Masteroppgave

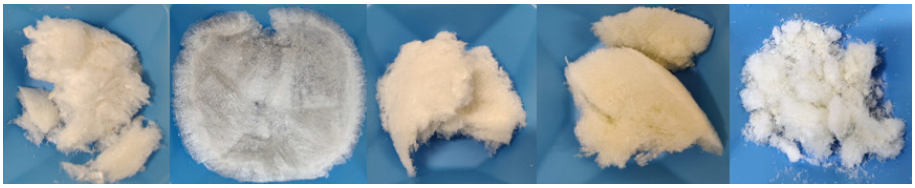
NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for bioteknologi og matvitenskap

Sine Marie Moen Kobbenes

Effect of green extraction methods on quality of gelatin from skins, heads and backbones from European plaice (*Pleuronectes platessa*)

Masteroppgave i Industriell kjemi og bioteknologi
Veileder: Jørgen Lerfall
Medveileder: Sophie Kendler

Februar 2023



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Preface

This master's thesis is written for the department of Biotechnology and food science at the Norwegian University of Science and technology (NTNU). The thesis is building on a project written for TBT4500, conducted in spring 2022, and is a part of the PhD-project of Sophie Kendler which is a part of the project OPTiMAT at NTNU.

I want to thank my supervisor Jørgen Lerfall and co-supervisor Sophie Kendler for all support during this master thesis. Jørgen, for guiding, helping sorting data and motivating the progress, especially during the last weeks of the project. Sophie, for all you have guided and helped at lab, been available for questions and for sharing encouragement to the project.

I also want to give a thanks to Turid Rustad for sharing advices and showing interest for my experiments, and to Siri Stavrum for support at lab and HPLC-analyses. Many other people at lab deserve a thanks for assistance and for making the laboratories at Akrinn and Gløshaugen to positive workplaces.

Lastly, I want to thank my friends for the very appreciated time spent together, at coffee and lunch breaks, and all memories making the study time in Trondheim great. Thanks to Martine for a being such a supportive roomie the last year, and thanks to family for sending strength over the telephone line, it helped, thank you.

Abstract

About 50 % of the worlds annual catch from the global fish production is either wasted or used for low value products such as animal feed, biogas and fertilizers. These fractions, usually composed of heads, bones, blood and viscera, contains valuable components that can be valorized for high value products. There are several reasons for utilizing these fractions, including environmental, political and economical aspects. European plaice (*Pleuronectes platessa*) is a fish below the maximum sustainable yield (MSY) can be utilized to a higher degree than today. It generates about 50 % rest raw materials that should be utilized to secure a sustainable food processing.

The main aim of this master thesis was to investigate different green extraction methods to valorize rest raw material fractions of European plaice. To complete this aim, gelatin extracted by microwave and ultrasound technology from different raw material fractions was conducted and the quality and yield of the resulting gelatin was evaluated. Raw material fractions included backbones, skins and heads. Then, each raw material fraction was divided into a not pre-treated part, a part pre-treated by salt wash and a part pre-treated by enzymatic hydrolysis. The durations of the extractions were 15 and 35 minutes, and a water bath of 150 minutes was used as a control extraction for comparison.

The quality was measured by considering the collagen yield, purity, amino acid profiles and molecular size of the gelatin samples produced. The purity was evaluated by looking at the protein and collagen concentration together with the amino acid profiles. SDS-page was run on the gelatin samples to analyze the molecular size. Lastly, the effect of the different extraction procedures was tested by General Linear Model (GLM) using Tukey's multiple comparison test with significance level of 95 % ($p<0.05$).

The results showed that skin gave gelatin with highest yields compared to the heads and backbones. For the pre-treatments, salt wash gave in general gelatin with lower yields and enzymatic hydrolysis gave higher yields than gelatin extracted from not pre-treated material. Pre-treatment by salt wash also gave presence of β-chains in the gelatin which gives better gel strength. Microwave and ultrasound assisted extraction affected the quality and yield after 35 minutes, and the green extraction methods showed that the collagen content in gelatin samples extracted for 35 minutes ($48.8 \pm 16.2\%$) did not differ significantly ($p<0.001$) from the collagen content of a 150 minutes water bath (47.4 ± 23.7 %). This indicates that longer extraction times could give better yield and higher collagen purity. The results showed that there are potential in utilizing microwave and ultrasound assisted technologies for extraction of gelatin from plaice rest raw material, but that several quality parameters can be investigated to know more about potential application areas.

Sammendrag

Omtrent 50 % av verdens årlige fangst fra den globale fiskeproduksjonen går enten til spille eller brukes til lavverdiprodukter som dyrefôr, biogass og gjødsel. Disse fraksjonene, vanligvis sammensatt av hoder, bein, blod og innvoller, inneholder verdifulle komponenter som kan brukes til høyverdige produkter. Det er flere grunner til å bruke disse fraksjonene, inkludert miljømessige, politiske og økonomiske aspekter. Europeisk rødspette (*Pleuronectes platessa*) er en fisk som i dag kommer fra en bærekraftig bestand og dermed kan utnyttes i høyere grad enn den gjør i dag. Den genererer ca. 50 % restråstoff som bør utnyttes for å sikre et bærekraftig fiske.

Hovedmålet med denne masteroppgaven var å undersøke ulike grønne utvinningsmetoder for å utnytte restråstoff av Europeisk rødspette. For å nå dette målet ble gelatin ekstrahert med mikrobølge- og ultralydteknologi fra ulike restråstoffraksjoner, og kvaliteten og utbyttet av den gelatinen ble evaluert. Råvarefraksjoner bestod av ryggrad, skinn og hoder. Deretter ble hver råvarefraksjon delt inn i en ikke forbehandlet del, en del forbehandlet ved saltvask og en del forbehandlet ved enzymatisk hydrolyse. Varigheten av ekstraksjonene var 15 og 35 minutter, og et vannbad på 150 minutter ble brukt som kontrollekstraksjon for sammenligning.

Kvaliteten ble målt ved å vurdere kollagenutbyttet, kollagenrenheten, aminosyreprofilene og molekylstørrelsen til de resulterende gelatinprøvene. Renheten ble evaluert ved å se på protein- og kollagenkonsentrasjonen sammen med aminosyreprofilene. Gelatinprøvene ble så analysert ved SDS-PAGE å evaluere molekylvektfordelingene. Til slutt ble effekten av de forskjellige ekstraksjonsprosedyrene testet av General Linear Model (GLM) ved bruk av Tukeys sammenligningstest med signifikansnivå på 95 % ($p<0,05$).

Resultatene viste at skinn ga gelatinprøver med høyeste utbytter sammenlignet med gelatin fra hode og ryggrad. Av forbehandlingene ga saltvask generelt gelatin med lavere utbytte, og enzymatisk hydrolyse ga høyere utbytter enn gelatin ekstrahert fra ikke forbehandlet materiale. Forbehandling med saltvask ga også høyere innhold av β-kjeder i gelatinen, noe som gir bedre gelstyrke på gelatinen. Mikrobølge- og ultralydassistert ekstraksjon påvirket kvaliteten og utbyttet etter 35 minutter, og de grønne ekstraksjonsmetodene viste at kollageninnholdet i gelatinprøver ekstrahert i 35 minutter (48,8 $\pm$ 16,2%) ikke var signifikant forskjellig ($p<0,001$) fra kollageninnholdet i et 150 minutters vannbad (47,4 $\pm$ 23,7 %). Dette indikerer at lengre ekstraksjonstider kan gi bedre utbytte og høyere kollagenrenhet. Resultatene viste at det er potensiale i å utnytte mikrobølge- og ultralydassisterte teknologier for utvinning av gelatin fra restråstoffrasksjoner fra rødspette, men at flere kvalitetsparametere kan undersøkes for å vite mer om potensielle bruksområder.

Contents

1 Introduction

Of the 179 million tonnes the global fish production reached in 2018, about 50 % of the catch was generated as rest raw material[1]. Rest raw material is defined as the part of the catch not considered as the main saleable product, usually composed of fractions like skins, heads, viscera, backbones and blood[2]. For processed fish the amount of rest raw material can reach up to 70 %. Today, most of these products are mainly either discarded or utilized for low-value products such as animal feed, biogas and fertilizers[2] [1]. However, extensive research over the past decades studying these fractions, have found that rest raw material fractions contain valuable components for humans such as polyunsaturated fatty acids, proteins, enzymes and minerals giving them a great potential to be used for human consumption[3].

In recent years, several factors affecting economical, political and environmental issues that point to an increased need for utilizing rest raw material fractions from the fish industry. A growing population leading to an increased need for food and the decrease of sustainable fish stocks leading to a shrinking amount of food resources, lay the foundation to finding ways on how to novelize rest raw material fractions. In addition, increased industrialization of the food sector involving more variety of food products, have led to more processed fish products, which generate larger amounts of generated rest raw material[3].

The need for a more sustainable global food production has led the EU commission to launch a Circular Economy Action plan in 2020 to accelerate already implemented strategies from the European Green Deal in 2015. The plan aims to facilitate a framework to make sustainable products the norm and to lead the way to implement the 2030 Sustainable Development Goals[4]. Additionally, the scientific research on valorization of waste/by-products has increased about four times the last decade[3]. New technology like enzymatic hydrolysis to improve protein quality and green technologies for extraction of bioactive components from marine rest raw materials are research examples that have reached great attention.

Extraction of gelatin is one way to utilize rest raw material further. The clear, water soluble product extracted from the protein collagen has several beneficial properties for the food and technological industry which give gelatin from marine sources a potential to novelize fish rest raw materials[5].

European plaice (*Pleuronectes platessa*) is a wild caught fish in the North sea. Plaice in the North sea is caught wild, and the spawning biomass is larger than the level that can produce the maximum sustainable yield (MSY)[6]. The harvest from the North sea can therefore be considered as a sustainable food source. However, rest raw material (heads,

backbones, skin and viscera) makes up 51-55 % of the total weight[7]. This corresponds to up to 81 303 tons of wasted material based on the fishing quota in 2021[8]. To secure sustainable processing of European plaice, the utilization of the whole fish should be maximized.

1.1 Aims of the study

This master thesis is a part of the PhD-project of Sophie Kendler working with Little utilized marine food resources, which is a part of the NTNU-funded project Optimal Utilisation Of Marine Food Resources (OPTiMAT). The goal of the OPTiMAT project are to optimize the utilization of marine resources along the whole value chain.

The main objective of this master thesis was to explore the possibility of using green extraction technologies to valorize different rest raw material fractions originating from European plaice. More precisely, to extract gelatin from three different fractions, being the heads, backbones and skins by applying microwave or ultrasound assisted extraction and enzymatic hydrolysis or washing with salt as pre-treatment prior to extraction. Following sub-goals were looked into closer:

1. to study the effect of the raw material fraction on the quality and yield of the gelatin
2. to determine if a pre-treatment with salt or enzymatic hydrolysis show an effect on the quality and yield of the gelatin
3. to investigate whether the extraction method as well as duration of extraction affects the quality and yield of the gelatin

2 Background

2.1 Collagen

Collagen is the source of gelatin and is the most abundant protein class in vertebrate animals. It belongs to connective tissue proteins and plays important roles for structural and functional properties in tissues and cell growth[9]. Per 2011, 28 different types of collagen have been classified based on their polypeptide structure and amino acid distribution. Common for all types are the linear, fibrious structure and the presence of a triple helix[10][11]. Of the 28 collagen types, Type I, II and III are the most frequent in animal tissues. Type I is found in connective tissues like skin, bones and tendons, type II is mostly found in cartilage tissue and type III is present in blood and skin[10]. For fish, type I and II are the ones typically found as the dominating types, where type I is abundant in fish skin, and type II is found in fish cartilage[12].

The primary structure of type I-collagen is the α-chains made up of the amino acid chain aligned as "Gly-X-Y", illustrated in Figure 2.1 (a). Every third amino acid is glycine (Gly), the "X-position" is usually occupied by proline (Pro) and "Y" is occupied by a non-polar amino acid, usually hydroxyproline (Hyp). The rest of the 20 amino acids that collagen consists of, are distributed on the remaining X- and Y-positions [10] [13]. In the secondary structure, Figure 2.1 (b, left), the polypeptide chains form two α-1-chains and one α-2-chain coiling into a left-handed helix with about three amino acids per turn. The tertiary structure, Figure 2.1 (b, right), is composed of the three α-chains twisting and forming a right-handed super-helix held tightly together by hydrogen bonds [14]. The non-polar side chains in α-structure together with the tight structure, makes the collagen molecule to be completely insoluble in water. The collagen helices are staggered into collagen-fibril sheets in the quarternary structure, illustrated in Figure 2.1 (c).

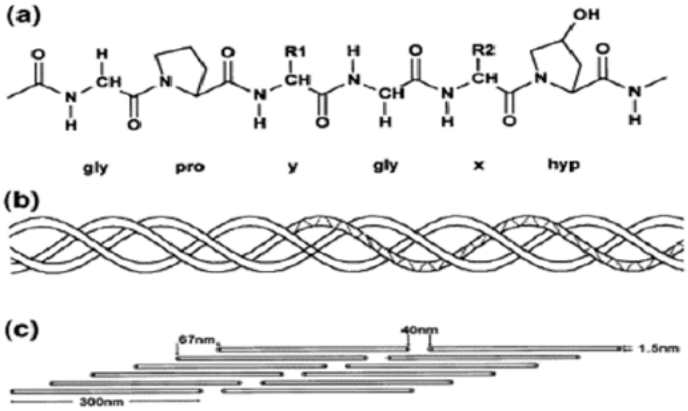

Figure 2.1: Primary (a), secondary (b, left side), tertiary (b, right side) and quaternary structure (c) of type I collagen [15].

The stability of collagen gives it the functional properties that are suitable for connective tissues and bone tissues and comes from the cross-links between the sheets and hydrogen bonds packing the tertiary structure. Thermal stability is also influenced by the amount of the imino acids proline and hydroxypoline [14]. Pyrolidine rings of proline and hydroxyproline contribute to stabilization of the collagen by limiting flexibility of the polypeptide chain. In addition, the hydroxyl group of hydroxyproline have been shown to enable formation hydrogen bonded water-bridges, which stitch together the triple α-helix, shown in Figure 2.2 [16]. The amount of the two imino acids and therefore the thermal stability of the collagen varies with species and tissue. Collagen in mammalian skin contains for instance more imino acids than collagen from cod skin, making mammalian skin collagen more stable in higher temperatures than fish skin collagen [17].

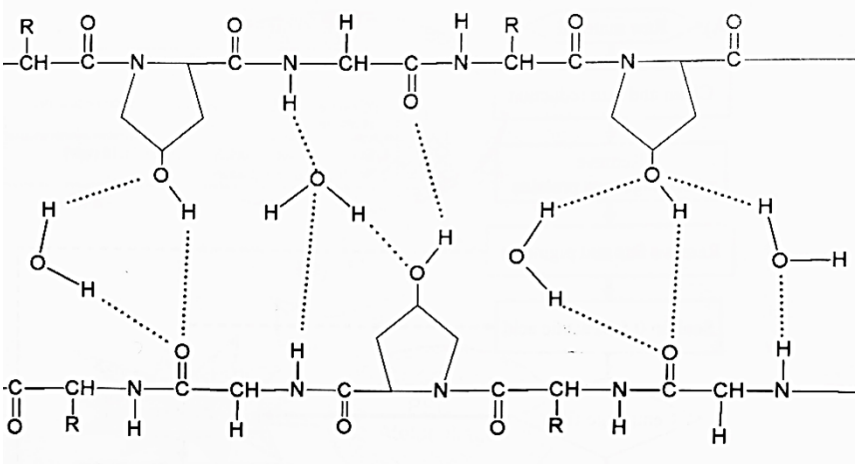

Figure 2.2: Bonds holding the triple helix structure in collagen together [endre figurtekst] [17]

2.2 Gelatin and properties of gelatin

Gelatin is referred to as the main product from irreversible degradation of collagen under the presence of water and mild heating [5]. When collagen is degraded, hydrogen bonds and the hydrophobic linkages from the hydroxyl group break and the collagen turns into water soluble gelatin, consisting of free polypeptide chains of varying molecular size [13] [5]. The three dominating chains in gelatin are called α- β- and γ-chains with different molecular weight. α-chains are the smallest of the three main chains, with molecular weights of 90-110 kDa, but by depolymerization, even smaller α-sub-chains can arise. β-chains have molecular weights of 180-220 kDa and are composed of two α-chains covalently linked, while γ-chains, composed of three α-chains are the largest of the three chains with molecular sizes of 270-300 kDa [13].

The different molecular weight distributions of the chains, structure and amino acid compositions give gelatin properties that vary with the conditions of the surroundings like temperature, pH, ionic strength and presence of other components [13] [10]. When the charged, polar and non-polar amino acids of the α-chains interact, gelatin gets the ability to form colloidal solutions with water, making gelatin a hydrocolloid. Important physio-chemical properties are related to gelling and surface active properties. Gelling properties include gel formation, texturizing, thickening and water binding, while the surface active properties include the ability to form emulsions, colloid effects, foaming, film forming and gluing effects [10].

The gel forming properties of gelatin are unique because of the stronger reversible effect compared to other hydrocolloids [13]. At lower temperatures, intramolecular hydrogen bonds are formed and the α-chains cluster together creating a gel solution. At higher temperatures, the process is reversed as the bonds break leading the gelatin solution to be dissolved, shown in Figure 2.3. The ability to hold on the gel, that is gel strength, is

mainly dependent on the molecular weight distribution where larger molecules give better gel strength[10]. Furthermore, the molecular weight distribution depends on factors related to the extraction process, like raw material source, treatment of the raw material before extraction (pre-treatment) and method of extraction. The gelling stability is affected by the stability of collagen and thus the amount of proline and hydroxyproline, shown in Table 2.1, making cold water fish gelatin to have poorer gel strength than collagen from bovine hide[13].

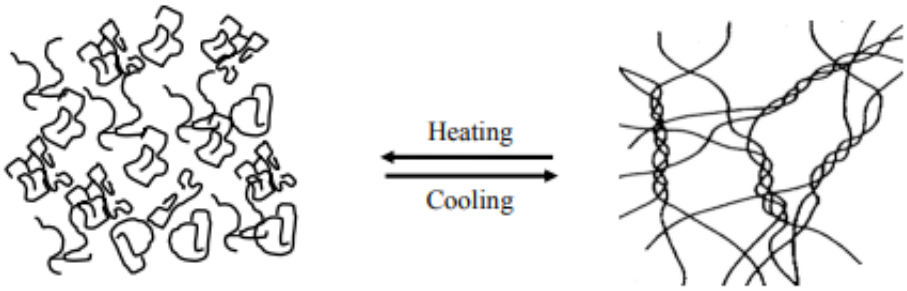

Figure 2.3: Upon cooling of gelatin, intramolecular hydrogen bonds are formed creating a gel and when heating, the process is reversed (Haug and Draget (2009))[13]

While the gelling properties are dependent on the size of the α-chains present in the gelatin, the surface active properties are dependent on the amino acids, or more specifically, their charged or polar groups in the side chains[10]. Charged or polar groups make the amino acids hydrophilic, listed in Table 2.1, which makes them to be attracted to the surface, reducing the tension and stabilize the formed surfaces. Stabilization of surfaces gives film forming and foaming effecst as well as the ability to form emulsions. Additionally, the isoelectric point of the amino acids also affect the surface active properties. The amino acid composition of the gelatin is dependent raw material source, pre-treatment and extraction method, and the surface active properties will vary with these. Based on the most common pre-treatments, two main types, type A and type B gelatin, with different properties can be formed[10]. Type A is gelatin from acid treated raw material and type B is extracted after alkali pre-treatment. The amino acid composition is not affected by the acid treatment and the composition of type A is more or less similar to the collagen source, giving the same isoelectrical point (pI) of 7-9.4. Type B, on the other hand, is missing glutamine and aspargine due to alkali deamidation, making the gelatins more acidic and having a pI of 5.7-7.4[13]. Followingly, the surface active properties of the two types will be different based on the pH of their solvents. The amino acid compositions of collagen type I and the two gelatin types from bovine hide, can be seen in Table 2.1.

Table 2.1: Composition of hydrophilic and hydrophobic amino acids residues per 1000 residues of Type I collagen, type A and B gelatin and gelatin from cold water fish gelatin. Hydrophilic and hydrophobic amino acids taken from Schrieber and Gareis (2007), and composition in gelatins from Haug and Draget(2009) [10] [13]

Amino acid	Hydrophilic/hydrophobic	Type I collagen from bovine	Type A gelatin	Type B gelatin	Cold water fish gelatin
Alanine	Hydrophobic	114	112	117	112
Arginine	Hydrophilic	51	49	48	49
Aspargine	Hydrophilic	16	16	46	48
Aspartic acid	Hydrophilic	29	29		
Glutamine	Hydrophilic	48	48	72	72
Glutamic acid	Hydrophilic	25	25		
Glycine	Hydrophobic	332	330	335	347
Histidine	Hydrophilic	4	4	4	11
4-Hydroxyproline		104	91	93	60
Hydroxyproline	Hydrophobic	5	6	4	5
Isoleucine	Hydrophobic	11	10	11	11
Leucine	Hydrophobic	24	24	24	21
Lysine	Hydrophilic	28	27	28	28
Methionine	Hydrophobic	6	4	4	3
Phynalalanine	Hydrophobic	13	14	14	13
Proline	Hydrophobic	115	132	124	96
Serine	Hydrophilic	35	35	33	63
Threonine	Hydrophilic	17	18	18	24
Tyrosine	Hydrophilic	4	3	1	9
Valine	Hydrophobic	22	26	22	18

[eventuelt]

2.3 Extraction of gelatin

The extraction process from raw animal tissue to applicable gelatin is generally composed of three steps, shown in Figure 2.4. First, the raw material is prepared and pre-treated, then the gelatin is extracted, followed by recovery or purification [18]. The parameters of pre-treatment, extraction conditions and recovery varies with the desired properties of the gelatin end product. Since gelatin has many different application areas, there are no universal desired properties of the end product. For example, the surface active properties like water absorption and retention properties will make the gelatin suitable for medical applications, while gelatin with better gelling properties are exploited in the food industry [12] [10]. However, the quality parameters collagen yield and purity are parameters that should be high to be able to maximize the utilization of the desired properties as well as to make it feasible for industrialized processes.

The first step in the gelatin extraction process involves preparation and pre-treatment of

the raw material. Per 2008, the most used raw materials were pig skin, bovine skin and bovine bones, accounting for 98.5 % of the global gelatin production, while other sources like fish and poultry made up 1.5 % of the annual production[19]. The raw material is either minced or used without further preparation. Mincing can improve the effect of washing and has been showed to give better yield for fish raw material[17] [20]. After preparation, the material is washed for impurities, fats, and non-collagenous proteins to improve the purity. Then, the material is treated usually with with acid or alkali solutions to open collagen structure and weaken intramolecular cross-linkages, called "swelling", and also to release additional impurities[21]. Other pre-treatment solvents, like NaCl-solutions and water washing have also been applied[17] [22]. Applying salt solutions on the raw material will increase the pI of proteins making them salt soluble which will contribute to removal of non-collagen proteins[].

During the second step, the extraction step, the collagen in the raw material is degraded into gelatin usually by heating in a water bath at 50-100 °C for 2-5 hours[10] [13]. Traditionally, the extraction process is a batch process where the gelatin solution is dissolved from inside to outside, but continuous and semi-continuous processes have also been applied in industries[10]. Different temperatures have been tried out to improve gelatin yield, and in general the extraction temperature of fish collagen is lower than the mammalian collagen due to the low hydroxyproline content[17].

In the recovery process, to prepare the extracted gelatin for marked, the soluble gelatin is separated from the rest of the raw material and subsequent dried, usually done by vacuum drying, hot air drying or freeze drying[23]. To remove minerals occuring from the pre-treatment, the product is normally additionally purified by demineralization and filtration[10].

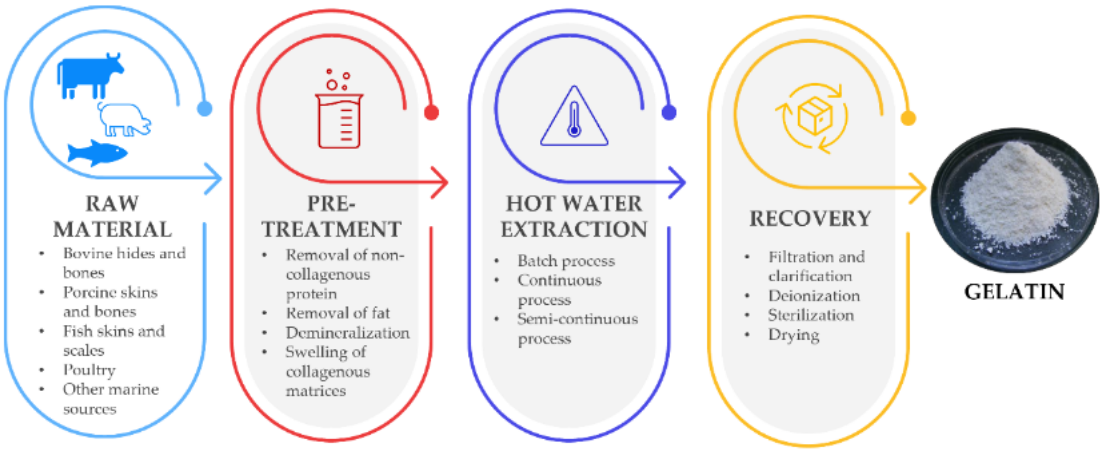

Figure 2.4: Illustration of general gelatin extraction process from raw material to gelatin, from Noor et al. (2021)[18]

2.4 Alternative green extraction methods

In the last years, alternative pre-treatments and extraction methods, next to the traditional acid- and alkali pre-treatments and hours-long water baths, have been introduced to not only improve yield and purity, but also to reduce the environmental footprint by making the extraction procedures more energy efficient and to use less water and chemicals[18]. New extraction technologies can involve enzymatic treatment before or during extraction, ultra-high pressure technology, use of alternative solvents, as well as ultrasound- and microwave technology[3] [24]. Ultrasound and microwave assisted extraction have gained attention in the previous years and poses a promising green technology for efficient extraction.

2.4.1 Ultrasound assisted extraction

Ultrasound assisted extraction uses ultrasound waves which are acoustic waves beyond the human capacity to hear (>20 kHz)[25]. When applied on a liquid solution, the mechanical energy generated from the ultrasound waves will make the pressure to fluctuate. The pressure can then fall below the vapor pressure of the liquid which causes creation of small bubbles, called cavitation bubbles[26]. When the ultrasound waves are further applied on the medium, the cavitation bubbles will expand and contract because of the pressure fluctuations. If the intensity of the waves are high enough, the bubbles will expand until reaching an unstable size and collapse, as illustrated in Figure 2.5[25].

The ultrasonic technology facilitates several chemical mechanisms that are exploited in the food industry. Applications involve for instance enhancing emulsification of oil-water mixtures, alternative methods to drying, change in food texture and for extractions[27]. The principle behind the effect of ultrasound waves in extraction of gelatin is that the collapse of the cavitational bubbles interrupt the three fold α-chain structure of the collagen causing the intramolecular and covalent bondings to break. This can reduce the extraction time and increase the yield[28] [18].

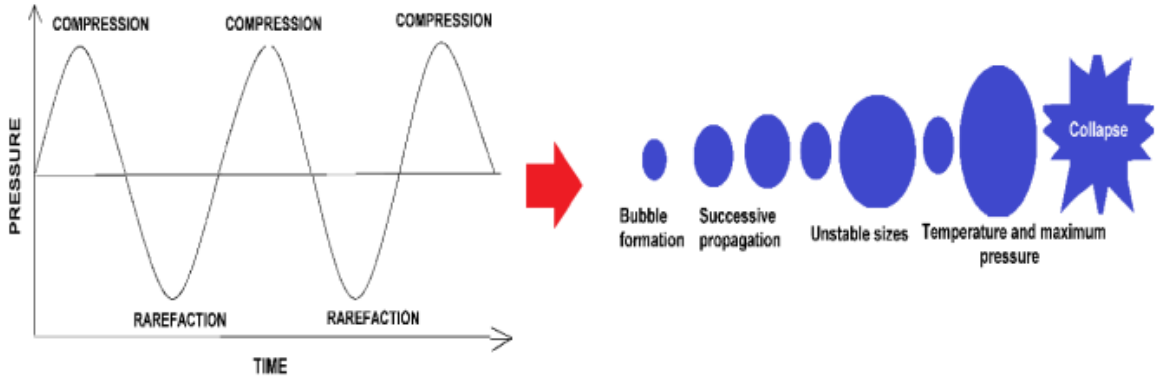

Figure 2.5: Ultrasonic waves cause formation of bubbles in the solvent that will collapse due to cavitation. Modified by Medina-Torres et al. (2017) from Soria and Villamiel (2010) [29] [27].

2.4.2 Microwave assisted extraction

Microwave extraction is another promising extraction method, which is more and more studied and applied gradually in recent years. Microwaves are electromagnetic waves with wave lengths between 1 mm and 30 cm and cover the area between infrared waves and radiowaves [30]. When applied, the microwaves create a magnetic and an electric field which oscillate perpendicularly [31]. The electric field causes creation of energy by the dipolar rotation and ionic conduction, depending on the molecules having a dipole moment or electric charge. Polar molecules with a dipole moment will try to align to adapt the change in the magnetic field which will make rotate, called dipolar rotation [31], shown to the left in Figure 2.6. Ionic conduction is caused when positively or negatively charged molecules are disrupted by the change in the field, making them move towards the negative and positive side of the field and colliding with each other and other components, shown to the right in Figure 2.6. The rotation and colliding from the two mechanisms will release heat energy.

Like ultrasound technology, microwave technology is utilized in several application areas in the food industry, such as cooking, drying and pasteurization [32]. The microwave technology is mainly used as an alternative to traditional heating and is popular in industry due to low energy consumption, more even heating on the material and ease of use [24]. The principle of using microwaves for gelatin extraction is to use less energy on heating. In addition, vibrating water molecules caused by microwaves, will contribute to breakage of the covalent cross linking in the collagen structure, facilitating more time efficient extraction [24].

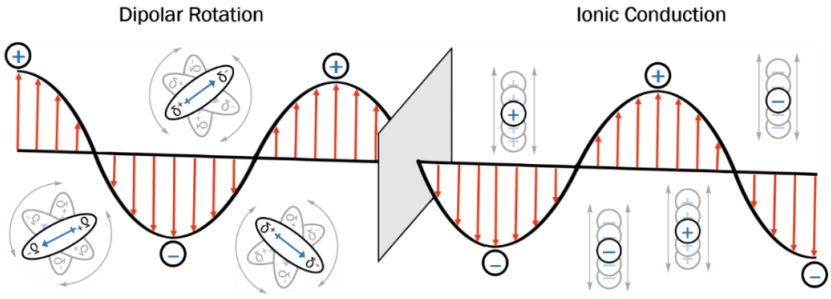

Figure 2.6: Microwaves cause the two mechanism dipolar rotation or ionic conduction depending on the molecules [33].

3 Materials and methods

Gelatin from not pre-treated and pre-treated skins, heads and backbones from European plaice was extracted for 15 and 35 minutes by microwave assisted extraction (MWAE) and ultrasound water bath extraction (UWAE). As a control for a traditional extraction method, an extraction for 150 minutes in a water bath was conducted additionally. All raw material fractions were minced before any pre-treatment. The two pre-treatments conducted were salt pre-treatment and enzymatic hydrolysis. Furthermore, the pre-treated material and gelatins were freeze dried and analyzed for protein content, hydroxyproline content, amino acid profiles and molecular size distribution. The experimental design is illustrated in Figure 3.1, and an overview of each sample and procedure can be found in Table A.1 in Appendix A.1.

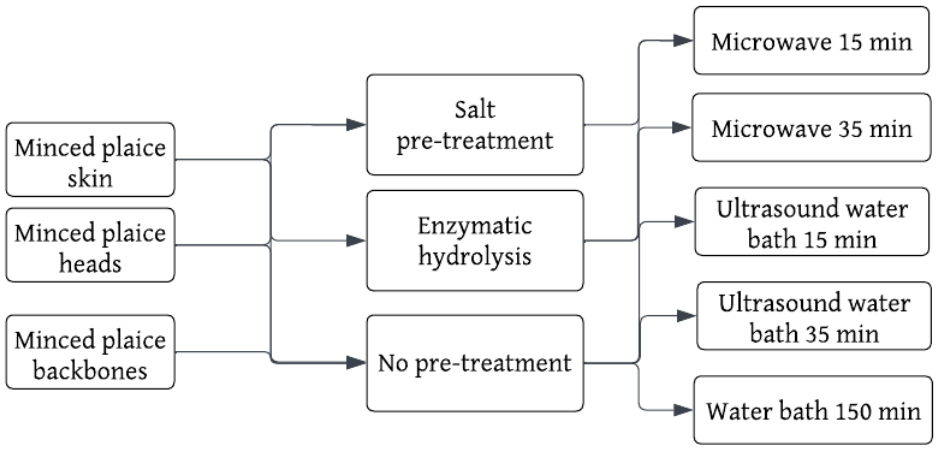

Figure 3.1: Flowchart illustrating the pre-treatments and gelatin extractions of the rest material.

3.1 Preparation of raw material

The rest raw material fractions used for the experiments originated from more than 20 individuals caught caught in the fishing area 2.a.2, as classified by FAO Major Fishing Area (1990-2021) and described in detail by Kendler et al. (2022) [34] [7]. The material was stored at -80 °C until thawed in 4 °C and homogenized during spring 2022. A floor model meat grinder was used for homogenizing the heads and backbones, and the skin was homogenized in a Robot Coupe food processor (Robot-Coupe Inc.). After homogenizing, the material was frozen at -80 °C until any pre-treatment or extraction conducted autumn 2022.

3.2 Pre-treatments

3.2.1 Salt pre-treatment

The salt pre-treatment procedure conducted was based on the method of Kolodziejska et al. with modifications from the master thesis of Rebeca Alvarez[22] [35] and is shown as a flowchart in Figure 3.2. Minced rest raw material was thawed to 4 °C the day before the salt pre-treatment. A 0.45 M NaCl-solution (6:1 v/w) was added to the material and the mixture was shaken for 10 minutes at 4 °C. Then, the solution was filtered and the salt wash weighted and discarded. A filter placed in a tract was used to wash the material. This step was repeated two more times followed by washing in cold tap water for two minutes. The remaining material was shaken in a 10 % ethanol solution (6:1 v/w) for 30 minutes, filtered and washed in cold tap water for two minutes. Some of the ethanol wash was saved for dry matter and ash determination and the rest was discarded. The salt treated material was stored at 4 °C until extractions conducted the same or the next day.

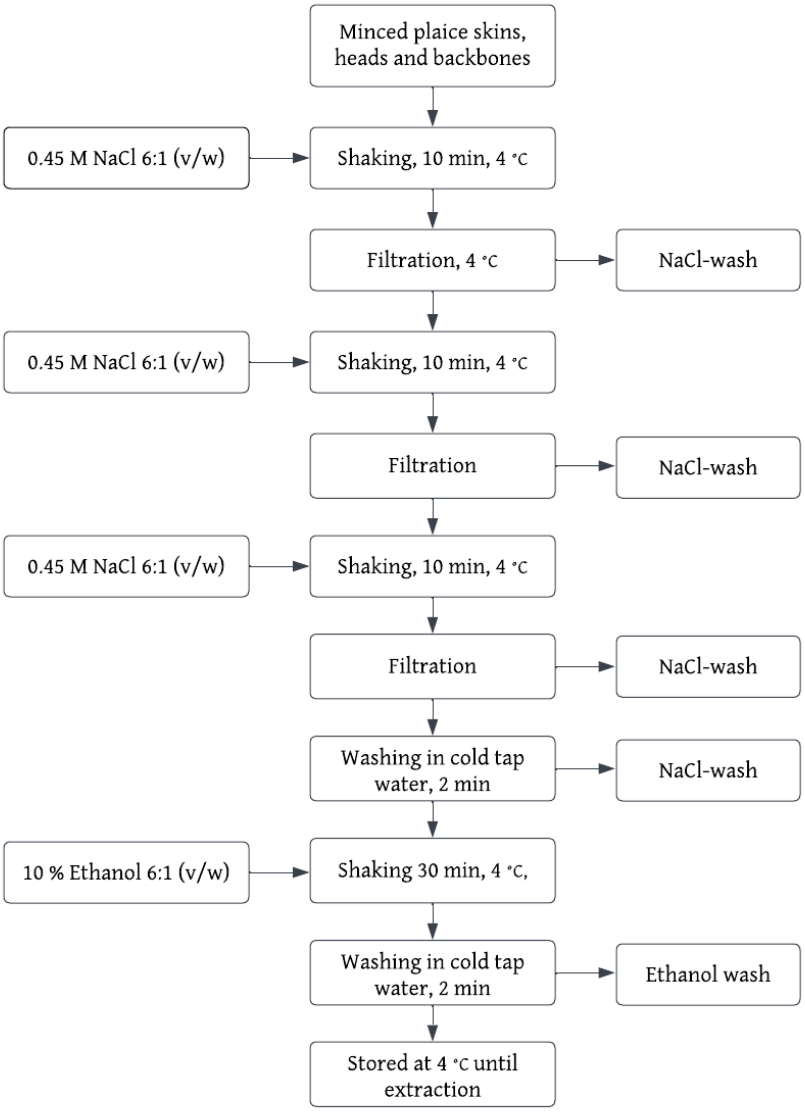

Figure 3.2: Flowchart illustrating the procedure of the salt pre-treatment of the rest raw material before extractions. The procedure is based on a method of Kolodziejska et al. with modifications from the master thesis of Alvarez [22] [35]

3.2.2 Enzymatic hydrolysis using Alcalase 2.4 L

The enzymatic hydrolysis was done together with PhD candidate Sophie Kendler. The method was following the procedure from the doctoral thesis of Veronica Hjellnes [36] using the peptidase Alcalase 2.4 L (Novozymes A/S, Bagsvaerd, Denmark) as catalyst for the hydrolysis. An Atlas bioreactor of model Scorpion Drive was used for the hydrolysis. The bioreactor contained a stirrer, a pH-probe and a temperature probe for measuring pH and temperature during the procedure.

An equal amount of pre-heated water (50 °C) and rest raw material was added to the reactor and the stirrer was turned on. When the mixture reached 50 °C, a portion of

the sample was taken out as a 0-sample (having no added enzyme) and deactivated in a 95 °C water bath for 5 minutes while stirring vigorously. Time and pH of the remaining sample was noted down. If pH was below 7.0, the pH was adjusted by adding NaOH. Alcalase 2.4 L was then added (0.1 % of the remaining sample weight) to the reactor and the stirring was started. A 30-sample was taken out after 30 minutes and inactivated in the same way as the 0-sample. pH was adjusted and the reactor turned on. After a total time of 60 minutes, the rest of the mixture was taken out and inactivated.

All samples were frozen at -40 °C in centrifuge tubes over night. The next day the samples were separated into the three fractions fat, hydrolysate and sediment. The total weight of each fraction was noted down for calculation of yield and the sediment and protein hydrolysate fractions were freeze dried for extraction and analysis. Both protein hydrolysate and sediment were analyzed for hydroxyproline content to decide which part to use for extraction.

3.3 Extractions

3.3.1 Water bath extraction

Both untreated and pre-treated material was extracted by water bath extraction. As for the salt pre-treatment, the water extraction procedure was based on the method of Kolodziejska et al. with modifications from the master thesis of Alvarez [22] [35]. Deionized water (6:1 v/w) was added to the material and shaken for 1 minute. The mixture was then put in an Erlenmeyer flask and extracted in water bath at 45 °C for 150 minutes, shaking every 30 minutes. After extraction, the material was centrifuged (9000 rpm, 10 min, 20 °C) and the sediments and soluble gelatin solution was separated and weighed. The gelatin solution was then freeze dried for analyses.

3.3.2 Microwave assisted extraction

Microwave assisted extraction was conducted with a MARS 6 microwave Digestion System (CEM Corporation, NC) instrument equipped with TFA digestion vessels. About 30-40 g raw material was homogenized in with a Kinematica Polytron PT3100 homogenizer (10 000 rpm, 1 min) and mixed with deionized water (6:1 v/w). For hydrolysates, 2-3 g freeze dried weight was used and deionized water was added according to six times the weight of the wet weight of the hydrolysate. When freeze dried material used, the hydrolysate and water mixture was not homogenized before extraction, but vortexed to mix the water and hydrolysate. After mixed, the material was added to the digestion vessels and a "Classic method" was run with with the parameters listed in Table 3.1.

Table 3.1: Parameters for microwave assisted extraction of the plaice rest raw material fraction using a Mars 6 microwave Digestion System (CEM, Corporation, NC). The parameters were applied on a "Classic" method.

Parameters for microwave extraction with Mars-6 Xpress	
Ramp time	5 minutes
Hold time	15/35 minutes
Temperature	40 ℃
Power	350 W
Stirring	On, medium speed

3.3.3 Gelatin extraction in ultra sound water bath

For the ultrasound water bath extraction, an Ultrasonic cleaning bath, USC-T (VWR) was used. The material was prepared in the same way as for the microwave extraction procedure, described in Section 3.3.2. Centrifuge tubes were used for extraction. The ultrasound water bath was pre-heated to 40 °C and the centrifuge tubes were placed in the water bath when the temperature reached 35 °C to obtain the ramp time of 5 minutes similar to the microwave extraction. After extraction, the samples were centrifuged (9000 rpm, 10 min, 20 °C) and the sediments and soluble gelatin solution separated and weighted. The gelatin solution was then freeze dried for further analyses.

3.4 Analyses

3.4.1 Protein determination by Kjeldahl method

The total protein content was determined by the Kjeldahl method, using a Digest Automat K-438 and a KjelMaster-375 with sample changer KjelSampler K-376 [37]. The method was also done during spring 2022 in the pre-project of this study, and the following method description is based on the method description from the specialization project.

About 0.5 g freeze dried material was weighted accurately on digestion papers which were crunched and put into the sample tubes. The two first tubes were blank with empty digestion papers and the third and last tube contained digestion paper with 1 g glycine as the reference substance. 15 mL of sulphuric acid (H_2SO_4) and two titanium tablets were added to each sample tube before the digestion was performed in the Digest Automat according to parameters from Table 3.2. After digestion, the rack with the samples was placed in the distillation apparatus of the KjelMaster. The program was set with accurate weight of each sample and run with program parameters listed in Table 3.3 and sample-specific protein parameter 6.25. The nitrogen and protein content were given as output values from the KjelMaster in percentage of wet weight.

Table 3.2: Temperature parameters for digestion with Digest Automat K-438 when determining crude protein content with Kjeldahl method [37].

Step	Temperature [°C]	Time
Preheating	320	-
1	420	120
Cooling	-	30

Table 3.3: Parameters for distillation and titration when determining crude protein content by the Kjeldahl method. A KjelMaster K-375 aparatus with KjelSampler K-376 was used for the analysis [37].

Method parameters KjelMaster K-375			
H_2O volume	50 mL	Titration solution	H_2SO_4, 0.1 mol/L
NaOH volume	60 mL	Sensor type	Potentiometric
Reaction time	5 s	Titration mode	Standard
Distillation mode	Fixed time	Measuring mode	Endpoint pH
Distillation time	180 s	Endpoint pH	4.65
Stirrer speed distillation	5	Stirrer speed titration	7
Steam output	100 %	Titration start volume	0 mL
Titration type	Boric acid	Titration algorithm	Optimal
Receiving solution vol.	50 mL		

3.4.2 Determination of total amino acid

Determination of total amino acid composition was performed based on the Blackburn method (1978) with acid hydrolysis [38]. The following description is repeated from the description of total amino acids from the pre-project of this master thesis, conducted during spring 2022 [39].

About 50 mg of freeze dried samples were weighted out in triplicates in glass tubes with screw top and flat ends. 1 mL 6 M HCl was added to each triplicate. Then, the tubes were placed with loose screw tops in a heating cabinet at 105 °C for 22 hours. The screw tops were tightened after 30 minutes. After cooling, the solutions were transferred to a 10 mL beaker glass and neutralized with NaOH. The solutions were filtered using suction through Whatman glass microfiltre GF/C. 10 mL of each filtered sample was transferred to a measuring flask before dilution to 1:500 with deionized water. The samples were again filtered through 0.22 µm filters and 0.205 mL of the solutions were transferred into vials for HPLC-analysis. The HPLC-analysis was performed by department engineer Siri Stavrum. The HPLC machine was a Dionex Thermo Scientific UltiMate 3000 system. A silica-based column of type Waters Nova-Pak C18 WAT086344 with size 3.9 mm x 159 mm based on 4 µm particle technology was used. The detector was a digital RF 2000 fluorescence detector.

3.4.3 Determination of free amino acids

To determine the free amino acid profile of the gelatin samples, the method of Osnes and Mohr (1985) was used[40]. Like for the total amino acid composition, Section 3.4.2, the description of determination of the free amino acids is a based on the description from the project conducted in spring 2022[39].

Water soluble protein extract was prepared by homogenizing 0.25 g freeze dried sample and 0.5 mL deionized water using a Kinematica Polytron PT3100 homogenizer (10 000 rpm, 1 min). The samples were then centrifuged and 1 mL of the supernatant was transferred to an Eppendorf tube. 0.25 mL sulphoasalisylic acid (10 % $C_7H_6O_6S$) was added and the samples were shaken vigourously. The samples were stored at 4 °C for 30 minutes before they were centrifuged for 10 minutes (10 000 rpm). After centrifuging, the supernatant was diluted (1:25) and filtered through 0.22 µm Syringe filters. Lastly, 0.205 mL filtered sample were transferred to vials for HPLC-analysis, which were performed by department engineer Siri Stavrum. The HPLC-instrument used is described in Section 3.4.2.

3.4.4 Determination of hydroxyproline and collagen content

The hydroxyproline content is based on the method Neuman and Logan, modified by Leach (1960)[41].

An L-hydroxyproline stock solution was made by dissolving 0.05 g hydroxyproline in 400 mL distilled water, adding 20 mL of concentrated HCl and adding distilled water to the volume reached 500 mL. From the stock solution, four standards of 2.5, 5.0, 10.0 and 15.0 µg hydroxyproline/mL was prepared.

Hydrolyzed samples made during determination of total amino acids, described in Section 3.4.2, was diluted 1:40 (skins) or 1:20 (heads and backbones) with deionized water. First, 0.5 mL blank, standards and diluted samples were mixed with 0.5 mL 0.05 M $CuSO_4$ and 2.5 M NaOH and heated in water bath (50 °C, 10 min). After heating in water bath, 0.5 mL 6 % H_2O_2 was added and shaken. After heating in water bath (50 °C, 10 min), the samples were cooled, 2 mL 1.5 H_2SO_4 and 1 mL 5 % *p*-dimethylbenzaldehyd in 1-propanol were added and the sample tubes were shaken immediately. A last heating in water bath (70 °C, 16 min) were conducted followed up by cooling and shaking. The standard and sample solutions should now be pink and the absorbances were measured against the blank at a spectrophotometer at 555 nm.

To calculate the concentration of hydroxyproline in each sample, a standard curve based on the absorbance of the standards was used, shown in Equation – in Appendix. The collagen content was then determined by multiplying the hydroxyproline concentration

with the collagen factor for flounder, which is found to be 10.417 by Sikorski et al. (1985) [42].

3.4.5 Determination of molecular size by SDS-PAGE

The molecular size distribution of the freeze dried gelatin samples were found using a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) Dual Cool Electrophoresis System to separate peptides based on size in a polyacrylamide gel. The principle behind the SDS-PAGE is that the smaller peptides migrate faster than the larger in a uniform voltage and at constant pH 8.

The freeze dried samples were dissolved in deionized water to a concentration of 0.5 mg/mL and 1.0 mg/mL before it was mixed with an NuPAGE LDS sample buffer containing 400 mM DL-Dithiothreitol. After mixing, the samples were placed in water bath (70 °C, 10 min), cooled, added 10 % glycerol and mixed. Then, 4-12 % NuPAGE Bis-Tris gels were placed in electrode chambers. SDS running buffer made of 40 mL MOPS SDS Running Buffer buffer (NuPAGE) and 760 deionized water was filled into the inner and outer chamber. The samples (10 µL) and a PageRuler Broad Range Unstained Protein Ladder standard (Thermo Fisher Scientific Inc.) were loaded to the wells of the gels before the gel electrophoresis was run at 200 V on a Hoefer PS300B power supply (Hoefer Inc.). After the gel electrophoresis was finished, the gels were stained using Bromophenol blue in a genscript to visualize the peptides in the gels.

3.5 Statistics

Chemical analyzes of the raw material was performed in triplicates (n=3). Differences between the groups were tested by two factor t-test in Microsoft Excel. Data from the analyzes were tested by General Linear Model (GLM) in SPSS statistics , using Tukey's test as the post-hoc comparison test ($P<0.05$).

4 Results and discussion

In this study, the yield and quality of gelatin from plaice rest raw material fractions, pre-treated and extracted with various procedures, was evaluated. The following parameters during the procedures were conducted:

- **Rest raw material fractions**: Skins, heads, backbones
- **Pre-treatments**: No pre-treatement, salt wash, enzymatic hydrolysis
- **Extraction methods**: Microwave assisted extraction (MWAE), ultrasound water bath extraction (UWAE) and water bath extraction
- **Extraction times**: 15 and 35 minutes (MWAE and UWAE), 150 minutes (water bath)

The yield and quality was evaluated by looking at the collagen content, protein content, amino acid profiles and molecular size. The effects of procedures on the protein and collagen purity were tested by applying a General Linear Model (GLM) using Tukey's multiple comparison test with significance level of 95 % ($p<0.05$) to compare the difference within the groups.

4.1 Pre-treatments

Before the extraction processes, all pre-treated material was analyzed for protein and hydroxyproline content to look at the independent effect of each pre-treatment.

4.1.1 Enzymatic hydrolysis

The soluble protein hydrolysate and sediments from the enzymatic hydrolysis were analyzed for hydroxyproline content which was further converted into collagen content by the conversion factor for flounder, explained in Section 3.4.4, to determine which part to use for extraction. The reason for analyzing both the sediment and protein hydrolysate was to examine which part the collagen content was highest to determine what part use for gelatin extraction. Table 4.1 shows the collagen content from the sediment and protein hydrolysate after 60 minutes of enzymatic hydrolysis.

The collagen content in the sediment fractions of skins (28.3 ± 3.0 %) and heads (13.2 ± 4.8 %) were significantly lower ($p<0.05$) than for their respective protein fraction with protein content of 52.1 ± 13.0 % and 34.6 ± 12.1 % for skins and heads, respectively. For backbones, the collagen content did not vary significantly ($p>0.05$) between the sediment (12.0 ± 1.7 %) and protein hydrolysate fraction (26.2 ± 6.4 %). Since the intramolecular bondings holding the collagen structure together, it would be expected that all the collagen should be found in the sediment fraction if the hydrolysis was conducted at low

temperature. During the hydrolysis, the temperature rose above 50 °C, which is above the degradation temperature of collagen, which means that most likely, the collagen was turned into gelatin during the hydrolysis. The reason for why not 100 % of the collagen was broken down to gelatin, seen from the amount of collagen in sediment fraction, could be due to that 60 minutes of hydrolysis was not sufficient to turn all the collagen into gelatin. Due to the general highest collagen content, the protein hydrolyates from all the raw material fractions was chosen for extractions conducted in the project.

Table 4.1: Collagen content of sediment and protein fraction obtained from enzymatic hydrolysis. The statistical analyses was performed in triplicates (n=3). Different superscript letters (a, b, c) indicates significantly different values ($p<0.05$).

	Collagen content [%dw.]	
	Sediment	Protein
Skins	$28.31^{bc} \pm 3.00$	$52.07^{a} \pm 13.00$
Backbones	$12.03^{c} \pm 1.73$	$26.21^{bc} \pm 6.38$
Heads	$13.16^{c} \pm 4.76$	$34.63^{ab} \pm 12.06$

4.1.2 Protein and collagen content of pre-treated raw material

Table 4.2 shows the collagen and protein content of freeze dried raw material not pre-treated, pre-treated with salt wash and protein hydrolysates obtained from enzymatic hydrolysis. The protein content of not pre-treated material was found in spring 2022 [39].

Table 4.2: Collagen and protein content in plaice backbones, skins and heads with no treatment, salt treatment and protein hydrolysates from the respective fractions. The standard deviation is calculated with n=3 for all samples except the protein content for backbones, skins and heads with no pre-treatment that were calculated with n=4, 2 and 4, respectively.

	Protein content [% dw.]	Collagen content [% dw.]
No pre-treatment		
Backbones	63.19 ± 0.40	18.71 ± 1.12
Skins	82.84 ± 0.83	32.54 ± 0.36
Heads	62.61 ± 0.08	12.73 ± 0.18
Salt pre-treatment		
Backbones	58.27 ± 2.06	32.48 ± 0.94
Skins	73.82 ± 0.16	72.52 ± 5.98
Heads	63.84 ± 0.18	29.81 ± 0.90
Hydrolysates		
Backbones	84.58 ± 0.11	26.21 ± 6.38
Skins	96.57 ± 0.07	44.62 ± 2.10
Heads	86.41 ± 0.48	27.97 ± 4.99

4.2 Collagen yield

The collagen yields were calculated based on the weight of collagen in the freeze dried gelatin compared to the dry weight of the raw material used for extraction, shown in Equation A.1 to A.4 in Appendix A. The results are presented according to the pre-treatment used. Figure 4.1 shows the yields obtained for the different raw materials with no pre-treatment, Figure 4.2 shows the yield for the gelatin of raw material pre-treated by salt washing, marked "salt" and Figure 4.3 shows the yields from gelatin extracted from protein hydrolysates, marked "EH". For the salt pre-treated skins with water extraction, the yield is not representative due to loss of material before freeze drying. Additionally, the sample material from head hydrolysates were too small to conduct the microwave extraction for 15 minutes, which leads to missing results from this sample.

As gelatin is composed of dissolved collagen molecules, the collagen yield plays an important role for knowledge about gelatin yield. A higher yield shows more efficient extraction and thus a more profitable product, making the material valuable for gelatin extraction. For the raw materials, the highest collagen yield for any pre-treatment, extraction method and extraction time, was found for skins, with highest yield of 50.60 % (EH UWAE15). Backbones gave in general higher collagen yields than the heads where the highest yields were 24.25 % (EH MWAE15) and 19.30 % (EH MWAE15) from backbones and heads, respectively.

The leap between the yields was clearly larger between skins and heads and backbones than between heads and backbones. When looking at the collagen and protein content from Table 4.2 in Appendix A, the differences in yields are corresponding with the differences in collagen content. A higher yield in backbones gelatin compared to the heads corresponds to the findings on gelatin yield Kolodziejska et al. found in a study on salt pre-treated backbones (74 $\pm$ 10.2 %) and heads (57 $\pm$ 2.4 %) from cod extracted in water bath for 120 min at 45 ℃. However, the difference in collagen yields for heads and backbones were closer for the rest raw material fractions in the present study than for the findings of Kolodziejska. The high yield for skin in the can also be due to the form the material when performing the extraction. Unlike the heads and backbones, the skin raw material was homogeneous and viscous, and a little amount of material went through the filter during the salt wash procedure. The heads and backbones consisted of several particles due to the presence of bones, flesh and tissues, which more easily went through the filter. This loss of particles could contribute to less protein.

For the pre-treatments, there is a clear tendency showing that the enzymatic hydrolysis had an effect on the yield. All raw materials gave the highest collagen yield when extracting from hydrolysates. The yields from the hydrolysates were calculated in a different way (Equation A.4) than for the other pre-treatments, which affect the ability to compare

the yields. The salt extraction gave the lowest yield for all raw material fractions, which can be due to loss when filtering during the salt wash and ethanol wash.

For the extraction methods, gelatin from both no pre-treated and salt pre-treated skins had the highest yields (41.05 % and 27.95 %, respectively) when extracted in microwave for 35 minutes. Gelatin extracted from hydrolysates had a more even yield distribution across extraction methods. This can be due to different calculations when calculating the yield from the hydrolysates.

The extraction times had an effect of the collagen yields for the gelatin samples extracted from not pre-treated and salt pre-treated skins. It is clear that for both microwave extraction and ultrasound assisted extraction, the yields increased after 35 minutes, but the yield was not affected after 15 minutes of extraction compared to water bath. Increasing yield with increasing time corresponds with the results in a study done by Feng et al. (2022) where pre-treatment by microwave for a longer time (60 min) gave higher yield [24].

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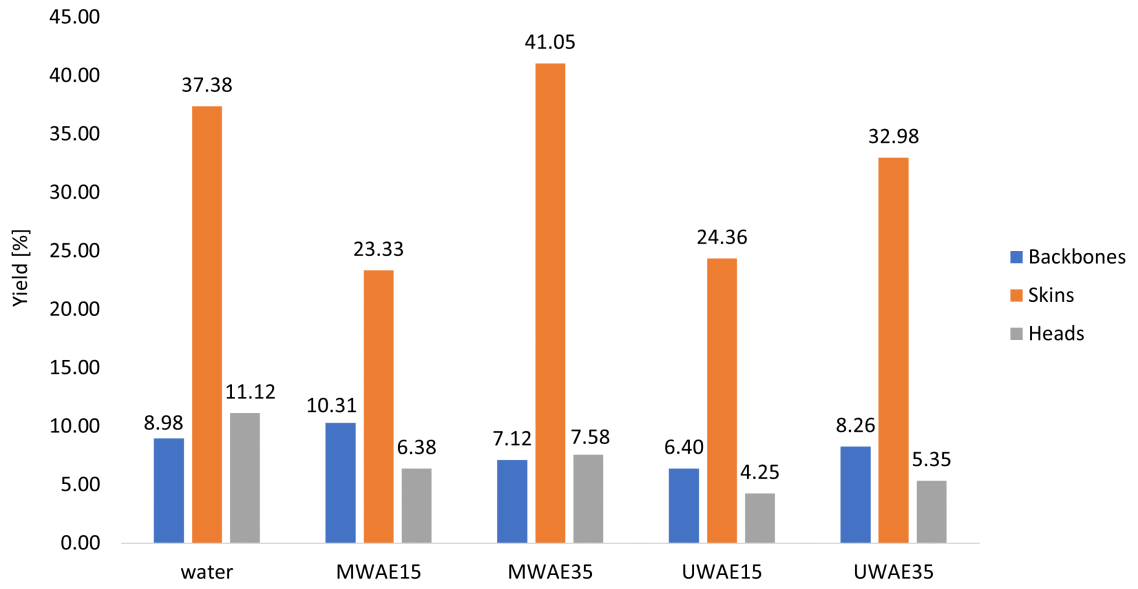

Figure 4.1: Collagen yield for gelatin samples with no pre-treatment. "water150" indicates gelatin extracted in water bath for 150 minutes, "MWAE15" and "MWAE35" are gelatin extracted in microwave for 15 and 35 minutes, respectively, and "UWAE15" and "UWAE35" are gelatin extracted in ultrasound water bath for 15 and 35 minutes, respectively.

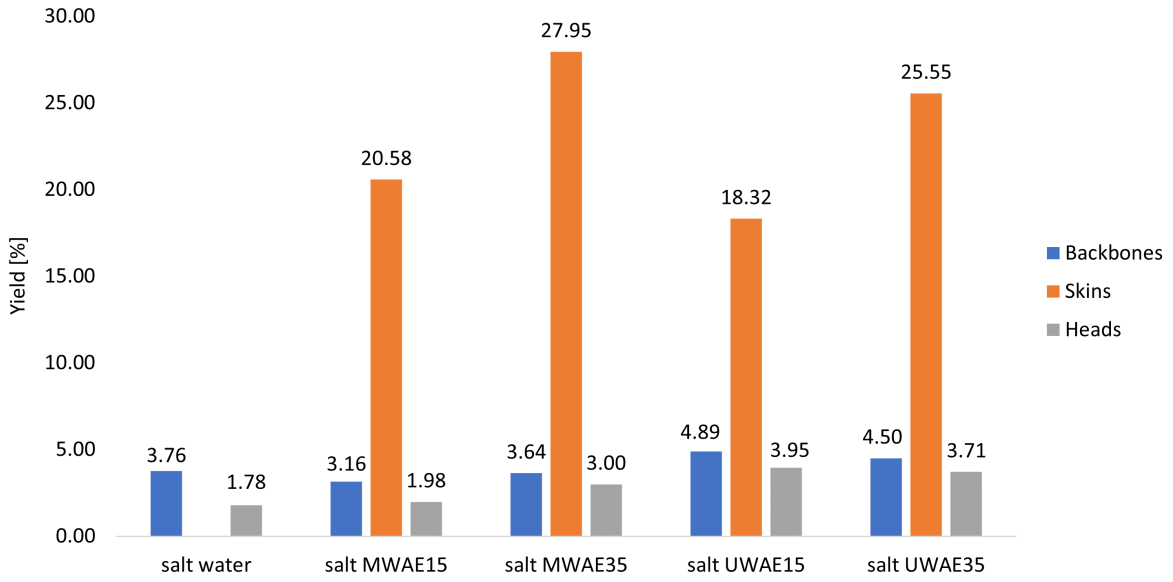

Figure 4.2: Collagen yield for gelatin samples with salt pre-treatment. "water150" indicates gelatin extracted in water bath for 150 minutes, "MAE15" and "MAE35" are gelatin extracted in microwave for 15 and 35 minutes, respectively, and "UWAE15" and "UWAE35" are gelatin extracted in ultrasound water bath for 15 and 35 minutes, respectively.

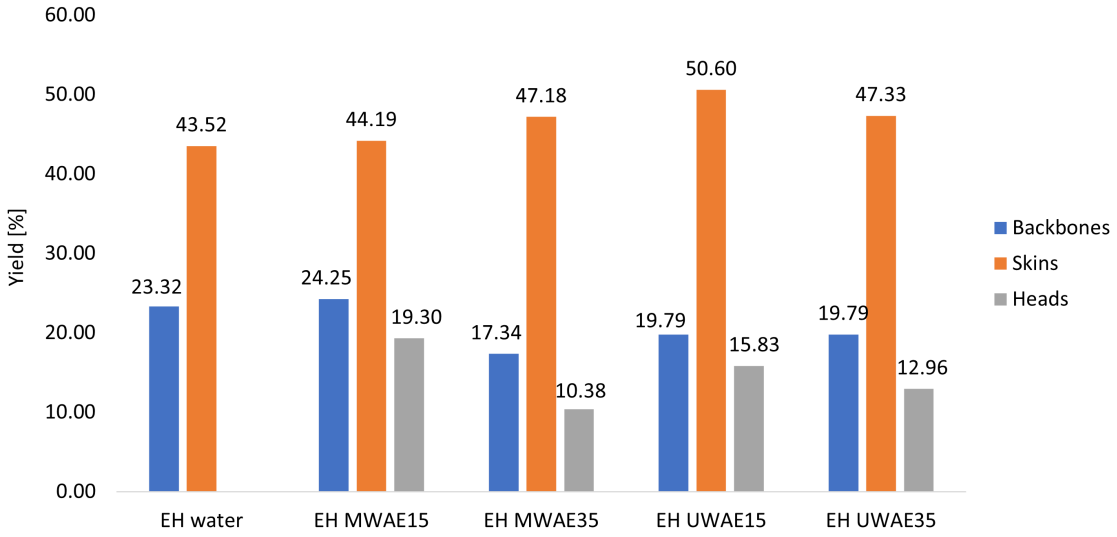

Figure 4.3: Collagen yield for gelatin samples extracted from hydrolysates. "water150" indicates gelatin extracted in water bath for 150 minutes, "MWAE15" and "MAE35" are gelatin extracted in microwave for 15 and 35 minutes, respectively, and "UWAE15" and "UWAE35" are gelatin extracted in ultrasound water bath for 15 and 35 minutes, respectively.

4.3 Collagen purity

Table 4.3 presents the effect the four factors raw material, pre-treatment, extraction method and extraction time had on the collagen purity on the freeze dried gelatin. Since the water bath extraction was meant to be used as a standarized extraction method to

compare to the two other extraction methods, only one water bath extraction of 150 minutes was performed, and the 150 minutes is only representative for the water bath extraction.

The collagen purity varied significantly ($p<0.001$) within the raw material fractions, where the highest average collagen content was found in skin (65.3 ± 15.5 %), and a lower content were found in backbones (32.9 ± 9.43 %) and heads (30.9 ± 9.9 %). The pre-treatments was also affecting the collagen purity significantly where pre-treatment by hydrolysis the highest (47.5 ± 18.4 %) and no pre-treatment gave the lowest purity (39.6 ± 21.0 %). For the extraction methods, microwave extraction and ultrasound water bath did not influence the collagen purity, but the water bath gave significant higher purity ($p<0.001$) than for the two other extraction methods. The collagen purity was not influenced before after 35 minutes of extraction with average collagen content of 48.8 ± 16.2 % for 35 minutes and 47.3 ± 23.7 % after 150 minutes. For the water bath, it is not possible to tell whether it is the extraction method or extraction time that influenced the purity since the water bath was not run for 15 and 35 minutes. However, it is clear that the extraction time had an influence for the two other extraction methods, and that the ultrasound water bath or microwave assisted extraction for 35 minutes did not differ significantly ($p<0.05$) from collagen content as for a water bath of 150 minutes. The similarity between the 35 minutes of green extraction and 150 minutes water bath shows that the green extraction methods are more time efficient with respect to collagen content than the water bath.

In addition, since it is most common to extract gelatin for 2-5 hours (120-300 min) it is conceivable that the time rather was influencing the collagen content for the water bath. Testing out microwave extraction and ultrasound water bath for a longer time could therefore be relevant to investigate whether increased time will increase the collagen content. To improve the experiment, a water bath of 15 and 35 minutes could be included to see how the microwave and ultrasound affected the collagen content independently of time.

Table 4.3: Main effect of the experimental factors (skin, heads, backbones), pre-treatments (salt washing, enzymatic hydrolysis and no pre-treatment) and extraction methods (microwave, ultrasound water bath, and normal water bath) on collagen content of freeze dried gelatin samples.

	Collagen content [% of freeze dried gelatin]	n
Raw material		
Skins	$65.25^{a} \pm 15.52$	53
Heads	$30.85^{c} \pm 9.90$	45
Backbones	$32.94^{b} \pm 9.43$	53
Effect of raw material (p-value)	<0.001	
Pre-treatment		
Salt wash	$44.00^{b} \pm 19.93$	50
Enzymatic hydrolysis	$47.45^{a} \pm 18.38$	50
No pre-treatment	$39.61^{c} \pm 20.95$	51
Effect of pre-treatment (p-value)	<0.001	
Extraction method		
No extraction	$38.41^{c} \pm 23.61$	25
Microwave	$46.94^{a} \pm 20.31$	50
Ultrasound water bath	$40.94^{b} \pm 14.02$	49
Water bath	$47.37^{a} \pm 23.73$	27
Effect of extraction method (p-value)	<0.001	
Time of extraction		
No extraction	$38.41^{b} \pm 23.61$	25
15 min	$39.82^{b} \pm 17.98$	53
35 min	$48.75^{a} \pm 16.18$	46
150 min	$47.37^{a} \pm 23.73$	27
Effect of extraction time (p-value)	<0.001	

4.4 Total protein content

Table 4.4 presents the effect the factors had on the total protein content in the gelatin samples. The total protein content compared to the collagen content in the samples will give an indication on the amount of not-collagen proteins present. A high protein content and low collagen content can indicate impurities present in the samples.

All experimental factors had effect on the protein content, which is similar to the collagen content. For the raw material, the skins showed the highest protein content of (90.1 ± 7.65 %). The backbones gave significantly higher ($p<0.001$) protein content (79.1 ± 9.0%), but lower collagen content (32.9 %) compared to heads with protein content of 76.5 ± 11.5 %. For the pre-treatments, the statistical analysis showed that pre-treatment with salt wash gave significant higher ($p<0.001$) protein content (79.5 ± 11.2%) than for the not pre-treated material. Additionally, enzymatic hydrolysis increased the protein content

significantly ($p<0.001$). As for the collagen content, the extraction time influenced the protein content after 35 minutes for both microwave and ultrasound extraction. It should again be mentioned that the normal water bath was only set for 150 minutes which means that it is not possible to tell whether the water bath or extraction time had an influence on the extraction.

The high collagen and protein concentration in the salt pre-treated material indicates that the general protein purity is higher for the salt pre-treated material. By putting the average collagen content and average protein content up against each other, it can be seen that the salt pre-treatment increased ($p<0.001$) the collagen content from no pre-treatment to salt pre-treatment, but that the salt pre-treatment and no pre-treatment gelatin did not differ significantly ($p>0.05$) for protein content. The high protein purity in material treated with salt might be due to the salt treatment procedure removing non-protein impurities because of the washing process. This can be further investigated by examine the ash content of the samples to see whether minerals are removed. However, gelatin from salt extraction usually contain minerals as salt. It would also be expected that the salt pre-treatment removed salt soluble proteins due to the decrease of isoelectric point [43].

Table 4.4: Main effect of the experimental factors raw materials (skin, heads, backbones), pre-treatments (salt washing, enzymatic hydrolysis and no pre-treatment), extraction methods (microwave, ultrasound water bath, and water bath (water)) and time (15, 35, 150 min) had on protein content of freeze dried gelatin samples.

	Protein content [% of freeze dried gelatin]	n
Raw material		
Skin	$90.11^{a} \pm 7.65$	52
Heads	$79.07^{b} \pm 8.97$	49
Backbones	$76.51^{c} \pm 11.52$	52
Effect of raw material (p-value)	<0.001	
Pre-treatment		
Salt wash	$79.48^{b} \pm 11.23$	54
Enzymatic hydrolysis	$86.71^{a} \pm 9.69$	48
No pre-treatment	$79.66^{b} \pm 11.22$	51
Effect of pre-treatment (p-value)	<0.001	
Extraction method		
No extraction	$75.91^{d} \pm 11.13$	24
Microwave	$83.03^{b} \pm 10.57$	52
Ultrasound water bath	$79.14^{c} \pm 11.95$	50
Water bath	$89.91^{a} \pm 3.97$	27
Effect of extraction method (p-value)	<0.001	
Extraction time		
No extraction	$75.91^{c} \pm 11.13$	24
15 min	$76.92^{c} \pm 13.13$	54
35 min	$86.15^{b} \pm 6.24$	48
150 min	$89.91^{a} \pm 3.97$	27
Effect of extraction time (p-value)	<0.001	

4.5 Amino acid compositions

The total and free amino acid compositions were analyzed by HPLC to obtain information about the purity, and physical and chemical properties of the gelatin samples.

4.5.1 Effect on sum of total amino acids

The sum on total amino acids will give indications of the protein purity of the gelatin samples where pure gelatin samples are expected to contain 1000 mg amino acids/g sample. Table 4.5 shows the statistical effect the extraction parameters had on the sum of total amino acids present in the samples. None of the samples was composed of pure gelatin where the highest content was found as 820.2 ± 73.1 mg AA/g sample in gelatin extracted from skin hydrolysate in ultrasound water bath for 35 min and the lowest content was 355.2 ± 31.3 mg AA/g sample in gelatin from not pre-treated

heads extracted in ultrasound water bath for 15 minutes. This can indicate non-protein impurities in the sample, as suggested for the protein content or a high amount of free amino acids indicating degradation of the proteins.

The effect Table, Table 4.5, shows no significant variations ($p=0.133$) between the average amino acid content in gelatins from skins, heads and backbones. Furthermore, it shows that the pre-treatments salt wash and hydrolysis gave significantly ($p<0.05$) higher average contents of amino acids than from the not pre-treated, with 611.1 ± 165.9 and 551.7 ± 253.5 mg AA/g sample for salt wash and hydrolysis, respectively. The extraction methods did not affect ($p=0.914$) the amino acid contents within the extraction methods. However, ultrasound water bath (575.0 ± 187.1) and microwave (574.3 ± 173.6) increased the content significantly ($p<0.05$) from not extracted material (470.7 ± 104.3). For the duration of the extractions, 35 minutes gave significantly ($p<0.05$) higher amino acid purity (590.0 ± 187.1) than 150 minutes (494.8 ± 277.1), and 35 minutes did not differ ($p<0.05$) from 15 minutes (559.0 ± 181.0). Interestingly, the amount of amino acids decreased ($p<0.05$) when the time of extraction went from 35 to 150 minutes, and the 150 minutes and 15 minutes did not differ significantly ($p<0.05$). This can indicate that the proteins are more degraded when extracted for a longer time. Additionally, 35 minutes of extraction still gives higher amino acid purity than not extracted samples. However, high standard deviation (± 277.1) for the 150 minutes, makes it too uncertain to conclude. To determine what time of extraction that gives the highest purity, more extraction times can be investigated for the two green extraction methods.

The sum of total amino acids are lower when looking at the ratio between the given amino acids and 100 mg compared to the protein content in Table 4.4 in Section 4.4. For instance was the total protein content of skin 90.11 %, while the sum of amino acids only showed a ratio of 582.8/1000 giving 58.2 % total amino acids per g sample. Since the Kjeldahl method is based on the amount of nitrogen in the sample, the not corresponding content of amino acids and proteins can indicate a high amount of free amino acids and non-protein nitrogen compounds present in the samples. Mariotti et al. (2008) have also discussed the that the Kjeldahl conversion factor should be lowered, which also could make the total amino acid content to be closer to the protein content [44].

Table 4.5: Main effect of the experimental factors(skin, heads, backbones), pre-treatments (salt washing, enzymatic hydrolysis and no pre-treatment), extraction methods (microwave, ultrasound water bath, and water bath) and extraction times (15, 35 and 150 min) on sum of total amino acids of freeze dried gelatin samples.

	Sum of total amino acids [mg amino acid/g freeze dried sample]	n
Raw material		
Skins	$582.8^{a} \pm 219.5$	53
Heads	$523.8^{a} \pm 186.3$	53
Backbones	$531.8^{a} \pm 171.5$	53
Effect of raw material (p-value)	0.133	
Pre-treatment		
No pre-treatment	$473.1^{b} \pm 111.9$	52
Salt wash	$611.1^{a} \pm 165.9$	54
Enzymatic hydrolysis	$551.7^{a} \pm 253.5$	53
Effect of pre-treatment (p-value)	<0.001	
Extraction method		
No extraction	$479.7^{b} \pm 104.3$	25
Microwave	$574.3^{a} \pm 173.6$	53
Ultrasound water bath	$575.0^{a} \pm 187.1$	54
Water bath	$494.8^{ab} \pm 237.8$	27
Effect of extraction method (p-value)	0.914	
Time of extraction		
No extraction	$479.7^{b} \pm 104.3$	25
15 min	$559.0^{ab} \pm 181.0$	53
35 min	$590.0^{a} \pm 178.7$	54
150 min	$494.8^{b} \pm 277.1$	27
Effect of extraction time (p-value)	0.245	

4.5.2 Total amino acid compositions

This section will discuss the amino acid compositions of gelatins extracted for 35 minutes, that is the time that showed to influence the collagen and amino acid contents, discussed in Section 4.5 and 4.5.3. To investigate the results from the 35 minutes microwave and ultrasound water bath extractions, the total amino acid compositions of gelatin samples extracted from not pre-treated, salt treated and hydrolysates from skins, heads and backbones are presented.

The total amino acid composition of the gelatin samples are presented according to raw material fraction in Figure 4.4 (skins), Figure 4.5 (backbones) and Figure 4.6 (heads). Common for all fractions is the abundant amount of glycine/arginine (Gly/Arg). Glutamic acid (Glu), Aspartic acid (Asp), Serine (Ser) and Alanine (Ala) had also a higher concentration than the other amino acids present in the samples. The higher content of

Gly, Asp, Ala and Ser corresponds to the distribution of amino acids found in collagen type I and cold fish collagen from Table 2.1 in Section 2.2, but glutamic acid was more dominating in the gelatin from the present study than for the general cold fish collagen. This can indicate a higher amount of other proteins than collagen present in the samples, which is reasonable if the average collagen contents are varying from 30.85 ± 9.90 (heads) to 65.25 ± 15.52 % (skins) seen in Table 4.3 in Section 4.3. To know more precisely what amino acids that were the dominating in gelatin samples from the present study, the data from amino acid compositions should be analyzed statistically.

The distribution of the total amino acids seemed to be somewhat similar across all the raw material fractions. For the backbones (Figure 4.5) and heads (Figure 4.6), the concentration of glycine was higher for microwave extraction than ultrasound bath than for the skins (Figure 4.4. From the effect of collagen content, Table 4.3, it can be seen that extraction in microwave gave higher ($p<0.001$) average collagen content that the ultrasound water bath. By comparing the amino acid content with the collagen content, it can be conceivable that the microwave only affects salt pre-treated heads and backbones and that the ultrasound water bath influence the collagen content of salt pre-treated skins. However, this does not apply to the gelatin extracted from hydrolysates or untreated material of any of the raw material fractions. For hydrolysates and not pre-treated material, the glycine content was somewhat similar between the two extraction methods, except from for the hydrolysates from untreated heads where the microwave gave higher glycine concentration.

When looking at the amount of hydrophobic (Gly, Ala, Val, Leu, Ile, Pro, Phe, Met, Trp) and hydrophilic (Tyr, Glu, Thr, Ser, Asp), the general amount of hydrophobic amino acids seemed to be higher for hydrolysates than for the not pre-treated samples. The presence of hydrophobic amino acids can improve foaming abilities, found in a study on gelatins from fish skins and calf hides by Alfaro et al. (2015)[45]. Foaming abilities are beneficial in the food industry and can be applicated for a various of foods, like in gums and different beverages[10]. Furthermore, in a study on foaming stability of gelatin, gelatin from fish showed better foaming stability than from beef skin[46]. It should be mentioned that the study was done on warm water fish, and that the structure and amino acid compositions varies between fish species. It could still be relevant to look deeper into the foaming abilities of the plaice gelatin.

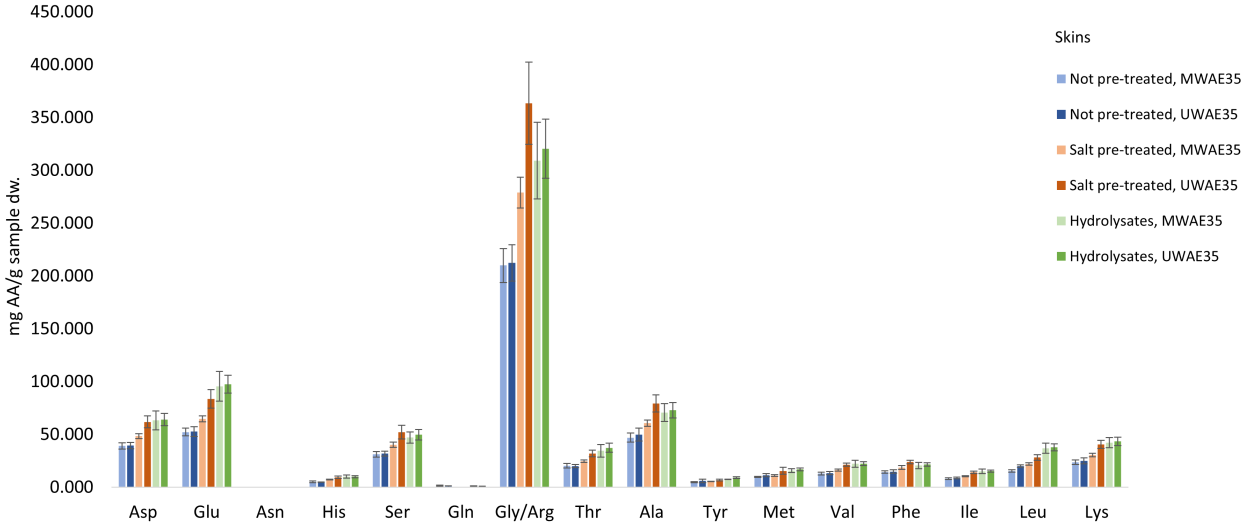

Figure 4.4: Composition of total amino acids of gelatin from skins that are not pre-treated, salt treated and treated by hydrolysis and extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35).

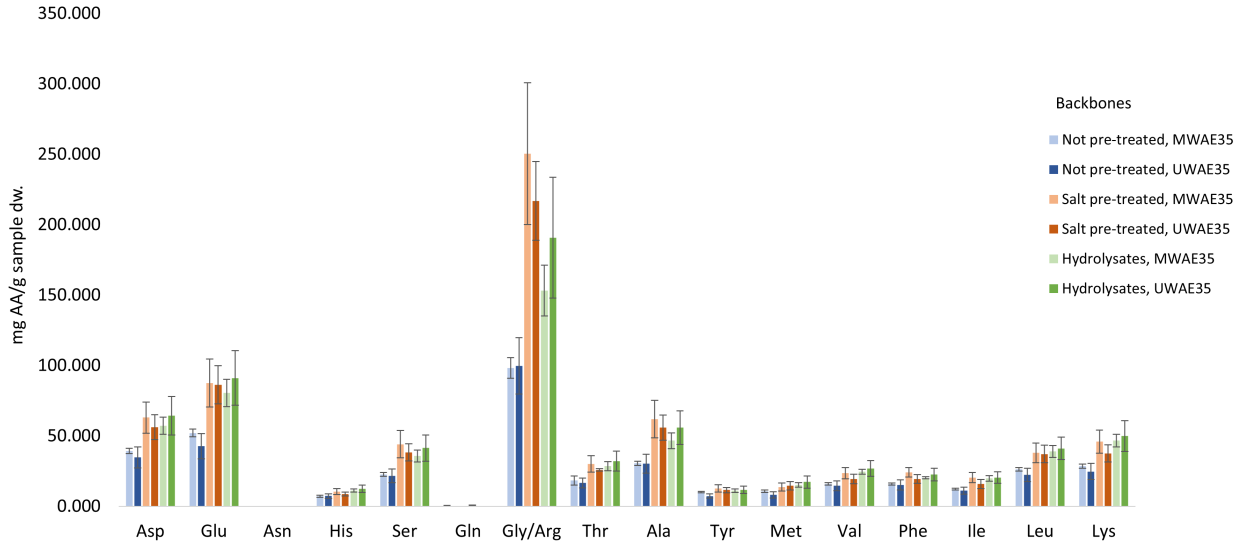

Figure 4.5: Composition of total amino acids of gelatin from backbones that are not pre-treated, salt treated and treated by hydrolysis and extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35).

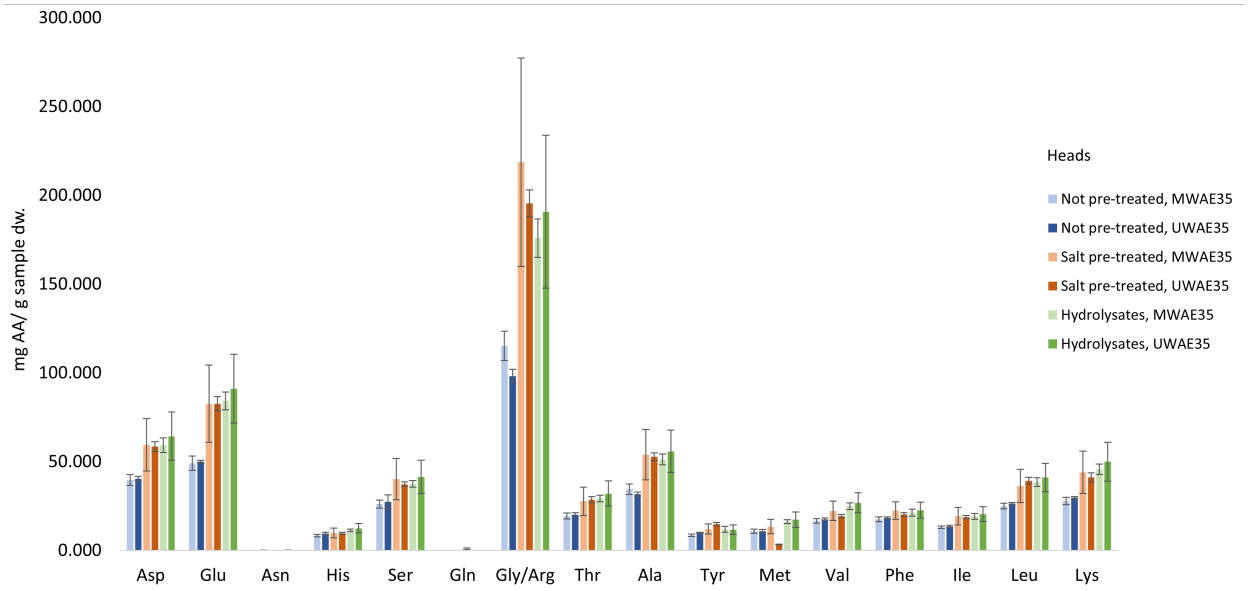

Figure 4.6: Composition of total amino acids of gelatin from heads that are not pre-treated, salt treated and treated by hydrolysis and extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35).

4.5.3 Sum of free amino acids

The effect on the sum of free amino acids in the gelatin samples are presented in Table 4.6. A high amount of free amino acids present in the sample indicates degradation of the polypeptide chains. The sum of free amino acids are in general affected by oxidation . In addition, free amino acids are important for physical properties, like taste and smell. In general, all the factors had effect on the sum of free amino acids present in the samples. Gelatin from backbones had the highest ($p<0.001$) amount of free amino acids of 28.03 $\pm$ 23.02 %. Enzymatic hydrolysis was the pre-treatment giving the highest ($p<0.001$) amount of free amino acids (37.29 $\pm$ 20.27 %), while salt pre-treatment gave the lowest (7.25 $\pm$ 10.27 %, $p<0.001$). For the extraction methods, ultrasound water bath gave higher ($p<0.001$) amount of free amino acids (23.78 $\pm$ 19.21) than gelatin extracted in microwave (16.79 $\pm$ 16.01 %) and water bath (19.82 $\pm$ 18.94 %).

Higher content of amino acids in gelatin extracted in ultrasound water bath indicate that ultrasound water bath leads to higher degradation of proteins. The microwave did not seem to affect the amount of free amino acids from the not extracted samples ($p>0.05$). Extractions that lasted for 35 minutes (21.33 $\pm$ 18.12 %) gave higher ($p<0.001$) concentration of free amino acids than the 150 minutes which could be due to the ultrasound bath influencing release of free amino acids. Lower temperature (4 °C) when conducting the salt pre-treatment and higher temperature (50 °C) during the enzymatic hydrolysis could be the reasons for the varying free amino acid contents between between the two pre-treatments. The temperature increase during the hydrolysis have likely led to protein degradation and release of free amino acids.

Table 4.6: Main effect of the experimental factors (skin, heads, backbones), pre-treatments (salt washing, enzymatic hydrolysis and no pre-treatment) and extraction methods (microwave, ultrasound water bath, and normal water bath) on sum of free amino acids of freeze dried gelatin samples.

	Sum of free amino acids [mg amino acid/g freeze dried sample]	n
Raw material		
Skins	$12.70^{c} \pm 10.55$	54
Heads	$17.63^{b} \pm 17.43$	54
Backbones	$28.03^{a} \pm 23.02$	54
Effect of raw material (p-value)	<0.001	
Pre-treatment		
Salt wash	$7.25^{c} \pm 10.27$	54
Enzymatic hydrolysis	$37.29^{a} \pm 20.27$	54
No pre-treatment	$13.82^{b} \pm 6.72$	54
Effect of pre-treatment (p-value)	<0.001	
Extraction method		
No extraction	$15.78^{c} \pm 21.90$	27
Microwave	$16.79^{c} \pm 16.01$	54
Ultrasound water bath	$23.78^{a} \pm 19.21$	54
Water bath	$19.82^{b} \pm 18.94$	27
Effect of extraction method (p-value)	<0.001	
Time of extraction		
No extraction	$15.78^{c} \pm 21.90$	27
15 min	$19.23^{b} \pm 17.88$	54
35 min	$21.33^{a} \pm 18.12$	54
150 min	$19.82^{b} \pm 18.94$	27
Effect of extraction time (p-value)	<0.001	

4.5.4 Free amino acid composition

The free amino acids profiles of gelatin samples extracted in microwave and ultrasound water bath for 35 minutes from skins, heads and backbones are presented in Figure 4.7, 4.8 and 4.9, respectively. The variations of distributions reflect the high standard deviations in the sample sizes in the effect table, when comparing the sum of free amino acids in Table 4.6 with the distributions in Figure 4.7 to 4.9,

Figure 4.7 shows that hydrolysates extracted in microwave gave gelatin with highest amount of each amino acid. This does not correspond to the other samples extracted in microwave or to the results from Table 4.6, where microwave extraction showed no statistical effect on the release of free amino acids. On the days the experiments were done, several extractions were done which caused some fractions to wait in room temperature. Exposing the material to room temperature for a longer time can lead to release of free

amino acids. For the not pre-treated and salt pre-treated skin gelatin, the amount of free amino acids was relatively low.

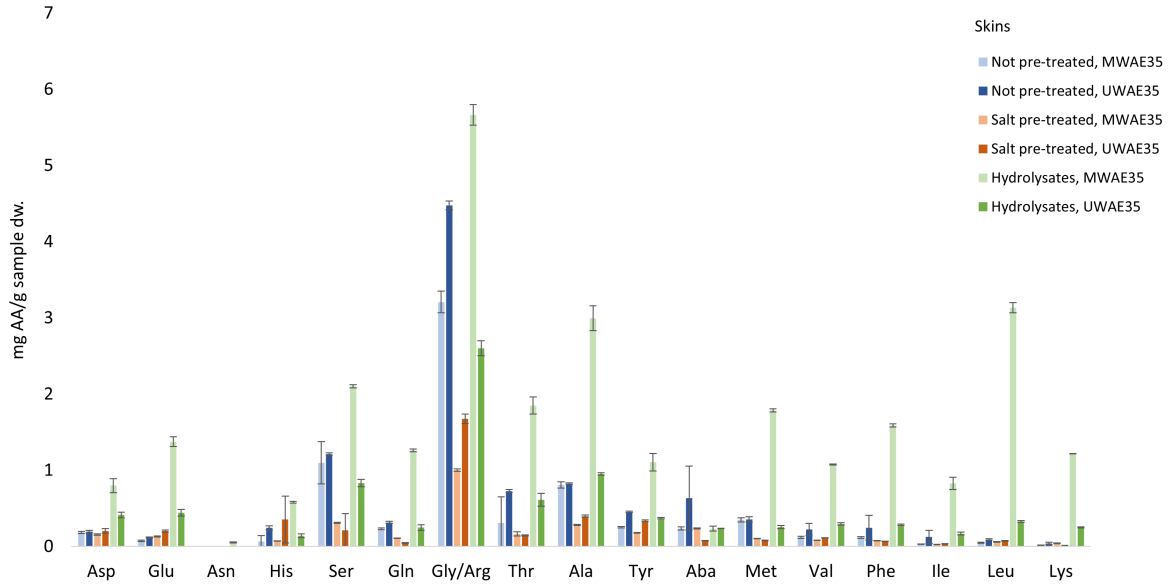

Figure 4.7: Free amino acid profiles for freeze dried gelatin samples from not pre-treated, salt pre-treated and hydrolysates from plaice skins, extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35). The chemical analyses were conducted in triplicates (n=3).

For the gelatin extracted from backbones, Figure 4.8, the amount of free amino acid was higher than for the skins, which also can be seen from Table 4.6. Higher content of free amino acids contribute to odour and color changes which is not suitable for industry and gives the gelatin lower quality. If gelatin from plaice backbones are going to be utilized, the high amount of free amino acids should be taken into account. Additionally, high amount of glycine indicates degradation of collagen which is reflected in the significantly lower ($p<0.001$) average collagen content for gelatin from backbones (32.9 ± 9.4) compared to gelatin from skins (65.25 ± 15.52) seen in Table 4.3 in Section 4.3.

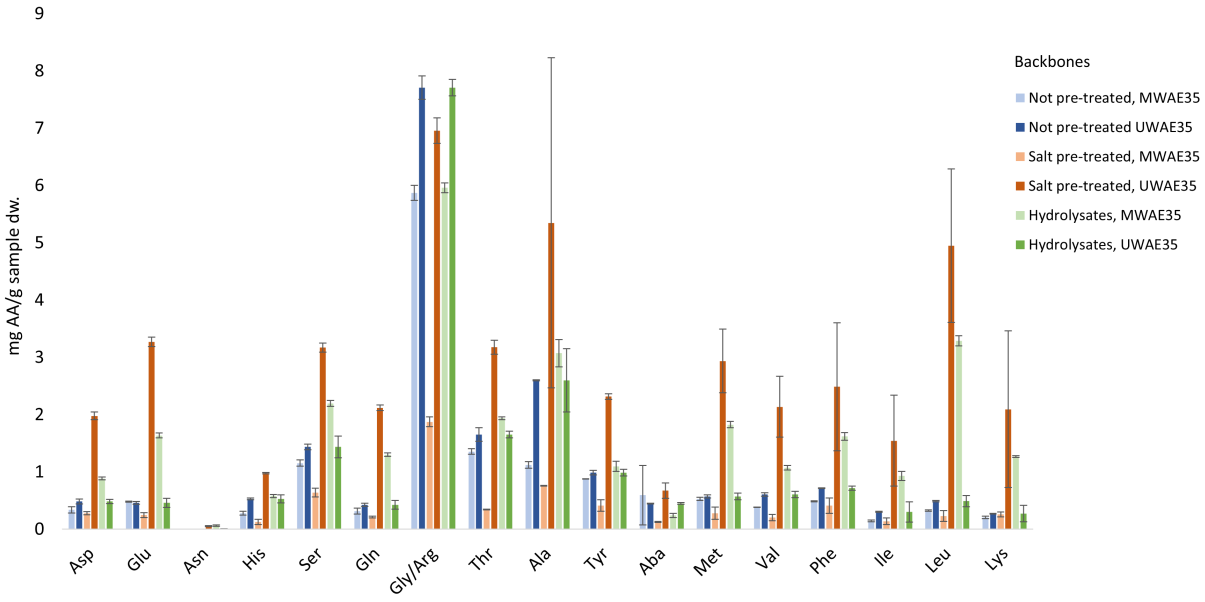

Figure 4.8: Free amino acid profiles for freeze dried gelatin samples from not pre-treated, salt pre-treated and hydrolysates from plaice backbones, extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35). The chemical analyses were conducted in triplicates (n=3).

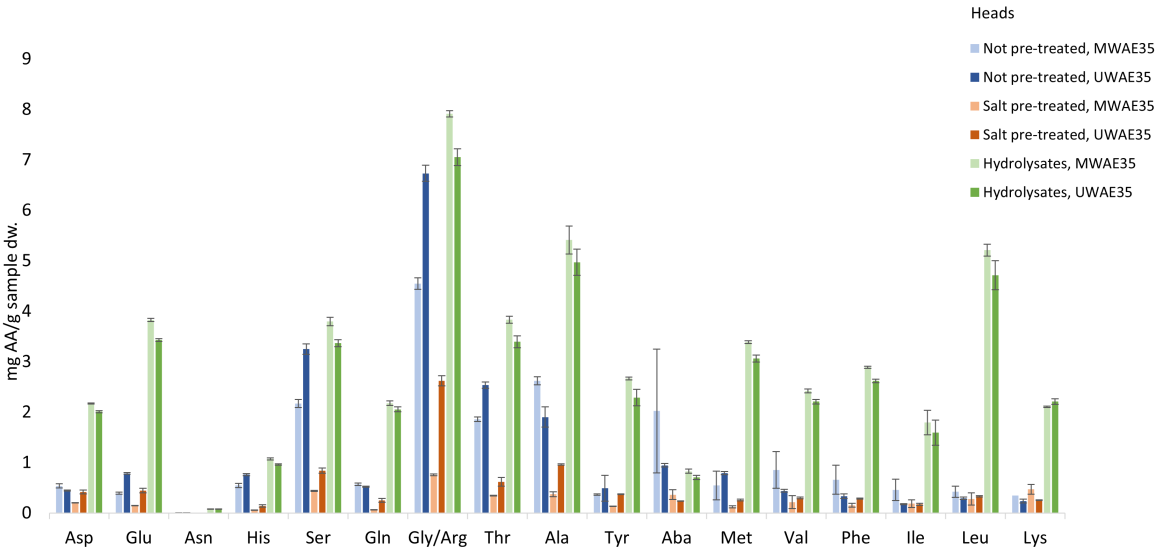

Figure 4.9: Free amino acid profiles for freeze dried gelatin samples from not pre-treated, salt pre-treated and hydrolysates from plaice heads, extracted in microwave for 35 minutes (MWAE35) and ultrasound water bath for 35 minutes (UWAE35). The analyses were conducted in triplicates (n=3).

4.6 Molecular size distribution

The molecular size distribution of the not pre-treated, pre-treated material and gelatin samples were analyzed on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to investigate the effect on degradation of the molecules. A PageRuler Broad Range Protein Ladder standard and ruler (5-250 kDA) and was used to measure the resulting molecular weights. In the present study, the standard was visible from 10-250 kDA and 15-250 kDA. The protein hydrolysates from the raw material fractions, labeled 5, 6 and 7 (skins, backbones, heads, respectively) in Figure 4.10, did not show any bands on the gel. This indicates that the peptides were too small to be seen, which is reasonable because enzymatic hydrolysis degrade peptide linkages in protein[47]. Due to the small sizes of the peptides in the hydrolysates, it was assumed that gelatin samples extracted from the hydrolysates also were too small to be visualized on the polyacrylamide gel, and the electrophoresis was not run on gelatin samples extracted from hydrolysates. When conducting the SDS-PAGE on salt pre-treated samples, the were difficulties that made the gel not to be stained properly and the resulting gel is not presented. This was also the issue for gelatins extracted from salt pre-treated material and extracted in water bath and ultrasound water bath for 35 minutes.

The raw material fractions head and backbones, 3 and 4 in Figure 4.10 show more or less similar molecular weight distributions where peptides of 20-30 kDA, 45-50 kDA and 125 kDA were present. A higher concentration of 20-30 kDA than the larger sizes was seen by the stronger color of the bands. Collagen has molecular size of 300 kDa and the presence of collagen or larger proteins than 250 kDA will not be visible in the gel[48]. Gelatin from salt pre-treated heads extracted for 15 minutes in ultrasound water bath are labeled 1 in Figure 4.11 and shows in general higher concentration of all peptide sizes than the corresponding not extracted raw material, which can be due to less amount of impurities as discussed in Section 4.4.

There were also observed peptides of larger molecular sizes in the gelatin from salt pre-treated heads extracted for 15 minutes, and the stronger bond on 250 kDA indicates that there was presence of β-chains in the sample[13]. There are studies that have shown that gelatin composed of polypeptides with higher molecular weight has higher gel strength, which is a property indicating high quality because of the various possibilities of use in the food industry[49] [10].

Number 9 to 14 in Figure 4.11 show the molecular weight distributions of gelatin extracted from not pre-treated material, with highest molecular weight of about 150 kDA for all the samples. Number 16 to 20 and number 22 in Figure 4.12 show the molecular weight distribution of gelatin from salt pre-treated material, where the highest molecular weight was 250 kDA for all salt pre-treated samples. By comparing the pictures, it can be seen

that the salt pre-treatment in general gave presence of polypeptide chains with higher molecular weight than for gelatin extracted from not pre-treated material.

Number 14 and number 11 in Figure 4.11 shows the bands from gelatin extracted by ultrasound water bath from not pre-treated heads for 15 and 35 minutes, respectively. The gelatin extracted for 15 minutes had one band of 150 kDA that the sample extracted for 35 minutes was lacking. The same pattern coulld be seen for the gelatin from salt pre-treated heads extracted in microwave for 15 minutes (number 17, Figure 4.12) and 35 minutes (number, 20, Figure 4.12. In the study by Feng et al. (2021) on pre-treatment of gelatin in microwave, the longer extraction time gave decrease in gel strength [24]. The lack of the 150 kDA bond after 35 minutes can indicate that the gel strength decrease after longer time for both ultrasound water bath and microwave assisted extraction as well. However, this should be looked into closer using several extraction methods and several duration of extractions.

The bands from the skins samples were somewhat weak, seen as number 2 in Figure 4.10 and number 8 in Figure 4.11. This could have been due to that the gelatin concentration in the dilutions that were run on the electrophoresis were lower (0.5 mg/mL) than for heads (1.0 mg/mL) and backbones (1.0 mg/mL) when conducting the experiment. The faint bands for skin gelatin are therefore not necessary due to lower content of α and β-chains present in the gelatin, but more likely due to lower amount of gelatin sample added. It can be seen from number 15 and 18 in Figure 4.12 that the gelatin from skin samples differs from the other samples by lacking the two bonds of 15 and 20 kDA.

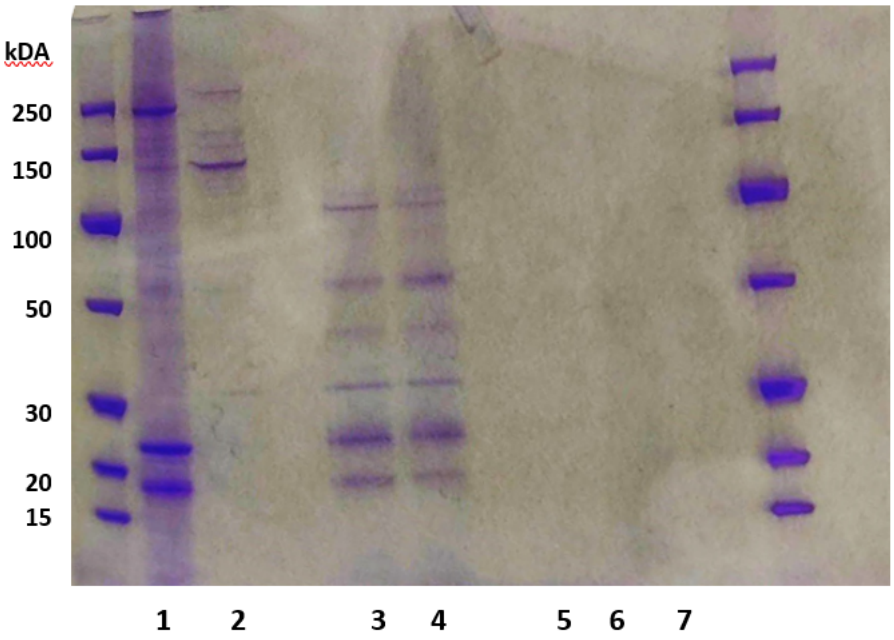

Figure 4.10: Picture of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel with gelatin extracted from skins, backbones and heads with various extraction protocols, raw material fractions and protein hydrolysates from raw material. 1: salt pre-treated heads extracted in ultrasound water bath for 15 min. 2: skins raw material. 3: backbones raw material. 4: heads raw material. 5: skins hydrolysates. 6: backbones hydrolysates. 7: heads hydrolysates. 12, 13 and 14 are not visible due to too small peptides.

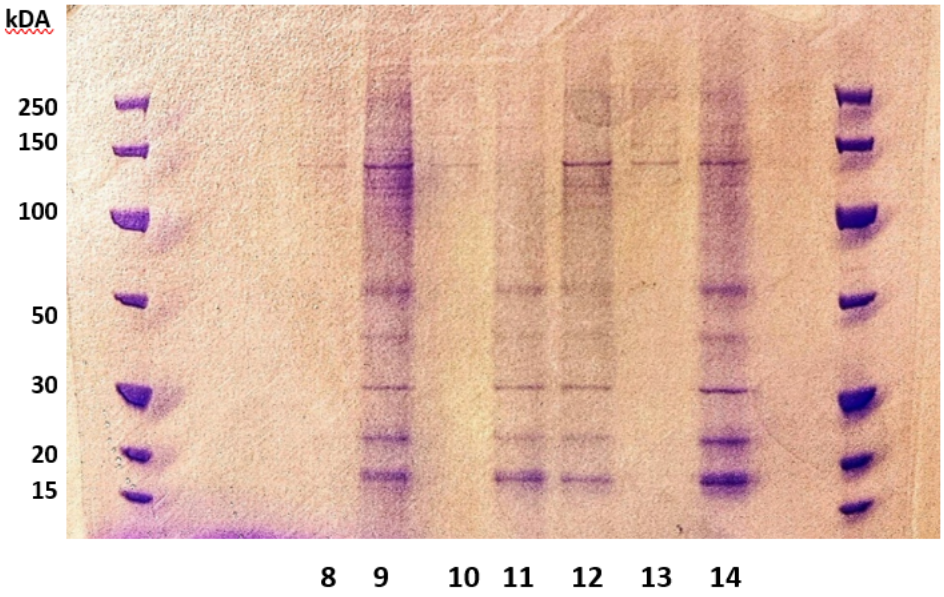

Figure 4.11: Picture of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel with gelatin extracted from skins, backbones and heads with various extraction protocols. 8: salt pre-treated skins extracted in ultrasound water bath for 15 min. 9: not pre-treated heads extracted in water bath for 150 min. 10: not pre-treated skins extracted in water bath for 150 min. 11: not pre-treated heads extracted in ultrasound water bath for 35 min. 12: not pre-treated backbones extracted in ultrasound water bath for 35 min. 13: not pre-treated skins extracted in ultrasound water bath for 35 min. 14: not pre-treated heads extracted in ultrasound water bath for 15 min.

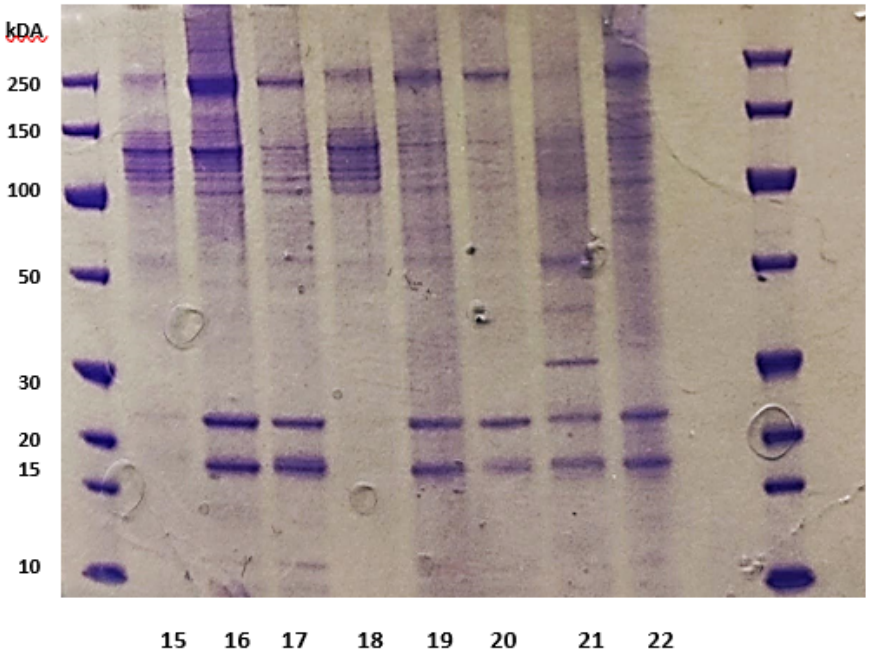

Figure 4.12: Picture of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel with gelatin extracted from skins, backbones and heads with various extraction protocols. 15: salt pre-treated skins extracted in microwave for 15 min. 16: salt pre-treated backbones extracted in microwave for 15 min. 17: salt pre-treated heads extracted in microwave for 15 min. 18: salt pre-treated skins extracted in microwave for 35 min. 19: salt pre-treated backbones extracted in microwave for 15 min. 20: salt pre-treated heads extracted in microwave for 35 min. 21: not pre-treated backbones extracted in water bath for 150 min. 22: salt pre-treated backbones extracted in ultrasound water bath for 15 min.

5 Conclusion

This study was conducted to explore the possibilities of applying two green extraction technologies, microwave and ultrasound technology, to valorize rest raw material fractions from European plaice. The green technologies were applied to extract gelatin from the rest raw material fractions skins, heads and backbones not pre-treated or pre-treated by salt wash or enzymatic hydrolysis. Furthermore, the possibilities of the green extraction methods were explored by looking into how they affected quality and yield of gelatin as well as how the raw material fractions, pre-treatment and duration of extraction affected the quality and yield. The parameters used for evaluating the quality of gelatin were collagen content, protein content, amino acid profiles and molecular weight distribution. The two green extraction technologies were conducted at 15 and 35 minutes and compared against a water bath extraction for 150 minutes.

The results showed in general that plaice skins had highest content of collagen and gave highest yield compared to the other rest raw material fractions. Skins also had lower content of free amino acids which indicates less degradation of the polypeptide chains of gelatin. For the pre-treatments, salt wash in general gave lower yield, but higher ($p<0.001$) collagen purity (44.0 $\pm$ 19.9 %) and concentration of total amino acid purity (611.1 $\pm$ 165.9 mg AA/g sample) than gelatin from not pre-treated material. This is most likely is due removal of non protein impurities during the salt wash. Pre-treatment with salt wash also showed highest amount of β-chains with higher molecular weight, giving better gel strength. Gelatin extracted from protein hydrolysates obtained from enzymatic hydrolysis contain hydrophobic amino acid that gives foaming properties that are applicable for the food industry. On the other hand, the high amount of free amino acids decrease the quality.

For the extraction methods, the two green extraction methods gave the same effects on yield, as both extraction methods were most efficient after 35 minutes. Gelatin samples from microwave assisted extraction gave higher ($p<0.001$) collagen content (46.4 $\pm$ 20.3 %) than the ultrasound water bath (23.78 $\pm$ 19.21 %). The time of extraction seemed to affect the quality and yield first after 35 minutes. Extraction of 35 minutes showed the two green extraction methods to be as efficient with respect to collagen content as for a 150 minutes water bath. However, extraction for 15 minutes gave molecular weight distributions with higher peptides indicating that the gel strength decreases with time. To further investigate this, several extraction times can be investigated.

Based on the results from the study, applying microwave and ultrasound technology can be efficient for gelatin extraction, and by combining the extraction methods with salt pre-treatment, the collagen purity and gelling properties can give gelatin product applicable for the industry.

6 Future work

The present study showed that there is a potential for applying microwave and ultrasound assisted extraction for extraction of gelatin by looking at the quality parameters: yield, collagen purity, amino acid composition and molecular size distribution. However, other quality parameters like gel strength, water holding capacity and sensory analyses could be beneficial to investigate and therefore determine the commercial potential of plaice gelatin.

The yields were calculated from only one parallel, which makes it hard to draw good comparisons between the yields. In order to achieve significant values between the yields, several extractions should be applied. To better compare the microwave and ultrasound water bath with a normal water bath, a water bath of 15 and 35 minutes should be applied. Additionally, it could be interesting to look into green extraction methods extracted for a longer period of time since quality parameters seemed to increase with the time of extractions.

Studies have also combined green extraction methods to increase yield and quality of gelatin[12]. Further work where different extraction methods can be applied as pre-treatments would be interesting to look deeper into efficient extraction procedures.

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A Appendix A

A.1 Overview of samples

All individual sample IDs for gelatins from each raw material fraction and extraction procedure are listed in Table A.1. H_{EH} is listed as a sample, but not extracted due to insufficient sample size.

Table A.1: Sample ID of each gelatin sample extracted and analyzed. EH stands for pre-treatment by enzymatic hydrolysis. H_{EH} water was not conducted due to insufficent sample size.

Sample ID	Raw material fraction	Pre-treatment	Extraction method	Time [min]
S water	Skins	No treatment	Water bath	150
S MWAE15	Skins	No treatment	Microwave	15
S MWAE35	Skins	No treatment	Microwave	35
S UWAE15	Skins	No treatment	Ultrasound water bath	150
S UWAE35	Skins	No treatment	Ultrasound water bath	15
S_salt water	Skins	Salt wash	Water bath	150
S_salt MWAE15	Skins	Salt wash	Microwave	15
S_salt MWAE35	Skins	Salt wash	Microwave	35
S_salt UWAE15	Skins	Salt wash	Ultrasound water bath	15
S_salt UWAE35	Skins	Salt wash	Ultrasound water bath	35
S_EH water	Skins	EH	Water bath	150
S_EH MWAE15	Skins	EH	Microwave	15
S_EH MWAE35	Skins	EH	Microwave	35
S_EH UWAE15	Skins	EH	Ultrasound water bath	15
S_EH UWAE35	Skins	EH	Ultrasound water bath	35
H water	Heads	No treatment	Water bath	150
H MWAE15	Heads	No treatment	Microwave	15
H MWAE35	Heads	No treatment	Microwave	35
H UWAE15	Heads	No treatment	Ultrasound water bath	150
H UWAE35	Heads	No treatment	Ultrasound water bath	15
H_salt water	Heads	Salt wash	Water bath	35
H_salt MWAE15	Heads	Salt wash	Microwave	15
H_salt MWAE35	Heads	Salt wash	Microwave	35
H_salt UWAE15	Heads	Salt wash	Ultrasound water bath	15
H_salt UWAE35	Heads	Salt wash	Ultrasound water bath	35
H_EH water	Heads	EH	Water bath	150
H_EH MWAE15	Heads	EH	Microwave	15
H_EH MWAE35	Heads	EH	Microwave	35
H_EH UWAE15	Heads	EH	Ultrasound water bath	15
H_EH UWAE35	Heads	EH	Ultrasound water bath	35
B water	Backbones	No treatment	Water bath	150
B MWAE15	Backbones	No treatment	Microwave	15
B MWAE35	Backbones	No treatment	Microwave	35
B UWAE15	Backbones	No treatment	Ultrasound water bath	150
B UWAE35	Backbones	No treatment	Ultrasound water bath	15
B_salt water	Backbones	Salt wash	Water bath	35
B_salt MWAE15	Backbones	Salt wash	Microwave	15
B_salt MWAE35	Backbones	Salt wash	Microwave	35
B_salt UWAE15	Backbones	Salt wash	Ultrasound water bath	15
B_salt UWAE35	Backbones	Salt wash	Ultrasound water bath	35
B_EH water	Backbones	EH	Water bath	150
B_EH MWAE15	Backbones	EH	Microwave	15
B_EH MWAE35	Backbones	EH	Microwave	35
B_EH UWAE15	Backbones	EH	Ultrasound water bath	15
B_EH UWAE35	Backbones	EH	Ultrasound water bath	35

A.2 Calculation of yields

The yields of the gelatin with No treatment and salt pre-treatment were calculated according to Equation A.1 using the weight of collagen in the gelatin sample and the dry weight of the raw material used before any pre-treatment. The dry weight of the raw material was calculated using the average dry matter content of each fraction found in Table **??**.

$$\text{Yield (\%)} = \frac{\text{Weight of collagen in freeze dried gelatin (g)}}{\text{Dry weight of raw material fraction}} \cdot 100\% \tag{A.1}$$

To calculate the yields of the gelatins extracted from the raw material with hydrolysis as pre-treatment, the yield of the hydrolysate was firstly calculated and then multiplied with the gelatin yield based on the hydrolysate. The yield of the hydrolysate is based on the dry weight of the raw material used and the weight of the freeze dried protein hydrolysate obtained from the hydrolysis, by Equation A.2.

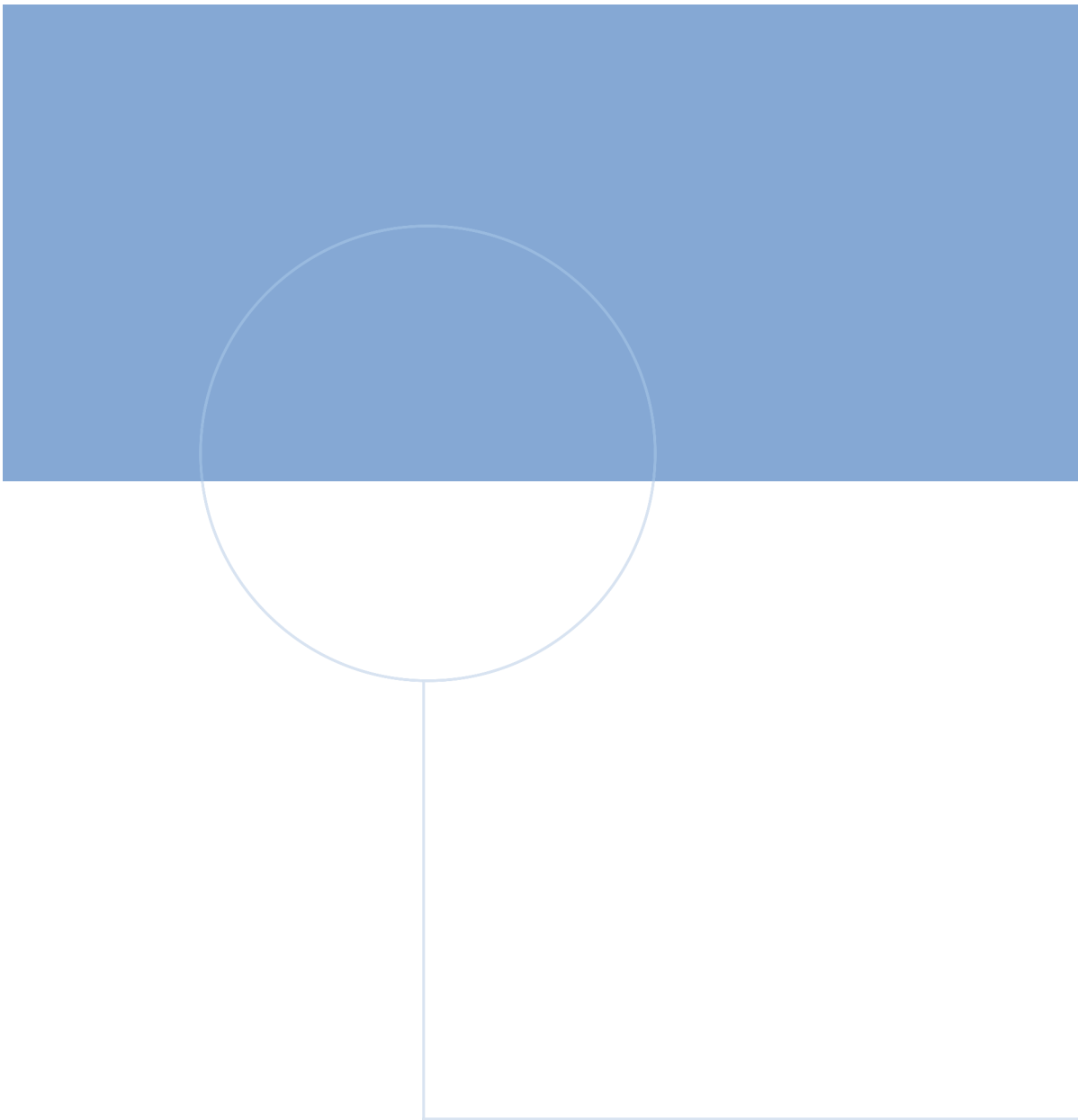
$$\text{Hydrolysate yield (\%)} = \frac{\text{Weight of freeze dried hydrolysate (g)}}{\text{Dry weight of raw material used for hydrolysis (g)}} \tag{A.2}$$

Then, the yield of the gelatin based on the hydrolysate was calculated using Equation A.3

$$\text{Yield based on hydrolysate (\%)} = \frac{\text{Weight of collagen in freeze dried gelatin (g)}}{\text{Weight of freeze dried hydrolysate (g)}} \cdot 100\% \tag{A.3}$$

Lastly, the gelatin yield based on raw material for the hydrolysis pre-treated sample was calculated by multiplying the two yields, following Equation A.4.

$$\text{Yield (\%)} = \frac{\text{Hydrolysate yield (\%)} \cdot \text{Yield based on hydrolysate (\%)}}{100} \tag{A.4}$$



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