2h-65°	2.05 ± 0.34

Sensory analysis and gel formation

Sensory analysis

As expected all the gelatin extracted had a distinct fish smell. There was no washing or purification done on the gelatin extraction, apart from running it through a fine mesh sieve. Including a washing/purification step could probably reduce the odor of the samples. However, this was not done because of time constraints. The smell was characterized relatively to the other sample smells, and is divided into three levels, weak-, medium- and strong smell. These were denoted as 1, 2 and 3 respectively in *Table 6.* To obtain more accurate results, a sensory analysis by a professional panel should be conducted. For the single participant in this test however, using these levels seemed reasonable.

Table 6: The result of the simple sensory analysis done on the freeze-dried gelatin samples. The samples were sorted by their relative fish smell. The scale had three levels, where 3 was a strong fish smell relative to the other samples and 1 was a weak smell. The sample abbreviations are explained in Table 4.

Sample	**Smell**	**Sample**	**Smell**
Basic I	3	*4h-65° I*	2
Basic II	2	*4h-65° II*	2
PT-10 I	3	*4h-65° III*	3
PT-10 II	3	*4h-65° IV*	3
PT-10 III	2	2h-56° I	3
PT-10 IV	1	2h-56° II	2
PT-15 I	1	2h-56° III	2
PT-15 II	1	2h-56° IV	2
PT-15 III	1	*2h-65° I*	1
PT-15 IV	2	*2h-65° II*	1
4h-56° I	2	*2h-65° III*	1
4h-56° II	1	*2h-65° IV*	1
4h-56° III	3		
4h-56° IV	1		

The gelatin was also visually assessed and all the samples were found to have a white and pure appearance, with no sign of discoloration or impurities. There were some differences in the gelatin powder after maceration. Some formed a quite fine powder, while others formed a powder with larger particles. This is a result of the chosen way of macerating the gelatin, a hand mixer with two blades. It does not seem like the particle size correlates with the extraction method, but gelatin of lower weight was harder to macerate into a fine powder due to it not having enough resistance against the blades. With a better tool for macerating, such

as more blades or less room for the gelatin to move in, this would not be an issue. The finely macerated gelatin was easier to dissolve in water but it was highly electrostatic and thus harder to work with. The coarser gelatin however did not dissolve as easily but appeared much less electrostatic, most likely due to the individual weight of the gelatin particles. Commercial gelatin is sometimes stabilized in a salt structure which makes it less soluble but a lot easier to work with. It can also be pressed into gelatin sheets, which is more common in Europe. Both ways can easily be done with the gelatin extracted in this project.

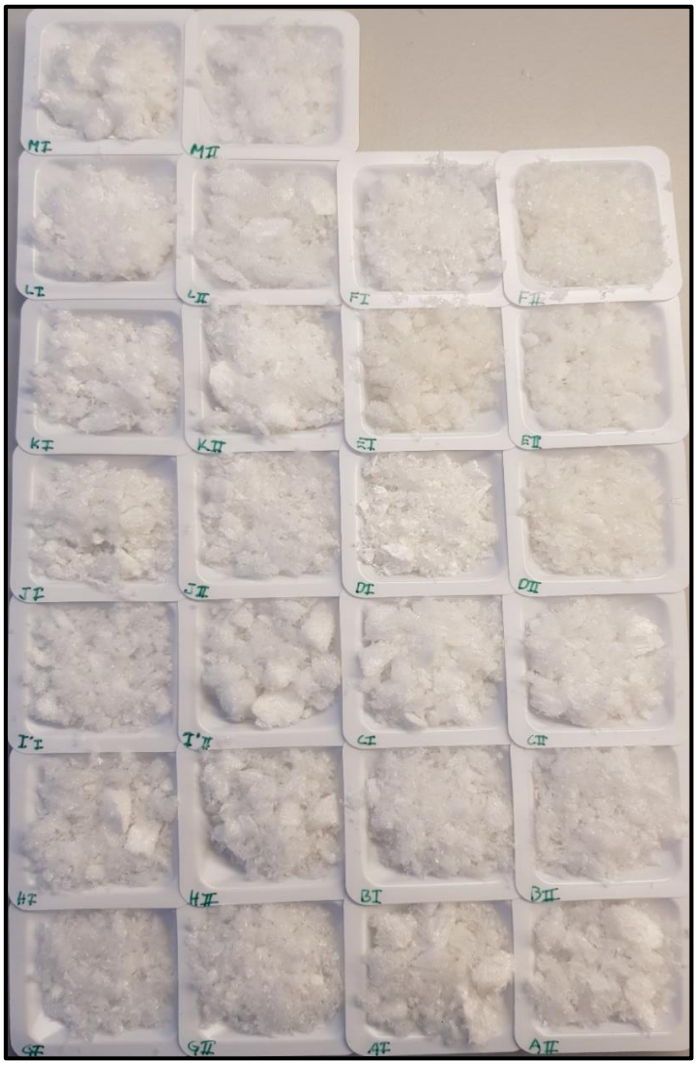

Figure 9: Freeze-dried gelatin powders from all experiments. All samples were white, clean and pure in powder form. Sample A was the normal extraction method without any modifications, BC was extracted with 10 minutes shorter pre-treatment steps, sample DE was extracted with 15 minutes shorter pre-treatment steps, FG was extracted at 56 °C for 4h, HI was extracted at 65 °C for 4h, JK was extracted at 56 °C for 2h and LM was extracted at 65 °C for 2h. The sample abbreviations are shown in Table 4.

Figure 10: Sample 4h-56° II shown as an example of fine powdered freeze-dried gelatin. The gelatin is lightweight and highly electrostatic in this form. The gelatin was easier to dissolve in water compared to the gelatin powder with larger particles. The gelatin was extracted at 56 °C for 4h with all the pre-treatment steps as the basic method.

Figure 11: Sample 4h-65° shown as an example of freeze-dried gelatin in larger pieces. The gelatin is lightweight and there are large amounts of air in each piece of gelatin., the form of the gelatin made it appear less electrostatic, probably because of the individual weight of the pieces. This gelatin was extracted at 65 °C for 4h with all pre-treatment steps equal to the basic method.

Gel formation

All gelatin powders formed a gel when mixed with warm water and then cooled. The gel strength was not measured, but the melting point of the gel was investigated and found to be consistent for all gels (15 °C). The melting point at 15 °C is too low for the gelatin to be a suitable replacement for pork and bovine gelatin. The gel kept its shape below its melting point even during handling. The gel was quite opaque and had an odor of fish even when solid. The opaqueness and smell could probably be resolved somewhat by purifying the gelatin after extraction.

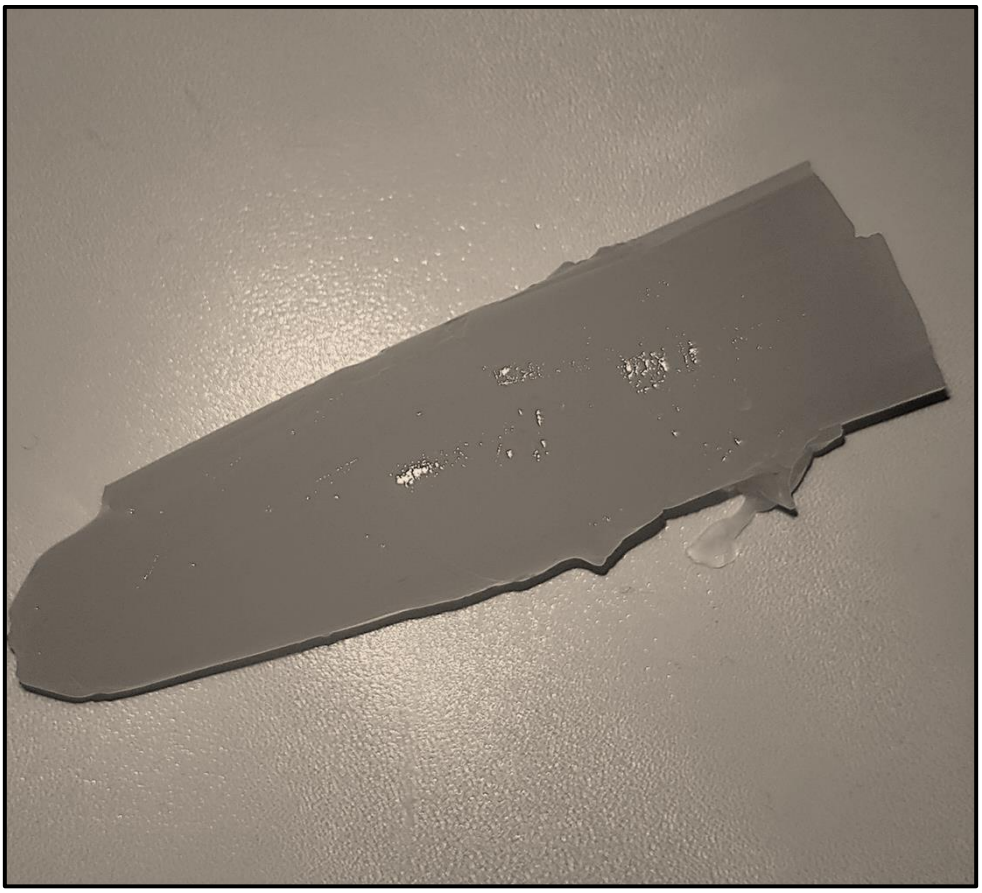

Figure 12: A gel cast in a 50 ml centrifuge tube from the gelatin extracted. The gel has a 6.67% (w/w) concentration of gelatin and distilled water.

Molecular weight distribution using gel electrophoresis

All gelatin extracts were run on three different polyacrylamide gels. The first gel was run with two wells containing standard on each outer edge, and samples *Basic I* to *PT-15 IV* between. The leftmost well in *Figure 13* is the HMW standard and the samples are deposited one in each well following until the last well which were HMW standard. Well number two was sample *Basic I* and well number two were sample *Basic II* etc. until well number eleven which was sample *PT-15 IV*. The HMW standard and the concentration was appropriate and the gels gave clear bands in the top range of the standard. This indicates that the gelatin has a high molecular weight, and long helixes. The *PT-15 III* and *IV*. samples lack bands at around 669000 Mw. The other gels, shown in *Figure 14* and *Figure 15*, showed almost identical results but the development of the color was much better. With such high molecular weight, the theoretical gel forming properties of the gelatin is in agreement with the results found in the gel forming experiment.

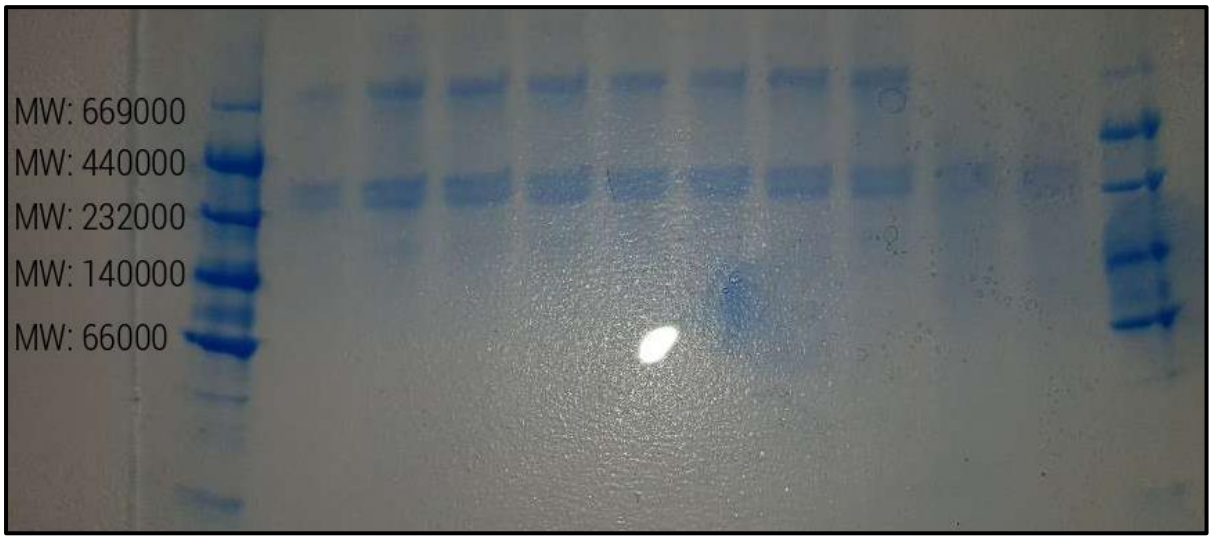

Figure 13: The resulting gel from the gel electrophoresis done with a high molecular weight (HMW) standard, starting at 669000 Mw and ending at 66000 Mw. The samples on this gel was sample "Basic I" to sample" PT-15 IV" from left to right, and each outer well had the HMW standard.

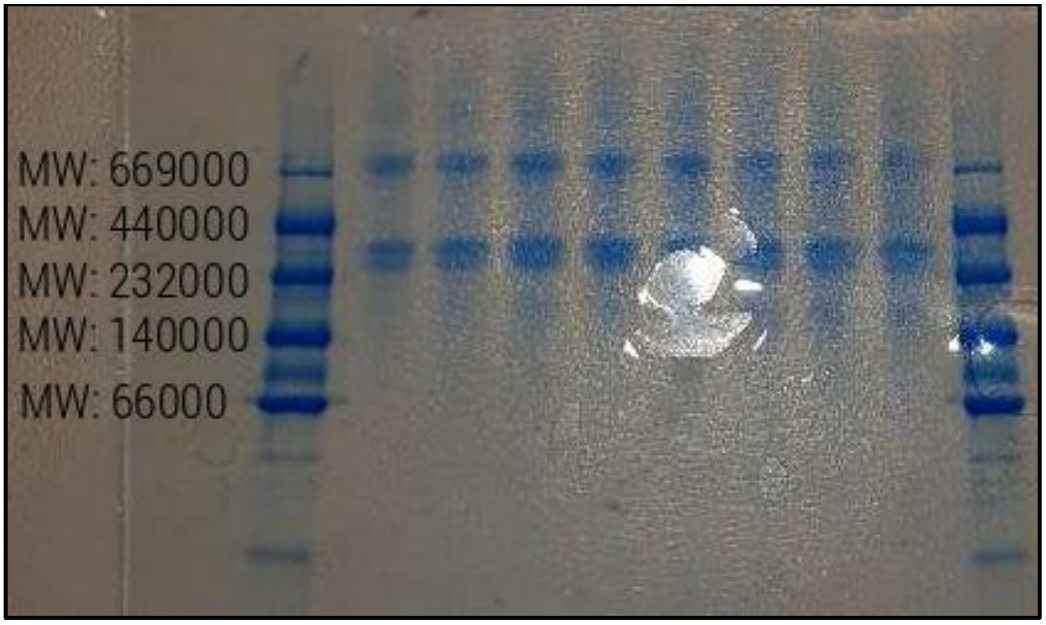

Figure 14: The resulting gel from the gel electrophoresis done with a high molecular weight (HMW) standard, starting at 669000 Mw and ending at 66000 Mw. The results show that the gelatin have a lot of gelatin strands with a Mw of around 669000 Mw and between 440000 Mw and 232000 Mw. The samples on this gel was sample "4h-56° I" to sample "4h-65° IV" from left to right, and each outer well had the HMW standard.

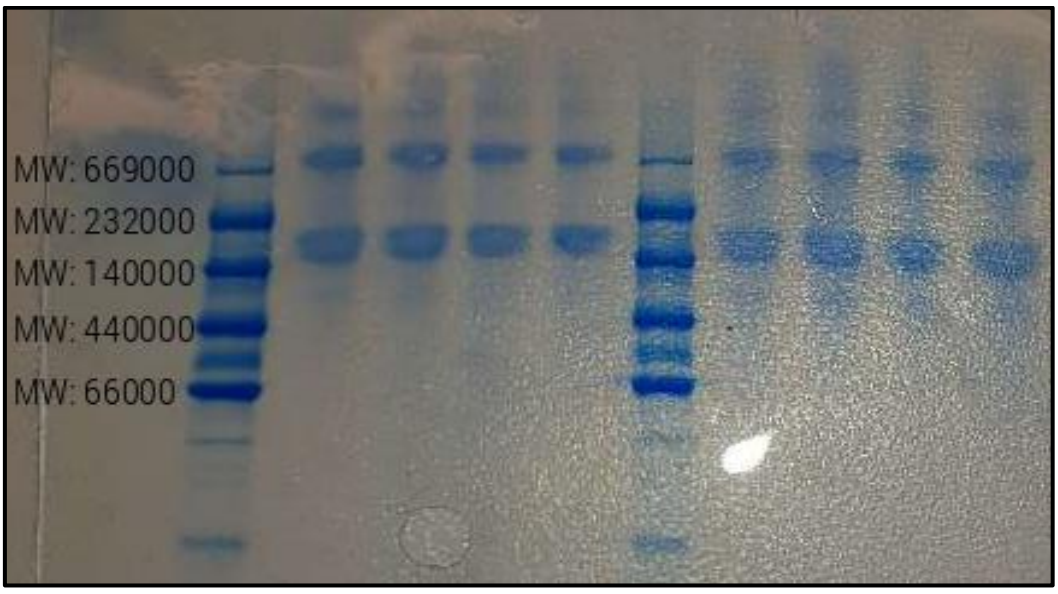

Figure 15: The resulting gel from the gel electrophoresis done with a high molecular weight (HMW) standard, starting at 669000 Mw and ending at 66000 Mw. The results show that the gelatin have a lot of gelatin strands with a Mw of around 669000 Mw and between 440000 Mw and 232000 Mw. The samples on this gel was sample "2h-56° I" to sample "2h-65º IV" from left to right, and the leftmost and center well had the HMW standard.

Hydroxyproline and gelatin sample purity

The hydroxyproline content varied slightly between each gelatin sample, with a purity of approximately 80% gelatin for all extraction methods. Gelatin content was calculated by multiplying content of hydroxyproline by a factor, specific for salmon collagen, of 11.42 (Eckhoff, Aidos, Hemre, & Lie, 1998). Results of this analysis are presented in *Figure 16*. The lowest sample purity was found for method *4h-56°* (75.5%) and *2h-65°* (76.0%), where gelatin had been extracted at 56 °C for four hours and 65 °C for two hours respectively. The highest gelatin purity was found for sample *2h-56°* (83.3%), which was extracted for 2h at 56 °C. For sample *4h-56°* four parallels were discarded and for *2h-56°* two parallels were discarded because of a large deviation from the other parallels. The collagen content is directly related to the gelatin content because gelatin is the hydrolyzed product of collagen.

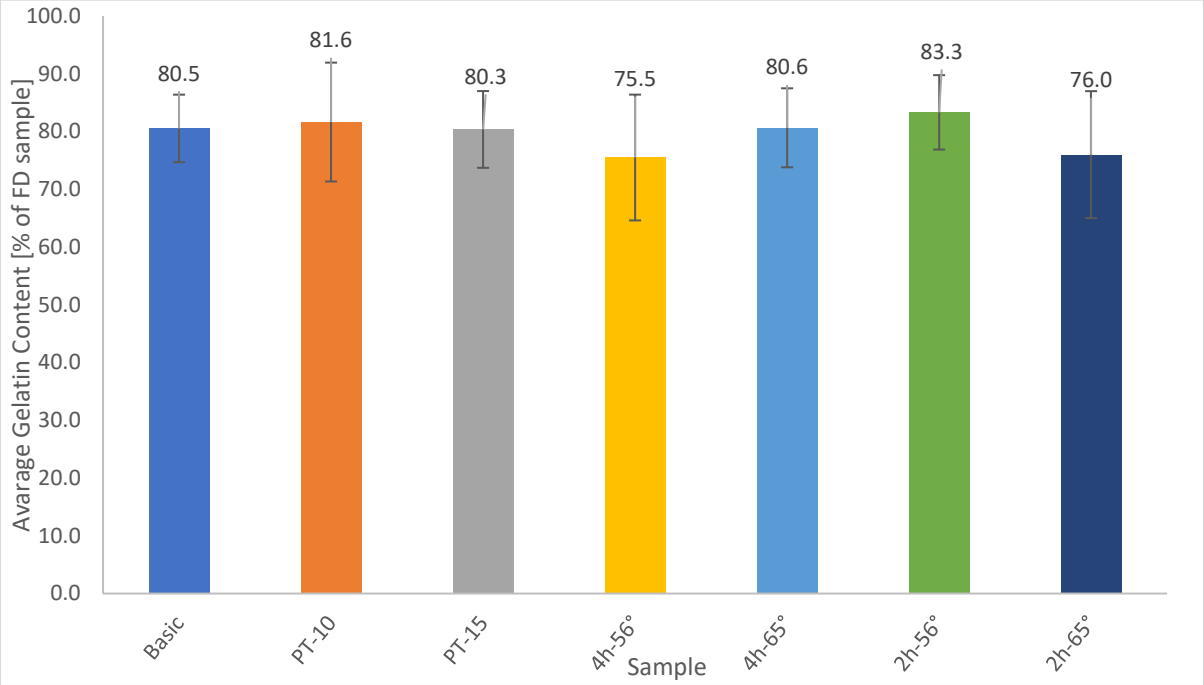

Figure 16: The average gelatin content of the different gelatin extracted with different methods. The collagen content was analyzed by determining the hydroxyproline content in the samples by spectrophotometer, and then calculating the content. Multiplying the amount of HyP with 11.42 gives the collagen content. The different methods are explained in Table 4. (For Basic: n=8, for 4h-56°: n=12, 2h-56°: n = 14 the rest have: n=16)

Gelatin yield

The yield of gelatin was calculated based on the dry matter yield and the gelatin sample purity obtained from the hydroxyproline content. All extraction methods gave similar yields, sample *PT-10* was found to have the highest yield with an average yield of 34.2% of theoretical maximum and sample *4h-65°* was found to have the lowest yield at an average yield of 25.9% of theoretical maximum, shown in *Figure 17*. Four parallels were discarded for sample *4h-56°* and for *2h-56°* two parallels were discarded because of a large deviation from the other parallels. The amount of gelatin in 150 grams of raw material is 13.27 g (Speilberg, 2018).

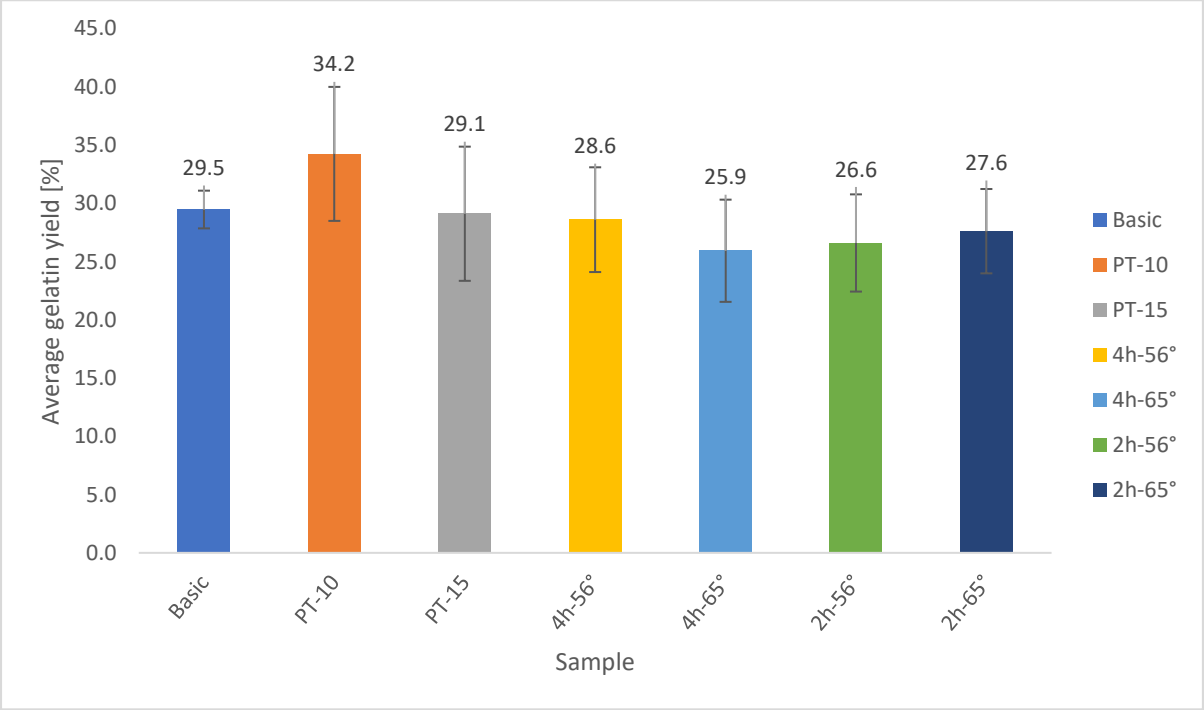

Figure 17: Average gelatin yield for each extraction presented in percent of theoretical yield from raw material. The different methods are explained in Table 4. (For Basic: n=8, for 2h-56°: n=12, 4h-56°: n = 14 the rest have: n=16)

Total amino acid composition

Analysis of the total amino acids content in the different samples showed very similar results, with only small variations between the individual samples. Most samples had the same composition of amino acids, with Glycine/Arginine being the most common and Histidine being the least common. When hydrolyzing under acidic conditions, the two amino acids Gln and Asn are converted to Glu and Asp respectively. Sample *4h-56°* had an overall higher content of amino acids than the others, and a much higher glutamic acid content. *Figure 18* shows the amino acid content of all samples sorted by which amino acid is most prevalent in fish bones as presented in *Table 2*. Proline and hydroxyproline cannot be detected by the UHPLC column, but hydroxyproline content was later determined by the method from AA. Leach (Leach, 1960).

The total amino acid content was found to be close to 1000 mg AA/g sample (DW) for all samples. A high amino acid content indicates a high purity of the sample. For some samples the amino acid content was found to be above 1000 mg AA/g sample, which is due to uncertainties in the sample preparation procedure or the HPLC analysis itself. Sample *4h-56°* was the method that had the highest total amino acid content, with 1284 mg AA/g sample. Sample 2*h-65°* was found to have the lowest amino acid content at 981 mg AA/g sample. The uncertainties vary between ±40.7 and ±103.4, for sample *PT-15* and *2h-65°* respectively, and are larger for the higher values.

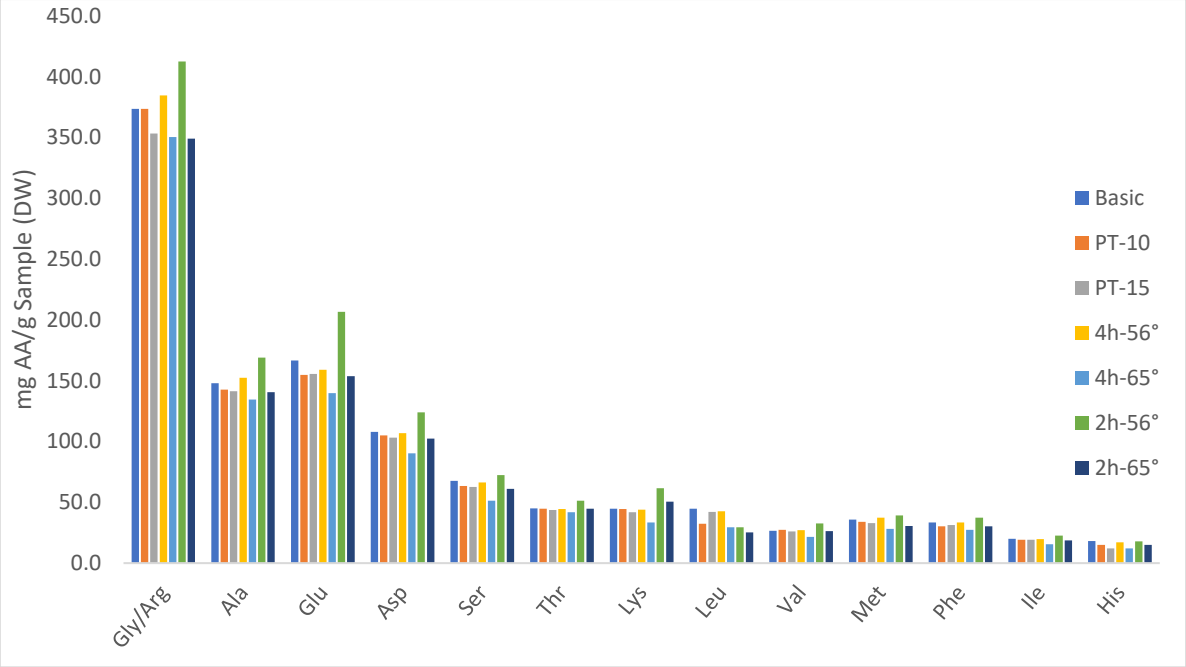

Figure 18: The amino acid content of the different samples. Each sample was analyzed using a UHPLC, and run by Siri Stavrum at IBT. The amino acids are sorted after the most prevalent amino acids in cold water fish, from left to right. The different methods are explained in Table 4. (For Basic: n=4, for the rest: n=8)

Discussion

Dry matter

The gelatin extracts were found to consist of between 1.5-2.5% dry matter, with dry matter content of each sample presented in *Table 5*. The dry matter was found to mainly consist of gelatin, as can be observed from *Figure 16*. Gelatin is highly soluble in warm water and can be dissolved to a concentration of up to 34% before saturation. During extraction, the concentration of gelatin does not exceed 2.5%, which is insufficient for the extract to reach saturation. Production of dry gelatin or gelatin sheets requires removal of the large amount of water from the primary extract. For industrial purposes, removal of water by evaporation involves a large economic cost. Reducing the amount of water used for extraction could therefore decrease the energy required to dry the extract, and reduce the production cost of dry gelatin production. The water content must however not be reduced to a point where the extraction liquid is near the saturation point before all the available gelatin is extracted from the raw material. This could lower the yield, and reduce the effectivity of the procedure.

Since analysis of the extraction product indicated a high degree of purity, and at the same time a high water content, content of mineral components was expected to be very low. In addition, previous experiments showed a very low ash content (Speilberg, 2018). Based on this, further analysis of ash content in the extract was not conducted.

Sensory analysis and gel formation

A simple sensory analysis was conducted, with results shown in *Table 6*. The odor of each sample was graded relative to each other, on a scale from 1 to 3. All samples were analyzed at the same time. All freeze-dried gelatin extracts had a distinct fishy odor, with varying intensity. One explanation for the varying intensity of the smell observed for each sample may be length of storage. However, this does not seem to be the main factor influencing the intensity as samples with a longer storage time were observed to have both strong and weak smell. The same was true for samples that had been stored a shorter amount of time. Samples impurities, like residual oil and fat, is probably the main cause of the detectable fishy odor. This is something that would be reduced if a purification step was included, as is the case for production of commercial gelatin. A purification step was not included in this experimental

procedure in order to save time and because it was regarded as unnecessary since the gelatin was not made for human consumption.

To investigate the gel forming capabilities of the product, gels were prepared using freeze-dried gelatin. One gel per sample were prepared by adding a small amount of gelatin (2% w/w) to equal amounts of water. The gelatin showed very good gel formation capabilities, and formed seemingly strong gels at low temperature (4 °C). A visible reduction of the viscosity was observed when the temperature of the gel reached 15 °C, after which it quickly melted. The observed low melting point was expected, due to the lower proline and hydroxyproline content in cold water fish gelatin compared to gelatin from mammalian animals (Pranoto, et al., 2016). The extraction method used did not seem to influence the gel forming capabilities, as all gels melted at the approximately same temperature and produced seemingly strong gels. To obtain a more accurate measure of gel strength, the Bloom number should be determined, but was not conducted in this project due to lack of time.

Molecular weight distribution and applications

Gel electrophoresis (SDS-PAGE) was run on three different gels over two days. The results are presented in *Figure 13*, *Figure 14* and *Figure 15*.They indicate that all samples have approximately the same MWD. All samples, except samples *PT-15 III & IV*, showed distinct bands right above 669000 Mw. Bands between 440000 Mw and 232000 Mw were also visible for all samples. The absence of bands at 669000 Mw for samples *PT-15 III & IV* can probably be explained by an uneven coloring of the gel by the *InstantBlue ™* dye. The gelatin extracted with the same conditions at the same time, samples *PT-15 I & II*, did however produce a band at 669000 Mw. It is therefore unlikely that samples *PT-15 III & IV* did not contain gelatin of the same Mw. The results from the gel electrophoresis, with bands appearing at the same Mw for all samples, supports the assumption that the MWD is not influenced by different extraction conditions or pre-treatment time used in this project. The MWD is narrow and concentrated around 669000 Mw, and size between 440000 Mw and 232000 Mw. A wide MWD have been found to have a negative effect on some functional properties (Yau, Kirkland, & Bly, 1979 - Not seen, cited after Taheri, Abedian Kenari, A., & Behnam, 2009). Based on the results of gel electrophoresis, this is not likely to be a problem for the fish gelatin extracted in this project.

Based on the results found for the gel forming capabilities and the MWD, the gelatin extracted in this experiment could have several potential applications. The MWD of fish gelatin influence the mechanical properties (Eysturskarð, Haug, Ulset, & Draget, 2009). If a larger fraction of the gelatin has a low molecular weight, the gelatin will not have as good mechanical properties as if it is a small fraction. The potential applications include soft gel capsules, thickener in cosmetics and food, and embedding of oil-based vitamins. There are however some limitations for the extracted gelatin as a replacement for mammalian gelatin due to the low melting point. Some studies suggest that addition of coenhancers, such as magnesium sulphate, glycerol or transglutaminase, can increase several physical properties of the gel, such as gel strength, melting and gelling point, and thereby facilitate the use of cold water fish gelatin as a replacement for mammalian gelatin (Ahmed, 2017) (Fernández-Díaz, Montero, & Gómez-Guillén, 2001). Some research has been done on addition of filler particles to alter the texture (Dille, Draget, & Hattrem, 2015). By improving the most important properties of gelatin, the gelling and melting point, it is possible to increase number of potential applications for cold water fish gelatin.

Total amino acid composition, purity and gelatin yield

The amino acid content in the gelatin samples, shown in *Figure 18*, show only small variations between all samples and follows the expected amino acid content presented in *Table 2*. All samples had an amino acid content of approximately 1000 mg AA/g sample. This indicates very pure gelatin, which is further reinforced by the hydroxyproline and high gelatin content found for all samples.

The most prevalent amino acid in all samples was Gly/Arg, the least prevalent was His. This is in agreement with results from other studies that Gly is the most prevalent and His is among the least prevalent (Gómez-Guillén, et al., 2002). Results presented in *Figure 18* indicates that the purity of the extracted gelatin was not significantly influenced by the extraction parameters nor the time of the pre-treatment steps. There are some differences between the samples, *4h-56°* had the highest amino acid content at 1284 ± 96 mg AA/g sample (DW) and *2h-65°* had the lowest amino acid content at 984 ± 78 mg AA/g sample (DW). Sample *4h-56°* was extracted for four hours at 56 °C which can be a more optimal temperature for a high protein purity since there is less thermal degradation at lower temperatures. This fits well with the results found for gelatin content calculated from

hydroxyproline which found a high gelatin content in sample *4h-56°*. The results from the hydroxyproline analysis does not match the low purity found for some samples, the hydroxyproline analysis found the lowest purity for samples *2h-56°* and *4h-65°*. These somewhat contradicting results might come from uncertainties in the analysis of hydroxyproline or the HPLC. Another explanation could be the uncertainties in the preparation of samples, which could result from dilution of the sample and uncertainties in the instruments. Error from the HPLC could be due to errors in the program, column or standard. But this is more unlikely than an error in the preparation of the sample.

The highest gelatin purity was found for sample *PT-10,* which a gelatin content of 83.3±6.44%. A statistical analysis was performed and none of the samples was not found to be significantly different from the basic method, with $p>0.05$. The gelatin yield was found to be high for all methods, the only procedure that was found to have a yield higher than the basic was *PT-10* with a yield of 34.2±5.75% of theoretical yield. The other samples had lower yield than the basic method. None of these was found to have a statistical significant different from the basic method, with $p>0.05$.

Since none of the samples had a significant difference in yield or purity for the variations of the method, all variations could be chosen. The next potential variation that should be investigated is a combination of shorter pre-treatment, 10 minutes, and one-step extraction. This could reduce the expense of the procedure.

Market possibilities

The gelatin extracted in this project was found to have good physiochemical properties and several potential applications. This could make it suitable for commercial applications. Currently the marked value of fish gelatin is higher than gelatin from mammalian sources. A higher marked value product will make it easier to obtain profit, making it attractive for the industry. The salmon farming industry is an excellent source for a continuous supply of fresh rest raw materials, thus solving one of the larger issues of using marine rest raw materials. Currently, these rest raw materials are mainly used for low value products like silage, about 79%. Small quantities are being used for products for human consumption, such as fish oils or hydrolysates, but less than 20% (Richardsen & Nystøl, 2015). Gelatin extraction is an

excellent opportunity for utilizing marine rest raw materials for a high value product for human consumption.

The Muslim and Jewish population creates a demand for halal or kosher gelatin, which is currently an undersupplied product. As long as fish gelatin has approximately the same physical properties and taste as mammalian gelatin, the general marked is not likely to have any reluctance to use it. With potential areas of application within the food industry, pharma and cosmetics, fish gelatin extracted from rest raw material is something that should be considered a promising product.

Fish gelatin sells for approximately $2 for 28 grams of gelatin (Fish gelatin Price, 2018). The process in this project yielded around 5 grams from 150 grams of raw material, which converted to one metric tonnes of raw material yields 33 kilograms of gelatin. 33 kg gelatin could sell for approximately $2400.

Salmon backbone as a source of gelatin

Comparing the yield and purity of gelatin extracted from other fish species, both cold and warm water species, with the yield and purity with the gelatin extracted in this project the viability of salmon back bone as a source of gelatin can be determined.

In an experiment performed on Nile perch (*Lates niloticus*) bones done by Muyonga et al. they got a gelatin yield on dry ash free basis from bones from young fish (6.1%) and from old fish (11.5%) (Muyonga, Cole, & Duodu, 2003). This is considerably lower than the yield obtained in this project (25.9-34.2%). Their yield was much higher when extracting from skin (64.3%). Experiments performed on other species like seabass (L*ates calcarifer*), found a high yield when extracting from skin at 45 °C (51.6-57.3%) and at 55 °C (62.0-66.4%) (Sinthusamrana, Benjakula, & Kishimurab, 2014). In another experiment performed on skin from unicorn leatherjacket (*Aluterus monoceros*) they obtained different dry weight yields based on the acidic pre-treatment, 0.2 M acetic acid (5.23-9.18%) and 0.2 M phosphoric acid (6.12-11.54%) (Ahmad & Benjakul, 2011). In an experiment done by Kolodziejska et al. the gelatin yield of (71-75%) of available gelatin in fresh salmon (*Salmo salar)* skin and cod (*Gadus morhua)* back bone was obtained (Kołodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008). Another project performed on Atlantic cod (*Gadus morhua*) using

enzymatic hydrolysis to prepare the gelatin for extraction got a low yield (~5-8%) with poor physical properties and low MWD (Gildberg, Arnesen, & Carlehög, 2002). In an experiment performed by Taheri et al. gelatin was extracted from greater lizardfish (*Saurida tumbil*), they got different yield for skin (10.7%) and bone (5.1%) dry gelatin/raw material wet weight (Taheri, Abedian Kenari, A., & Behnam, 2009).

These results indicate that the extraction method used in this project is promising. The yield is excellent compared with the other extractions done from bone. A reason for the much higher yield in this project could be due to that there was no leaching procedure performed on the bones. The leaching procedure could result in a loss of collagen. This was done in Taheri et al. and Muyonga et al. a leaching was performed on the bones. This was not done in the experiment by Kolodziejska et al. which had a very high yield.

Comparing the yield from bones with skin shows that the yield is much lower, this is expected. The gelatin is more available in the skin, and the ash content is lower. Skin however makes up a much smaller proportion of the rest raw material produced from the production of fish products, compared to backbones. In addition, backbone contains some meat residues which if isolated could be used as a nutraceutical, either as meat or a hydrolysate.

Conclusion

All variations of the gelatin extraction method performed in this thesis shows promising results with high yield (~26-34%), and high purity (75.5-83.3%). Looking at the yield and purity presented in *Figure 17* and *Figure 16* respectively, all methods gives high yield and high purity close to the basic method. which is positive considering the increased production and reduced cost of production.

The results from the gel electrophoresis showed a similar MWD which indicates that this is not influenced significantly by the method. This analysis is however qualitative and a more quantitative analysis should be performed.

The highest yield (34.2%) was found for method *PT-10*, where each pre-treatment step was 10 minutes shorter. This method was also found to give a product with a purity (81.6%) that was higher than the purity obtained by the basic method. This leads to the conclusion that method *PT-10* will yield the best gelatin of the methods investigated in this procedure. The second-best method, *4h-56°*, with 4 hours extraction at 56 °C was found to have the highest purity (83.3%). However, the yield (26.6%) was lower than the basic method.

Due to the qualitative nature of the investigation of gel strength no direct conclusions can be made about which products it can be used for, to do this the bloom number must be determined. The gelatin from all extractions form a distinct gel at low temperatures (4 °C) and it keeps its structural integrity until ~15 °C, where it melts. This coincides with the high MWD of the samples, larger gelatin strands are considered beneficial for gel strength and gel formation. The low melting point could be offset by addition of coenhancers, which will expand the number of applications. Thus, a conclusion can be made that the gelatin extracted using the extraction methods of this project produce gelatin with good functional properties and promising areas of application.

Future work

The results obtained in this project gives a good basis for this specific pre-treatment and extraction procedure. There are however more optimizations that could be investigated, such as a combination of time reduction of pre-treatment and, in the extraction step, adjustment of the concentration of chemicals in the pre-treatment. In addition, there is a need for an investigation of the impact of upscaling the batch size. The project was performed in lab scale, which makes upscaling vital to fully investigate the potential of the procedure in the industry. There is also a need for a closer and more precise MWD determination of the extraction gelatin. Gel electrophoresis is not a quantitative analysis and the results are not numerical for MWD, but this can be done with analytical methods such as GC (Yau, Kirkland, & Bly, 1979).

The gelatin extracted in this experiment has only been investigated on a physiochemical and analytical level. So, there is a need to look at the potential applications within several aforementioned industries. Gel-sheets, film formation, bloom number and product tests are among the potential things that should be tested. The sensory analysis performed in this project was performed by an untrained subject and the need for an expert analysis is apparent, but before this is done there should be some investigation of the purification procedure that is performed after extraction. This was excluded in this project because of equipment requirements, time restraints, and because it was deemed unnecessary since it was not meant for human consumption. There have been some studies on purification via ultrafiltration, this is something that should be considered due to its benefits (Simon, Vandanjon, Levesque, & Bourseau, 2002). In addition to the purification of the gelatin, the ultrafiltration can be used to remove some of the smaller gelatin strands in the extract, and thereby increasing its gel forming capabilities. Ultrafiltration have also been found to be cheaper on an industrial scale, that regular evaporation and purification (Chackravorty & Singh, 1990). Another area that can be investigated is the use of coenhancers to increase the physiochemical properties, such as gel strength and melting point.

Analysis of minerals, such as calcium, on the remaining bones post extraction is also something that could be of interest. To even further increase the utilization of the rest raw material.

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Appendix

I: Equations

I.1 Yield

The yield of the collagen was found by multiplying the dry matter content in the raw material by 150 since this was the amount of raw material used for all extractions. Then multiplying the result by the average collagen content in the corresponding raw material, this gives the amount of collagen in 150 grams of raw material. To find the yield from each sample the amount of freeze dried sample was multiplied with the average collagen content in for the respective sample and then divided by the collagen content in 150 grams raw material.

$$Gelatin\ Yield = \frac{AvgColSamp \cdot InWeight}{\frac{DM \cdot 150 \cdot AvgColRawMat}{100}} \qquad \textit{Equation I.1}$$

I.2 Hydroxyproline

The hydroxyproline was calculated by comparing the OD of the samples with a standard curve prepared the same day. The hydroxyproline in undiluted sample was calculated using the dilution factor (*DF*), the OD_{555} and the values from the standard curve (*a* & *b*). This was multiplied by the volume (*V*) and divided by the weight of the sample that was hydrolyzed (*w*). And to convert to the collagen content multiplied with 11.42, which is equal to the amount of gelatin.

$$H_yP_{UDS} = \frac{(DF \cdot OD_{555}) - b}{a} \qquad \textit{Equation I.2}$$

$$H_yP\%_{FD} = \frac{H_yP_{UDS} \cdot V}{w \cdot 10^6} \cdot 100\% \qquad \textit{Equation I.3}$$

$$Col_{FD} = H_yP\%_{FD} \cdot 11.42 \qquad \textit{Equation I.4}$$

II: Tables and data

Table 7: Raw data dry matter, freeze dried gelatin and %DW freeze dried sample. The dry matter was analyzed gravimetrically by weighing out ~4 grams extract and drying in a heating cabinet for 24h at 105 °C and the %DW FD was calculated by weighing the freeze-dried sample. There is some loss when freeze drying due to difficulties when transferring from the freeze-drying vessels.

Sample		Tot DM [g]	Freeze Dried Sample [g]	% DW FD	% DM
Basic	**I**	5.09	4.48	2.2%	2.5%
	II	4.65	4.04	2.2%	2.5%
PT-10	**I**	6.24	5.46	2.1%	2.4%
	II	5.24	4.68	2.0%	2.3%
	III	5.64	5.78	2.4%	2.4%
	IV	5.14	5.14	2.1%	2.1%
PT-15	**I**	5.34	5.65	2.4%	2.2%
	II	6.27	6.16	2.5%	2.5%
	III	3.83	4.30	2.6%	2.3%
	IV	3.96	4.41	2.7%	2.5%
4h-56°	**I**	5.45	4.66	1.8%	2.1%
	II	4.97	5.34	2.3%	2.1%
	III	4.96	5.29	2.1%	2.0%
	IV	5.16	5.08	2.1%	2.1%
4h-65°	**I**	4.10	4.42	2.4%	2.2%
	II	3.64	3.29	2.0%	2.3%
	III	4.46	4.39	2.3%	2.3%
	IV	4.82	4.28	2.2%	2.5%
2h-56°	**I**	4.36	4.02	1.6%	1.7%
	II	3.98	3.81	1.5%	1.6%
	III	4.14	4.58	2.0%	1.8%
	IV	4.72	5.18	2.2%	2.0%
2h-65°	**I**	4.16	5.06	2.3%	1.9%
	II	4.20	4.01	1.6%	1.7%
	III	5.63	4.86	2.1%	2.5%
	IV	5.49	4.97	2.0%	2.2%

Table 8:Raw data for the amino acid content found using the HPLC. Presented with standard deviation. The basic sample had four parallels and the rest had 8 (Basic: n=4, the rest: n=8). The values are presented as mg AA/g sample ± SD.

Amino acid	**Basic**	**PT-10**	**PT-15**	**4h-56°**	**4h-65°**	**2h-56°**	**2h-65°**
Gly/Arg	373.6±29.0	373.5±29.4	353.4±25.7	384.8±29.8	350.4±26.8	412.6±25.6	349.1±27.9
Ala	148.0±8.3	142.7±8.2	141.4±6.9	152.4±6.8	134.6±8.7	169.0±10.4	140.6±11.9
Glu	166.8±9.2	154.7±8.1	155.5±7.6	159.0±7.0	139.9±14.3	206.7±15.7	153.8±17.6
Asp	108.0±4.3	105.1±4.8	103.2±4.6	106.8±4.4	90.3±7.6	124.0±10.5	102.4±11.5
Ser	67.8±3.5	63.6±6.7	62.5±6.2	66.3±6.9	51.3±5.8	72.4±9.3	61.2±9.8
Thr	44.9±2.6	44.8±3.5	43.6±4.8	44.4±4.1	41.8±5.0	51.3±5.5	44.8±5.1
Lys	44.6±3.9	44.5±7.6	41.9±6.7	44.0±5.2	33.5±5.5	61.7±7.1	50.5±7.6
Leu	44.8±2.0	32.5±6.6	42.2±7.3	42.7±8.2	29.4±8.5	29.5±4.5	25.2±5.2
Val	26.6±1.3	27.3±2.0	26.0±2.0	27.2±2.0	21.5±2.2	32.8±3.1	26.2±3.3
Met	35.8±3.2	34.1±4.2	33.0±3.9	37.3±3.7	28.2±4.6	39.4±5.5	30.5±5.8
Phe	33.4±1.9	30.2±3.1	31.5±2.9	33.5±2.9	27.3±3.0	37.3±3.4	30.3±3.7
Ile	20.1±0.9	19.3±0.7	19.3±0.7	19.7±0.7	15.6±1.5	22.8±2.2	18.8±2.4
His	18.1±1.2	15.1±6.5	12.2±6.0	17.2±8.1	12.1±8.1	17.9±7.4	14.9±7.2
Tyr	7.0±0.6	4.3±3.4	4.4±3.2	4.6±3.0	4.0±2.7	3.8±3.0	3.2±3.3
Gln	2.3±0.7	1.6±1.5	2.1±1.3	1.9±1.0	1.6±1.2	2.4±1.1	2.6±1.7
Aba	4.5±0.4	2.0±2.3	4.6±2.5	3.5±2.4	2.6.0±2.0	0.2±0.7	0.2±0.7
Asn	0.1±0.0	0.3±0.2	0.3±0.2	0.4±0.3	0.3±0.3	0.3±0.3	0.3±0.3
Total:	**1146.5±66.8**	**1095.5±46.6**	**1077.0±40.7**	**1145.8±41.2**	**984.2±77.8**	**1284.0±96**	**1054.5±103.4**

Table 9: Average purity calculated from the hydroxyproline content, the yield of gelatin calculated from gelatin purity and expected gelatin content in 150 g of raw material. Using Equation I.1. To convert the hydroxyproline content to gelatin, the HyP content was multiplied with a factor of 11.42 (Eckhoff, Aidos, Hemre, & Lie, 1998). (Average value ± SD, Basic: n=8, 4h-56° n=12, 2h-56° n=14, for the rest n=16)

SAMPLE	GELATIN PURITY	AVERAGE YIELD
BASIC	80.5±5.8	29.5±1.6
PT-10	81.6±10.3	34.2±5.8
PT-15	80.3±6.6	29.1±5.8
4H-56°	75.5±10.9	28.6±4.5
4H-65°	80.6±6.8	25.9±4.4
2H-56°	83.3±6.4	26.6±4.2
2H-65°	76.0±11.0	27.6±3.6