Master's thesis

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

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Gelatine extraction from Atlantic salmon *(Salmo salar)* skin, a comparison between two methods

Master's thesis in Ocean Resources (MSOCEAN)
Supervisor: Turid Rustad
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Abstract

In Norwegian aquaculture, Atlantic Salmon (*Salmo salar*) is one of the most imported species. As a result of slaughtering or other incidents where salmon perish (due to e.g., delicing, handling etc.), a considerable amount of rest raw material is created. Today, 93% of it is utilized, but mostly for different kinds of animal feed or biogas production. However, there are many valuable components to be retrieved from the rest raw materials like for instance collagen, other proteins, fish oil, fish meal, peptides etc.

The first aim of this thesis was to compare two different methods used to extract gelatine from salmon rest raw material based on the methods used by Fossen (2020) and Alvarez (2018), respectively. The aim was to see which method would give the highest gelatine yield and to compare the properties of the different gelatines. The two methods used different approaches to extract the gelatine; Alvarez (2018) used thermal treatment, while Fossen (2020) did not. There was also a difference in extraction time between the two methods: 2 hours with the method used by Alvarez (2018) and 12 hours with the method used by Fossen (2020). The second aim was to make an overview of different methods used to extract gelatine from different fish species.

The results of this study showed that the method used by Fossen (2020) had a much higher gelatine yield than the method used by Alvarez (2018). The gelatine yield for the method used by Fossen (2020) was 6,85% and the gelatine yield obtained from the method used by Alvarez (2018) was 0,46%. The collagen yield based on the freeze-dried samples was 80,8% for the method used by Fossen (2018) and 38,0% for the method used by Alvarez (2018). The amino acid composition of the extracted gelatine showed that the method used by Fossen (2020) yielded almost twice as much as the method used by Alvarez (2018). The SDS-PAGE showed a higher degree of degradation in the samples from the method used by Alvarez (2018), compared to a higher content of high weight molecules in the samples from Fossen (2020). Furthermore, the method used by Fossen (2020) gave a total hydroxyproline yield of 7,0%, while the method used by Alvarez (2018) only yielded 3,3%.

Sammendrag

I havbruksnæringen i Norge er atlantisk laks (*Salmo salar*) en av de viktigste artene. Som en konsekvens av at laksen blir slaktet eller dør på andre måter (som for eksempel ved avlusing, håndtering osv.) blir det produsert store mengder restråstoff. I dag blir 93% av dette restråstoffet utnyttet, men for det meste bare til ulike typer dyrefôr eller biogassproduksjon. På tross av dette finnes det mange verdifulle komponenter man kan hente fra restråstoffet som for eksempel kollagen, andre proteiner, fiskeolje, fiskemel, peptider osv.

Det første målet med denne oppgaven var å sammenligne to ulike metoder for å hente ut gelatin i restråstoff fra laks, basert på metodene som ble brukt av Fossen (2020) og Alvarez (2018). videre gikk målet ut på å finne ut hvilken metode som ga høyest gelatinutbytte, og samtidig sammenligne egenskapene til de ulike gelatinene. De to metodene brukte ulike tilnærminger for å hente ut gelatin; Alvarez (2018) brukte varmebehandling. Det gjorde ikke Fossen (2020). Det var også en forskjell i ekstraksjonstiden mellom de to metodene; 2 timer med metoden brukt av Alvarez (2018) og 12 timer med metoden brukt av Fossen (2020). Det andre målet med denne oppgaven var å lage en oversikt over forskjellige ekstraksjonsmetoder brukt til å ekstrahere gelatin fra forskjellige fiskearter.

Resultatene i denne oppgaven viste at metoden brukt av Fossen (2020) hadde mye høyere gelatinutbytte enn metoden brukt av Alvarez (2018). Gelatinutbyttet etter å ha brukt samme metode som Fossen (2020) var 6,85% mot 0,46% etter å ha brukt samme metode som Alvarez (2018). Kollagenutbyttet basert på de frysetørkede ekstraktene var 80,8% med metoden brukt av Fossen (2020), og 38,0% med metoden brukt av Alvarez (2018). Aminosyresammensetningen til det ekstraherte gelatinet viste at metoden brukt av Fossen (2020) gav et utbytte på nesten dobbelt så mye i forhold til metoden brukt av Alvarez (2018). Gel-elektroforesen (SDS-PAGE) viste høyere grad av nedbryting i prøvene basert på metoden brukt av Alvarez (2018) sammenlignet med tyngre molekyler i prøvene basert på metoden brukt av Fossen (2020). Videre viste hydroksyprolinundersøkelsene at metoden benyttet av Fossen (2020) gav et utbytte på 7,0%, hvorimot metoden brukt av Alvarez (2018) gav et utbytte på bare 3,3%.

Preface

This Master's thesis was written in the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU). It is the final piece in the puzzle, and completed it now makes up the picture of me as a finished Ocean Resources (MSOCEAN) student. Before starting with the thesis, I thought that I would only utilize what I already knew from before, but I have truly learned so much during this period, and I am sure that I will be able to "brief" with these skills and knowledge later in life.

I must start by thanking my supervisor Prof. Turid Rustad for being patient and guiding me from the beginning. She has kept her cool when I have not, and has always had an answer for every issue encountered in the lab or otherwise during the writing of the thesis. A very big thank you to the people in the lab, both students and employees who have helped an unknowing and stressed biology student, and who have guided me through many different methods and shown me the ropes. Siri and Emilie have been great to have when the methods did not work as planned. They have double-checked and tested again if the results turned out strangely.

My boyfriend also deserves a big thank you for listening to my frustrations and halfway explanations about various topics that are very far away from his own study field (he is thankful that it was only a 30-credits master and not 60).

Lastly, I hope that my parents are now proud and pleased that I have lived up to my academical potential, and that they from now on will accept that I will do what *I* want to do in life. I do however acknowledge all the opportunities and personal development that studying has given me.

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List of Abbreviations and symbols

α	Alfa
β	Beta
γ	Gamma
κ	Kappa
SDS-PAGE	Sodium Sodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SEC-MALLS	Size Exclusion Chromatography - Multi-Angle Laser Light Scattering
GPC	Gel Permeation Chromatography
w/v	Weight by volume

1 Introduction

1.1 Gelatine through the centuries

Gelatine has been used for centuries and was used for many different purposes by humans. It was discovered that it had good adhesive properties and was used as a "biological" adhesive in ancient times according to Schrieber & Gareis (2007). There is also evidence that people living in caves around 8000 years ago were able to produce glue-like compounds from different animal tissues (Schrieber & Gareis, 2007). In ancient Egypt around 5000 years ago, they discovered that glue produced from collagen could be used to glue wooden furniture (Schrieber & Gareis, 2007). At the English court around the year 1500, when Henry VIII was the king, they used to serve dishes with jelly from calves' hoofs at court (Schrieber & Gareis, 2007). Gelatine has also had an important role relating to medicinal purposes. It was already described in the 12th century by Hildegard von Bingen (1098-1179) as a source for relieving joint pains in humans in form of a calve hoof broth (Schrieber & Gareis, 2007). Later in history gelatine was used to produce capsules for easier medicine dosage, and following that, gelatine played a role in making photography available to more people in the 19th century, and not only the rich (Schrieber & Gareis, 2007). Later in the 19th century the dessert JELL-O entered the food market as a result of granulated gelatine becoming more available (Schrieber & Gareis, 2007). A large-scale industrial production of gelatine from pigs started in the 1930s, and nowadays gelatine is used in an array of products such as cosmetics, foods, fertilizers, pharmaceuticals, paints etc. (Boran, 2013). Pig gelatine is still the most important source, however extraction of gelatine from other sources like fish or insects has been notably more visible in research the last decade (Boran, 2013).

1.2 Gelatine sources

The gelatine available on the market today comes mostly from leather industries and meat industries (Hattrem & Draget, 2014). The gelatine can come from both agricultural and non-agricultural sources (Mariod & Adam, 2013). It is a valuable by-product from these kinds of productions. Porcine and bovine sources are preferred. The reason for this is that the supply is continual and that there are good systems for quality control. Another reason is tradition. (Hattrem & Draget, 2014). Currently, research on gelatine extracted from fish and insects is conducted. These products are both acceptable in halal food for Muslims and in kosher products for Jews (Mariod & Adam, 2013). Moreover, Muslims and Jews are forbidden to eat products containing gelatine from porcine sources (Mariod & Adam, 2013).

Halal gelatines from two insects (sorghum bug and melon bug) were tried out in Sudan, but were met with scepticism due to concerns related to health, and also socio-cultural conflicts (Mariod & Adam, 2013). However, the insects contained 16 different amino acids, had approximately 55% fat, and the gelatine yield was 1-3% (Mariod & Adam, 2013). It is more common to extract chitin from insects because the biopolymer is found in the shell of arthropods (Doucet & Retnakaran, 2012). Chitin extracted from green shield bug (*Palomena prasina*) showed 10% chitin content of the dry weight according to Kaya et al.

(2015). The chitin yield is normally much higher than what was achieved when extracting gelatine from insects. Therefore, it is more likely that the research efforts in the future will focus more on extracting compound that give a higher yield.

Additionally, Hindus consider bovines to be holy and do therefore not consume them, and naturally also not gelatine from bovine sources (Hattrem & Draget, 2014). There have also been concerns that gelatine products descending from bovine sources can be a source for illness caused by bovine spongiform encephalitis (BSE), however EFSA (2006) considers the risk to be small. Gelatine produced from poultry or fish only makes up 2-3% of the total gelatine production in the world according to Hattrem & Draget (2014). It is estimated that 41% of gelatine comes from pig skin, 29.5% from bovine bones, 28.5% from bovine hides and only 1.5% from fish raw materials (Milovanovic, 2018). There are several reasons for this; the availability of good quality raw material is limited as well as the fact that mammalian gelatines have better properties than cold-water fish gelatine, especially considering gel strength, gelling and melting temperatures (Hattrem & Draget, 2014). However, warm- water fish species contain gelatine with more desirable properties (e.g. melting temperature, gelling temperature, viscosity, Bloom strength etc.) and have the ability to replace the use of mammalian gelatine (Hattrem & Draget, 2014). One of the reasons for extracting gelatine from fish rest raw material is to produce compounds that can be used for human consumption instead of being used for animal feed. One way to do so is by for instance isolating or extracting valuable proteins. Alternatively, fish oil, fish meal, hydrolysates or peptides can be retrieved from rest raw material. More about this can be read in section 4.1. There is also an increasing number of pescatarians and vegetarians around the world, where the former would benefit from having a wider variety of products to choose from if fish gelatine was more available in food.

Hence, there is a considerable amount of the Earth's population that would benefit from alternative gelatine sources that are acceptable according to the rules of the different religions, diets and lifestyles.

2 Collagen

The protein that is in highest abundance in humans and animals is collagen. It is naturally found in all parts of the body, but the highest concentrations are found in bones, ligaments, skin and tendons (Bou-Gharios, Abraham & de Crombugghe, 2020). Today, there are a total of 28 different types of collagens which all have their own roman numerals, given based on the order that they were discovered (Ruggiero, 2021). Collagen provides stability in the structures where it resides, and is an insoluble protein (Kadler et al., 2007). Each of the different collagens have different functions, structures and distributions (Ramshaw et al., 2009). Normally we count three main types of collagens: I, II and III. Type I is the most common and is most often found in bones, muscle and skin (Gómez-Guillén et al., 2011). Type II is almost only found in cartilage tissue, and type III is most often found in skin and inner organs (Bella & Jordi, 2017). All the types have different peptide chains and occur in different areas of the body as shown in Table 1.

Table 1: Different collagen types, their molecular composition, and their tissue distribution. Table taken from Gelse, Pöschl & Aigner (2003).

Type	Molecular composition	Genes (genomic localization)	Tissue distribution
Fibril-forming collagens			
I	$[\alpha1(I)]_2\alpha2(I)$	COL1A1 (17q21.31–q22) COL1A2 (7q22.1)	bone, dermis, tendon, ligaments, cornea
II	$[\alpha1(II)]_3$	COL2A1 (12q13.11–q13.2)	cartilage, vitreous body, nucleus pulposus
III	$[\alpha1(III)]_3$	COL3A1 (2q31)	skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)
V	$\alpha1(V),\alpha2(V),\alpha3(V)$	COL5A1 (9q34.2–q34.3) COL5A2 (2q31) COL5A3 (19p13.2)	lung, cornea, bone, fetal membranes; together with type I collagen
XI	$\alpha1(XI)\alpha2(XI)\alpha3(XI)$	COL11A1 (1p21) COL11A2 (6p21.3) COL11A3 = COL2A1	cartilage, vitreous body
Basement membrane collagens			
IV	$[\alpha1(IV)]_2\alpha2(IV)$; $\alpha1-\alpha6$	COL4A1 (13q34) COL4A2 (13q34) COL4A3 (2q36–q37) COL4A4 (2q36–q37) COL4A5 (Xq22.3) COL4A6 (Xp22.3)	basement membranes
Microfibrillar collagen			
VI	$\alpha1(VI),\alpha2(VI),\alpha3(VI)$	COL6A1 (21q22.3) COL6A2 (21q22.3) COL6A3 (2q37)	widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils			
VII	$[\alpha1(VII)]_3$	COL7A1 (3p21.3)	skin, dermal–epidermal junctions; oral mucosa, cervix,
Hexagonal network-forming collagens			
VIII	$[\alpha1(VIII)]_2\alpha2(VIII)$	COL8A1 (3q12–q13.1) COL8A2 (1p34.3–p32.3)	endothelial cells, Descemet's membrane
X	$[\alpha3(X)]_3$	COL10A1 (6q21–q22.3)	hypertrophic cartilage
FACIT collagens			
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	COL9A1 (6q13) COL9A2 (1p33–p32.2)	cartilage, vitreous humor, cornea
XII	$[\alpha1(XII)]_3$	COL12A1 (6q12–q13)	perichondrium, ligaments, tendon
XIV	$[\alpha1(XIV)]_3$	COL9A1 (8q23)	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$[\alpha1(XIX)]_3$	COL19A1 (6q12–q14)	human rhabdomyosarcoma
XX	$[\alpha1(XX)]_3$		corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	$[\alpha1(XXI)]_3$	COL21A1 (6p12.3–11.2)	blood vessel wall
Transmembrane collagens			
XIII	$[\alpha1(XIII)]_3$	COL13A1 (10q22)	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$[\alpha1(XVII)]_3$	COL17A1 (10q24.3)	dermal–epidermal junctions
Multiplexins			
XV	$[\alpha1(XV)]_3$	COL15A1 (9q21–q22)	fibroblasts, smooth muscle cells, kidney, pancreas,
XVI	$[\alpha1(XVI)]_3$	COL16A1 (1p34)	fibroblasts, amnion, keratinocytes
XVIII	$[\alpha1(XVIII)]_3$	COL18A1 (21q22.3)	lungs, liver

Further, collagen can be divided into fibrillar and non-fibrillar collagens (Bella & Jordi, 2017). All the different types of collagens form a triple helix with three polypeptide chains (Gelse, Pöschl & Aigner, 2003). The α-chains can be made from three identical chains or with different chains (Gelse, Pöschl & Aigner, 2003). Every α-chain in the molecule forms a helix twisted to the left with 18 amino acids per turn (Hofmann, Fietzek & Kuhn, 1978). The α-chains contain 1000-1100 amino acids each (Eysturskarð, 2010). The chains are

further coiled around a central axis with rotation to the right and form the characteristic triple helix (Fraser, MacRae & Suzuki, 1979). The amino acid glycine is found in every third position of the polypeptide chains (Gelse, Pöschl & Aigner, 2003). Therefore, the structure results in infinite $(Gly\text{-}X\text{-}Y)_n$ rows as shown in Figure 1. (Gelse, Pöschl & Aigner, 2003). The structure is primarily stabilised by inter- and intra-chain hydrogen bonds (Asghar & Henrickson, 1982). Glycine is in the centre of the helix, while the side groups of the amino acids are on the outside (Gelse, Pöschl & Aigner, 2003). In the X position you will often find proline, while in the Y position hydroxyproline is most likely to occur (Gelse, Pöschl & Aigner, 2003). It is estimated that approximately 14% of the total amino acid content is comprised of hydroxyproline (Cundy, Reid & Grey, 2014). A unit of collagen fibrils is made up of four to eight collagen molecules (Gómez-Guillén et al., 2011). These are buttressed and stabilised by covalent bonds (Gómez-Guillén et al., 2011). To be able to extract collagen, a pre-treatment is needed in order to convert the collagen into a different form which is more suitable for extraction (Gómez-Guillén et al., 2011). There are thermal and chemical pre-treatments. The pre-treatment is often carried out in water that has a temperature above 45°C according to Gómez-Guillén et al. (2011). However, both Eysturskarð (2010) and Schrieber & Gareis (2007) say differently; 14-20°C for alkaline pre-treatments and 15-20°C for acid pre-treatments. If a chemical pre-treatment is used it will disarrange the structure of the protein and break the non-covalent bonds. This makes the collagen structure soluble and induces enough swelling (Gómez-Guillén et al., 2011). If more heat treatment is applied after this, the hydrogen and covalent bonds are cleaved and transform the triple-helix to a helix-to-coil transition (Gómez-Guillén et al., 2011). This will result in soluble gelatine (Gómez-Guillén et al., 2011). However, the degree of conversion is highly dependent on the pre-treatment including the extraction time, pH and temperature (Johnson-Banks, 1990).

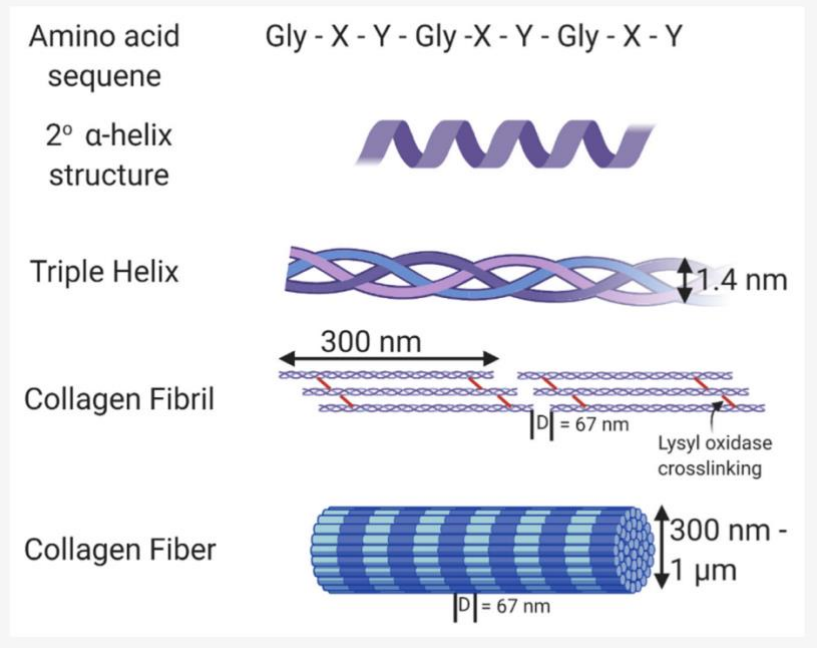

Figure 1: Collagen structure from amino acid sequences to collagen fibres. Figure taken from Walimbe & Panitch (2020).

3 Gelatine

Gelatine is a protein that can be obtained through a partial hydrolysis of collagen (Boran, 2013). This can be done with different treatments like high-temperature treatments, alkali treatments, acid treatments or with enzymatic hydrolysis (Schrieber & Gareis, 2007). To obtain the physical and chemical properties that are desired from the gelatine it is important to control the hydrolysis (Boran, 2013). Through this you can control colour, clarity, viscosity, gel strength and adhesiveness of the gelatine (Boran, 2013). More about this is section 3.3. Gelatine is most often recovered from skin and collagen deriving from connective tissues in mammals (Yang et al., 2022). Because gelatine is thermo-reversible, it melts in the mouth (Yang et al., 2022). The reason for this is that collagen forms a gel when dissolved in water (Yang et al., 2022). Historically gelatine has had a wide variety of applications, and never more diverse than today, as mentioned before. It is expected that the use of fish gelatine also will increase in the coming years as researchers have been experimenting with different extraction methods and an abundance of different fish species in the last two decades (Yang et al., 2022; Alipal et al., 2021; Koli et al., 2012; Jongjareonrak et al., 2010; Karim & Bhat, 2009; Gómez-Guillén et al., 2002).

3.1 Structural and chemical properties of gelatine

Gelatine has a relatively similar amino acid composition as a collagen; however, it does depend on the living conditions and temperatures of the organism (Akita et al., 2020). Mammalian gelatine can form thermo-reversible gels and can be dissolved in water (Boran, 2013). Gelatine also has a melting point which is close to human body temperature (Boran, 2013). This makes it a very versatile protein. The molecular weight of gelatine is usually between 15 and 400 kDa; however, this highly depends on the conditions that have been used and on the manufacturing process (Benjakul & Kittiphattanabawon, 2019). the value should be around 300 kDa, but if it is as low as 15, it means that the collagen is highly degraded.

3.2 Functional properties

When gelatine is manufactured, it is characterized based on many different properties like for instance gel strength, viscosity and melting point (Duthen et al., 2021). In the next paragraphs these will be explained further.

3.2.1 Gel strength in gelatine

Gel strength in gelatine is measured with a Bloom test (Duthen et al., 2021). It was named after Oscar T. Bloom which was an American scientist (Schrieber & Gareis, 2007). Bloom developed the method in order to be able to test the strength of different gelatines and

glues (Schrieber & Gareis, 2007). The result from such a test will give an indication of the gelling power and stiffness that a specific type of gelatine has (Duthen et al., 2021; Hanani, 2016). The Bloom scale usually has a range between 30 and 350 depending on the type (Duthen et al., 2021; Hanani, 2016). A number below 150 is considered to be a low value, 150-220 is considered to be a medium value and above 220 is considered to be a high value (Hanani, 2016). The higher the bloom value, the greater gelatine strength it indicates (Hanani, 2016). When determining the gel strength, a gelatine solution with a 6.67% (w/v) is made at 60°C and thereafter matured for 17 hours at 10°C. Next, a 4 mm standard plunger is used to measure the mass. (Gelatine Manufacturers Inst of America, 2019).

For fish gelatines this may not be a representative way of measuring gel strength according to Arnesen & Gildberg (2007). The reason for this is that fish gelatines will most likely show a strengthening rate that is higher than the one in porcine gelatine during storage (Arnesen & Gildberg, 2007). Due to hydrogen bonds forming between hydroxylated amino acids and water molecules, and reconstruction of helical structures between collagen peptide chains, gel strengthening during storage can occur (Arnesen & Gildberg, 2007). In addition to this the conditions that are described above cannot be used for some of the cold-water species due to the fact that several of those gelatines do not gel under such conditions (Arnesen & Gildberg, 2007).

Improving the gelling ability of fish gelatine is necessary in some cases and has been tried through various methods; high pressure, irradiation, enzymatic modification, mono- and disaccharide additions or through adding caffeic acid (Derkach et al., 2020). However, according to Derkach et al. (2020), it is both more common and effective to improve gelling ability by modifying the fish gelatine with κ-carragenan, chitosan, gum Arabic, sodium alginate, gellan or pectin. By adding κ-carragenan or gellan the gel strength increased, according to Pranoto, Lee & Park (2007). On the other hand, if salt is added to fish gelatine, both the gel strength and hardness of the gel will decrease (Tong, et al., 2022).

3.2.2 Viscosity

The molecular weight distribution determines the viscosity of gelatine (Boran & Regenstein, 2010). Measuring viscosity does not have to be expensive or complicated (Boran & Regenstein, 2010). It can be done by using a tubular viscometer made from glass (Boran & Regenstein, 2010). More advanced instruments may give a better accuracy, but the viscometer also has high accuracy (Boran & Regenstein, 2010). The correlation between viscosity and texture is not as high as for gel strength and texture (Boran & Regenstein, 2010). This means that if a gelatine extract has high viscosity, it does not automatically have a high Bloom value (Boran & Regenstein, 2010). In fish gelatine samples one can find high viscosity values, but at the same time have quite low Bloom values compared to pig skin (Boran & Regenstein, 2010). According to Boran & Regenstein (2010), it is not unusual that the viscosity in fish gelatines is lower if it is compared to gelatines from mammals that have a molecular weight distribution that resembles the one in fish gelatines (Boran & Regenstein, 2010). Fish gelatine can have high viscosity, but low gel strength (Boran & Regenstein, 2010). This means that fish gelatine can be used for flavour release enhancement, among other things (Boran & Regenstein, 2010). Depending on the product gelatine is added into, different viscosities are preferred in order to get the desired product texture (Schrieber & Gareis, 2007).

3.2.3 Molecular weight distribution

One of the most integral parameters in determining gelatine quality is the molecular weight distribution. It influences many different parameters; for instance gel strength and viscosity, as mentioned in the paragraph above (Olijve, Mori & Toda, 2001). The molecular weight distribution of gelatine is poly-dispersed, which means that the solution has particles in different sizes (Olijve, Mori & Toda, 2001). Further, this leads to a variation in the dispersity caused by different treatments and the use of different raw materials (Olijve, Mori & Toda, 2001). It is possible to reduce the dispersity by using fractionation, but it is not economically viable (Olijve, Mori & Toda, 2001). SDS-PAGE and chromatographic methods like SEC-MALLS or GPC are normally used to determine the molecular weight distribution in gelatines when the gelatine contains molecules with high molecular weight (Haug & Draget, 2009). According to Eysturskarð et al. (2009), "gelatin with low polydispersity and high weight average molecular weight is suggested as the optimal product with respect to the gelling properties" (p. 2320).

3.2.4 Gelling and melting points

Gelatine is a very important ingredient in numerous products, as previously mentioned. However, for the food industry some parameters are more important than others, like for instance melting or gelling temperatures. many manufacturers want their product to melt on the tongue, and therefore require certain types of gelatine that can in fact melt at body temperature (Wang et al., 2013). In addition to this gelatine can help enhance flavours and improve the texture of the food product (Wang et al., 2013). For some companies within the photography sector, setting time is essential to the process, and a gelatine which can set quickly is needed (Schrieber & Gareis, 2007). Depending on the gelation process, gelling temperatures and times can influence the gel structure (Fonkwe, Narsimhan & Cha, 2003). In fish there is considerable difference in gelling and melting points depending on the species and whether the species have their natural habitat in warm or cold water (Gómez-Guillén et al., 2011). This can be seen in table 2 in section 4.2.

3.3 Gelatine extraction methods

When obtaining gelatine from a raw material there are many different processes that have to be completed before getting gelatine as the final product. The steps include washing the raw material to remove impurities, a pre-treatment (either alkaline or acidic), gelatine extraction, filtration, concentration or evaporation, and lastly, sterilisation and drying (Eysturskarð, 2010). The reason for cleaning the raw material and utilizing a pre-treatment is because the purity of the gelatine can be improved through these processes (Ahmad et al., 2017). In addition to this, pre-treatments can break up the different collagen bonds (Ahmad et al., 2017). There are different types of gelatine, and depending on the pre-treatment, the result can be either type A gelatine or type B gelatine (Haug & Draget, 2011; Ahmad et al., 2017). Depending on the type of pre-treatment there are two types of gelatine that are normally obtainable; type-A gelatine and type-B gelatine (Gómez-Guillén et al., 2011). When utilizing acid pre-treatments, it will most likely result in gelatine type A because this treatment preserves glutamine and asparagine to a bigger extent than alkali pre-treatments (Haug & Draget, 2011). The properties of the extracted gelatine and the yield will depend on the acids that are used, temperatures, salts in the extraction media and the extraction pH (Boran, 2013).

3.3.1 Acid pre-treatment

When employing an acid pre-treatment it will most likely result in Type A gelatine with an isoelectric point between 6 and 9.5 (Hanani, 2016; Alfaro et al., 2014; Eysturskarð, 2010). Acid pre-treatments give a higher isoelectric point than an alkaline pre-treatment because the hydrolysis of the amino acids glutamine and asparagine is limited (Alfaro et al., 2014). This type of pre-treatment is often less time consuming and milder than an alkaline pre-treatment (Schrieber & Gareis, 2007). In addition to this, it works well on tissues where the collagen is weakly crosslinked, for example young pig skin or fish skin (Schrieber & Gareis, 2007; Karim & Bhat, 2009). Eysturskarð (2010) suggests soaking the raw material in inorganic acids with a concentration of 2-5% for a time period of 10-48 hours at 15-20°C. Thereafter, the pH is adjusted, normally to around 4 (Eysturskarð, 2010).

3.3.2 Alkaline pre-treatment

When employing an alkali pre-treatment, you will most likely get a type B gelatine with an isoelectric point between 4.8 and 5.2 (Hanani, 2016; Alfaro et al., 2014, Eysturskarð, 2010). As mentioned in the paragraph above, acid pre-treatments have a higher isoelectric point than alkaline pre-treatments. In an alkali pre-treatment the hydrolysis of glutamine and asparagine is not limited, in fact they are easily hydrolysed and turned into glutamic and aspartic acid, which in turn gives the lower isoelectric point (Alfaro et el., 2014). The isoelectric point of gelatine can be very important when looking at its application (Alfaro et al., 2014). It is also highly dependent on which pH range the product it is intended for has (Ward & Courts, 1977). As mentioned before, an alkaline pre-treatment is much more time consuming than an acidic pre-treatment; in fact, an alkaline pre-treatment can take anywhere from a few days to several months (Schrieber & Gareis, 2007). According to Schrieber & Gareis (2007), the pre-treatment can take a few days when using 1% sodium hydroxide at 20°C. The raw material can also be submerged in an alkaline solution for a longer period of time with some stirring, before it is washed until it is neutralised (Alvarez, 2018). Lastly the pH is adjusted with a diluted acid (Alvarez, 2018).

3.3.3 Defatting

High lipid content in the raw material used for gelatine extraction, can be considered negative. The reason for this is that these types of raw material often are prone to lipid oxidation during gelatine extraction, but also during storage. When the temperatures are higher, the risk for oxidation is even higher. This has a negative effect on the rheological properties of the final product. It can cause unsavoury flavours and odours in the gelatine. As a result of that, the application area is narrowed down (Sae- Leaw & Benjakul, 2015). These reasons are all incentives to defat the raw material before extraction. Some studies have shown that gel strength was lower when using raw material that had not been defatted (Sae-Leaw, Benjakul & O'Brien, 2016).

Defatting can be done in different manners with a variety of solvents; warm water, 10% butyl alcohol, 30% isopropanol or lipase extract (Muyonga et al., 2004; Eysturskarð, 2010; Sae-Leaw & Benjakul, 2018).

3.3.4 Extraction

When collagen fibres are heated, they shrink to one quarter of their initial size. During the heating process while the fibres are shrinking, the triple-helix disintegrates. The same thing

takes place when a collagen solution is heated at a lower temperature. When collagen is converted into gelatine, many of the bonds are broken. As a result of this the structure changes from a collagen helix to amorphous gelatine (Foegeding et al., 1996).

When extracting gelatine from cold-water species, room temperature will suffice, according to Eysturskarð et al. (2009). However, for warm-water fish species water with a temperature between 40°C and 80°C is normally used (Eysturskarð et al., 2009). When extracting gelatine, the process should be optimized in order to achieve the highest yield possible. However, it should be done with the wanted properties in mind (Johnston-Banks, 1990). If the pre-treatment conditions are sufficiently efficient, it will allow the extraction conditions to be of a milder sort (Johnston-Banks, 1990). This will most likely result in an end-product with more desirable properties (Johnston-Banks, 1990).

3.3.5 Post extraction

After extraction the gelatine has to go through more steps. The gelatine extract has to be filtered in order to remove undesirable matter or salts from the pre-treatments (Johnston-Banks, 1990). Next, the gelatine extract is sterilised and dried (Johnston-Banks, 1990). After drying, the gelatine can be made into particles with a size around 10 nm (Alvarez, 2018).

4 Rest raw material and fish gelatine

4.1 Rest raw material from aquaculture

The seafood industry in Norway has grown considerably the last decades and produced 3,55 million tons raw material in 2019 (Myhre et al., 2020). As a result of this there is a lot of rest raw material created in the process like for instance viscera, blood, bones and skin (Boran, 2013). Myhre et al. (2020), estimated that approximately 84% of the rest raw material from the whole seafood industry was utilized in some manner. The white-fish sector has the highest percentage of non-utilized rest raw material (Myhre et al., 2020). The reason for this is that the sector is still lacking technology and economic incentives to land the rest raw materials (Myhre., 2020). In this thesis the main focus has been skin from Atlantic salmon reared in aquaculture facilities, and the numbers will therefore reflect this. The aquaculture industry in Norway produced 446 000 tons of rest raw material in 2020, and 531 000 tons in 2021 (Barentswatch 2022; Myhre et al., 2022).The aquaculture industry managed to utilize 93% of the rest raw materials in 2020 and 94% in 2021 (Barentswatch, 2022; Myhre et al., 2022). These are high numbers; however, it is possible to extract more valuable components from most of the rest raw materials like fish oil, peptides, hydrolysates, gelatine, collagen etc. (Boran, 2013). However, it should be noted that fish which is found dead in the cages or fish dying during treatment can often not be used for human consumption because of food safety and quality parameters. Additionally, fish which has been killed with anaesthesia can also not be used for human comsumption before the residuals have left the fish. This would mean several days of waiting. Therefore, it is easier to make ensilage and thereafter biogas instead of waiting for the anesthesia to leave the system while the fish is degrading. Figure 2 shows what the rest raw materials from Norwegian salmon and trout farming are used for.

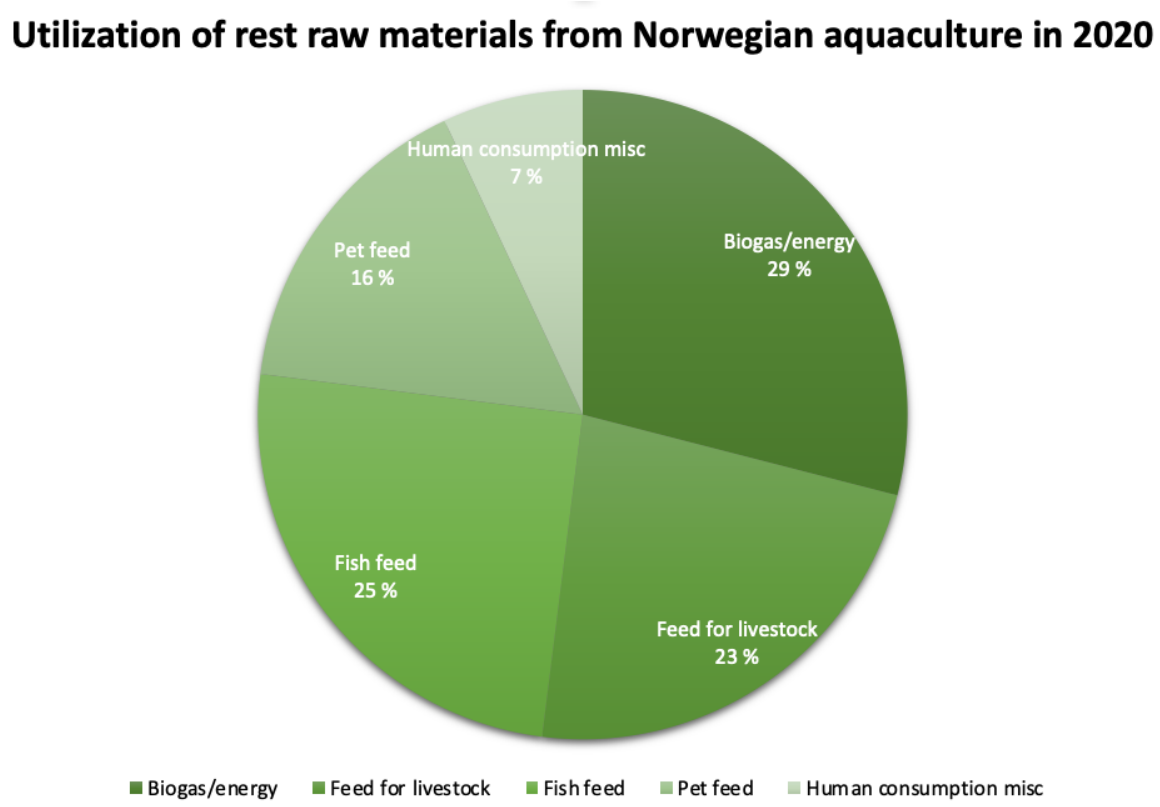

Figure 2: Utilization of rest raw materials from Norwegian aquaculture (salmon and trout) in 2020. Figure modified after Barentswatch (2022) with data from Winther, U., Myhre, M. S., Nystøyl, R. (2021).

A considerable amount of the rest raw materials are produced on land as a result of the slaughtering process. This means that there is good access to fresh rest raw materials that can be packaged and sent to further processing. For rest raw materials created at sea, e.g. fish that have succumbed due to operations such as delicing, sorting, illness etc., it is harder to send a fresh product to shore and extract valuable components from it. Today, these fish are made into ensilage and used in animal feed or used for biogas. Only 7% of the rest raw materials are used for human consumption. This is very low compared to 64% being used for animal feed. There is reason to believe that it is possible to make the percentage of rest raw material consumed by humans higher if the production rate of the examples mentioned above is higher and more profitable.

4.2 Fish gelatine

Fish gelatine is already used as an additive in a range of products, however there is still a great potential income for Norwegian salmon farmers if they decide to extract gelatin from fish skin. The skins of the salmon are approximately 5wt% of the fish (Fan, Dumont & Simpson, 2017). However, it should be noted that most of the salmon exported still has the skin attached for esthetic and economical reasons. Additionally, Karim & Bhat (2009) wrote that there are several moments of uncertainty for the further growth of the fish gelatine market, especially when concerning price volatility and control of natural quality traits in fish gelatine.

The length of the protein chains in gelatine determine the quality (Fan, Dumont & Simpson, 2017). When the molecular weight of the of the long protein chains is higher , the gelatin produced will have better physiochemical properties (Benjakul et al., 2012).

The extraction conditions have a large influence on the molecular weight distribution of gelatin (Goméz-Guillén et al., 2009). When lower temperatures and shorter reaction times are used for extraction, the protein is not as heavily degraded as it would be with more aggressive extraction conditions, and therefore gelatin with protein chains with a higher molecular weight are produced (Galea et al., 2000; Boran & Regenstein 2010). However, when utilizing mild extraction conditions it results in lower gelatin yields according to Boran and Regenstein (2010). Using higher temperatures and longer reaction times the gelatin yields are greater. However, the protein chains are shorter and there is a larger cleavage which gives a broader molecular weight distribution (Boran & Regenstein, 2010; Kittiphanttanabawon et al., 2010). In later years other pre-treatments have been used and has shown that gelatin yields can be improved even though milder extraction conditions are being used (Benjakul et al., 2012). By using a protease-aided pre-treatment with pepsin the gelatin yield has almost doubled in comparison to the pre-treatments without pepsin (Nalinanon et al., 2008).

Extraction of gelatin from numerous different fish species has been reported; sin croaker and shortfin scad (Cheow et al., 2007), New Zealand hoki (Mohtar et al., 2010; 2013), Atlantic salmon (Arnesen & Gildberg, 2007; Vázquez et al., 2021; Enrione et al., 2020), Alaska pollock (Zhou et al., 2006), brownstripe snapper and bigeye snapper (Kasankala et al., 2007; Nalinanon, Benjakul & Kishimura, 2008), Nile perch (Muyonga, Cole & Duodu, 2004), grass carp (Kasankala et al., 2007), yellowfin tuna (Cho, Gu & Kim, 2005), megrim (Montero & Goméz-Guillén, 2000), channel catfish (Liu, Li & Guo, 2008), African catfish (Alfaro et al., 2014), giant catfish (Jongjareonrak et al., 2010), cod (Goméz-Guillén et al., 2002; Arnesen & Gildberg, 2007; Gudmundsson & Hafsteinsson, 1997), North Sea horse mackerel (Badii & Howell, 2006), spotted golden goatfish (Chuaychan, Benjakul & Kishimura, 2016). Results from these studies and more can be seen in Table 2.

Table 2: Overview over experiments studying gelatine extraction methods in different fish species. Based on the tables of Karim and Bhat (2008), Alipal et al. (2021), Gómes-Guillén et al. (2009) and own literature search.

Species	Raw material	Gel strength (g)	Gelling temp (°C)	Melting temp (°C)	Pre-treatment	Temp (°C)	Duration	Extraction	Temp (°C)	Duration	Yield %	Reference
Atlantic Cod (*Gadus Morhua L*)	Skin	110-120	not reported	8-10	NaOH for 40 min and washed until pH 7	not reported	40 min x3	0,3% Sulfuric acid/ 0,7%citric acid and distilled water	45	overnight	11-14,3 %	Gudmundsson and Hafsteinsson (1997)
	Skin	71	10		Skin frozen at -20, Washed and incubated in 0,04 M NaOH, acid incubations 0,12 M sulfuric acid, 0,005 M citric acid	56/65	1+1 h	Extracted in water	56/65	2+2h	45 %	Arnesen and Gildberg (2007)
	Skin	72	13	13	NaCl and dilute NaOH, thereafter swelling with 0,05 M acetic acid	not reported	not reported	Extracted in water	45	overnight	not reported	Gómez-Guillén et al. (2002)
Baltic Cod *(Gadus Morhua)*	Skin	not reported	not reported	not reported	NaCl solution and water, stirring with water /centrifuged at 10,000 *g* for 30 min at 0°C	0	15-120 min	Extracted in water	30-60	15-120 min	12,30 %	Kołodziejska et al. (2004)
Spotted golden goatfish (*Parupeneus Heptacanthus*)	Scales	286,6	18,7-20,1	26,4-28,0	0,1 M NaOH / 0,75 M HCl	28-30	6 h	Extracted in distilled water	45/60/ 75	6 h / 12 h	2,3-2,6% /8,6-9,3% /9,9-10,1 %	Chuaychan, Benjakul & Sae-Leaw (2017)

Alaska Pollock (*Theragra chalcogramma*)	Skin	98 (10°C) /217 (2°C)	not reported	21,2/16,1	Ca(OH)2 with varying OH– conc for 60 min, rinsed with tap water 2 times, Thereafter varying conc of acetic acid for 45 min, rinsed again, All pretreatments performed at different temp			Extracted in distilled water	50	3 h	not reported	Zhou et al. (2006)
Yellowfin tuna *(Thunnus albacares)*	Skin	426	23,8/25,6	29,7/32,3	Frozen at −15 °C until used, 1–3% NaOH with shaking, Swollen neutralized with 6 N HCl	10	1-5 days	Hot water extraction	40-80	1-9 h	not reported	Cho et al. (2005)
	Skin	not reported	not reported	not reported	Frozen skin thawed at room temp for 1 h, Skin washed in 0,5 M NaCl (5 min, 5 °C), washing in tap water 3x, 0,1 M NaOH stirred (40 min, 20°C), The solution was washed 3x more with distilled water,	5/20	1 h/ 5 min/ 40 min	Placed in 0,1 N acetic acid solution for heating and stirring,	50	18 h	18 %	Rahman et al. (2008)
Bigeye snapper (*Priacanthus macracanthus*)	Skin	56	not reported	not reported	0,025 M NaOH, 0,2 M acetic acid	25-28/4	2 h/48 h	Treated skin mixture constantly stirred	45	12 h	not reported	Nalinanon et al. (2008)
	Skin	105,7	not reported	not reported	0,2 M NaOH at 4 °C with stirring, Washed until pH 7, 0,05 M acetic acid for 3 h at 25 °C with stirring,	4/25	3 h	Extracted in distilled water	45	12 h	not reported	Jongjareonrak et al. (2006)
Brownstripe red snapper (*Lutjanus vitta*)	Skin	218,6	not reported	not reported	0,2 NaOH at 4°C with stirring, Washed with tap water until pH 7, 0,05 M acetic acid	4/25	3 h	Extracted in distilled water	45	12 h	not reported	Jongjareonrak et al. (2006)

					3 h at 25 °C with stirring,							
African catfish (*Clarias gariepinus*)	Skin	not reported	not reported	22,1	0,2 % NaCl, 0,3 % NaOH, washing until pH 8, 0,3 % H2SO4, 0,7 % citric acid	room temp	5 min/ 80 min/ 80 min/ 80 min	Extracted in distilled water	45	12 h	5,85 %	Biluca, Marquetti & Alfaro (2011)
Channel catfish (*Ictalurus punctatus*)	Skin	243-256	15-18	23-27	0,05 M acetic acid	15	18 h	Extracted in distilled water	45	7 h	not reported	Liu et al. (2008)
Farmed giant catfish *(Pangasianodon gigas)*	Skin	153	not reported	not reported	0,2 M NaOH, 0,05 M acetic acid	1/ room temp	30 x 3 min /3 h	Extracted in distilled water	45	12 h	20,10 %	Jongjareonrak et al. (2010)
Carp (Cyprinus Carpio L)	Skin	5,68/158 ,7/267,0 8	not reported	28	Prereatment 1: 2,6% NaCl, Pretreatment 2: 0,1 N NaOH, ethanol 12 h, Pretreatment 3: 0,2% NaOH, 0,2% H2SO4, 1,0% C6H8O7	16/4/2 1	40 min/6h + 12 h/ 2 h+ 2h +2 h	Extracted with distilled water, constant stirring	45	1 h/4h/overn ight	5,21 %/10 ,47 % / 12,00 %	Tkaczewska et al. (2018)
Grass carp *(Ctenopharyngodon idella)*	Skin	267	19,5	26,8	0,1–3,0% HCl, Skin samples were washed once,	7	not reported	Extraction was done in a shaking hot water bath	hot water	40-80 min	10-19%	Kasankala et al. (2007)
Silver carp *(Hypophthalmichthys molitrix*)	Skin	not reported	not reported	27-29	Skin stored at −25 °C, 0,1 M NaOH, washed until pH 7, 10% butyl alcohol	not reported / not reported/	not reported	Extracted in distilled water	50	3 h	not reported	Zhang et al. (2012)

						overnight						
Red tilapia *(Oreochromis nilotica)*	Skin	128,1	not reported	22,4	0,2% NaOH, 0,2% H2SO4 acid, 1,0% citric acid. Rinsing with distilled water.	not reported	40 min	extraction in distilled water	45	12 h	not reported	Jamilah and Harvinder (2002)
Black tilapia *(Oreochromis mossambicus*)	Skin	180,7	not reported	28,9	0,2% NaOH, 0,2% H2SO4 acid, 1,0% citric acid. Rinsing with distilled water.	not reported	40 min	extraction in distilled water	45	12 h	not reported	Jamilah and Harvinder (2002)
	Skin	141,2	not reported		4% HCl	not reported	8/16/24 h	Extracted in distilled water	60	8 h	16,90 %	Nelson et al. (2016)
Tilapia	Skin	273 (10°C)/ 395(2°C)	not reported	25,4	Ca(OH)2 with varying OH– conc for 60 min, rinsed with tap water 2. Varying conc of acetic acid for 45 min, rinsed again.All pretreatments performed at different temp.	not reported	45 min	Extracted in distilled water	50	3 h	not reported	Zhou et al. (2006)
	Skin	101/701/ 725			Pretreatment 1: 0,1 M NaOH, 0,05 M CH3COOH, Pretreatment 2: 0,1 M NaOH, 50 mL hot water, Pretreatment 3: 0,1 M NaOH, 50 mL water, 1 M CH3COOH + 0,02g pepsin level 5 units/g	room temp	1 h/ 3h	Water bath	55	6 h	18,40 %	Zhang et al. (2020)

Tilapia (Oreochromis niloticus)	Skin	not reported	not reported	not reported	Stored at −50 °C, Thawed at 4 °C for 20 h, Tap water for 10 min, washed twice, Shaking for 4 min, 0,3 M NaOH for 1 h, Drained for 5 min, Process repeated 5x, Soaking in selected acid for 1 h, The acids used: citric acid (0,01, 0,02, 0,03, 0,04, 0,05, 0,07, 0,10 and 0,20 M), acetic acid (0,01, 0,03, 0,05, 0,10, 0,13, 0,15, 0,18 and 0,20 M) and HCl (0,01, 0,02, 0,03, 0,04, 0,05, 0,07, 0,10 and 0,20 M),	not reported	1 h	Extracted in distilled water	50	3 h	Citric acid: 10,52 % - 22,40 %, acetic acid: 1,92 % - 21,55 %, HCl: 4,47 %- 24,35 %	Niu et al. (2013)
Nile Tilapia *(Oreochromis niloticus)*	Skin	328	not reported	not reported	0.4 M NaOH, washed with running tap water, 0.4 M HCl. Washed again with running tap water until pH 7.	25	4 h /1 h/ 4 h	Extracted in distilled water	70	1,5 h	18,10 %	Songchotikunpan et al. (2008)

Young Nile perch		222	13,8	21,4	0,01 M sulphuric acid solution, washed twice	not reported	not reported	Extracted in water, cooked afterwards	50/60/ 70	5 h /5 h	12 %	Muyonga et al. (2004)
Adult Nile perch		229	19,5	26,3	0,01 M sulphuric acid solution, washed twice	not reported	not reported	Extracted in water, cooked afterwards	50/60/ 70	5 h/ 5 h	16 %	Muyonga et al. (2004)
Dover sole (*Solea vulgaris*)	Skin	350	18-19	19,4	NaCl and dilute NaOH, thereafter swelling with 0,05 M acetic acid	not reported	not reported	Extracted in water	45	overnight	not reported	Gómez-Guillén et al. (2002)
Megrim *(Lepidorhombrus boscii)*	Skin	340	18-19	18,8	NaCl and dilute NaOH, thereafter swelling with 0,05 M acetic acid	not reported	not reported	Extracted in water	45	overnight	not reported	Gómez-Guillén et al. (2002)
Hake *(Merluccius merluccius)*	Skin	110	11-12	14	NaCl and dilute NaOH, thereafter swelling with 0,05 M acetic acid	not reported	not reported	Extracted in water	45	overnight	not reported	Gómez-Guillén et al. (2002)
Sin croaker *(Johnius*	Skin	124,9	7,10	18,5	NaOH for 40 min and washed until pH 7	not reported	40 min x3	0,3% Sulfuric acid, 0,7% citric acid	40-50	12 h	14,30 %	Cheow et al. (2007)

dussumieri)								and distilled water				
Shortfin scad *(Decapterus macrosoma)*	Skin	176,9	9,90	24,5	NaOH for 40 min and washed until pH 7	not reported	40 min x3	0,3% Sulfuric acid, 0,7% citric acid and distilled water	40-50	12 h	7,25 %	Cheow et al. (2007)
Alaskan pink salmon *(Oncorhynchus gorbuscha)*	Skin	216	5,30		0,2 N sulfuric acid, 0,7% citric acid	not reported	not reported	Extracted in water	45	overnight	not reported	Avena-Bustillos et al. (2006)
Atlantic salmon (*Salmo salar*)	Skin	108	12	not reported	Skin frozen at −20 °C, Washed and incubated in 0,04 M NaOH, acid incubations 0,12 M sulfuric acid, 0,005 M citric acid	8	30 min x 4	water	56/65	2+2 h	39,70 %	Arnesen and Gildberg (2007)
	Skin	98±9,8	not reported	not reported	Skin frozen at −20 °C, P1: Treatment 1: 0,05 M NaOH, Treatment 2: 0,02 M H_2SO_4 , Treatment 3: 0,052 M citric acid	22	30 min	water	45	16 h	4,70 %	Vázquez et al. (2021)
	Skin	53,5±1	not reported	not reported	Skin frozen at −20 °C, P2: Treatment 1: 0,05 M NaOH, Treatment 2: 0,02 M H_2SO_4, Treatment 3: 0,052 M citric acid	4	30 min	water	45	16 h	4,60 %	
	Skin	0	not reported	not reported	Skin frozen at −20 °C, P3: Treatment: 1 0,8 M NaOH, Treatment 2: 0,2 M NaOH,	4/ 4/ 22	30 min/30 min/3 h	water	45	16 h	5,10 %	

					Treatment 3: 0,05 M acetic acid							
	Skin	44,5±2,9	not reported	not reported	Skin frozen at −20 °C, P4: Treatment 1: 0,1 M NaOH, Treatment 2: H3PO4 until pH 5-5,2	22	30 min/ 3 h	water	45		4,60 %	
	Skin	92±4,9	not reported	not reported	Skin frozen at −20 °C, P5: Treatment 1: 0,1 M NaOH, Treatment 2: H3PO4 until pH 5-5,2	22	30 min/ 3 h	water	45		4,80 %	
	Skin	not reported	2,8-10	not reported	0,1 NaOH solution 2x, repeated twice, 0,05 M acetic acid	10/ 10	1 h+ 1h/ 1 h	Acetic acid and NaOH with different pH (3, 4, 5).	60	3,5 h	not reported	Enrione et al. (2020)
Rainbow Trout *(Onchorhynchus mykiss)*	Skin	254	not reported	15,7	Frozen and thawed overnight at 4 °C. 0,01-0,21 M NaOH, 0,01–0,21 M acetic acid	7/ 7	1-3 h	Extracted in water	50	14-18 h	4,60 %	Shahari Tabarestani et al. (2014)
Horse mackerel (*Trachurus trachurus*)	Skin	230	8,1-18,8	not reported	Frozen at - 30 °C. 0,2% Sulfuric acid, 0,7% citric acid	4/ 4	15 h/ 18h	Extracted in distilled water	45	overnight	not reported	Badii and Howell (2006)
Sea bass *(Dicentrarchus labrax)*	Scales	305	not reported	not reported	0,1 M NaOH, 0,5 M EDTA-2Na, 0,5 M acetic acid	4	6 h/ 48 h/ 48 h	0,1 M acetic acid, distilled water	4	not reported	18,49 %	Dinçer et al. (2015)

Sea bass (Lates calcarifer)	Descaled skin	369	20	26,3-27	0,1 M NaOH, 0,05 M acetic acid	30 / 30	3 h/ 2 h	distilled water, continous stirring	45/55	3 h/6 h/12 h	45 degrees 3 hrs 51,6 %, 6hrs 55,7 %, 12 hrs 57,3 % / 55 degrees 3 hrs 62,0 %, 6 hrs 65,3 %, 12 hrs 66,4 %	Sinthusamram, Benjakul & Kishimura (2014)
Flounder *(Platichthys flesus)*	Skin	150	9	7,5-14	Frozen at −12°C and −20 °C for 15 days before use.	not reported	not reported	mild acid treatment, extraction in water	<50	not reported	not reported	Fernández-Díaz et al. (2003)

Skipjack tuna *(Katsuwonus pelamis)*	Caudal fin	126	not reported	not reported	0,025 M NaOH, 0,6 M HCl, 0,2 M acetic acid	room temp/ room temp/ room temp	1 h/ 5 days/ 3 h		50	12 h		Aewsiri et al. (2008)
Hoki *(Macruronus novaezelandiae)*	Skin	197		16,6	Frozen and thawed overnight at 4 °C. 0,8 M NaCl, 0,2 M NaOH, 0,05 M acetic acid	not reported/ 5 / not reported	not reported/ 30 min/ 3 h	Extracted in distilled water	45	12 h	17,60 %	Mohtar, Perera & Quek (2010)
Grey triggerfish *(Balistes capriscus)*	Skin	168,3			0,2 M NaOH, washed in tap water until pH 7, 0,05 M acetic acid	4/ 4	1,5 h/ 6 h	Extracted in distilled water, continous stirring	50	16 h	5,67 %	Jellouli et al. (2011)
Tiger-toothed croaker *(Otolithes ruber)*	Skin	170	not reported	20,3	Stored at −20 °C, Soaked with 0,2% sodium hydroxide, 0,2% sulphuric acid and 1,0% (w/v) citric acid, Repeated three times,	not reported	40 min x3	Extracted in distilled water	45	12 h	7,56 %	Koli et al. (2012)
Pink perch *(Nemipterus japonicus)*	Skin	140	not reported	19,2	Stored at −20 °C, Soaked with 0,2% sodium hydroxide, 0,2% sulphuric acid and 1,0% (w/v) citric acid, Repeated three times,	not reported	40 min x3	Extracted in distilled water	45	12 h	5,57 %	Koli et al. (2012)

European Eel (Anguilla anguilla)	Skin		14	21	0,025 M NaOH, 0,2 M acetic acid	25-28/ 4	2 h/48 h	Treated skin mixture constantly stirred	45	12 h	8,69 %	Sali et al. (2017)

4.2.1 Rheological properties

One of the main disadvantages with fish gelatines are that they often make gels that have less favourable rheological properties and seem to be less stable than gelatine extracted from mammals like pigs or cows (Gómez-Guillén et al., 2011). However, this is not the case for warm-water species, which seem to have more similar properties to those of land mammals, of course depending on the processing conditions, species and the raw material being used (Karim & Bhat, 2009, Rawdkuen et al., 2010, Gómez-Guillén et al., 2009). Fish gelatines have different melting points and different gelling temperatures (Boran, 2013). The gel strength can also differ from mammalian gels (Boran, 2013). This is because of the difference in amino acids (Boran, 2013). Proline and hydroxyproline are two amino acids that are important for the stability of the collagen structure (Boran, 2013). The two amino acids form hydrogen bonds that twist the collagen structure sharply by stabilizing the triple-helix structure (Boran, 2013). Fish gelatine has a lower content of hydroxyproline and proline compared to the amounts found in gelatine from mammals (Derkach et al., 2020). Scientists have also found that gelatine from warm-water species is similar to that from pork skin and calf skin (Derkach et al., 2020). According to Karim and Baht (2009) and Gómez-Guillén et al. (2009), fish from colder water environments contain gelatines that have low gelling and melting temperatures. The gelling temperatures are normally between 4°C and 12°C, while the melting temperature is less than 17°C (Karim & Baht, 2009). For warm-water species gelling temperatures are thought to be between 18°C and 19 °C, while the melting temperature is as high as 24°C to 29°C (Karim & Baht, 2009). The gel strengths also differ from cold-water species and warm-water species; 100g or lower in cold-water species and normally more than 200g in warm-water species (Gómez-Guillén et al., 2009). Different gelling and melting temperatures in cold-water fish, warm-water fish and mammals can be seen in table 4.

The viscosity of fish gelatine varies over a wide range (Alfaro et al., 2014). The reason for this is that different extraction methods are used and thereby also influence the quality of the product (Alfaro et al., 2014). Additionally, it is also highly dependent on the fish species and the molecular weight (Alfaro et al., 2014). The amounts of β- and γ-components have been reported as the major factor governing the gelation of gelatine (Taheri et al. 2009).

Table 3: Amino acid composition in certain cold-water and warm-water fish species compared to pork skin and calf skin gelatine. Table taken from Derkach et al. (2020).

Source	Cold Water Fish Skin			Warm Water Fish Skin				
	Cod [10]	Hake [10]	Alaska Pollock [2]	Tilapia [1,2]	Tuna [46]	Black Carp [19,47]	Pork Skin [2]	Calf Skin [21]
Amino Acid Composition (Residues Per 1000 Total Amino Acid Residues)								
Glycine (Gly)	344	331	358	347	336	314	330	313
Basic groups	99	97	91	86	90	88	86	101
Lysine (Lys)	29	28	26	25	25	29	27	34
Hydroxylysine(Hyl)	6	5	6	8	6	2	6	11
Histidine (His)	8	10	8	6	7	4	4	5
Arginine (Arg)	56	54	51	47	52	53	49	51
Carboxylic groups	130	123	125	117	115	126	118	116
Aspartic acid (Asp)	52	49	51	48	44	48	46	45
Glutamic acid (Glu)	78	74	74	69	71	78	72	71
Hydroxylic groups	142	134	146	140	150	131	147	144
Serine (Ser)	64	49	63	35	48	37	35	37
Threonine (Thr)	25	22	25	24	21	25	18	18
Hydroxyproline (Hyp)	50	59	55	79	78	69	91	86
Tyrosine (Tyr)	3	4	3	2	3	0	3	3
Hydrophobic groups	286	314	280	309	321	336	322	326
Alanine (Ala)	96	119	108	122	119	119	112	114
Valine (Val)	18	19	18	15	28	22	26	22
Leucine (Leu)	22	23	20	23	21	22	24	25
Isoleucine (Ile)	11	9	11	8	7	12	10	11
Proline (Pro)	106	114	95	119	117	133	132	135
Phenylalanine (Phe)	16	15	12	13	13	14	14	13
Methionine (Met)	17	15	16	9	16	14	4	6

Although using fish skin from warm-water fish species should be a good opportunity to increase the production of fish gelatine, there are several limitations; it is very challenging to control and monitor the quality of the raw material (Hattrem & Draget, 2014). Secondly, there is a high chance that the skin is microbially contaminated (Hattrem & Draget, 2014). Furthermore, fish skins have not been considered valuable historically, therefore it can be difficult to change this perception in fishermen (Hattrem & Draget, 2014). The availability of the raw material will depend on quotas and regulations in fisheries and the availability of useable by-products (Hattrem & Draget, 2014). If warm-water fish are being used in aquaculture on a larger scale, it is possible that this can become a more reliable source of gelatine in the future compared to being reliant on fisheries.

The content of collagen in fish varies with the species (Boran, 2013). In addition to this the feeding behaviour of the fish and the maturation stage has an influence (Boran, 2013). The collagen in fish is different from the collagen found in pigs and cows (Boran, 2013). The concentration of essential amino acids is much/significantly higher in fish collagens than in collagen found in pigs and cows (Boran, 2013). The amount of hydroxyproline residues is much lower in fish collagen, like mentioned before (Boran, 2013). Table 3 shows the amino acid composition in gelatines from cold-water fish species, warm-water fish species and mammals.

Table 4: Comparison of melting and gelling temperatures of gelatin from mammals, cold-water fish and warm-water fish Figure taken from Darkach (2020).

Gelatin	Gelling Temperature, °C	Melting Temperature, °C
Cold water fish gelatin	4–8	16–18
	7–11	11–19
	4–5	12–13
	4–12	<17
	4–10	13–16
		16–21
	7–9	18–20
	12	14–21
Warm water fish gelatin	21–22	28–29
	15–20	20–27
	18–19	24–29
		22
		22–29
	19–22	24–25
Mammalian gelatin	26–27	33–34
	20–25	28–31
		29

4.3 Aim of the project

The aim of this project was to compare the methods used by Fossen (2020) and Alvarez (2018) to extract gelatine from Atlantic salmon skin and viscera. The aim was to compare yield and properties of the extracted gelatines. The method used by Alvarez (2018) was tested on salmon viscera only. However, in this project it was decided this method should also be tested on salmon skin. The method used by Fossen (2020) was originally only tested on salmon skin, and the same was done in this experiment. The experiments have been carried out on a smaller scale than the experiments mentioned above because of time restraints. Furthermore, an overview over different gelatine extraction methods in different fish species was to be created as a part of this thesis.

5 Materials and methods

5.1 Preparation of raw material

The salmon skins used were obtained from Nutrimar AS. The fresh skins were received in April 2022 at the Department of Biotechnology and Food Science at NTNU. The remaining skins were washed and rinsed in distilled water. The skins were cut into 2 cm^2 squares with a scalpel and measured to weigh 10.00g ± 0.03g. They were stored in a fridge at the lab at 4°C between processes. The rest of the raw material was divided into portions and frozen at -20°C degrees for possible further experimentation.

5.2 Gelatine extraction method

The extraction methods used in this thesis were based on the extraction methods used by Fossen (2020) and Alvarez (2018). The methods were adjusted and the flowcharts of the two different extraction processes are shown in Figure 3 and Figure 4. In Fossen (2020) only fish skin was tested, while viscera were tested in Alvarez (2018). The two methods are slightly different; whereas Fossen (2020) goes straight to removal of non-collagenous proteins, Alvarez (2018) has a short thermal treatment first. Several of the steps are repeated more often in the method used by Fossen (2020) compared to the one used by Alvarez (2018). The extraction is also longer in the method used by Fossen (2020) (12 h in Fossen (2020) compared to 2 h in Alvarez (2018)). Stronger concentrations of chemicals than Fossen (2020) used, were utilized in this experiment due to a reading error. All solids and liquids from the process were kept in in separate containers in the freezer in case of further use.

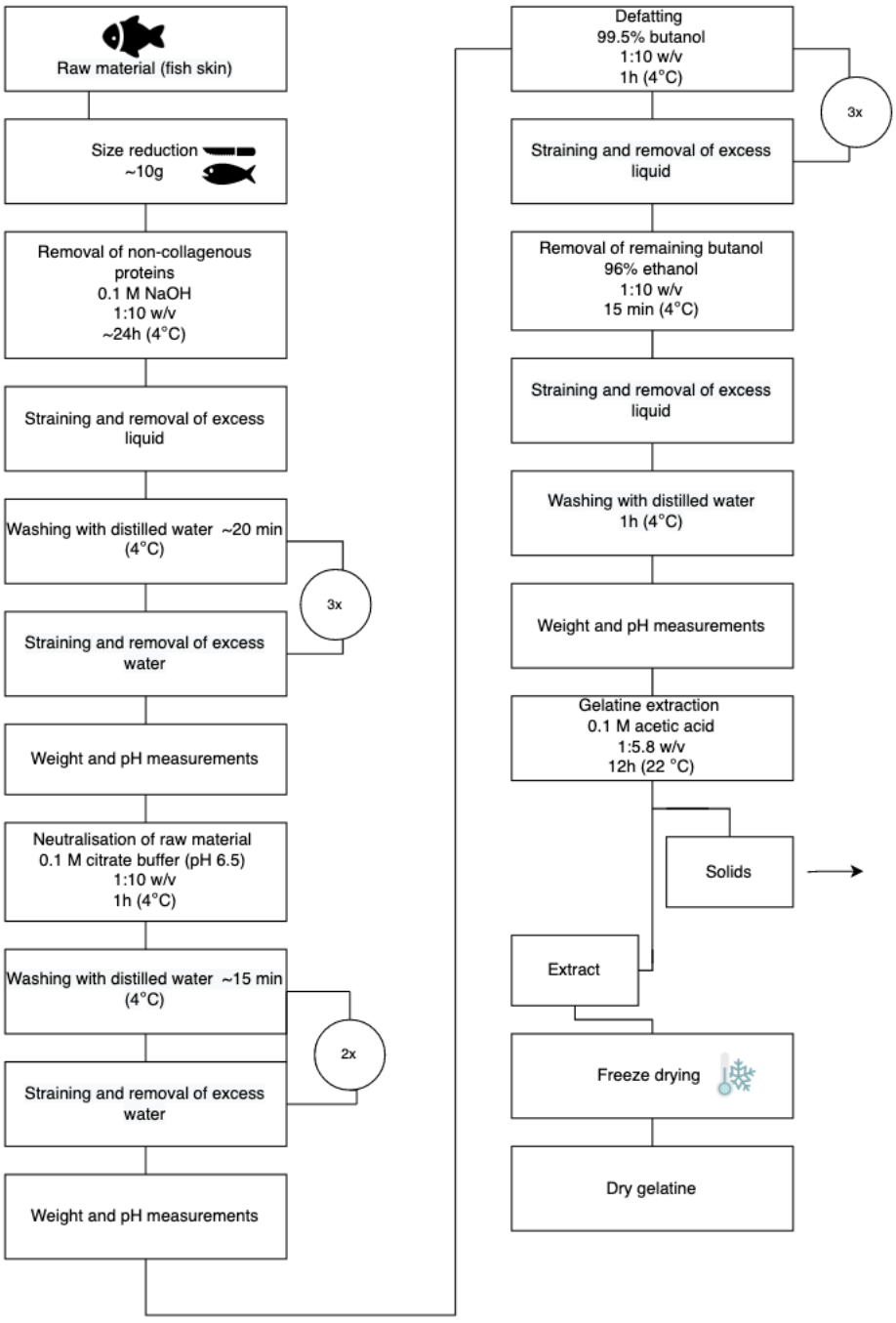

Figure 3: Flowchart based on Fossen (2020) showing the gelatine extraction process without heat treatment.

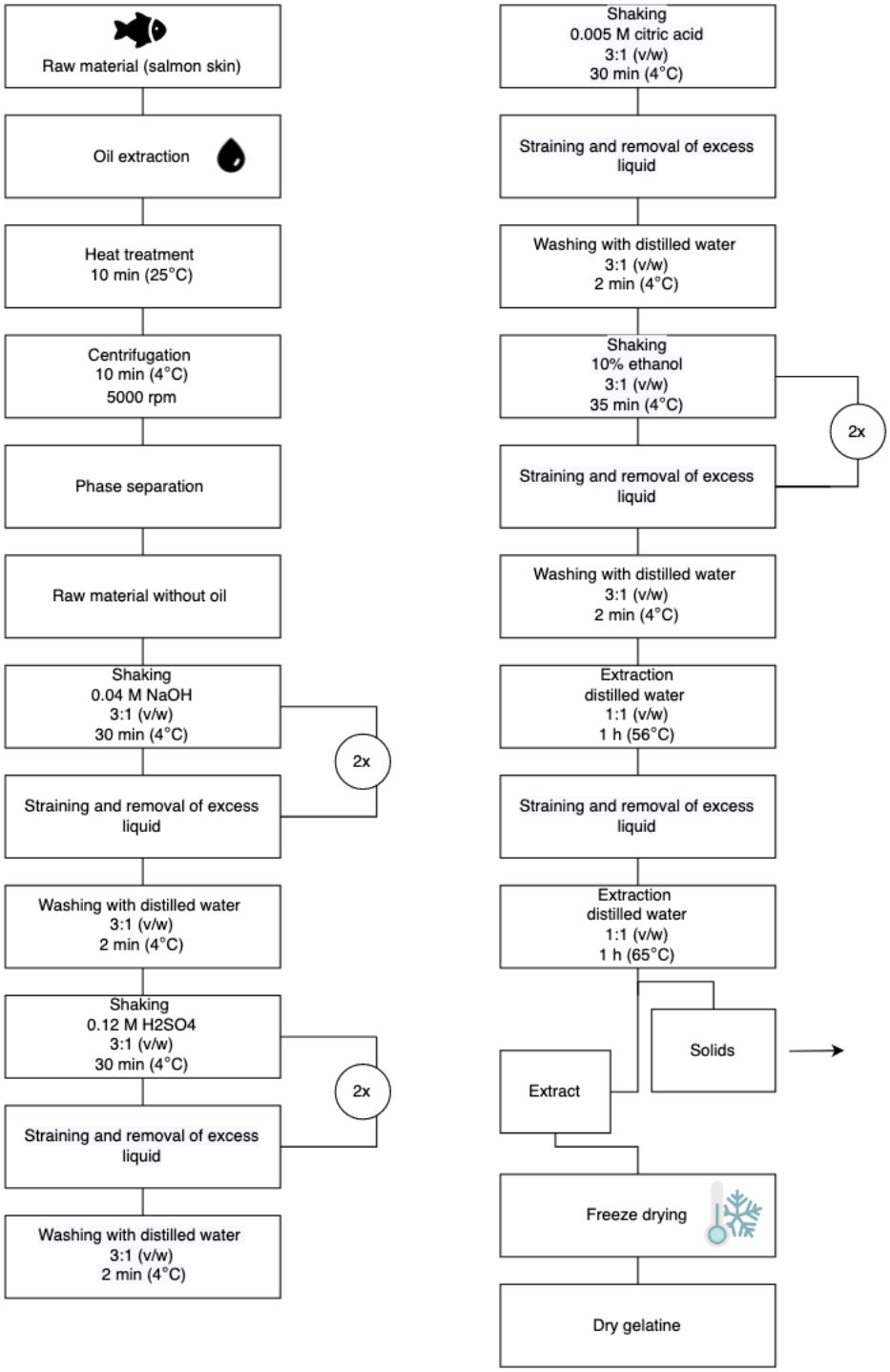

Figure 4: Flowchart based on Alvarez (2018) showing the gelatine extraction process with heat treatment.

5.2.1 Pre-treatment with acid

The pre-treatments were used to remove non-collagenous proteins from the salmon skins, and to make the gelatine available for extraction by breaking up the bonds in the collagen molecules (Schrieber & Gareis, 2007).

Following the method of Fossen (2020) the salmon skins were put in 0.1 M NaOH with a ratio of 1:10 weight by volume (w/v) (10 g raw material and 100 mL NaOH). The skins were gently shaken for 24 hours at 4°C (Heidolph Unimax 1010). Following the alkali pre-treatment the skins were put in a sieve with a cheese cloth to separate it from the NaOH solution. The excess NaOH was squeezed out and put in the freezer. In order to reach a neutral pH in the skins they were submerged into distilled water while being gently shaken for three cycles of 20 minutes. After each wash the liquid was strained using a sieve and cheesecloth. The liquid was put in the freezer. The next step was to treat the raw material with 0.1 M citrate buffer (pH 6.5, 1:10 w/v) for the duration of one hour at 4°C. the raw material and the solution was separated by using the sieve and cheesecloth again. Lastly the raw material was submerged in distilled water for two cycles of 15 minutes. The excess liquid wrung out with a cheesecloth. The skins were then weighed and put into sealed plastic bags. Thereafter they were stored in the freezer at (-20°C).

Following the method of Alvarez (2018) the raw material was first given a heat treatment for 10 min at 25°C. Thereafter the skins were centrifuged (Heraeus Multifuge X1R) at 5000 rpm/ 7334 g for 10 minutes in 4°C. The oil that had come out during centrifugation was separated from the skins and frozen at (-20°C). The skins were then put in 0.04 M NaOH with a 3:1 (v/w) ratio. They were shaken (Heidolph Unimax 1010) for 30 min at 4°C in two cycles. In between the cycles a sieve was used to strain of the liquid and a cheesecloth to squeeze out the excess liquid. Thereafter the raw material was washed in distilled water with a 3:1 (v/w) ratio for 2 minutes. The liquid was strained, removed and frozen. In the next step 0.12 M H2SO4 was added to the skins with a 3:1 (v/w) ratio. The solution was shaken for 30 minutes in two cycles at 4°C. After each cycle, the liquid was strained and put in the freezer. Following this, the skins were washed again for 2 minutes in distilled water with a 3:1 (v/w) ratio. The liquid was strained again before moving on to the next step.

5.2.2 Defatting

Following the method of Fossen (2020) the raw material was defatted by using 99.5% butanol in a 1:10 (w/v) ratio at 4°C. This was done three times in total for 1 hour each time. Between the cycles the excess liquid was strained and removed with a sieve and a cheesecloth. After this the salmon skins were submerged in 96% ethanol for 15 min at 4°C in a 1:10 (v/w) ratio.

Following the method of Alvarez (2018) 0.005 M citric acid was added to the raw material with a 3:1 (v/w) ratio. Next the skins were washed for two minutes in distilled water before straining and removing the excess liquid. Thereafter 10% ethanol was added to the raw material and was shaken for 35 minutes in 4°C in two cycles. Between and after the cycles the liquid was strained and removed. Thereupon the skins were washed for 2 minutes in distilled water with a 3:1 (v/w) ratio.

5.2.3 Extraction

Following the method of Fossen (2020) gelatine was extracted from the salmon skins using 0.1 M acetic acid with a 1:5.8 (w/v) ratio for 12 hours in room temperature. Solids and liquids were separated after the extraction using a sieve and cheesecloth. The liquid was transferred into vessels suitable for freeze-drying. Solids were put in the freezer at -20°C.

Following the method of Alvarez (2018) distilled water was added to the salmon skins in a 1:1 (v/w) ratio and put in a warm bath for 1 hour on 56°C. Thereafter the excess liquid was taken out and put in the freezer before adding 1:1 (v/w) ratio of water again. The raw material was then in a warm bath for another hour on 65°C. The solids and liquids were separated after the extraction using a sieve and cheesecloth. The liquid was transferred into vessels suitable for freeze-drying.

5.3 Analyses

5.3.1 Hydroxyproline content

The content of hydroxyproline in the gelatine samples was determined by using the method of Leach (1960) (which is modified based on Neuman and Logan 1950). The standard used was L-hydroxyproline (Sigma Aldrich, Oslo, Norway). The measuring range was 5, 10 and 15 µg/ml. The samples from the method used by Alvarez (2018) were diluted 1:10 and the samples from the method used by Fossen (2020) were diluted 1:25. There were three replicates for each sample.

A blank was made from doubly distilled water. In addition to this, standards were made. First 0.5 ml CuSO4 and 0.5 ml NaOH was added to the blank, the standards and the samples. They were shaken immediately afterwards to give a blue solution. Marbles were placed on the top of the test tubes and placed in a water bath at 40°C for 5 min. Thereafter they were taken out and 0.5 ml 6% H2O2 was added before the test tubes were shaken immediately after. Next the tubes were put back into the water bath at 40°C for 10 min with marbles on the top. The tubes were gently shaken. The solution turned green. The samples were cooled in running water until they had approximately reached room temperature. Thereafter the samples were placed in the fume hood and 2 ml of 1.5 M H2SO4 and 1 ml 5% p-dimethylaminobenzaldehyde in 1-propanol were added to the blank, standards and samples. They were shaken immediately, and the solution turned blank. The marbles were put on top of the test tubes again, and the test tubes were put back into the water bath at 70°C for 16 min. The solution turned pink. After this the samples were cooled to room temperature and shaken. Optical density (OD) was measured at 555 nm with a spectrophotometer (Pharmacia Biotech Ultrospec 2000). The blank (doubly distilled water) was used as reference.

From the values of the spectrophotometer, a standard curve was made. The slope and intercept were used to calculate the concentration of hydroxyproline in the samples. A factor of 11.42 was used to convert the amount of hydroxyproline into gelatine/collagen content (Sato et al., 1991).

5.3.2 Lipid content

The lipid content was measured in the raw material (salmon skins) based Bligh and Dyer's (1959) method. The whole process before centrifugation was done under a fume hood. 5-10 g of raw material was weighed and put in a centrifuge bottle. Both the samples and the

chloroform were put on ice to prevent a high degree of evaporation. To the centrifugation bottles 16 mL of distilled water, 40 mL of methanol and 20 mL of chloroform was added. This was homogenized for 2 min (IKA T25 digital Ultra Turrax). Thereafter 20 mL of chloroform was added and homogenized for 40 seconds. Next 20 mL of distilled water was added and homogenized for 40 seconds. Following this the caps were put onto the centrifugation bottles and they were transferred to the centrifuge (Multifuge X Pro Series). Here they were centrifuged at 9000 rpm/ 13202 g for 10 min at 4 °C.

The chloroform phase was pipetted out of the centrifugation bottles and put into pre-weighed and pre-dried tubes with caps. 2 mL was put in each tube. There were two parallels. The parallels were placed in a heat block (Pierce 18780 Reacti-Vap) at 60°C for 5 min. while being flushed out. The samples were flushed with nitrogen gas. Thereafter the caps were put on the tubes and they were placed in a desiccator until they had cooled down. Next, the tubes were weighed and the percentage of lipids was calculated using Equation 1.

$$total\ lipid\ \% = \frac{a \text{ x } b \text{ x } 100}{c \text{ x } v}$$

Equation 1: Where a = evaporated fat (g), b = chloroform added (mL), c = evaporated chloroform (mL) and v = extraction sample (g)

5.3.3 SDS-PAGE

0.1 g of freeze-dried gelatine was mixed with 1 mL doubly distilled water. The sample was moved to an Eppendorf tube and 0.5 ml of buffer was added. It was mixed for a few seconds before being placed in a water bath at 70°C for 10 min. thereafter 0.1 ml 10% glycerol was added, and the sample was mixed. 100 µl distilled water was added to the container with buffer and tracker dye (High molecular weight standard). ClearPAGE SDS Gel 4-20% was used in the apparatus. The running buffer was made from 40 ml ClearPAGE SDS Standard TEO-Tricine Running Buffer and 760 ml distilled water. The inner chamber was filled above the gel wells. The rest of the buffer was added to the outer chamber. 10 µl with sample was then put into the wells, and 10 µl of standard was added to well 1 and 12. Electrophoresis was then performed at 180V, 25 W and 180 mA for 40 min. At this point the standards had reached the steel thread at the bottom of the gel. The gel was taken out and the running buffer was put into glass containers. The gel was taken out of the plastic container and put onto the tray. Over it a sheet of damp paper was placed. It was then closed and put into the colouring machine. There the gel was dyed for 9,5 minutes. When the dyeing was completed, the tray was taken out and the paper was removed. The gel was put in a Petri dish with distilled water to rinse off some of the dye.

5.3.4 Dry matter

The amount of dry matter was determined by firstly drying and weighing the crucibles. Thereafter 2-5 grams of raw material was weighed and put in the pre-weighed crucibles. Next the crucibles with material were placed into an oven at 105°C. The samples were left in the oven for approximately 24 hours. After this the samples were removed from the oven and put in a desiccator. The samples stayed there until reaching room temperature. Thereafter they were weighed and the percentage of dry matter was calculated using Equation 2:

$$Dry\ matter\ (\%)\ =\ \frac{(b-D)}{s}\ \text{x}\ 100$$

Equation 2: Where b = the weight of the crucible and the dry raw material (g), D = the weight of the crucible (g) and s = the sample (g)

5.3.5 Ash content

The ash content was found as a continuation of the process in 5.3.4 (dry matter). After that the samples had been in the oven and had been weighed, they were put in an ash oven for 12 hours at 550°C. Next, they were taken out of the oven and put in a desiccator until they had reached room temperature again. The samples were then weighed, and the ash content was calculated using the same equation as for dry matter (Equation 2).

5.3.6 Mass balance, yield and extraction efficiency

To determine the mass balance, the amount of dry matter removed from the salmon skins, the amount of gelatine and the amount of protein was measured. The result of this was compared to the dry matter content in the raw material (salmon skins). When determining the amount of dry matter removed during the pre-treatments, the approximated input of NaOH was removed from the total.

To calculate the gelatine yield, the data from hydroxyproline determination was used. This was based on the sample purity and total extraction yield.

To calculate the extraction efficiency, the amount of freeze-dried gelatine was compared to the weight of the dry matter in the raw material. This was done with an equation based on the work of Arnesen & Gildberg (2007) Equation 3:

$$\text{Extraction efficiency}\ =\ \frac{Weight\ of\ freeze{-}dried\ gelatine\ (g)}{Weight\ of\ dry\ matter\ in\ raw\ material\ (g)}\ \text{x}\ 100$$

Equation 3: Extraction efficiency

5.3.7 Amino acid composition

To learn what the amino acid composition of the freeze-dried gelatine samples were, Reversed-Phase Ultra High Performance Liquid Chromatography was used (RP-HPLC) (Thermoscientific Dionex UltiMate 3000) together with column from Nova-Pak. The pre-column derivatization used the o-phthalaldehyde method, also known as OPA.

Samples from both extraction methods were hydrolysed in triplicates following the method by Blackburn (1978). For the method of Fossen (2020), 50 mg of freeze-dried gelatine was used for hydrolysis, while for Alvarez's (2018) method only 25 mg was used. All the samples were hydrolysed for 22 hours at 105 degrees after adding 1 ml of 6 M HCl. The samples were cooled and transferred to 10 ml beakers using doubly distilled water. Thereafter the pH was adjusted with NaOH until it reached between 6.5-7.5. Next the samples were filtered through Whatman filters (Whatman glass microfibre filter GF/C, 1.2 µm) while using suction. After filtering the samples were transferred to 10 ml flasks and were filled up until 10 ml with doubly distilled water. The samples were diluted 1:500 and filtered through 0.22 µm filters (Whatman, 0.2 µm, F30/0.2 CA-S) with a syringe. Thereafter 0.205 ml of the samples were transferred to HPLC sample glasses.

Siri Stavrum at the Department of Biotechnology and Food Science (NTNU) performed the RP-HPLC procedure. Furthermore, it should be noted that RP-HPLC cannot detect certain amino acids; cysteine, proline, tryptophan, hydroxylysine and hydroxyproline respectively. This is because the OPA method is used. It can also not differentiate between arginine and glycine. There is also a chance that the amino acids threonine and serine are destroyed before the amount can be determined. This is due to that these amino acids are acid labile (Darragh et al., 1996). To compensate for the losses of these amino acids a correction factor was used; 1.03 for threonine and 1.14 for serine (Bunka et al., 2009).

6 Results and discussion

6.1 Gelatine

6.1.1 Yield

Based on Equation 4, the yield was calculated to be 6,85% for the method used by Fossen (2020) and 0,46% for the method used by Alvarez (2018).

$$Yield\% = \frac{Weight\ of\ freeze\ dried\ gelatine\ (g)}{Weight\ of\ initial\ raw\ material\ (g)} \text{ x } 100$$

Equation 4: Yield

If the experiment had been on a larger scale, it would have been easier to investigate more parameters. With the scale that was used for this particular experiment, each test had to be carefully considered in order to make the freeze-dried gelatine last as long as possible. The results would most likely have been more interesting if it was possible to experiment and fail more. The reason for the small scale was time constraints. This is also why methods were not tested more thoroughly.

If more heat had been applied, it could have been that the yield had been higher as was seen in Sinthusamram, Benjakul & Kishimura (2014). This because the bonds between the a-chains in the collagen of the salmon skin had been more effectively destabilised. As a result of this, the triple-helix structure will become more amorphous and ultimately lead to a higher yield. However, seeing that Atlantic salmon is a cold-water fish, it should not need to be subjected to very high temperatures to get a high gelatine yield compared to a warm-water fish.

6.1.2 pH

The pH values in a gelatine solution can indicate if the gelatine type that has been obtained is either type A or type B. within a range of pH values it can be determined which type of gelatine has been obtained; pH 3,8-5,5 for type A gelatine and pH 5,0-7,5 for type B gelatine (GMIA, 2019).

pH was only measured for the method of Fossen (2020) because Fossen (2020) has included this in the flowchart for the method which was followed in this thesis. Alvarez (2018) did not measure pH and therefore it was not done for the entirety of the experiment. In hindsight pH should of course have been measured here as well, but when the realization hit, it was already too late. The pH from the method used by Fossen (2020) throughout the whole experiment and the pH from the liquid extract in the method used by Alvarez (2018) can be seen in Table 5.

Table 5: Variation of pH during the experiments, from untreated raw material to liquid extract.

	pH				
Parallel	Untreated salmon skin	After pre-treatment	After washing and neutralisation	After defatting	Liquid extract
E1	7,1	11,4	7,3	7	6,7
E2	7,1	11	7,1	6,8	6,6
E3	7,1	11,1	6,8	6,8	6,6
R1	NR	NR	NR	NR	6,6
R2	NR	NR	NR	NR	7,1
R3	NR	NR	NR	NR	6,9

From the methods used by Fossen (2020) and Alvarez (2018), it was possible to decide which type of gelatine that was obtained during the gelatine extraction process. Gelatine type B was most likely obtained from both methods used in this experiment.

6.1.3 SDS-PAGE

Like mentioned in 5.3.3, SDS-PAGE was used to analyse which molecules where present in the samples. The results from the SDS-PAGE can be seen in Figure 5. Compared to the results in Alvarez (2018) there seems to be a larger presence of high molecular weight components in this experiment. One of the reasons for this could be that Alvarez (2018) used viscera in the experiment, while skin has been used in this experiment. Viscera is a type of raw material that has active enzymes which could in turn explain the degradation of the gelatine which was seen in the SDS-PAGE in Alvarez (2018). In the gel pictured in Alvarez (2018), the majority of the bands are in the bottom, indicating molecules with a low molecular weight. This also indicates that the degradation of collagen was lower during this experiment. The topmost faint bands in E1 could represent γ-chains (Enrione et al., 2020). At 220 kDa it is most likely β-chains that can be seen. The two bands seen at 100 kDa and directly above could represent α-chains, which is expected according to Boran & Regenstein (2010). Compared to the results of Enrione et al. (2020) (Figure 6), the results from this experiment very similar.

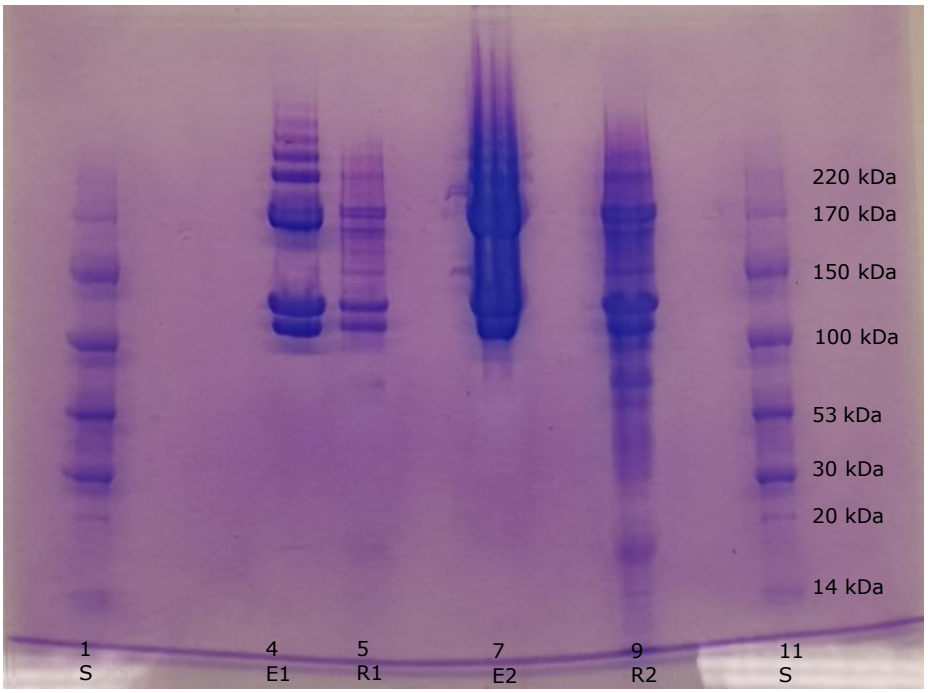

Figure 5: SDS-PAGE made with freeze-dried gelatine extract from salmon skin with acid pre-treatments. Lane 1 is a standard, lane 4 is sample E1 with 1:10 dilution, lane 5 is sample R1 with 1:10 dilution, lane 7 is sample E2 with 1:2 dilution, lane 9 is sample R2 with 1:2 dilution and lane 11 is also a standard. E is based on the method used by Fossen (2020) and R is based on the method used by Alvarez (2018).

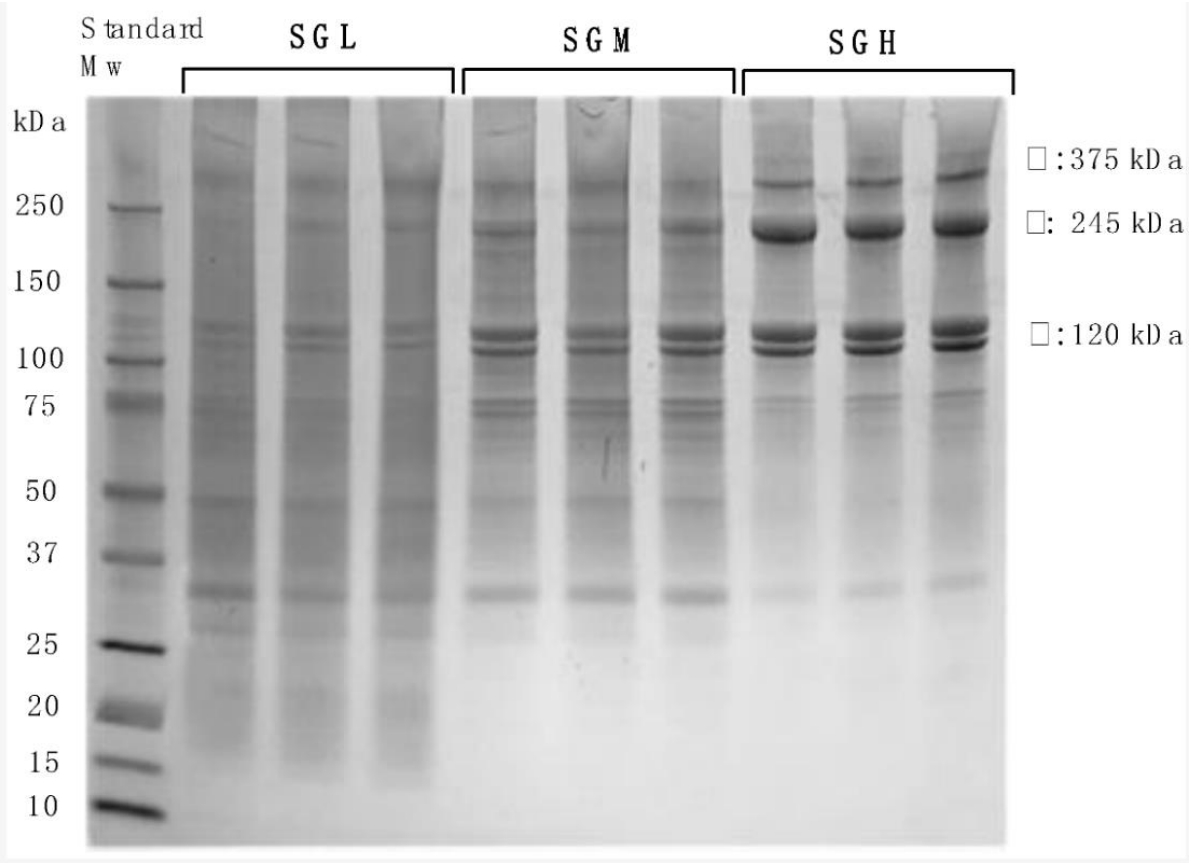

Figure 6: SDS-PAGE made with dried salmon gelatine (SG) with low (SGL) medium (SGM) and high (SGH) controlled molecular weight. Figure taken from Enrione et al. (2020).

After several failed attempts at getting any patterns in the gel, a new solution with gelatine and doubly distilled water was made in hope of getting readable patterns. It was evident that there was not a lot of proteins left in the samples, nevertheless there was enough to get some patterns given that the dilution and the concentration of the sample was right. The perfect degree of dilution was never found, but 10 mg/ml seemed to work well. However, this is several times higher than what the protocol suggests. The suggested amount is 0.04-0.06 mg/ml given that the protein content is 100%. Of course, it would be wrong to assume that these samples have 100% protein content because they have been washed with alcohols and acids in several different steps. Due to this, most of the non-gelatine proteins have most likely been washed and strained away. It is also possible that some of the gelatine has been lost in the process.

The chamber that the gel was put into was often not completely sealed, and therefore a lot of the buffer solution leaked into the outer chamber. On some occasions it was not discovered before the buffer had been filled into the outer chamber as well. The buffers from the inner and outer chamber are not supposed to be mixed and the gel-electrophoresis had to continue. After a certain amount of time the buffer in the inner chamber would have too low levels, and thereby stopping the gelatine solution in the wells from being dragged downwards through the gel. Naturally, this led to invalid results. Different types of gels were also tried out, but there was no manual, and it was a learning process with no results that could be used. The gel-electrophoresis method was not written in enough detail, and therefore caused a lot of failed attempts.

6.1.4 Amino acid composition

To determine the amino acid composition in the gelatine extracted from the salmon skin, RP-HPLC was used as described in 5.3.7. The raw data can be found in Appendix A and Figure 7 shows the average values.

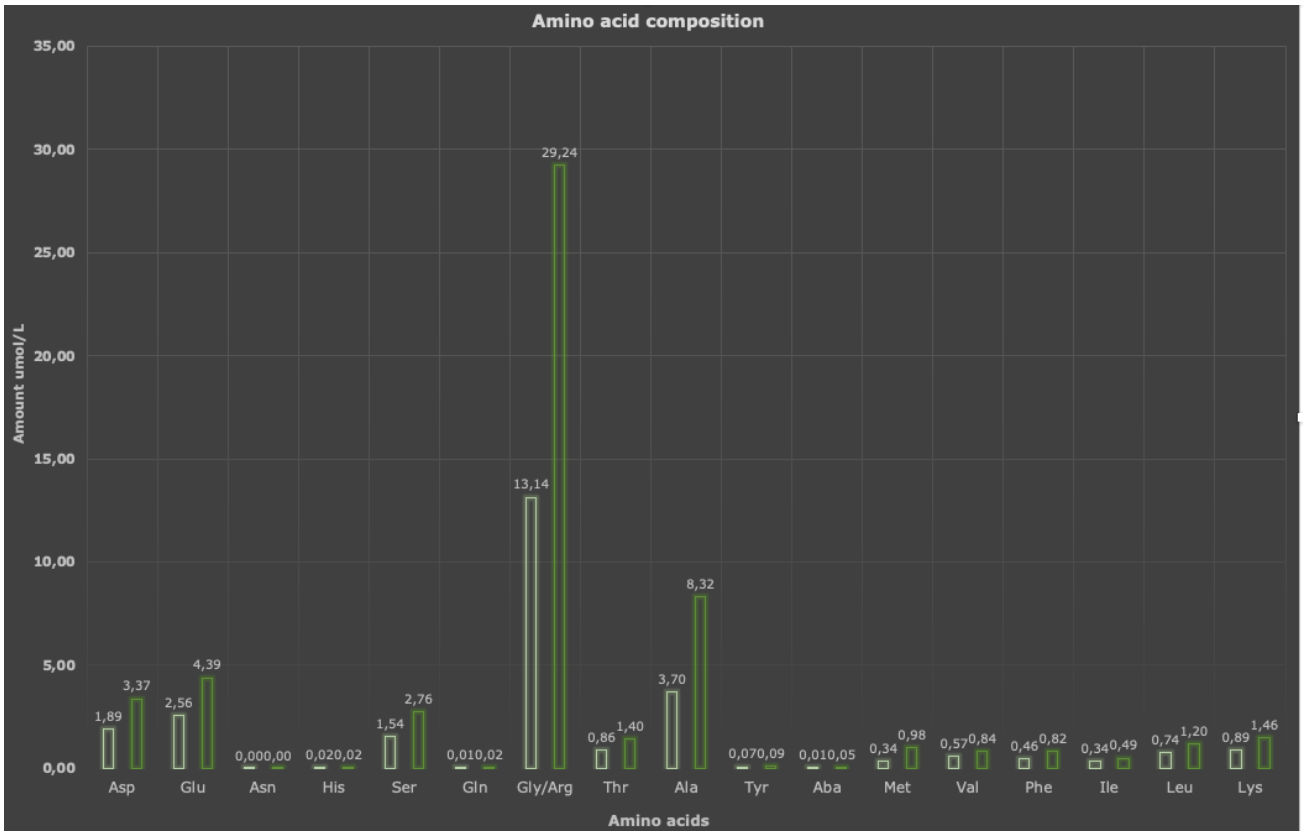

Figure 7: Amino acid composition in the two compared methods of Fossen (2020) and Alvarez (2018). Dark green values are the average from the method used by Fossen (2020) method and the light green values are from the method used by Alvarez (2018).

Glycine and arginine cannot be detected separately by the RP-HPLC method (Li et al., 2012). However, it has been estimated that glycine represents 80% of the total amount represented in the combined column in Figure 7 (Karim & Bhat, 2009). This is because glycine normally makes up more than 30% of the total amount of amino acids in gelatine (Karim & Bhat, 2009). Arginine on the other hand, only makes up around 5% (Karim & Bhat, 2009). Figure 6 shows that glycine is the most common amino acid in salmon skin gelatine. This corresponds with the findings of Fossen (2020). Overall, there is a higher content of amino acids in the gelatine after using the method of Fossen (2020) compared to the method of Alvarez (2018), even though harsher chemicals were used in the method used by Fossen (2020). In both the samples no asparagine was detected. Following this, histidine and tyrosine had the lowest levels in the method used by Fossen (2020). In the method used by Alvarez (2018), glutamine and amino butyric acid had the lowest values. Fossen (2020) had high levels of glycine and alanine. This correlates to the results from this experiment. Compared to the results of Vázquez et al. (2021) shown in Table 6, the values from this experiment are quite similar.

Table 6: Amino acid composition in salmon skin using 5 different treatments. The values are given in % or g/100 g total amino acid. OHPro is hydroxyproline. Pr: % of protein. P1: 0,05 M NaOH, 0,02M H_2SO_4, 0,052 M citric acid at 22 °C. P2: 0,05 M NaOH, 0,02M H_2SO_4, 0,052 M citric acid at 4°C. P3: 0,8 M NaOH, 0,2 M NaOH, 0,05 M acetic acid at 4 and 22 °C. P4: 0,1 M NaOH, H_3PO_4 at 22°C. P5: 0,1 M NaOH, H_3PO_4 at 22 °C. Table taken from Vázquez et al. (2021).

AA	P1	P2	P3	P4	P5
Asp	6.61 ± 0.09	6.84 ± 0.21	6.81 ± 0.08	6.71 ± 0.29	6.79 ± 0.04
Thr	2.87 ± 0.01	2.84 ± 0.03	2.73 ± 0.03	2.93 ± 0.19	2.74 ± 0.09
Ser	4.80 ± 0.13	4.92 ± 0.10	4.82 ± 0.31	4.85 ± 0.12	4.83 ± 0.04
Glu	10.44 ± 0.17	11.01 ± 0.04	10.61 ± 0.03	10.95 ± 0.09	10.40 ± 0.04
Gly	21.74 ± 0.10	21.27 ± 1.66	22.58 ± 0.12	21.49 ± 1.18	22.46 ± 0.25
Ala	9.12 ± 0.12	9.54 ± 0.38	9.43 ± 0.02	9.63 ± 0.09	9.41 ± 0.03
Cys	0.41 ± 0.23	0.25 ± 0.10	0.39 ± 0.10	0.27 ± 0.02	0.31 ± 0.02
Val	2.02 ± 0.29	1.83 ± 0.07	1.98 ± 0.05	1.80 ± 0.09	1.77 ± 0.04
Met	2.86 ± 0.34	2.46 ± 0.10	2.72 ± 0.13	2.47 ± 0.01	2.43 ± 0.02
Ile	1.51 ± 0.25	1.22 ± 0.08	1.40 ± 0.01	1.22 ± 0.01	1.32 ± 0.03
Leu	2.55 ± 0.05	2.54 ± 0.01	2.50 ± 0.01	2.69 ± 0.30	2.45 ± 0.01
Tyr	0.60 ± 0.02	0.51 ± 0.01	0.56 ± 0.09	0.53 ± 0.03	0.60 ± 0.09
Phe	2.20 ± 0.06	2.11 ± 0.13	2.25 ± 0.05	2.09 ± 0.08	2.35 ± 0.03
His	1.40 ± 0.04	1.43 ± 0.07	1.44 ± 0.07	1.42 ± 0.04	1.45 ± 0.02
Lys	3.52 ± 0.02	3.83 ± 0.30	3.92 ± 0.24	3.77 ± 0.18	3.58 ± 0.03
Arg	8.67 ± 0.16	8.50 ± 0.47	8.34 ± 0.17	8.84 ± 0.57	8.36 ± 0.11
OHPro	8.19 ± 1.11 [a]	7.50 ± 0.17 [a]	7.29 ± 0.25 [a]	7.57 ± 0.18 [a]	7.75 ± 0.21 [a]
Pro	10.53 ± 0.07 [a]	11.39 ± 0.22 [b]	10.20 ± 0.09 [c]	10.75 ± 0.31 [ad]	11.01 ± 0.04 [d]
Pr (%)	91.0 ± 3.5 [a]	91.5 ± 2.7 [a]	82.2 ± 2.9 [b]	88.9 ± 3.3 [a]	89.3 ± 2.6 [a]
TE/TA (%)	27.6 ± 1.2 [a]	26.8 ± 1.0 [a]	26.4 ± 0.5 [a]	27.2 ± 0.7 [a]	26.6 ± 0.3 [a]

To decide the collagen and gelatine content in the freeze-dried extractions, hydroxyproline was measured. As previously mentioned, hydroxyproline constitutes a large part of the total amount of amino acids in gelatine, and is therefore important to measure. The factor for trout was used to calculate the hydroxyproline content (Sato et al., 1991). Fossen (2020) also used this factor, and the results are therefore easier to compare. The average hydroxyproline content was calculated to be 7,0% of the freeze-dried gelatine in the gelatine extracted using the same method as Fossen (2020), and 3,3% using the same extraction method as Alvarez (2018). This shows that the gelatine extracted following the same method as Alvarez (2018) has a lower purity. The impurities can be other proteins or ash. Compared to Dave et al. (2019) it is roughly in the same range. Dave et al. (2019) had two different values for the hydroxyproline content in Atlantic salmon skin: one for wet tissue (1,49%) and one for dry matter (3,43%). The percentage of collagen in the freeze-dried gelatine was 80,8% for the method used by Fossen (2020) and 39,8% for the method used by Alvarez (2018). Fossen (2020) found 764,4±130,8 mg amino acid/g sample. In this experiment following the same method as Fossen (2020) the average was 748,3 mg amino acid/ g sample. Following the method of Alvarez, the average was 376,7 mg amino acid/ g sample. This is close to half of the amount found using the same method as Fossen (2020). The protein content in the freeze-dried gelatine, could also have been decided by using measuring chain weight (CN).

6.1.5 Dry matter and ash

The average ash content was calculated to be 4,02%. However, there might have happened some sort of measuring error during the process because the two parallels have

very different values (1,71% and 6,33%, respectively). Głowacz-Rózynska et al. (2016), found 2,3% ash content in salmon skins, which is considerably lower than the average in this experiment. Dave et al. (2019), found an ash content of 0,79% in their study of Atlantic salmon skin.

6.1.6 Lipid content

Using the Bligh and Dyer (1959) method the lipid content in the raw material (Atlantic salmon skin) was determined. The average lipid content found in this experiment was 23,7%. Głowacz-Rózynska et al. (2016), found 20,3% lipid content in their study of lipid content in Atlantic salmon skin. The lipid content highly depends on the fish size and the method used for removing the skin (Sila et al., 2017). A fair amount of the fat is stored right under the skin of the fish. Ahmmed et al. (2021) did a study on Chinook salmon (Oncorhynchus tshawytscha) and found only 13,4 % lipid content in the skin. Another study done on Atlantic salmon found an even lower lipid content; 11,20% (Dave et al., 2019).

7 Conclusion

As a result of the aquaculture production there is a significant amount of rest raw material which becomes available after slaughter. This can be used to extract valuable components that can be useful in many different industries. Gelatine is one such component, and it can be utilized in an array of products.

In this thesis two different methods of gelatine extraction from Atlantic salmon skin have been tested and compared to see which processes gave the highest gelatine yield and the highest purity. The method used by Alvarez (2018) involved mild heat treatment, while the method used by Fossen (2020) did not. The method used in the thesis of Fossen (2020) had the highest yield and the least degradation.

The results of this study showed that the method used by Fossen (2020) had a much higher hydroxyproline yield than the method used by Alvarez (2018). Based on the freeze-dried gelatine a yield of 6,85% was found for the method used by Fossen (2020), and 0,46% was found for the method used by Alvarez (2018). The amino acid composition of the extracted gelatine showed that the method used by Fossen (2020) yielded almost twice as much as the method used by Alvarez (2018). The SDS-PAGE showed a high degree of degradation in the samples from the method used by Alvarez (2018), compared to more high weight molecules in the samples from Fossen (2020). Regarding the hydroxyproline content, the method used by Fossen (2020) gave a total collagen yield of 7,0% based on the extracted freeze-dried gelatine, while the method used by Alvarez (2018) had a collagen yield of only 3,3%.

8 Suggestions for further work

There is still much knowledge to be obtained and discovered if these methods are tried out further. It would be interesting to see how much of the valuable components disappear and are washed away during the treatments. It would also be interesting to make hydrolysates from some of the liquids or solids that are left after each step.

Furthermore, it would be desirable to know if reducing the amount of washing has a big impact on the properties of the extracted gelatine. Like Fossen (2020) mentioned, it would be interesting to see if defatting with milder solvents would be effective enough. More tests should be made on the rheological properties of salmon skin in order to truly learn if gelatine extracted from salmon skin is a product that will do well in the future.

For future master students it could also be interesting to try out this comparison on a larger scale with more raw material and more parallels. This could make it easier to determine the parameters for the gelatine extracted.

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Appendix

Appendix 1:
Raw material
RP-HPLC

E1

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,547	7,849	69,380	6,22	11,20	2,5854
2	Glu	2,112	10,423	40,172	8,27	6,48	3,3291
3	Asn	3,482	0,006	0,058	0,00	0,01	0,0018
4	His	4,368	0,033	0,335	0,03	0,05	0,0180
5	Ser	4,712	4,877	21,166	3,87	3,42	1,5910
6	Gln	4,978	0,094	0,931	0,07	0,15	0,0284
7	Gly/Arg	8,048	67,724	262,489	53,71	42,36	24,8945
8	Thr	8,662	2,258	9,504	1,79	1,53	0,9435
9	Ala	13,198	18,272	91,030	14,49	14,69	7,8033
10	Tyr	14,558	0,099	0,722	0,08	0,12	0,0377
11	Aba	16,525	0,502	3,529	0,40	0,57	0,1488
12	Met	18,145	2,886	24,093	2,29	3,89	0,9333
13	Val	18,442	1,790	14,519	1,42	2,34	0,5495
14	Phe	18,940	1,962	16,791	1,56	2,71	0,7145
15	Ile	19,913	1,156	9,663	0,92	1,56	0,3465
16	Leu	20,263	3,084	27,490	2,45	4,44	1,0594
17	Lys	21,952	3,074	27,751	2,44	4,48	1,2162
Total:			**126,088**	**619,623**	**100,00**	**100,00**	

E2

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,548	9,073	80,226	7,08	12,69	2,9888
2	Glu	2,105	12,420	47,363	9,70	7,49	3,9671
3	Asn	3,678	0,001	0,014	0,00	0,00	0,0004
4	His	4,408	0,067	0,715	0,05	0,11	0,0367
5	Ser	4,720	8,325	39,285	6,50	6,21	2,7159
6	Gln	5,063	0,061	0,473	0,05	0,07	0,0186
7	Gly/Arg	8,053	65,140	247,524	50,86	39,15	23,9444
8	Thr	8,662	2,866	12,163	2,24	1,92	1,1977
9	Ala	13,202	15,534	77,356	12,13	12,23	6,6337
10	Tyr	14,562	0,245	1,864	0,19	0,29	0,0936
11	Aba	16,628	0,024	0,124	0,02	0,02	0,0071
12	Met	18,143	2,427	20,114	1,90	3,18	0,7850
13	Val	18,445	2,513	20,830	1,96	3,29	0,7715
14	Phe	18,942	1,926	16,802	1,50	2,66	0,7011
15	Ile	19,913	1,504	13,581	1,17	2,15	0,4507
16	Leu	20,263	2,889	26,073	2,26	4,12	0,9925
17	Lys	21,950	3,056	27,781	2,39	4,39	1,2089
Total:			**128,071**	**632,290**	**100,00**	**100,00**	

E3

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,552	13,781	125,130	6,81	12,60	4,5395
2	Glu	2,102	18,413	73,665	9,10	7,42	5,8814
3	Asn	3,450	0,003	0,021	0,00	0,00	0,0010
4	His	4,298	0,031	0,000	0,02	0,00	0,0170
5	Ser	4,715	12,215	61,281	6,03	6,17	3,9850
6	Gln	5,133	0,011	0,000	0,01	0,00	0,0034
7	Gly/Arg	8,043	105,766	390,301	52,25	39,31	38,8780
8	Thr	8,658	4,905	20,085	2,42	2,02	2,0501
9	Ala	13,203	24,661	122,498	12,18	12,34	10,5316
10	Tyr	14,570	0,380	2,729	0,19	0,27	0,1453
11	Aba	16,442	0,005	0,068	0,00	0,01	0,0016
12	Met	18,153	3,767	31,328	1,86	3,15	1,2182
13	Val	18,453	3,902	32,836	1,93	3,31	1,1982
14	Phe	18,948	2,863	25,902	1,41	2,61	1,0424
15	Ile	19,922	2,279	21,171	1,13	2,13	0,6830
16	Leu	20,272	4,509	40,994	2,23	4,13	1,5488
17	Lys	21,957	4,945	44,975	2,44	4,53	1,9562
Total:			**202,438**	**992,986**	**100,00**	**100,00**	

R1

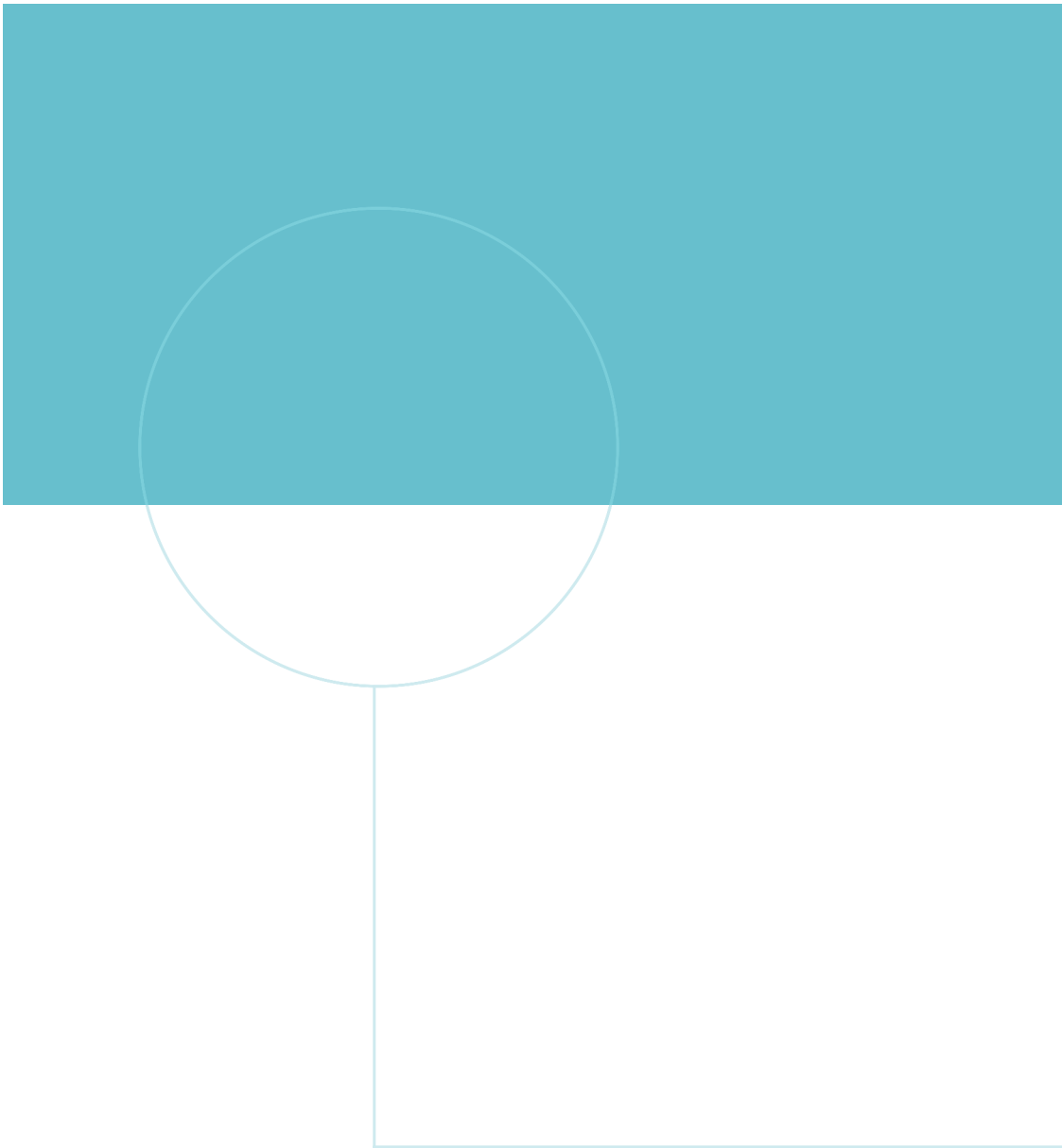
No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,555	7,103	62,681	7,68	13,53	2,3398
2	Glu	2,132	9,996	38,028	10,81	8,21	3,1929
3	Asn	3,515	0,010	0,065	0,01	0,01	0,0029
4	His	4,240	0,029	0,000	0,03	0,00	0,0159
5	Ser	4,727	5,655	27,735	6,12	5,99	1,8447
6	Gln	5,238	0,017	0,030	0,02	0,01	0,0050
7	Gly/Arg	8,072	43,748	161,916	47,32	34,94	16,0813
8	Thr	8,683	2,700	10,073	2,92	2,17	1,1284
9	Ala	13,220	10,635	52,951	11,50	11,43	4,5418
10	Tyr	14,577	0,238	1,724	0,26	0,37	0,0910
11	Aba	16,470	0,034	0,298	0,04	0,06	0,0100
12	Met	18,148	1,174	9,705	1,27	2,09	0,3798
13	Val	18,450	2,337	19,201	2,53	4,14	0,7175
14	Phe	18,943	1,610	13,544	1,74	2,92	0,5861
15	Ile	19,917	1,427	12,961	1,54	2,80	0,4276
16	Leu	20,263	2,754	25,288	2,98	5,46	0,9459
17	Lys	21,953	2,995	27,171	3,24	5,86	1,1848
Total:			**92,461**	**463,372**	**100,00**	**100,00**	

R2

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,563	5,079	45,210	7,63	13,47	1,6731
2	Glu	2,122	7,108	26,602	10,68	7,93	2,2705
3	Asn	3,515	0,007	0,061	0,01	0,02	0,0020
4	His	4,462	0,065	0,894	0,10	0,27	0,0353
5	Ser	4,725	4,400	20,916	6,61	6,23	1,4354
6	Gln	5,035	0,048	0,354	0,07	0,11	0,0146
7	Gly/Arg	8,068	31,767	119,991	47,72	35,75	11,6773
8	Thr	8,670	1,667	6,991	2,50	2,08	0,6965
9	Ala	13,213	7,711	38,297	11,58	11,41	3,2931
10	Tyr	14,575	0,192	1,392	0,29	0,41	0,0733
11	Aba	16,455	0,050	0,336	0,08	0,10	0,0149
12	Met	18,153	0,947	7,868	1,42	2,34	0,3063
13	Val	18,453	1,611	13,489	2,42	4,02	0,4946
14	Phe	18,950	1,128	9,679	1,69	2,88	0,4108
15	Ile	19,923	0,982	9,027	1,47	2,69	0,2942
16	Leu	20,273	1,912	17,366	2,87	5,17	0,6569
17	Lys	21,957	1,901	17,196	2,86	5,12	0,7520
Total:			**66,576**	**335,668**	**100,00**	**100,00**	

R3

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1,558	5,026	44,286	7,63	13,38	1,6557
2	Glu	2,128	6,985	26,585	10,60	8,03	2,2310
3	Asn	3,513	0,004	0,040	0,01	0,01	0,0012
4	His	4,237	0,001	0,000	0,00	0,00	0,0007
5	Ser	4,722	4,098	20,114	6,22	6,08	1,3369
6	Gln	5,242	0,046	0,013	0,07	0,00	0,0139
7	Gly/Arg	8,068	31,712	119,390	48,12	36,07	11,6567
8	Thr	8,673	1,808	6,931	2,74	2,09	0,7557
9	Ala	13,213	7,637	38,213	11,59	11,54	3,2612
10	Tyr	14,578	0,122	0,839	0,19	0,25	0,0468
11	Aba	16,453	0,061	0,423	0,09	0,13	0,0182
12	Met	18,155	1,034	8,572	1,57	2,59	0,3344
13	Val	18,457	1,602	13,200	2,43	3,99	0,4920
14	Phe	18,952	1,090	9,460	1,65	2,86	0,3968
15	Ile	19,923	0,959	8,767	1,45	2,65	0,2873
16	Leu	20,275	1,830	16,901	2,78	5,11	0,6287
17	Lys	21,958	1,889	17,271	2,87	5,22	0,7474
Total:			**65,905**	**331,003**	**100,00**	**100,00**	



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