Master's thesis

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

Sophie Kendler

Effect of oxidised rest raw materials on the quality of fish protein hydrolysates

Master's thesis in Food Technology and Nutrition
Supervisor: Turid Rustad
June 2019

NTNU
Norwegian University of
Science and Technology

Sophie Kendler

Effect of oxidised rest raw materials on the quality of fish protein hydrolysates

Master's thesis in Food Technology and Nutrition
Supervisor: Turid Rustad
June 2019

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

NTNU
Norwegian University of
Science and Technology

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Trondheim, June 2019

Sophie Kendler

Preface

This master thesis is part of the MSc programme Food Technology and Nutrition from the University of Applied Sciences Upper Austria, Campus of Engineering, Wels. The work was carried out during an exchange year at the Norwegian University of Science and Technology at the Department of Biotechnology and Food Science. The work was conducted as a part of the PhD project of Kristine Kvangarsnes and will be part of a publication in a scientific paper.

I would like to express my highest gratitude to my supervisor Professor Turid Rustad, without whome I would not have been able to stay a full year in Trondheim and who gave me the opportunity to write my Master Thesis in the food chemistry laboratory at NTNU. My research would have been impossible without the guidance and invaluable help Professor Turid Rustad offered me throughout the process. Furthermore, I would like to thank PhD student Kristine Kvangarsnes for her practical tips and her positive feedback as well as postdoctoral fellow Janna Cropotova for her kindness and help in the laboratory.

Trondheim, June 2019

Sophie Kendler

Abstract

The growing world population demands a solution for the utilisation of rest raw materials from primary production. There is a huge potential to make aquaculture and fishery more sustainable in the various application of rest raw materials from catches. Especially in Norway, where the fishing industry has a long tradition, it is of great importance to investigate sustainable and future orientated solutions for marine by-products. Studies on achieving a full protein recovery of fish rest raw materials have been a main focus in the past years. Fish protein hydrolysates deliver a high amount of easily digestible proteins and have a good nutritional value as well as functional properties.

In this study, fish protein hydrolysates made out of trout heads were investigated. The aim of the thesis was to study the effect of oxidised rest raw materials on the quality of fish protein hydrolysates. The raw material varied in its oxidative status and was either fresh, oxidised or oxidised with added antioxidant. The raw material and the thereof produced hydrolysates were analysed. To evaluate the properties and quality of the rest raw material and hydrolysates, different parameters were determined. These include the determination of protein content, molecular weight distribution, total and free amino acid composition, soluble and acid-soluble proteins, collagen as well as the determination of the degree of hydrolysis. Ultramembrane filtration was performed and molecular weight distributions as well as water solubility was performed on the retentates and permeates of the hydrolysates.

All hydrolysate samples showed a high content of essential amino acids and high solubility, thus exhibiting a high nutritional value and digestibility. The combination of the good nutritional value and high solubility of the proteins lead to the conclusion that the hydrolysates are a good source of dietary proteins. Furthermore, the results of the degree of hydrolysis and acid-soluble peptides showed no significant difference between the samples. The molecular weight distribution of the hydrolysates showed a high amount of small sized peptides and the ultramembrane filtration lead to two fractions of peptides.

The results show, that the overall quality of the hydrolysates are influenced by the oxidation status of the raw material. No linear correlation was found between oxidative status and free amino acid concentrations, but a correlation between degree of hydrolysis and free amino acid content of three out of the four hydrolysate samples was found. The linear correlation was found in the three samples, that were hydrolysed using the same hydrolysis condition. The hydrolysate, where the antioxidant was added directly into the hydrolysis tank, did not fit in the correlation. This indicates that the time of adding the antioxidant plays a crucial role for the outcome and quality of the hydrolysate. Furthermore, a correlation between oxidation indicators and protein solubility of three of the four hydrolysates was observed. It was found that a higher oxidation in the raw material leads to a decreased solubility in the hydrolysates, but does not necessarily mean a high oxidation in the hydrolysates. This implicates, that the ongoing oxidation process of not correctly stored rest raw material does not have a negative effect on the nutritional value of the hydrolysates, but changes the digestibility of proteins.

Table of Contents

List of Tables

List of Figures

Abbreviations

ACE	=	Angiotensin converting I enzyme
Da	=	Dalton
DH	=	Degree of Hydrolysis
FAA	=	Free amino acids
FPH	=	Fish Protein Hydrolysate(s)
FPI	=	Fish Protein Isolate(s)
FAC	=	Fat Absorption Capacity
FPLC	=	Fast Protein Liquid Chromatography
HPLC	=	High Pressure Liquid Chromatography
kDa	=	Kilo Dalton
MWCO	=	Molecular weight cut-off
RRM	=	Rest Raw Material
ROS	=	Reactive oxygen species
SD	=	Standard deviation
TAA	=	Total amino acids

CHAPTER 1

Introduction

As the world population is growing steadily and the worldwide fish resources are limited, the importance of using as much of the catch as possible and therefore optimizing manufacturing processes, is increasing. A balanced diet demands a constant supply of proteins and polyunsaturated fatty acids as important macro nutrients. By providing 17 % of the need of animal proteins and 6.7 % of the total required protein supply, fish should not be underestimated as an import protein source (Rustad et al., 2011; FAO, 2018).

Marine by-products or in other words rest raw materials show an enormous potential to provide high quality marine proteins and lipids for the world population. Besides being future-oriented by sustainably using rest raw materials from catches, by-products like viscera, heads, cut-offs, bone, skin and damaged fish can be processed to highly valuable consumer goods (Rustad et al., 2011).

Marine rest raw materials are an important value-adding resource in the Norwegian fishery and aquaculture industry. Many companies focus on exploiting by-products as good as possible. Nevertheless, there is great potential for increasing the utilization rate, especially from demersal fish catches (Richardsen et al., 2017).

Many investigations on the processing and application of rest raw material have been made in the past few years, both from industry and academia. There are still hurdles to overcome, but an increased utilization in the future seems possible.

1.1 Aim of the thesis

The aim of the thesis was to study the effect of oxidised rest raw materials on the quality of fish protein hydrolysates. The thesis is part of the project of PhD student Kristine Kvangarsnes.

Different rest raw material samples, either fresh, oxidised or oxidised with antioxidant added, were used for this study. The aim was to get information about possible differences, arising from the status of the raw material on the thereof produced hydrolysates. The focus of the planned analyses was to obtain information about the protein profile of the raw material and hydrolysate samples. As hydrolysates are high in protein and can contain from 73.8 % (Šližytė et al., 2016) to 93 % (Venugopal, 2016) of protein, it is of great importance to get information about the protein profile. This gives knowledge about the functionality and nutritional value of the samples as well as their bioactivity.

Analyses, determining the acid-soluble, water soluble and total protein concentration, as well as the molecular weight of peptides were conducted. Moreover, amino acid analyses, showing

the total amino acid content and composition as well as the amount of free amino acids in the samples were performed. Furthermore, the hydrolysates were filtrated with ultramembrane filtration in order to see if they can be fractionated based on their molecular weight. Additionally, the oxidation progress was analysed by measuring thiol and carbonyl groups. .

Fish proteins are high in essential amino acids and exhibit great nutritional values. Therefore, it is important to get information about possible correlations arising from the status of the raw material and the effect of this on the protein structure of the hydrolysates.

This study of different statuses of the raw material and the effect of these on the produced hydrolysates will hopefully give crucial knowledge about how fast rest raw material needs to be processed or frozen after the catch in order to yield high value protein hydrolysates that maintain their functionality and nutritional value.

CHAPTER 2

Background

Since 2013, salmon and trout are the top traded seafood consumer goods when it comes to value terms. They made up around 18 % of the overall price of worldwide traded fish and fish products in the year 2016. The second most exported commodities in 2016 were shrimps and prawns counting for approximately 16 %, followed by ground fish like cod, haddock or Alaska Pollock with 10 % and tuna counting 9 %. In contrast, by-products such as fishmeal and fish oil only make up respectively 3 % to 1 % of the total export value (FAO, 2018).

In the year 2013, approximately 19.8 kg of fish was eaten per person worldwide. In total, 3.2 billion people get around 20 % and 5.1 billion people approximately 10 % of their regular per capita intake of animal protein from fish. Researches indicate that the trend of getting proteins through fish and fish products will increase in near future. Furthermore, fish supply through aquaculture is estimated to be higher than through offshore fishery (FAO, 2018).

The capture production of trouts, salmons and smelts show a slight decrease in the number of catches from the year 2010 with 979 660 t to the year 2016 with 937 470 t. Whereas fish production from aquaculture is slightly increasing (FAO, 2018). As the numbers show, the salmon and trout industry has reached a very high amount of catches per year. A significant amount of the caught salmons are sold head on gutted, but a large number is also sold as fillets. Fillets of a 5 to 6 kg fish usually account for approximately 59 to 63 % of the fish wet weight, whereas trimmings (1 to 2 %), frames (9 to 15 %) and the head (10 to 12 %) make up the considerable rest (Liaset et al., 2003).

The rising amount of caught and harvested fish leads to a high amount of rest raw materials such as heads, backbones, trimmings, fins, frames and viscera. It is of high importance to not discard these high value commodities, since they offer great chances to produce various products (Nurdiani et al., 2015).

Using fish by-products has a long tradition in Norway. Earlier fish skin was used for clothes, carrier bags or as isolation on windows (Bekkevold and Olafsen, 2007). Later industrial processes were developed to produce fertilizer and animal feed out of dead fish (Rustad et al., 2011). Figure 2.1 shows the application of marine rest raw material in Norway in the year 2016. It can be seen that the majority of by-products in Norway are used for the production of silage. The production of fish silage makes use of formic acid which is added in a concentration of 2 to 3 % to minced or chopped fish (Toppe and James, 2018). A pH of 3.5 is favored, to ensure stable and storable products (Toppe and James, 2018). Fish silage finds use in animal feed, being the primary protein source in the feed of pigs and poultry (Dong et al., 1993).
A considerable amount of 143 164 t (20.8 %) of discarded fish is used for the production of fishmeal/-oil and 19.9 % of the rest raw material is used for the production of oil- and protein products based on fresh raw material.

Approximately only 10 % of the rest raw material is used for direct human consumption, including seafood products as well as cod liver oil and extracts.

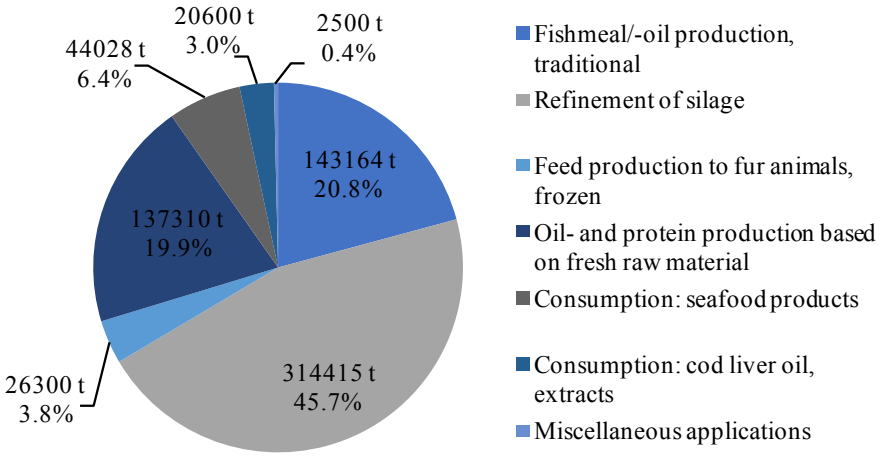


Figure 2.1: Application of marine rest raw material in Norway in the year 2016 in tons and percentage [%] adapted from (Richardsen et al., 2017).

2.1 Utilization of Rest Raw Material

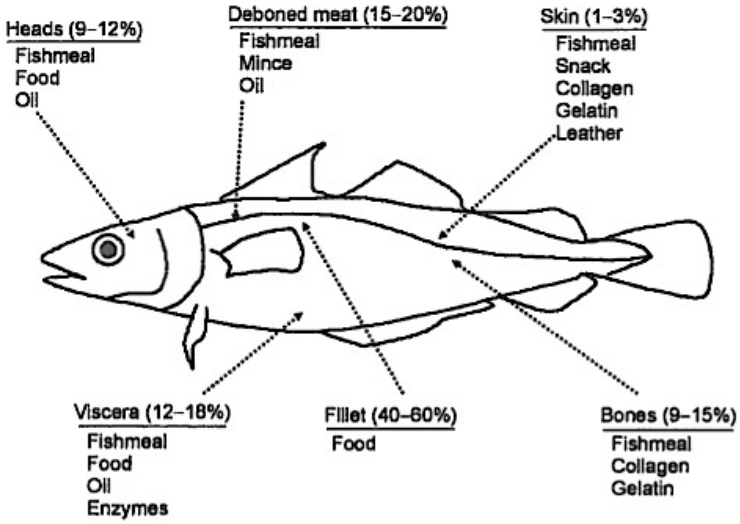


Figure 2.2: Fish by-products and their possible use as illustrated from Guerard and Shahidi (2007).

Due to the high nutritional value by-products have, they deliver important nutrients like essential polyunsaturated fatty acids such as omega-3 and omega-6 fatty acids, different vitamins and minerals and a considerable amount of protein (Nurdiani et al., 2015). This is particularly important in terms of fighting malnutrition of important micronutrients in third world countries (FAO, Fisheries and Aquaculture Department, 2014). To be able to further use marine rest raw material, correct treatment and processing is very important. According to Rustad et al. (2011) it is from high importance to directly process by-products after catching/ harvesting. Only with

rapid processing and hygienic handling of the raw material, unwanted microbial growth can be avoided and a high-quality standard can be reached (Rustad et al., 2011).

As Figure 2.1 shows, only a small amount of fish by-products are used for direct human consumption. There are fish rest raw materials that do not require any pre-treatment before they are used for human consumption. Some examples are fish roe, liver, milt and cleaned stomachs (Rustad et al., 2011), which are known as delicacies in some cultures. The food market includes a lot of different marine products, where many of these are made out of by-products. Applying easy preparation technologies like drying, preservation (canning) or salting (Rustad et al., 2011), these rest raw materials are turned into highly appreciated food commodities.
Figure 2.2 shows the different products that can be made out of different parts of fish as well as the approximate percentage of these parts (Guerard and Shahidi, 2007). The fillet accounts for the highest percentage of the fish weight (40 to 60 %). Whereas the rest of the fish can be seen as by-product, counting for approximately half of the fish weight. The fish head makes up 9 to 12 % of the total fish weight. For now, most of the fish heads are used to produce fishmeal for animals, food (seen as delicacies in some countries) and oil. There is potential to produce hydrolysates out of the head and therefore decrease the application of this high value fraction for animal feed. Moreover, the application of hydrolysates made out of heads would mean an increased offer in products with high nutritional and functional properties. Nevertheless, the interest in the application of fish head for human consumption is yet not as high as for other fractions of fish.

Some of the main fractions used for food and feed production are:

- Blood
- Marine lipids
- Protein fractions

2.1.1 Blood

Fish blood is a valuable and simply received rest component. The separation from the fish is simple as the fish is bleeded after killing, which makes the use of blood for further applications very easy. For different applications, the blood can be further separated into the red hemoglobin and colourless plasma. There are various fields of applying blood. The main application is the utilisation as pet food ingredient, giving both color and delivering a high amount of long chain polyunsaturated fatty acids (Rustad et al., 2011). Furthermore, there are trials, where salmon blood is applied instead of pork or cattle blood, but the scale is now limited to research and development (Rustad et al., 2011). For industrial application, further research must be conducted (Rustad et al., 2011).
The company Sea Run Holdings Inc. is leader in developing products based on salmon blood. Some of their products are used as clotting factor (Wang et al., 2000), fibrin glue (Laidmäe et al., 2010), blocking agents for diagnostic applications as well as for wound healing (Laidmäe et al., 2006; Rustad et al., 2011).

2.1.2 Marine lipids

A well-known example for a highly appreciated rest raw material is the application of oil (mainly delivered from cod liver) (Rustad et al., 2011). It was earlier used to overcome Vitamin D and Vitamin A deficiencies, mainly during long and dark winter months (Rajakumar, 2003). Now it serves as an important source of long chain polyunsaturated fatty acids (Rustad et al., 2011). These long chain polyunsaturated fatty acids are why fish oil capsules are highly appreciated from consumers to compensate deficiencies.
At the moment, fish oil is one of the primary products received out of fish rest raw materials. It also finds application in animal feed, where it delivers a high amount of healthy long chain polyunsaturated fatty acids (Rustad et al., 2011). Fish oil is a main supplier for the omega-3 polyunsaturated fatty acids, eicosapentaenic acid (EPA) and docosahexaenic acid (Liaset et al., 2003). Studies have shown, that the intake of EPA and DHA is connected with the reduced risk for cardiovascular diseases (Damodaran and Parkin, 2017), why it shows good cardioprotective properties.
With enzymatic hydrolysis, it is possible to gain 80 % of the total amount of lipids from the rest raw material (Liaset et al., 2003). By using this efficient process, it is possible to produce high quality products and separate the lipids from the protein fractions, which can be further used for other products such as protein concentrates or hydrolysates (Rustad, et al., 2011).

2.1.3 Protein fractions

Fish contain highly edible protein fractions which find their application in different products (Rustad et al., 2011). It is not only the fish fillet that is high in protein, also frames and cut offs, which are seen as rest raw materials, show high amounts of valuable proteins (Kim and Mendis, 2006). These frames and cut-offs are primary not considered for human consumption (Kim and Mendis, 2006). Nevertheless, is it for instance possible to produce fish mince, surimi or products based on surimi out of flesh and cut-offs (Rustad et al., 2011). This is only possible if the end-product is stable and fulfills the requirements for human consumption. Therefore, it is from high importance to remain a high quality standard throughout the whole process, from catching, processing until handling of the mince or surimi (Rustad et al., 2011). Fish mince is used in ready-made products like fishcakes or fish balls. Surimi can be seen as a type of fish mince, which is washed several times to remove all sarcoplasmic proteins (Kim et al., 2007). When surimi gets mixed with cryoprotectants, it shows good functional properties and storage stability (Rustad et al., 2011).

Raw material consist of insoluble not digestible proteins, as well as water and salt soluble proteins that are digestible. These soluble proteins are able to exhibit bioactive properties. Cut-offs and frames contain highly digestible proteins and a good amino acid profile (Kim and Mendis, 2006). Therefore, there is a great interest in producing new products out of these soluble protein fractions. It is possible to recycle protein fractions from by-products and to further use them for the production of fish protein isolates (FPI) and hydrolysates (FPH) (Nurdiani et al., 2015). These FPI and FPH show lots of favored properties for the application as functional and nutritional ingredients (Nurdiani et al., 2015). Fish protein isolates are highly concentrated protein products, where the dry material shows a protein content from 90 % and above. This suggests FPI as a source to deliver a great amount of highly digestible proteins (Rustad et al., 2011).
Furthermore, there is a high interest in the organoleptic, nutritional and functional properties of

FPH (Rustad, et al., 2011). The production of fish protein hydrolysates from different parts of fish is of great interest for the food industry, as they show proteins with high bioactivity. Researches focus on the exploitation of proteins from rest raw materials, that would otherwise be discarded or used as animal feed. The characteristics and importance of the application of fish protein hydrolysates can be seen in Section 2.2.2. Guerard and Shahidi (2007) show a flowsheet of the production of hydrolysates and crude oil from by-products. This flowsheet can be seen in Figure 2.5 in Section 2.2.1.

Rustad et al. (2011, p. 2011) writes "*to ensure better utilisation of fish by-products for human consumption, we need to*

(i) ensure high quality of by-products;

(ii) increase the yield of desirable products;

(iii) develop controlled processes accounting for variation in raw material;

(iv) providing stable, healthy and high-quality products;

(v) provide documentation of properties, including bioactive properties; and

(vi) standardize the process and properties of the marine ingredients."

This statement covers challenges that have to be considered when planning further utilisation of marine by-products. Nevertheless, there is a great possibility to increasingly apply by-products in the production of food and food ingredients in the future. Current researches in the field of marine rest raw material utilisation in different food applications are promising for an increased application of these products in industrial scales in the future.

Protein Quality

The essential amino acid content in a protein is the main indicator when defining the quality of a protein (Damodaran and Parkin, 2017). A high-quality protein exceeds the essential amino acid content suggested from FAO/WHO/UNO illustrated in Figure 2.3 from Damodaran and Parkin (2017). The intake of essential amino acids can be increased by combining foods that show different amounts of essential amino acids and therefore a balanced intake can be reached. Excessive intake of any of the essential amino acids can lead to toxicity or antagonism, meaning that the amino acid is not digested properly. Over-consumption of an essential amino acid may lead to higher requirements in other amino acids or even functions as growth inhibitor (Damodaran and Parkin, 2017).
The quality of a protein does not only depend on the quantity of essential amino acids it contains. It is also from great importance to which extent these amino acids are used in the body and therefore to which extent they are digested. This digestibility and availability in the body is called bioavailability and affects the quality of proteins. Therefore, a high-quality protein needs to show a high digestibility next to a balanced essential amino acid profile, which can be compared to the one from egg white or milk proteins. In general, animal derived proteins show a good bioavailability and digested amino acids are readily available in the body parts where they are needed (Damodaran and Parkin, 2017).

In the study of Bimal Mohanty (2014), fish of 27 species showed good compositions of total

amino acids (TAA) and high amounts of essential amino acids (EAA). Fish like *Oncorhynchus mykiss* (rainbow trout) showed a great EAA profile. Furthermore, Wilson and Cowey (1985) report high contents of EAA and a good TAA composition in Atlantic salmon and rainbow trout. In these studies, the requirements for most of the EAA for adults are reached or exceeded, suggesting fish as a good source of EAA.

Recommended Essential Amino Acid Pattern for Food Proteins

	Recommended Pattern (mg g^{-1} Protein)			
Amino Acid	**Infant (2–5 Years)**	**Preschool Child (10–12 Years)**	**Preschool Child**	**Adult**
Histidine	26	19	19	16
Isoleucine	46	28	28	13
Leucine	93	66	44	19
Lysine	66	58	44	16
Met + Cys	42	25	22	17
Phe + Tyr	72	63	22	19
Threonine	43	34	28	9
Tryptophan	17	11	9	5
Valine	55	35	25	13
Total	434	320	222	111

Figure 2.3: Essential amino acid requirements as illustrated from Damodaran and Parkin (2017), referring to the suggestions from FAO (1985).

2.2 Hydrolysis of proteins

By hydrolysing proteins with proteolytic enzymes, frunctional properties of proteins are enhanced. Partial hydrolysis leads to an improvement of solubility, dispersibility, foaming and emulsifying of proteins. The good functional and nutritional properties as well as the high digestibility of protein hydrolysates are reasons for an increased application of these in the special food market. Protein hydrolysates are ingredients in infant formulas, sports nutrition as well as geriatric and diet foods (Damodaran and Parkin, 2017).

As Figure 2.4 shows, peptide bonds get cleaved through proteases in enzymatic hydrolysis. Due to the peptide cleavages, carboxyl and amino groups are released. The result of a full proteolytic hydrolysis is a mixture of all amino acids in the protein. Whereas partly hydrolysed proteins result in polypeptides of different sizes, as they are not completely cleaved (Damodaran and Parkin, 2017). The degree of hydrolysis gives information about the percentage of peptide cleavages in the protein and will be further discussed later.

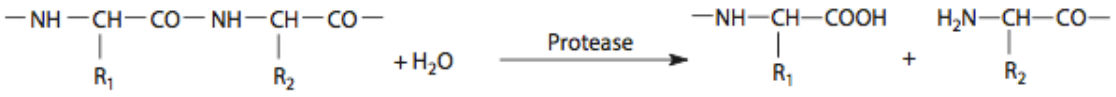


Figure 2.4: Reaction during proteolytic hydrolysis as illustrated from Damodaran and Parkin (2017).

2.2.1 Enzymatic hydrolysis

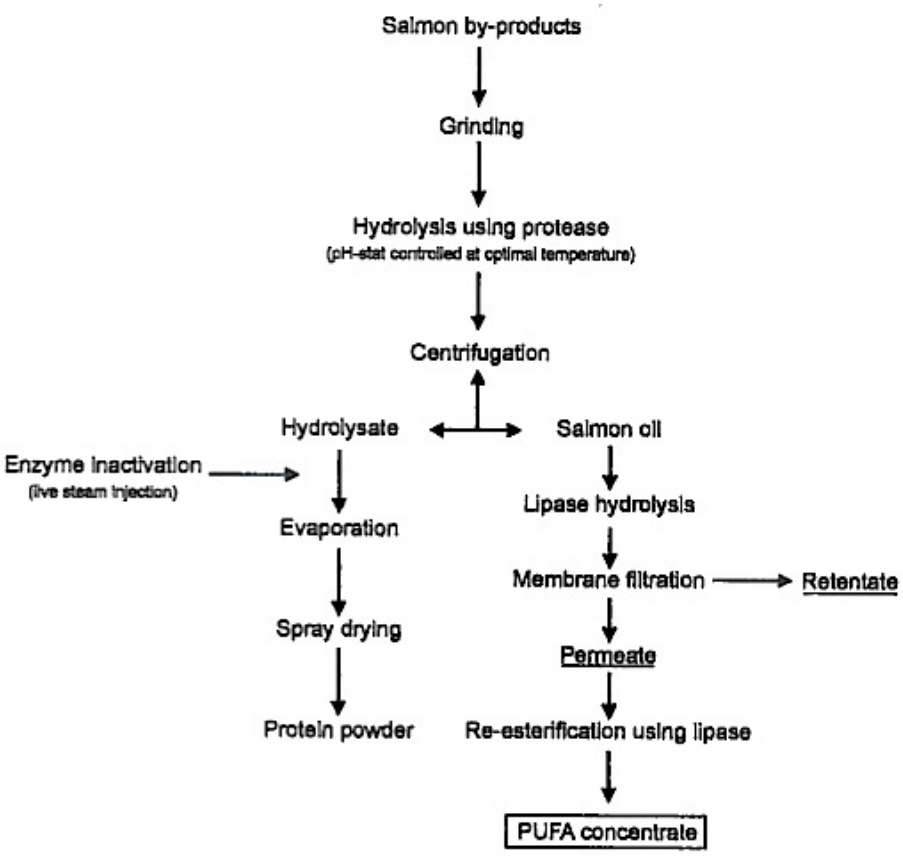


Figure 2.5: Flowsheet for the production of hydrolysate and crude oil from salmon by-products as illustrated from Guerard and Shahidi (2007).

Hydrolysis can be performed using chemical or biological agents (Ovissipour et al., 2012). Using biological means, enables the production of controlled end-products with high nutritional value (Nurdiani et al., 2015). Additionally, enzymes are not as invasive to the products as using chemicals in the process. Biological hydrolysis takes use of endogenous enzymes of the fish itself or more commonly, industrially produced enzymes (Nurdiani et al., 2015). By adding enzymes to the rest raw material and by filtrating and centrifuging, proteins can be extracted and recovered from the otherwise discarded waste material (Kristinsson, 2007). Figure 2.5 shows the flowsheet of a typical hydrolysis process.

Through enzymatic hydrolysis, the separation of different fractions of the raw material is pos-

sible. The raw material is separated into the oil fraction, the emulsion fraction, the aqueous fraction (which is rich in soluble nitrogen) and the insoluble nitrogen fraction (the sludge). The FPH are located in the aqueous fraction (Liaset et al., 2003). The separation into different fractions is due to the addition of proteases. Some proteolytic enzymes which are commonly used are be papain, bromelain, corolase, trypsin or protamex (Šližytė et al., 2016). These enzymes focus on a high yield in peptides that exhibit good bioactive properties in the end-products (Šližytė et al., 2016). To gather a stable, high quality fish protein hydrolysate, the enzymes must be inactivated after hydrolysis.
Furthermore, the enzymatic process is conducted under mild conditions and applies gentle pHs and temperatures (Diniz and Martin, 1997). In addition to the pH and temperature, the properties of hydrolysates are also dependent on the type and quality of enzymes used as well as controlled and stable conditions during the process (Nurdiani et al., 2015).
Moreover, the hydrolysates have to be dehydrated. Drying of the samples leads to a low moisture protein powder (Guerard and Shahidi, 2007). These mild conditions positively affect the quality of the end-products and retain the high nutritional value of the raw material (Nurdiani et al., 2015). After hydrolysis, the products are composed of high amounts of free amino acids and short chain peptides (Chalamaiah et al., 2012).

Enzymatic hydrolysis significantly increases the solubility of proteins. It promotes peptide bond cleavages that lead to smaller proteins, which are found to be either acid-soluble peptides or free amino acids. Hydrolysates are freeze dried powders made from the aqueous fraction that is received from hydrolysis. This fraction is high in water soluble proteins that contain small peptides, free amino acids and low molecuar weight compounds (Erdmann et al., 2008; Kousoulaki et al., 2012). Short peptides with a low molecular weight are known to exhibit nutritional values (Kousoulaki et al., 2009).
Therefore, adding enzymes to hydrolyse fish proteins significantly enhances biochemical and functional properties (Klomklao et al., 2013). It modifies the amino acid composition and concentration of the product and furthermore builds peptides with desired molecular weights (Lee, 2007).

Guerard and Shahidi (2007) write that the application of commercial enzymes significantly decreases the time of receiving similar degree of hydrolysis's and achieving the desired peptide size. In addition to that, enzymatic hydrolysis is a well controlled process. An advantage over acid or alkaline hydrolysis is, that no hydrolytic degradation products through racemization are produced (Guerard and Shahidi, 2007).
Some advantages as well as disadvantages of enzymatic hydrolysis can be seen as pointed out from Guerard and Shahidi (2007):

Advantages:

- Control of the molecular weight
- Mild reaction conditions
- Attractive functional product characteristics (solubility, dispersibility, foaming, capacity and foam stability)
- Control of the resulting products

- Few side reactions
- No destruction of amino acids
- High nutritional value

Disadvantages:

- Cost of enzyme(s)
- The need for subsequent deactivation of the enzyme(s)
- Complex process

It is a big challenge to overcome economic obstacles the hydrolysis process implicates, but it is necessary for implementing enzymatic hydrolysis in industrial scales. Further challenges in the application of FPH are described in Section 2.4.

2.2.2 Ultramembrane filtration

Ultramembrane filtration is an effective tool to fractionate peptides in their molecular weight and often used after hydrolysis. By applying ultramembrane filtration with the right molecular weight cut off, peptide fractions with desired molecular weights and high densities in bioactivity can be achieved (Kim and Mendis, 2006). Usually membranes with a molecular weight cut off (MWCO) of 4-8 kDa are used to fractionate bioactive peptides. Whereas peptides get concentrated by applying membranes with a MWCO of around 0.2 kDa (Cheung et al., 2015). By filtrating peptides, the desired sizes for different fields of application can be achieved.

2.3 Characteristics of Fish Protein Hydrolysates

The peptides present in fish protein hydrolysates are known to possess nutraceutical and functional properties which are due to a high content of peptides that are able to exhibit bioactive properties. The size and chemical properties of the peptides have a significant impact on their function (Kristinsson, 2007). Hence, biological activities and properties of FPH are highly dependent on the molecular weight and amino acid distribution of the contained peptides (Kim and Mendis, 2006). Dependent on the field of application, different peptide sizes are desired in FPH.

To produce hydrolysates with desired nutritional and therapeutic properties, low molecular weight peptides with only little free amino acid residues are desired (Guerard and Shahidi, 2007). According to García-Tejedor et al. (2014); Fernàndez-Musoles et al. (2013); Ruiz-Ruiz et al. (2013) and Wattanasiritham et al. (2016) peptides in a molecular range lower than < 10 kDa show greater antioxidative and hypertensive properties than peptides with higher molecular weights. Moreover Šližytė et al. (2016) state, that peptides with a molecular weight lower than 1000 Da, and amino acid residues in the range of 2 to 20 show good ACE inhibitory effects. On the other hand, peptides with a large molecular weight, therefore exerting more than 20 amino acid residues, are said to enhance the functional properties of hydrolysates (Guerard and Shahidi, 2007). This points out again, that dependent on the field of application, peptides with smaller or larger molecular weights are desired.

2.3.1 Functional properties

The increasing consumer awareness and the uncertainty about the safety of applying artificial ingredients in food, raises the interest of using natural additives with physiological and functional properties (Bernardi et al., 2016a). The good nutritional (Šližytė et al., 2005) and functional (Kristinsson, 2007) properties of FPH are promising for utilising them as ingredients in food production as they exhibit a variety of favored characteristics (Šližytė et al., 2016).
The desired functional properties, that hydrolysates exhibit, can be modified by using different enzymes in the hydrolysis process. This is because enzymes show different characteristics and lead to different sizes and physicochemical properties of the resulting polypeptides (Damodaran and Parkin, 2017). Whereas the solubility of protein hydrolysates is only little affected by the enzymes used, as they enhance great solubility regardless to the applied enzymes (Damodaran and Parkin, 2017). Normally, hydrolysis leads to a protein solubility of 90 to 100 % (Kristinsson, 2007), where a greater degree of hydrolysis (DH) leads to a higher solubility (Damodaran and Parkin, 2017).

Water-binding and water-holding properties

Fish are rich in greatly digestible proteins and the thereof made hydrolysates show an even greater amount of digestible and soluble proteins (Samaranayaka and Li-Chan, 2011). Hydrolysates differ from intact myofibrillar proteins in the fact, that they are readily soluble at a wide range of pH values, while intact myofibrillar proteins show a limited solubility at a wide range of pH values and ionic strenght (Kristinsson, 2007). Due to hydrolysis, proteins get broken down into a large amount of peptides with different sizes. Therefore, the amino and carboxyl groups of hydrolysed proteins are exposed and show a great ability of interacting with water. Due to the high solubility of FPH, applying them in sea food for enhancing the water-binding capacity is considered (Kristinsson, 2007). According to Kristinsson and Rasco (2000a) adding FPH to minced salmon muscle decreased the drip loss on thawing compared to samples where no FPH were added. Hydrolysates are high in their water-holding capacity and can be used to improve food systems. Furthermore, studies from Shahidi et al. (1995) have shown that the cooking yield of minced pork increases with adding fish hydrolysates.

Surfactant properties

Hydrolysates show good surfactant abilities and are able to act in the interface of oil-water or air-water phases (Samaranayaka and Li-Chan, 2011). Due to the amphiphilic structure, proteins possess both hydrophilic and hydrophobic groups that allocate themselves at the oil-water interface (Klompong et al., 2007). This structural specialty is the reason why hydrolysates can be used as emulsifying agents in products like margarine, various dressings and marinades (Kristinsson, 2007).
Another surfactant property is the ability of forming and stabilizing foams (Thiansilakul et al., 2007), which rely on the amphiphilic structure as well (Kristinsson and Rasco, 2000b). Furthermore, the solubility of the proteins is from great importance for their surfactant properties. In general, all hydrolysed proteins show good foaming and emulsifying properties. Moreover, partially hydrolysed proteins show a higher foaming and emulsifying property (Damodaran and Parkin, 2017). Furthermore, the DH plays an important role for surfactant properties. A DH of $< 10\,\%$ increases and a DH of $> 10\,\%$ decreases the surfactant properties (Damodaran and Parkin, 2017). Although protein hydrolysates show good foaming and emulsifying properties,

the original, intact protein exhibit greater properties. This is because long polypeptides are able to form cohesive viscoelastic films and show greater interactions at the oil-water or air-water interfaces than smaller peptide fractions (Damodaran and Parkin, 2017).

Cryoprotective properties

Furthermore, Jenkelunas and Li-Chan (2018) found out that FPH can be used as cryoprotectants during frozen storage of food or as cryoprotectants for repeated freeze-thawed products. According to Nikoo et al. (2016), the cryoprotective effects include surface hydrophobicity, water holding capacity, gel strength and texture enhancement. This makes the application of FPH in frozen fish products very attractive and it would become a remarkable alternative to the currently used sugar-based cryoprotectants (Jenkelunas and Li-Chan, 2018).

Another functional propery is the fat absorption capacity (FAC) as the FAC significantly influences and enhances the taste of a food product (Jenkelunas and Li-Chan, 2018). Furthermore, Nikoo et al. (2016) write that proteins from fish hydrolysates retard the formation of oxidation products such as carbonyls and protein cross-links. This leads to an inhibition of the oxidation process of myofibrillar proteins.

2.3.2 Nutraceutical properties

The word *nutraceutical* is a combination of nutrition and pharmaceutical (Cheung et al., 2015). According to Cheung et al. (2015) nutraceuticals are health promoting products that are naturally or enzymatically derived components from food. Despite their nutritional function, nutraceuticals exhibit a physiological effect on the body (Suarez-Jimenez et al., 2012). Cheung et al. (2015, p. 4007) write that nutraceuticals "*are usually claimed to prevent chronic diseases, enhance the immune system, manage stressful conditions, control body weight, regulate the blood glucose level, improve cognitive function, delay the aging process or increase life expectancy, etc.*". This points out how important the application of peptides derived from hydrolysis, in the implementation of different areas, is.

Research has shown that FPH exhibit high DPPH scavenging activities as well as the ability to chelate iron and other highly useful functions (Šližytė et al., 2016). Some of the nutraceutical properties FPH exhibit are following:

- antioxidative
- antitumor
- cardioprotective (antihypertensive, antiatherosclerotic and anticoagulant)
- antithrombic
- immunomodulatory
- anti-diabetic
- neuroprotective

functions (Kim and Mendis, 2006) and (Cheung et al., 2015).

Antioxidative propterties

It is well studied that the peptides in fish protein hydrolysates possess antioxidant activities (Suarez-Jimenez et al., 2012). According to Chen et al. (2010), the molecular weight and the amino acid composition of FPH are connected to their antioxidant properties. Therefore, FPH fractions with different molecular weight represent different antioxidative activities. It was found that a molecular weight range of 383 to 1492 Da most likely show a positive effect on the antioxidant activity in FPH (Suarez-Jimenez et al., 2012). Moreover, Jeon et al. (2000) state, that peptide fractions below 5 kDa are effective to decrease peroxidation of linoleic acid.
Antioxidants in the human body lower oxidative stress and damage. This leads to a lowered risk of developing related diseases. They inhibit the production of free radicals and therefore directly scavenge free radicals (Šližytė et al., 2016). A good antioxidative activity is also an advantage for extending the shelf-life when used in different food products (Šližytė et al., 2016). It slows down protein and lipid oxidation and preserves sensory attributes such as taste, aroma and color of the food (Parvathy U., 2018) and (Bernardi et al., 2016b).

Cardioprotective properties

The ability of FPH to exhibit antihypertensive activities leads to a decrease in chronic hypertension and this results in a significant reduction of mortality caused through cardiovascular diseases. The effect is based on inhibiting Angiotensin converting enzymes (ACE) (Šližytė et al., 2016). ACE convert angiotensin I to angiotensin II which leads to an increase in blood pressure (Damodaran and Parkin, 2017). The inhibition relies on the relative quantity of active peptides. These peptides are formed by amino acids in the range of 2 to 20 (Šližytė et al., 2016). Kristinsson (2007) writes, that peptides of cod FPH show a reduction in the ACE activity. The posibility of reducing ACE activity was dependent on the molecular weight of the peptides. According to Kristinsson (2007) and Jeon et al. (2000) smaller peptides have a better inhibitory effect and effects are suggested to be in following order $3\,\mathrm{kDa} > 5\,\mathrm{kDa} > 10\,\mathrm{kDa} > 30\,\mathrm{kDa}$ of molecular weight. This leads to the result, that ACE inhibitory effects of peptides are strongly dependent on the molecular weight of peptides (Kristinsson, 2007).
Furthermore, in vitro test have shown that peptides from enzymatically derived FPH possess antiplatelet as well as anticoagulant properties. These results indicate that fish peptides can retard coagulation factors in the intrinsic pathway of coagulation (Rajapakse et al., 2005). These peptides combat the platelet-membrane glycoprotein integrin and are able to inhibit platelet aggregation. Antiatherosclerotic peptides from FPH inhibit inflammatory responses in histamine-stimulated endothelial cells, which can be a reason for the early stage of atherosclerosis (Cheung et al., 2015).

Immunomodulatory properties

The mechanisms for immunomodulatory effects are not known at the moment, but researches show that fish hydrolysates possess high immunoregulatory functions in animals. The research from Yang et al. (2009) points out stimulating effects on the lymphocyte proliferation, the cytokine secretion and an improvement of the cytotoxic activity on the natural killer cells in mice. These effects were observed on chum salmon hydrolysates (Yang et al., 2009). Further investigations are made to prove these effects on humans as well.

Anti-diabetic properties

Type-2 diabetes mellitus is known as a widespread disease, especially in western countries. Developments for new therapeutic agents facing the complications of finding solutions for preventing and inhibiting type-2 diabetes mellitus are a challenge. Some fish protein hydrolysates could be used in regulating hyperglycaemia additionally to conventional therapies. In vivo tests have shown, that some marine peptides exhibit glucose uptake-stimulating activities. Hence, they can improve the glucose tolerance (Cheung et al., 2015).

Neuro- and neuroprotective properties

Furthermore, marine protein hydrolysates possess peptides that show opioid-like effects, meaning that they positively effect motivation, emotion, stress and behavior. These opioid-like peptides get advertised as dietary supplements for anxiety and for controlling stress. Applying these natural peptides gives a safe alternative to opioid drugs, which are known to show addictive, dependence and tolerance effects on patients (Cheung et al., 2015).
There are also neuroprotective effects of marine peptides like the prohibition of several neurodegenerative diseases. These peptides suppress the development of multiple sclerosis or Parkinson's and Alzheimer's disease. According to Ryu and Kim (2013) the peptides directly interact with enzyme/ion channels from molecular and cellular targets.

Enzymatically derived fish peptides already found their use in different pharmaceutical applications. For example Adcetris®, a marine peptide-derived drug from sea hare which is applied in cancer treatments or the Katsuobushi oligopeptide used in antihypertensive capsules. The bioactive peptides are a part of nutraceutical products and get active when entering the gastrointestinal tract, where they get transported into the system and apply their numerous functions (Cheung et al., 2015).

2.4 Challenges in the applications of FPH

Fish protein hydrolysates face some challenges, which make their field of application in spite of their great ability of exhibiting bioactive and functional properties, limited.
One of the biggest disadvantage or limitation is definitely the bitter taste, hydrolysis often provokes. This makes the produced hydrolysates undesirable and limits their application in products. It is necessary to overcome this limitation for a successful implementation of FPH in food industry (Rustad et al., 2011).

2.4.1 Bitterness

Bitterness is partly dependent on the molecular size of peptides, as well as on their hydrophobicity (Dauksas et al., 2004). Bitter tasting peptides are formed due to proteolytic cleavages, caused through extensive hydrolysis (Pedersen, 1994). Damodaran and Parkin (2017) state, that peptide residues with a mean hydrophobicity of more than 1.4 kcal mol^{-1} show great bitterness, whereas peptides with a mean residue hydrophobicity of less than 1.3 kcal mol^{-1} are not bitter. Furthermore, the bitterness of hydrolysates depends on the enzyme used as well as on the sequence and composition of amino acid residues (Damodaran and Parkin, 2017).
In order to produce FPH of high palatability, bitter tasting peptides must be minimised. The

degree of hydrolysis (DH) is a crucial factor and must be controlled in order to reach a high consumer acceptance (Pedersen, 1994). According to Adler-Nissen (1984) the DH should have low values of 3 to 5 % in order to significantly lower the bitterness. A complete hydrolysis on the other hand meaning a high DH, produces a great mixture of free amino acids, which decrease the bitterness (Dauksas et al., 2004). Furthermore, Dauksas et al. (2004) state, that a DH of 4 to 40 % indicates the highest probability of bitterness.
Pedersen (1994) suggests a treatment with exopeptidases and Dauksas et al. (2004) recommend the application of Flavourenzyme® that combines exopeptidase and endopeptidase activities, to overcome bitterness. These endo- and exopeptidases lead to a reduction of bitter tasting peptides to fractions smaller than 1.3 kcal mol^{-1} mean residue hydrophobicity (Damodaran and Parkin, 2017). It is possible to make use of chemical treatments in the hydrolysis process to minimize bitterness. In the research from Dauksas et al. (2004), butanol (BUOH) and cholestyramine resin (CAR) were both used in hydrolysis to show if they can positively influence the bitterness of the outcome. Both treatments with butanol and cholestyramine resin have shown to decrease bitterness, where CAR showed the largest reduction and BuOH lead to a plastic-like taste in the hydrolysates. Applying chemicals might increase the field of application for human consumption, but it also leads to a reduced yield of FPH (Dauksas et al., 2004).

2.4.2 Oxidation

Another problem is the oxidation process, especially occurring in fatty fish like salmon, herring or trout (Kristinsson and Rasco, 2000b). Producing FPH of fatty fish might lead to unstable products. Lipid oxidation leads to darkening of the product and provokes bitter and rancid taste (Rustad et al., 2011). This is due to deterioration of fats evoked by free-radical induced chain reactions (Ramis-Ramos and Caballero, 2003). To gain stable end products, the fat has to be extracted and separated to continue controlled hydrolysis (Kristinsson and Rasco, 2000b). Furthermore, the application of antioxidants (Kristinsson and Rasco, 2000b) or the washing with chemicals such as BuOH or CAR (Rustad et al., 2011) are possible ways to overcome or retard the oxidation process. The oxidation mechanism and indicators for lipid oxidation are described below.

Oxidation mechanism

The reaction of unsaturated fatty acids with oxidants such as molecular oxygen, metal ions or UV light, lead to the formation of free oxygen radicals. The oxidation mechanism for protein oxidation is similar to the mechanism of lipid oxidation, where ROS are formed. The primary product of lipid or protein oxidation is hydroperoxide, which leads to further reactions that result in new low molecular weight volatile oxidation products like aldehydes, ketones and acids (Ramis-Ramos and Caballero, 2003). The oxidation process can be described in three main steps: initiation, propagation and termination (Damodaran and Parkin, 2017).

In the initiation phase, an alkyl radical (L·) is formed by extracting a hydrogen molecule from a fatty acid. This alkyl radical is stabilised by the shift of its double bonds. In polyunsatturated fatty acids like in fish, the delocalization of double bonds lead to conjugated double bonds in cis or trans conjugation. Free fatty acid radicals are more likely to be formed in unsaturatted fatty acids and the ease of building these radicals increases with higher unsaturation (Damodaran and Parkin, 2017).

In the propagation phase, oxygen is added to the formed alkyl radical and builds a peroxyl radical (LOO·). These peroxyl radicals are high in energy and lead to the extraction of hydrogen molecules from other molecules. Unsaturated fatty acids are very susceptible to the exposure of peroxyl radicals. Peroxyl radicals can easily attack the carbon-hydrogen covalent bond. The abstraction of the hydrogen and the addition of it to the peroxyl radical, leads to the formation of a fatty acid hydroperoxide (LOOH) and furthermore a new alkyl radical on another fatty acid is built. This reaction causes a proliferation from fatty acid to fatty acid (Damodaran and Parkin, 2017).
In the termination phase, two radicals unite and result in a nonradical. If oxygen is present, the termination reaction will occur between two peroxyl radicals. Whereas in environments with low oxygen concentrations, the termination will most likely happen between alkyl radicals, which form fatty acid dimers (Damodaran and Parkin, 2017).

Oxidation indicators

The oxidation process is an important indicator for the quality of products. Protein oxidation can be caused through reactive oxygen species (ROS) that are formed due to lipid oxidation or oxidative stress (Estévez, 2011). Naturally occuring components in fish meat tissue such as unsaturated lipids, heme pigments and transition metals promote the formation of ROS (Xiong et al., 2000). The onset of oxidation from both raw material and the thereof produced hydrolysates is studied in this thesis. Known oxidation indicators in meat are the formation of carbonyl groups and the loss in thiol groups.
Carbonyl groups are built by the oxidation of amino acid side chains. The side chains of arginine, lysine , threonine and proline are reported to form carbonyl residues due to metal-catalyzed reactions (Lund et al., 2011). These chemical modifications cause changes in the physical and functional properties of proteins such as a loss in solubility as well as fragmentation and aggregation (Xiong et al., 2000).
Cysteine and methionine form derivatives that contain sulfur residues and lead to the loss of thiol groups. In the presence of hydrogenperoxide, they are highly susceptible to oxidation. The oxidation of thiol groups results in new products such as sulfenic acid (RSOH), sulfinic acid (RSOOH) and disulfide cross-links (RSSR) (Lund et al., 2011). The oxidation of essential amino acids leads to a decreased nutritional value as well as decreased functional properties of the product.

CHAPTER 3

Materials and Methods

The material analysed in this study was trout heads and hydrolysates made out from these trout heads. The aim was to determine if the quality of the rest raw material affects the properties of the fish protein hydrolysates. For the performed analysis, both the rest raw material and the produced hydrolysates were used. The preparation of the rest raw material and the hydrolysis were carried out in Ålesund at the Department of Biological Sciences by PhD student Kristine Kvangarsnes and then sent to Trondheim for further analyses.
Heads of farmed trout (*Oncorhynchus mykiss*) of similar grade and freshness was donated by HOFSETH AQUA on the 28^{th} of November 2018 and transferred to NTNU Ålesund. The fish weight was about 1 to 2.7 kg and the heads had a mean weight of $200 \pm 40\,\text{g}$. The mincing of the whole heads was done the same day as the collecting using a HOBART A 200N meat mincer (Illinois Tool Works Food Equipment Group, USA) which was adjusted to a 4.5 mm hole size. After mincing, the heads were immediately frozen in 1 kg batches at $-80\,^{\circ}\text{C}$. Further information about the process of the hydrolysis is given in 3.1.1.

3.1 Description and preparation of samples

The rest raw material used for the hydrolysis was either crude (RRM1), oxidised (RRM3) or oxidised with antioxidants added (RRM4). A description of the samples can be seen in Table 3.1 and 3.2 below. The rest raw material was numbered as RRM 1, 3, 4 and the hydrolysates otained from these raw materials were named H 1-4.

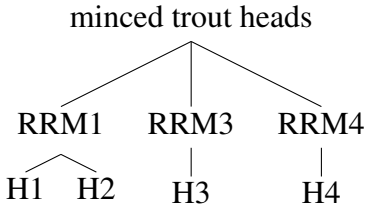


Figure 3.1: Overview of samples made from original raw material.

RRM1 is the crude minced trout heads used for the hydrolysis of H1, which can be seen as the *control hydrolysate* and sample H2, where an antioxidant was added directly into the hydrolysis tank.

RRM3 and RRM4 were oxidised and to RRM4 an antioxidant was added additionaly.
The preparation of the samples was done as following:
For both RRM3 and RRM4, 2.5 kg of the raw material was used, placed into a bucket and covered with a lid.
RRM3: 3.48 g iron sulphate (iron sulphate heptahydrate $FeSO_4$) + 4.4 ml cumene hydrogen-

peroxide ($C_9H_120_2$).

RRM4: 3.48 g $FeSO_4$ + 4.4 ml $C_9H_120_2$ + 200 ppm Butylhydroxytoluol (BHT).
After thoroughly stirring the solution, the the buckets were closed and placed at $4\,^{\circ}\mathrm{C}$ in a cold room.
Before starting the hydrolysis, the samples were stored in a cold room. The storage period was a total of 7 days and the solutions were stirred multiple times within this period.

Table 3.1: Rest Raw Material (minced)

RRM1	Crude raw material
RRM3	Oxidised raw material ($C_9H_120_2 + FeSO_4$), storage at $4\,^{\circ}\mathrm{C}$ for 7 days before hydrolysis
RRM4	Oxidised raw material ($C_9H_120_2 + FeSO_4$) + antioxidant BHT, storage at $4\,^{\circ}\mathrm{C}$ for 7 days before hydrolysis

Table 3.2: Hydrolysates

H1	Crude raw material hydrolysate
H2	Raw material (RRM1) + antioxidant BHT added directly into the hydrolysis tank
H3	Oxidised raw material ($C_9H_120_2 + FeSO_4$)
H4	Oxidised raw material ($C_9H_120_2 + FeSO_4$) + antioxidant BHT added

3.1.1 Hydrolysis

The hydrolysis was carried out by Kristine Kvangarsnes. Before starting hydrolysis, the frozen, minced rainbow trout heads were thawed overnight at $4\,^{\circ}\mathrm{C}$. To start with the process, 2 kg of minced heads and 2 litres of water were added into each hydrolysis tank. The mixture was stirred at 150 g and the hydrolysis was performed at $52\,^{\circ}\mathrm{C}$ for 60 minutes. The used enzymes were papain and bromelain and were both added at a total level of 1 % (w/w) of the raw material (0.05 % + 0.05 %). After the hydrolysis process, the enzymes were inactivated by heating at $90\,^{\circ}\mathrm{C}$ for 10 minutes using a microwave.
Next, the samples were cooled before centrifuging at 3500 g at $20\,^{\circ}\mathrm{C}$ for a total time of 30 minutes. After the centrifugation, three phases could be noticed: the lipid fraction on the top, the aqueous fraction containing the proteins in the middle and the sludge at the bottom. A picture showing the three fractions can be seen in Figure 3.2. To separate the lipid and protein fractions, a separating funnel was used. The fat and sludge was removed and discarded. The desired aqueous fraction was freeze dried for a total of 72 hours. The powdered and dehydrated hydrolysate samples were frozen at $-80\,^{\circ}\mathrm{C}$ until further utilization.

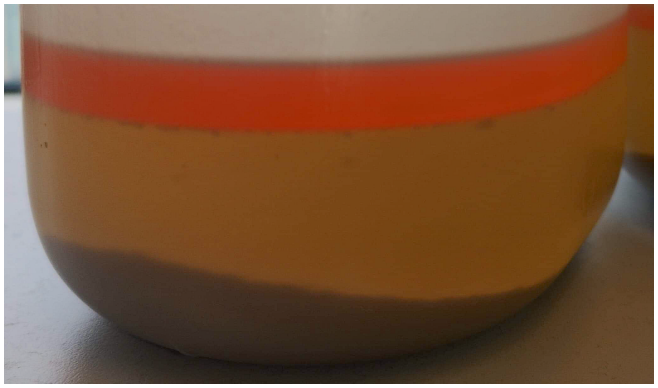


Figure 3.2: The received three fractions after hydrolysis was performed. From top to bottom: lipid fraction, aqueous fraction and sludge.

3.1.2 Storage of samples

The raw material samples were transported to Trondheim, frozen in centrifuge tubes and packed in bubble wrap with freezing elements to prevent the samples from thawing. They were stored temporarily in a freezer at $-20\,^{\circ}\mathrm{C}$. Then, frozen at $-80\,^{\circ}\mathrm{C}$ and a small amount was kept in the $-20\,^{\circ}\mathrm{C}$ freezer to be used for the first analysis. The hydrolysates were as well transported in centrifuge tubes and put into the $-80\,^{\circ}\mathrm{C}$ freezer. Physical characteristics as well as pictures of the samples can be seen in Appendix A.

3.2 Chemical analysis

The performed analyses were mainly conducted at the food chemistry laboratory located at Campus Gløshaugen and in the laboratory facilities located at Campus Kalvskinnet.
An overview of the analyses is illustrated in 3.3. It shows which analyses were done on the rest raw material samples and which ones on the hydrolysates.

Table 3.3: Overview of Analyses

Analysis	Rest Raw Material	Hydrolysates
Dry matter + ash content	*x*	
Soluble protein	*x*	*x*
Nitrogen + protein content	*x*	*x*
Molecular weight distribution	*x*	*x*
Degree of Hydrolysis	*x*	*x*
Total AA amount	*x*	*x*
Free AA amount	*x*	*x*
Collagen (hydroxyproline)	*x*	
Fractionation of hydrolysates		*x*
Acid soluble peptides	*x*	*x*
Protein oxidation (thiols)		*x*
Protein oxidation (carbonyls)		*x*

Additionally, the received permeates and retentates (H1-4/P and H1-4/R) from fractionating the

hydrolysates with ultramembrane filtration were analysed. The performed analyses can be seen in Table 3.4.

Table 3.4: Performed analyses on permeates and retentates

Analysis	Permeates	Retentates
Soluble protein	*x*	*x*
Molecular weight distribution	*x*	*x*

3.2.1 Determination of dry matter and ash content

The dry matter and ash content were determined according to the method of AOAC (1990). Both dry matter and ash content were measured in triplicates.
About 2 g of samples were weighed in porcelain crucibles (the exact weight of the crucible and sample was written down).
As for the dry matter, the samples were put into a drying cabinet at $105\,^{\circ}\mathrm{C}$ for 24 hours. After 24 hours the samples were put into a desiccator to cool them to room temperature. After cooling down, the samples got weighed.
To determine the ash content, the samples were put into a muffle furnace at $550\,^{\circ}\mathrm{C}$ overnight. The samples were cooled in a desiccator before reweighing.

3.2.2 Determination of soluble protein by the Lowry method

The amount of soluble protein was determined by the Lowry method according to Lowry et al. (2017). The Lowry method can be used to determine the quantity of soluble proteins in the samples.

Seven standards with a final concentration of 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml and 300 µg/ml of BSA were prepared. A total of 0.5 g of the samples were dissolved in 10 ml doubly distilled water, homogenized and centrifuged. The supernatant was used for diluting the samples. For the procedure 0.5 ml of each standard, 0.5 ml of a blank (doubly distilled water) and 0.5 ml of the diluted samples were pipetted into test tubes. Each sample was analysed in triplicates. Alkaline copper was added in a volume of 2.5 ml to each test tube and mixed immediately with a vortex mixer. After 10 minutes at room temperature, 0.25 ml Folin-Coicalteu reagents was added and mixed thoroughly with the solutions. After 30 minutes of incubation, the absorbance was read at 750 nm using a GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc., USA) .

3.2.3 Determination of nitrogen and protein by the Kjeldahl method

To get information about the nitrogen and therefore the total protein concentration in the hydrolysates and the rest raw material samples, the Kjeldahl method was performed. The calculation for the nitrogen and protein content can be seen in Appendix B. The apparatus' used for the digestion are a KJELDIGESTER K-449 with a Srubber type K-415 and for distillation and titration a KJELMASTER K-375 and KJELSAMPLER K-376 from Büchi Switzerland have been used. The procedure was performed according to the Application Note from Büchi Switzerland

(2013).

The rest raw material samples were first weighed in a range between 1 to 2 g and the hydrolysates between 0.5 to 1 g using WHATMAN KJELDAHL WEIGHING BOATS. The samples were prepared in triplicates and the exact weight was written down. As a reference substance, glycine was used by weighing in 19 g of it into a weighing boat. As blank samples, empty weighing boats were used. All samples were transferred into 300 ml sample tubes. In the fume hood 2 titanium tablets and 15 ml of sulfuric acid (conc. 98 %) were added in all sample tubes and shaken gently. The rack with 20 sample tubes was placed into the KJELDIGESTER in the cooling position. The Digester then had to heat up to $280\,^{\circ}\mathrm{C}$ and by reaching $280\,^{\circ}\mathrm{C}$ the rack was shifted down into the digestion position. Step 2 of the digestion was heating at $320\,^{\circ}\mathrm{C}$ for 20 minutes and then at $420\,^{\circ}\mathrm{C}$ for 90 minutes. In the last step, the rack was shifted up to the cooling position for cooling down, which took 35 minutes. The samples should be cooled down to room temperature before starting distillation and titration.
Before starting the distillation, the necessary sample information was registered into the system of the KJELMASTER and the method *fish meat* was selected. The rack was then inserted into the KJELMASTER to start distillation and titration using the parameters set in Table 3.5. The process first started with pre-heating, priming and calibration of the electrode. Then distillation and titration was performed and results are automatically calculated.

Table 3.5: Method parameters for KJELMASTER K-375 (Büchi Switzerland)

H_2O	50 ml	Titration solution	H_2SO_4, 0.25 mol/l
NaOH volume	60 ml	Sensor type	Potentiometric
Reaction time	5 s	Titration mode	Standard
Distillation mode	Fixed time	Measuring mode	Endpoint pH
Distillation time	180 s	Endpoint pH	4.65
Stirrer speed distillation	10	Stirrer speed titration	9
Steam output	100 %	Titration start volume	4 ml
Titration type	Boric acid	Titration algorithm	Optimal
Receiving solution vol.	70 ml		

3.2.4 Determination of molecular weight distribution by FPLC

To determine the molecular weight distribution of peptides, gel filtration was approached using a Fast Protein Liquid Chromatography (FPLC) system. This approach is also called *size exclusion chromatography* and separates the peptide molecules of samples according to their different sizes. The sample passes a gel filtration medium packed in a column. Depending on the molecular weight of the peptides, they elute faster or are retained in the column longer (GE Healthcare, 2010).

The applied apparatus for this analysis is an AKTAPURIFIER SYSTEM (GE Healthcare Life Science, Sweden) and for separation, a SUPERDEX PEPTIDE 10/300 column is used. The separation of peptides is most exact in a range of a molecular weight from 100 up to 7000 Da, with an exclusion limit at 20 000 Da. As mentioned before the retention time of each peptide defines their molecular weight. Large peptides have a shorter retention time, while smaller peptides are

retained longer in the column as they show higher affinity to the stationary phase (GE Healthcare, 2010).

A 0.05 M sodium acetate buffer was made by weighing in 4.102 g of sodium acetate and dissolving it in distilled water. The pH was adjusted to 5 using hydrogene chloride and the flask was filled up with distilled water to 1 l. The solution was filtered through a NALGENE RAPID-FLOW 75 mm Bottle Top filter (Thermo Fisher Scientific Inc., USA) by applying suction. An amount of 100 mg of hydrolysates were dissolved in 4 ml of the prepared buffer and filtered through a 0.22 µm WHATMAN filter into EPPENDORF tubes. For the raw material 5 g were dissolved in 10 ml buffer, homogenized and centrifuged. The supernatant was filtered through a 0.22 µm WHATMAN filter into EPPENDORF tubes. The permeates and retentates did not need any preparation and were used as received from ultramembrane filtration. The FPLC apparatus and the UNICORN programme on the computer were started. A flow rate of 0.5 ml/min and a wavelength of 280 nm were set. 100 µl of each sample was carefully injected into the FPLC machine using an injection needle. One run per sample, including the pre-cleaning, took 80 to 90 minutes. The process was the same for each sample.

3.2.5 Determination of the Degree of Hydrolysis by Formoltitration

The degree of hydrolysis (DH) was analysed as the proportion (%) of free amino groups with regard to the total nitrogen in the sample (Taylor, 1957). The analysis (Taylor, 1957) described below delivers results for the free amino groups. The nitrogen results gained from performing the Kjeldahl method were used to complete the calculation of the DH.

An amount of 1.5 g of the sample was weighed into a beaker and filled up to 50 g with distilled water. The pH was adjusted to 7.0 using 0.1 M NaOH and then 10 ml formaldehyde titrated to a pH of 8.5 was added into the beaker. The beaker was covered with aluminium foil and put on a magnetic stirrer for 5 minutes. For the titration, a TITROLINE 7000 titrator (SI Analytics, Xylem Analytics Germany Sales GmbH & Co. KG, Germany) was used. The titrator was rinsed 3 times before starting the titration. Furthermore, the titration was set to pH 8.5 with stopping automatically when reaching a pH of 8.5. The samples were titrated with 0.1 M NaOH and the used amount of NaOH was noted down.

3.2.6 Determination of total amino acid composition by HPLC

To determine the composition of amino acids in the samples, High Performance Liquid Chromatography (HPLC) was performed according to Blackburn (1978).

A total amount of 5 to 8 g of RRM 1, 3, 4 were freeze dried for analysis of amino acid composition using a ALPHA 1-4 LD PLUS freeze dryer (Glems-Technik, Germany). The dried samples were stored in centrifuge tubes in the $-20\,^{\circ}\mathrm{C}$ freezer. 50 mg of the freeze dried raw material samples and 50 mg of the hydrolysates were weighed into glass tubes and 1 ml 6 M HCl was added. The analysis was performed in triplicates. The screw tops were tightened thoroughly and the glass tubes were put into the heating cupboard for approximately 22 hours at $105\,^{\circ}\mathrm{C}$. After 30 minutes the screw tops were tightened again. The next day, the samples were put out, the screw tops were loosened and the samples cooled to room temperature. Next, the samples were transferred into beakers and the glass tubes were rinsed with small amounts of doubly dis-

tilled water. The pH was adjusted to 7 (6.5 to 7.5) by using a pellet of NaOH and then different concentrations of NaOH (0.1 M, 0.4 M and 2.5 M). The samples were filtered through a WHATMAN glass microfiber filter GF/C using suction and transferred to a measuring flask of 10 or 20 ml and filled up to the meniscus with doubly distilled water. Dilutions of 1:500 were made and filtered through a 0.22 µm WHATMAN filter. 0.205 ml of the samples were pipetted into vials and provided for the HPLC. The HPLC was run by the university employee Siri Stavrum.

3.2.7 Determination of free amino acids by HPLC

The determination of free amino acids on the freeze dried raw material and hydrolysates was performed according to Osnes and Mohr (1985).

Two parallels of each sample where made by transferring 1 ml of water soluble protein extract (0.5 g sample in 10 ml doubly distilled water) into EPPENDORF tubes and by adding 0.25 ml of 10 % sulphosalisylic acid. The samples were shaken to mix the solutions evenly. Next, they were put into a cold room at $4\,^{\circ}\mathrm{C}$. After 30 minutes, the samples were centrifuged with an EPPENDORF 5415 R centrifuge (Eppendorf AG, Germany) for 10 minutes at 10 000 g. After the centrifugation, one sample was picked to detect if all protein was precipitated. In case of this sample, 1 ml of the supernatant was mixed again with 0.25 ml of sulphosalisylic acid, put into the cold room for 30 minutes and centrifuged for 10 minutes. No precipitate could be seen. Therefore the intended samples could be taken for the further procedure. The supernatant was diluted to 1:25 using doubly distilled water and then filtered through a 0.22 µ WHATMAN filter. 0.25 ml of each filtered sample was pipetted into vials and provided for the HPLC. The HPLC was run by the university employee Siri Stavrum.

3.2.8 Determination of hydroxyproline

Collagen almost only consists of hydroxyproline, which therefore gives a good conclusion of how much collagen is in the raw material samples. The method was conducted according to Leach (1960).

In order to start the determination, the stored hydrolysed samples from the determination of total amino acids were used. A dilution factor of 10 was chosen and each sample was diluted in triplicates. A standard stock solution of L-hydroxyproline was diluted in triplicates to 5 µg/ml, 10 µg/ml, 15 µg/ml and 20 µg/ml. All needed reagents were already prepared. Only the 6 % H_2O_2 and the 5 % p-dimethylamino-benzaldehyde in 1-propanol had to be prepared before performing the analysis. Therefore 1 part of 30 % H_2O_2 was mixed in 4 parts of doubly distilled water and p-dimethylaminobenzaldehyde was dissolved 1-propanol in a ratio of 5 %. Test tubes were prepared and 0.5 ml of blank, standard and samples were transferred. 0.5 ml of 0.05 M $CuSO_4$ as well as 0.5 ml 2.5 M NaOH were added. Each tube was immediately mixed with a vortex mixer and the colour of the samples changed from transparent to blue. The tubes were covered with marbles and put into a water bath at $50\,^{\circ}\mathrm{C}$. After 10 minutes the tray was taken out and 0.5 ml of 6 % H_2O_2 added and mixed. The colour changed to green and then brown. The tubes were again covered with marbles and put into the water bath at $50\,^{\circ}\mathrm{C}$. After 10 minutes the test tubes were cooled to room temperature under running water. In the next step 2 ml of 1.5 M $CuSO_4$ and 1 ml of p-dimethylaminobenzaldehyde in 1-propanol were pipetted into the test tubes and immediately shaken. The solutions now turned from brown to transparent. The

last step was to put the test tubes covered with marbles into the water bath once more. This was done for 16 minutes at a temperature of 70 °C. After this, the samples were cooled down to room temperature, mixed and left at room temperature for 2 minutes. The absorbance of the samples was determined at a wavelength of 555 nm using a GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc., USA).

3.2.9 Fractionation of hydrolysates by ultramembrane filtration

To seperate the peptides in smaller and larger units, ultramembrane filtration was performed. Therefore H1-4 were prepared by dissolving 1 g sample/100 ml doubly distilled water. Then solutions were homogenized and centrifuged. The supernatant was filtered through a 0.7 µm WHATMAN Glass Microfiber filter using suction.
The cut off of the membrane filtration system is 4 kDa, meaning that the peptides in the permeates will be located at < 4 kDa and the peptides of the retentates at > 4 kDa. The used filtration system is a TRIPLE SYSTEM (MMS Membrane Systems, Switzerland) and was run with a pressure of 5 bar. The exact volume of sample was measured and written down before the filtration was started. By setting the pressure and system parameters, the filtration starts. The dead volume in the system constitutes of 50 ml, which is the minimum volume where the filtration has to be stopped. One sample run took around 30 to 40 minutes. The yielded volume of permeate and retentate was written down. The samples were filled into centrifugation tubes and frozen at -20 °C before using them for further analyses. The permeates and retentates will be further labeled H1-4/P and H1-4/R. H1-4/P and H1-4/R were analysed for their soluble protein content as described in Section 3.2.2 and their molecular weight distribution as described in 3.2.4.

3.2.10 Determination of acid-soluble peptides

The solubility in acid got determined and performed according to Hoyle and Merritt (1994).

Water soluble extracts (0.5 g sample in 10 ml doubly distilled water) were prepared, homogenized and centrifuged. The supernatant was filtered using a 0.2 µm WHATMAN filter. Of these filtered samples, 2 ml were mixed with 2 ml of 20 % trichloroacetic acid and incubated for 30 minutes at room temperature. The samples were then filtered using filter paper circles in funnels. The determination of acid-soluble peptides was done in triplicates for each sample by perfoming lowry method as described in 3.2.2. H1-4 were therefore diluted with 10 % TCA, RRM 1,3,4 did not need any dilution. The absorbance was read at 750 nm using a GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc., USA).

3.2.11 Determination of protein oxidation of thiols

The determination of carbonyl and thiol concentrations in the raw material samples were conducted by Kristine Kvangarsnes and results can be seen in Section 4.4.

The determination of thiol groups in the hydrolysate samples was conducted according to Ellman (1959). Thiol groups that are in their free form will undergo a thiol-disulfide interchange reaction with the aryl disulfide 5.5‘dithiobis (2-nitrobenzoate) (DTNB) (Ellman, 1959). The more thiol-disulphide bonds, mean less free thiols and more oxidative stress.
For this procedure, the samples were analysed in triplicates. The protein extract with the known

protein concentration from the determination of soluble proteins was used and put out of the freezer the day of the analysis. A buffer containing of 0.1 M phosphate buffer (pH 7.4) with 1 mM Ethylenediaminetetraacetic acid (EDTA) had to be prepared. The preparation of the phosphate buffer was done according to the description of Cold Spring Harbor Laboratory Press (2006).

Preparation of 0.1 M phosphate buffer (pH 7.4):
800 ml of distilled water were filled into a 1 l flask and then 3.1 g of $NaH_2PO_4 \cdot H_2O$ and 10.9 g of Na_2HPO_4 were added and mixed with a magnet stirrer. The pH of the solution was then adjusted to 7.4 and the volume of the 1 l flask was filled with doubly distilled water up to the meniscus.

After preparation of the phosphate buffer, 0.292 24 g EDTA was added, therefore accomplishing buffer A. An 8 M urea solution was made by dissolving 48.048 g in 40 ml of buffer A. The solution was stirred for 1 hour and the volume expanded. When the solution was dissolved the pH was adjusted to 7.4 with HCl and the volume was filled up to 100 ml with buffer A.
A 0.1 % DTNB solution in buffer A was made and the pH was adjusted to 7.4.
The analysis started by pipetting 100 µl of sample into EPPENDORF tubes, which was done in triplicates for each sample. A blank was made using 100 µl doubly distilled water. 800 µl of urea solution and 100 µl of DTNB solution were then added into each tube and mixed evenly. An incubation of 30 minutes at room temperature followed, before centrifuging the EPPENDORF tubes in an EPPENDORF 5415 R centrifuge (Eppendorf AG, Germany) at 12 000 g for 3 minutes. Finally, the absorbance was read at 412 nm using a GENESYS 10S UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc., USA) .

3.2.12 Determination of protein oxidation of carbonyls by ELISA

The used protein carbonyl ELISA assay is a highly sensitive approach to detect carbonyl groups, which give information about the oxidative stress in the proteins of the samples. The determination of carbonyl groups in the hydrolysate samples was performed according to the Product Manual from Cell Biolabs, Inc. (2018).

For this procedure, the protein extract with the known protein concentration from the determination of soluble proteins was used. The extracts were stored at $-20\,^{\circ}\mathrm{C}$ and put out the day the enzyme-linked immunosorbent assay was performed. The extracts had to be diluted in 1X Phosphate Buffered Saline in order to contain a protein concentration of 10 g/ml. The samples were analysed as triplicates.
The samples were diluted in centrifugation tubes as following:

Table 3.6: Dilution of samples H1, H2, H3 and H4

Sample	Protein in protein extract [µl/ml]	Added amount of protein extract [µl/ml]	Added amount of 1X PBS [ml]
H1	39891.52	10	39.89
H2	43252.59	10	43.25
H3	36012.21	10	36.01
H4	38901.77	10	36.01

Before starting the analysis, different reagents had to be prepared. The preparation was done according to the Product Manual of Cell Biolabs, Inc. (2018).
The preparation of PBS was done according to the description of AAT Bioquest, Inc. (2018).

Preparation of 1X PBS (pH 7.4):
Two bottles with two litres of 1X PBS were prepared. Therefore, 1.6 l distilled water was added in each flask and 16 g of NaCl, 400 mg of KCl as well as 2.88 g of Na_2HPO_4 and 480 mg of KH_2PO_4 were added. The solution was thoroughly dissolved on a magnetic stirrer. The pH was adjusted to 7.4 using 0.4 M NaCl and the volume was filled up to 2 l with distilled water.

Protein Carbonyl BSA Standards were prepared adding oxidized BSA and reduced BSA in different amounts to get a final carbonyl concentration of 7.5 nmol/mg, 6.0 nmol/mg, 4.5 nmol/mg, 3.0 nmol/mg, 1.5 nmol/mg, 0.75 nmol/mg, 0.375 nmol/mg and 0 nmol/mg (used as a blank). To start the procedure, 100 µl of the prepared protein samples and BSA standards were pipetted into a 96-well Protein Binding Plate and had to be incubated overnight in the cold room at $4\,^{\circ}\mathrm{C}$. The next day, the wells were washed a total of 3 times with 1X PBS and after the last wash, they were tapped on a paper towel to remove excess PBS. 100 µl of DNPH working solution was added in each well and incubated at room temperature for about 45 minutes in the dark. After that, 250 µl of 1X PBS/Ethanol (1:1, v/v) were used to wash the wells 5 times in total. In between each washing, an incubation of 5 minutes on an orbital shaker was required. After the last wash, the plates were tapped on a paper towel and washed 2 times with 250 µl of 1X PBS. The next step required pipetting 200 µl of blocking solution into each well and an incubation for a total of 1 hour on the orbital shaker. After the incubation, the wells were washed 3 times with 250 µl of 1X wash buffer and tapped on a paper towel as before. Then 100 µl of diluted anti-DNP antibody was added, incubated for 1 hour on the orbital shaker and each well was washed 3 times with wash buffer. The excess buffer was removed by tapping on a paper towel. 100 µl of diluted HRP conjugated secondary antibody were pipetted into the wells and also incubated for 1 hour on the orbital shaker and then washed a total of 5 times with wash buffer. Excess wash buffer was removed with tapping the plate on a paper towel as done earlier. The last steps required to add 100 µl of substrate solution to each well and an incubation from 2 to 30 minutes, depending on the colour change. In this case the incubation was done for 30 minutes on the orbital shaker and a change in colour from transparent to light blue could be seen. The enzyme reaction was stopped by adding 100 µl stop solution. The absorbance of each well was immediately read with a plate reader after adding the stop solution. A wavelength of 450 nm was set for reading the absorbance.

3.3 Statistical analysis

Data processing and statistical analysis was performed with Microsoft Excel 2016. All analyses were at least performed in duplicates or triplicates. The results in Chapter 4 are given as mean values with standard deviations. To assess the relationships between the results in the group of hydrolysates or between the raw material and hydrolysate group, linear correlations analysis with regression were performed. Furthermore, to establish the linear correlation, the Pearson-Correlation coefficient was calculated.

CHAPTER 4

Results and Discussion

To get basic information about the raw material samples, the dry matter and ash content was analysed. Physical characteristics and pictures of the samples are found in Appendix A. As can be seen in Table 4.1, the samples consist of more than 50 % of water, with RRM1 having the highest moisture content with 58 %. The ash content of the samples range from 4.46 % (RRM1) to 4.98 % (RRM4) respectively.
In the study of Hussain et al. (2011) about the proximate composition of head from wild and farmed Catla catla (main Asian carp), the moisture content varied between 63.06 and 54.91 %. The results are in the same range as the moisture content of the studied samples, whereas a noticeable higher ash content of 12.26 to 12.40 % was found in the heads of Catla catla. Another study of Petrova et al. (2018) states that salmon heads are constituted of approximately 60 % of moisture and 6.0 % of ash.
In the study of Sabetian et al. (2012) about the proximate composition of rainbow trout fillets, an ash content of 1.33 % ± 0.1 and a total moisture content of 71.70 % ± 1.9 was reported. Fillets are deboned whereas the analysed trout heads were minced as whole. The higher ash contents in the trout head samples are due to the higher proportion of bones in the head than in fillets.

Table 4.1: Water and ash content [wt.%] in raw material samples given as mean, standard deviation given (n=3).

Sample	water [wt.%]	ash [wt.%]
RRM1	58.44 ± 0.39	4.46 ± 0.03
RRM3	51.91 ± 0.52	4.82 ± 0.28
RRM4	55.48 ± 2.15	4.98 ± 0.47

4.1 Total Protein content

The total protein content was determined by the Kjeldahl method. The Kjeldahl method is known to be the primary reference method for protein analysis (Sáez-Plaza et al., 2013). In this method the total nitrogen content is determined and can be further converted into the total protein content. The calculation can be seen in Appendix B.

The results of nitrogen and total protein can be seen in Table 4.2. Total protein concentrations of 14.28 % (RRM1), 14.95 % (RRM3) and 16.28 % (RRM4) were found for the raw material samples. A similarity to the results of the study of Hussain et al. (2011), where the analysed Catla catla heads show a protein concentration of 14.77 to 19.92 % can be seen. The

study of Petrova et al. (2018) states approximately 11.3 % protein in the heads of salmon which are exceeded in this study. The protein contents showed again lower results compared to the study of Sabetian et al. (2012) where the analysed trout fillets contain of 19.65 % protein.
The hydrolysate samples show high contents of protein, where H1 shows the lowest content (79.70 %) and H2 the highest (88.53 %). The protein contents in the hydrolysates are higher compared to results from Šližytė et al. (2016) (73.8 % in salmon hydrolysates) and within the the range of the results from Šližytė et al. (2005) (75.0 % to 91.6 % in cod hydrolysates).

Table 4.2: Nitrogen and Total Protein [wt.%] in the samples as determined by the Kjeldahl method. Standard deviation given (n=3).

Sample	Nitrogen [wt.%]	Total Protein [wt.%]
RRM1	2.29 $\pm 0,02$	14.28 ± 0.14
RRM3	2.39 ± 0.09	14.95 ± 0.58
RRM4	2.60 ± 0.14	16.28 ± 0.85
H1	12.75 ± 0.05	79.70 ± 0.33
H2	14.17 ± 0.05	88.53 ± 0.29
H3	13.40 ± 0.11	83.09 ± 0.68
H4	13.74 ± 0.03	85.90 ± 0.21

The enzymatic hydrolysis of the raw material yielded the four different hydrolysate samples. Table 4.3 illustrates the amount of protein for each hydrolysate as well as the protein yield [%]. A total of 100 g of raw material yielded 6.85 g (H1), 6.00 g (H2), 6.88 g (H3) and 6.25 g (H4) dry hydrolysate, with different protein concentrations. Due to performing the Kjeldahl method, the protein concentration per received hydrolysate could be calculated as can be seen in the third column of the Table (f.ex. 5.46 g protein in 6.85 g hydrolysate). Column four, the protein yield [%], shows how much protein of the raw material ends up as protein in the hydrolysates. The yield ranges from 33 % in H4 to 38 % in H3 and H1. Even though components such as skin and bones can interfere with enzymatic hydrolysis (Kristinsson, 2007), the yielded protein contents in the samples are suggested to be high.

Table 4.3: Yield from Hydrolysis.

Sample	Protein/ 100 g raw material [g]	Protein/ hydrolysate [g]	protein yield [%]
RRM1	14.28 g	5.46/6.85 g (H1)	38 %
RRM1	14.28 g	5.31/6.00 g (H2)	37 %
RRM3	14.95 g	5.71/6.88 g (H3)	38 %
RRM4	16.28 g	5.39/6.25 g (H4)	33 %

4.1.1 Water soluble protein

Lowry is widely used to determine soluble proteins, peptides as well as free amino acids. By using amino acid analyses, the proteins need to be hydrolysed and this leads to a destruction of some amino acids (Mæhre et al., 2018). Therefore approaching Lowry leads to a first impression about the soluble protein concentration. The determination of the soluble protein in

the hydrolysate samples was performed several times. It was evaluated which results are most reliable with regard to the standard curve and furthermore which absorbances are in the range of the ones from the stock solution. The following results in Table 4.4 are mean values of parallels with different dilution factors.
The minced raw material samples show average soluble protein values from 4.45 % in RRM1 and the highest values of 7.00 % in RRM4 with regard to the sample wet weight. The percentage of soluble proteins in the raw material samples (4.45, 5.55 and 7.00 %) are relatively low, compared to the results gathered from the Kjeldahl analysis (can be seen on page 30). This is because the Lowry method only determines soluble proteins. Since the samples were prepared as water extracts, only the water soluble proteins got determined. As the trout head samples also consist of high amounts of ash, it is assumed that the samples contain a relatively high amount of insoluble protein bound in the collagen of skin, collagen as well as hydroxyapatite in bones and some salt soluble proteins are also expected.

Table 4.4: Average water soluble protein (WSP) concentration of the samples [wt.%] determined by the Lowry method. Standard deviation out of several dilutions is given.

Sample	WSP [wt.%]
RRM1	4.45 $\pm$1.67
RRM3	5.55 $\pm$1.56
RRM4	7.00 $\pm$0.46

Table 4.5 shows the water soluble protein (WSP) for the hydrolysate samples. The goal of hydrolysis is, to receive a high yield in soluble proteins and that that soluble protein is predominant in FPH. Hydrolysate sample H3, which is produced from oxidised raw material, shows the lowest water soluble protein content of 74.18 %. It has to be mentioned, that the standard deviation of H3 with 22 % is very high, where the lowest soluble protein value was measured to be 53 % and the highest 97 %. This inaccuracy and variance in the results might be due to some interference with the reagents or different incubation times of the dilutions that lead to different products of the Lowry reaction and further show a different absorbance. Nevertheless, the average of H3 was calculated out of several dilutions and the protein concentration of 74.18 % seems reasonable compared to other studies about FPH. H2 has the highest value with 86.51 % of soluble protein. BHT as antioxidant was added directly into the hydrolysis tank, to retard oxidation for H2. It seems that the addition of BHT shows a positive effect on the yield of water soluble proteins. As can be seen in table 4.2, the total protein content of the hydrolysates almost only consist of soluble protein. In average, it shows less than 9 % of difference between the water soluble and the total protein content. Furthermore, it can be seen that H2, besides having the highest water soluble proteins, exhibits the highest protein yield as well. This indicates, that a high amount of insoluble proteins became soluble due to hydrolysis. The remaining 9 % of insoluble protein are assumed to be ash and dissolved minerals from the minced trout heads. Furthermore, as no dry matter and lipid content of the hydrolysates was determined, a low percentage is also expected to be lipids and remaining moisture in the hydrolysates.
Šližytė et al. (2016) writes that fish protein hydrolysates made out of backbones show total protein values from 73.8 % and Venugopal (2016) states, that the protein content can be as high as 93 % in FPH respectively. Drotningsvik et al. (2018) write, that the soluble protein fractions of fish protein lower the circulating cholesterol of obese humans. Therefore, the high amounts

of soluble protein in the hydrolysates can be associated with good cardioprotective properties. Furthermore, the high amount of soluble proteins in the hydrolysate show a great digestibility (Rustad et al., 2011). In general, it is difficult to compare protein results from fish protein hydrolysates made out of different rest raw materials, such as backbones, heads or intestines, as they differ in their ash contents and protein solubility.

Table 4.5: Average water soluble protein concentration (WSP) of the sample wet weight [wt.%] determined by the Lowry method. Standard deviation out of several dilutions is given.

Sample	WSP [wt.%]
H1	79.78 $\pm$0.8
H2	86.51 $\pm$5.4
H3	74.18 $\pm$22
H4	77.80 $\pm$2.1

The hydrolysates were filtrated in order to see if they can be fractionated based on their molecular weight. The ultramembrane filtration separates peptides into two fractions and leads to a concentration of proteins in the retentate. Table 4.6 shows the soluble protein contents of the two fractions, the retentates and permeates, received from ultramembrane filtration. The samples H1-H4/R are the retentates and H1-H4/P are the permeates of the hydrolysates. Large peptides ($>$ 4 kDa) are located in the retentate, whereas small peptides ($<$ 4 kDa) are found in the permeate. This up-concentration is pointed out in the results of WSP in the samples. The soluble protein contents of the retentates show higher values as the protein contents of the permeates. H1/P shows 1.68 mg/ml soluble protein, whereas the retentate H1/R has 9.64 mg/ml soluble protein. The retentates have approximately a ten fold higher protein value than the permeates. This indicates, that the ultramembrane filtration efficiently separated the peptides. The molecular weight distribution of the retentates and permeates can be seen in Section 4.3 illustrated in Figure 4.3.

Table 4.6: Average water soluble protein concentration (WSP) of the samples [mg/ml] determined by the Lowry method. Standard deviation out of several dilutions is given.

Sample	WSP [mg/ml]
H1/P	1.68 $\pm$0.19
H2/P	1.81 $\pm$0.48
H3/P	1.73 $\pm$0.10
H4/P	1.90 $\pm$0.21
H1/R	9.64 $\pm$0.08
H2/R	12.8 $\pm$0.11
H3/R	10.4 $\pm$0.13
H4/R	11.8 $\pm$0.06

4.1.2 Acid-soluble peptides

Table 4.7 shows the results from the determination of acid-soluble peptides in the raw material and the hydrolysate samples. The amount of acid-soluble peptides was determined by precipitating the samples in 10 % trichloroacetic acid (TCA). The method was performed according to

Hoyle and Merritt (1994) and measures the TCA-soluble nitrogen in the samples (Rutherfurd, 2010). This fraction is mainly composed of small peptides and amino acids (Rutherfurd, 2010). The samples were analysed in triplicates. RRM1, RRM3 and RRM4 show noticeable lower results in the total protein content and in water soluble proteins compared to the hydrolysates (H1-H4). Since the proteins in the raw material are not degraded, a significantly lower value in ASP can be expected. Due to the extended degradation of proteins in the hydrolysis process, the fraction of acid-soluble peptides increased more than 10 fold in the hydrolysates. The values from the raw material range from 0.44 % (RRM4) to 0.54 % (RRM3) of ASP per sample wet weight. Whereas a range between 25.00 % (H3) to 28.75 % (H2) can be seen in the hydrolysate samples. The results correlate with the higher solubility in the hydrolysates as pointed out in Table 4.5.
The method measures the amount of TCA-soluble peptides, it does not directly measure the peptide bond cleavages (Rutherfurd, 2010). Nevertheless it can be assumed, that the hydrolysis was effective and cleaved peptides into smaller acid-soluble protein units as the increase in ASP in the hydrolysates show. The cleavage of peptide bonds induced through hydrolysis was investigated by determining the degree of hydrolysis (DH). This was done by Formoltitration and results can be seen in Section 4.2.

Table 4.7: Average acid-soluble protein (ASP) concentration per sample wet weight [wt.%] determined by the Lowry method. Standard deviation for hydrolysates are given; n=2

Sample	ASP/Sample [wt.%]
RRM1	0.46
RRM3	0.54
RRM4	0.44
H1	28.44 $\pm$0.9
H2	28.75 $\pm$2.2
H3	25.00 $\pm$2.3
H4	27.99 $\pm$3.0

4.1.3 Collagen content

The high ash content and the relatively low protein content lead to the assumption, that the minced raw material samples are high in insoluble proteins. Connective tissues like bones and skins consist of a high amount of collagen. Hydroxyproline is a major component of fibrillar collagen of all types (Cundy et al., 2014). To determine the content of collagen in the samples, the concentration of hydroxyproline was measured. To convert the concentration of hydroxyproline into collagen, Sato et al. (1991) uses a multiplication factor of 11.42 for trout muscles. More recent studies of Tylingo et al. (2016) determined the conversion factor through different experiments to get the most suitable conversion factor. For salmon skin, Tylingo et al. (2016) applied a conversion factor of 12.45, which was determined through dialysis.
The conversion factor of 12.45 was chosen to be most suitable, since the samples are heterogeneous in skin, bones and muscle and the results can be seen in Table 4.8. The minced trout heads contain 4.01 % (RRM1), 5.90 % (RRM3), and 8.21 % (RRM4) of collagen. It can be seen that RRM4 contains almost double the amount of collagen compared to RRM1 and also a higher ash content then the rest of the samples. Despite the high efforts to homogenize the

samples evenly, it is possible that more of the insoluble protein fractions such as bone and skin are located in RRM4. In general, the results seem low compared to the study of Lee et al. (2016) of the collagen content in only trout skin (approximately 61 %). Since the samples are whole minced heads, they contain collagen fractions bound in bone and skin. But they also contain soluble protein in the meat and minor contents of fat. Therefore the lower collagen content compared to Lee et al. (2016) is reasonable. Moreover, the measured collagen content explains the relatively low soluble protein content in the raw material samples.

Table 4.8: Hydroxyproline content [mg/ml], Hydroxyproline and Collagen [wt.%] in the raw material samples (wet weight) RRM1, RRM3 and RRM4. Standard deviation from the absorbance values given: n=2 for RRM1, n=3 for RRM3 and RRM4).

Sample	Hydroxyproline [mg/ml]	Hydroxyproline [wt.%]	Collagen [wt.%]
RRM1	0.017 ± 0.08	0.32	4.01
RRM3	0.025 ± 0.03	0.47	5.90
RRM4	0.034 $\pm 0,07$	0.66	8.21

4.2 Degree of Hydrolysis

According to Rutherfurd (2010), the degree of hydrolysis is defined as the proportion of cleavages of peptide bonds in protein hydrolysates. When determining the degree of hydrolysis, the free NH_2 groups are quantified and the degree of hydrolysis is calculated as the number of cleaved peptide bonds of the total amount of N in the sample (Šližytė et al., 2010). The free amino groups in samples are indicators for the degree of hydrolysis and need to be determined in order to calculate the degree of hydrolysis. The calculation of the DH can be seen in Appendix B.

Results for the free amino groups and degree of hydrolysis can be seen in Table 4.9. The standard error was calculated from the standard deviations of the free amino groups and nitrogen results (n=3). The raw material samples show a significantly lower degree of hydrolysis compared to the hydrolysates, which indicates only a minor cleavage of peptides. Whereas the hydrolysis lead to a decrease in peptide bonds for the hydrolysates.
The DH of the hydrolysates only varied little within the samples. H1 shows the lowest value with 16.51 % followed by 16.85 %(H2) and 17.38 % (H3) and H4 having the highest DH with 18.21 %. The hydrolysates H1, H3 and H4 were subjected to the same hydrolysis condition. Only H2, differed in the addition of BHT as antioxidant in the hydrolysis tank before starting the hydrolysis. No significant difference was found between the DH of all hydrolysates, indicating that the oxidation of the raw material did not affect the DH of H3 and H4. Furthermore, H2 has a similar DH to the other hydrolysates, suggesting that BHT did not affect the DH.

In proteolytic hydrolysis, proteins are cleaved and shorter peptides and free amino acids are formed. By protein cleavage, the number of N-residues (NH_2) and C-residues (COOH) increase and the average size of the proteins decreases (Šližytė et al., 2010). Different constitutions within the peptide chain as well as different chain lengths can exhibit the same DH. Therefore, the DH provides information about the average sizes of the peptides in the hydrolysate samples,

but gives no information about amino acids residues. The possible relationship between the DH and the free amino acid content in the hydrolysates is further discussed in Section 4.6 on page 48.

Small peptides are associated with good organoleptic and functional properties, whereas hydrolysates with larger, hydrophobic oligopeptides are associated with bitterness (Gbogouri et al., 2004). According to Maehashi et al. (2008), peptides with a molecular size between 100 and 6000 Da and a high hydrophobicity exhibit bitter taste. Klompong et al. (2007) reported, that interfacial activities such as emulsifying and foaming properties decrease with increasing DH. Moreover, increased DPPH radical scavenging activity is associated with increased DH, whereas larger peptides show increased iron chelating properties, indicating a lower DH (Šližytė et al., 2016). Dauksas et al. (2004) states, that DH levels between 4 and 40 % most likely show a negative effect on the bitterness of hydrolysates. According to Adler-Nissen (1984), low DH's between 3 an 5 % significantly lower the bitterness. Furthermore, an extended degradation of the proteins into very small peptide fractions degreases bitterness as well (Šližytė et al., 2010). To have low levels of bitterness in the hydrolysates, it is therefore suggested to have a very low DH with large peptide fractions or a high DH, with very small amino acid residues. In general, it is difficult to compare DH results between different studies, as different analytical methods are applied. Thus, one has to be careful when comparing DH results.

Table 4.9: Free amino groups and Degree of Hydrolysis (DH) [wt.%] as determined by Formoltitration. Standard deviation given for free amino groups; standard error of SD is given for DH; (n=3).

Sample	Free Amino Groups [wt.%]	Degree of Hydrolysis [wt.%]
RRM1	$0.218 \pm 0,02$	9.54 ± 0.03
RRM3	0.171 ± 0.04	7.14 ± 0.08
RRM4	0.280 ± 0.03	10.75 ± 0.09
H1	2.106 ± 0.19	16.52 ± 0.14
H2	2.387 ± 0.04	16.85 ± 0.05
H3	2.311 ± 0.05	17.38 ± 0.09
H4	2.502 ± 0.02	18.21 ± 0.03

4.3 Molecular weight distributions

The determination of the molecular weight of peptides was done by FPLC, using a column with a separation range between 100 to 7000 Da, with an exclusion limit at 20 000 Da.

The molecular weight distribution of the samples can be seen in Figure 4.1, Figure 4.2 and Figure 4.3. The gel filtration chromatogram on the raw material samples shows a wide distribution of different peptide molecular sizes. RRM1 shows the highest peaks in the absorbance, showing two peaks at an elution volume of ~8 ml and ~11 ml, therefore having a high density of large peptides with a greater molecular weight than the known molecular weight of 12.4 kDa from Cytochrome C. A peak was also detected at an elution volume of ~17.5 ml indicating a molecular weight between 12.4 kDa and 6.5 kDa, showing a higher absorbance than RRM3 and RRM4 at this molecular weight. RRM3 and RRM4 both show two high peaks at a lower molec-

ular weight. Indicating that they have a higher amount of small peptides with a lower molecular weight than RRM1.

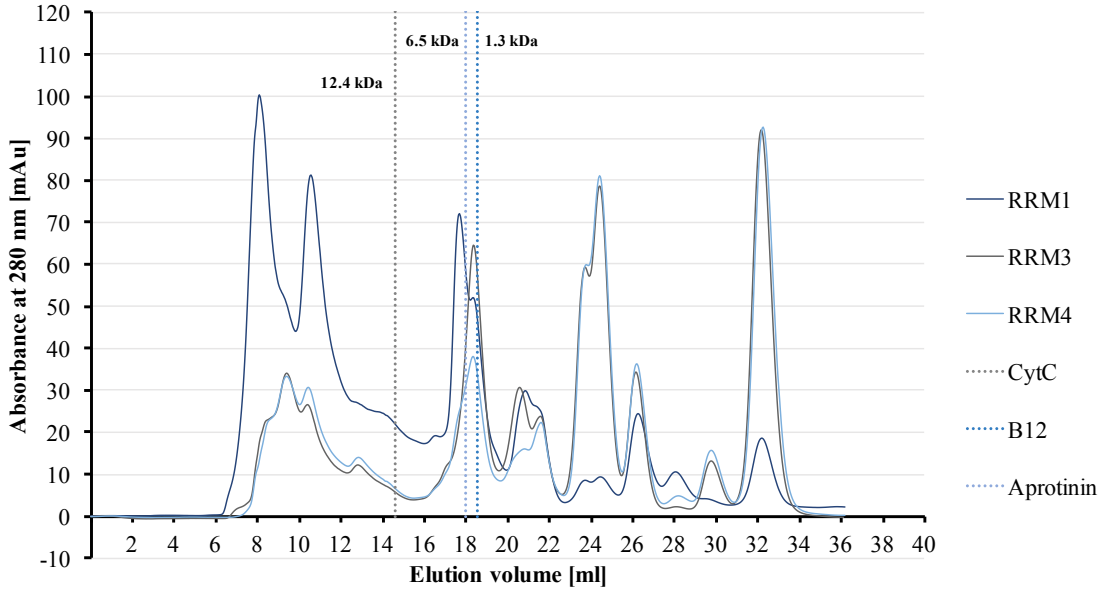


Figure 4.1: Gel filtration chromatogram showing the molecular weight distributions of RRM1, RRM3 and RRM4 compared to the peaks of known standards.

The chromatogram of the hydrolysates in Figure 4.2 shows a shift in the elution volume to the right compared to the chromatogram of the raw material. Suggesting, that the hydrolysate samples show more low molecular weight peptides than the raw material samples. The first peak can be observed at a elution volume of ~17.5 ml. H1 and H2 show a distinct higher absorbance at this elution volume than H3 and H4 and compared to the raw material, the absorbance is as well significant higher. Moreover, the hydrolysates show a high density of peptides smaller than 6.5 kDa and smaller than 1.3 kDa.

Small peptides, usually showing a molecular weight lower than 3 kDa, are able to exhibit bioactive (Hayes, 2013) as well as functional and nutritional properties (Gbogouri et al., 2004). Small peptides from myofibrillar proteins are said to be able to form hydrogen bonds with water to a higher degree than large proteins. This significantly increases the solubility. This ability is due to the fact that small peptides have proportionally more polar residues (Gbogouri et al., 2004). The results suggest, that the hydrolysates contain peptides in the molecular weight range where bioactive peptides are expected to be. Therefore, it can be assumed, that they exhibit a high nutritional value. Furthermore, these small peptides are able to enhance solubility.

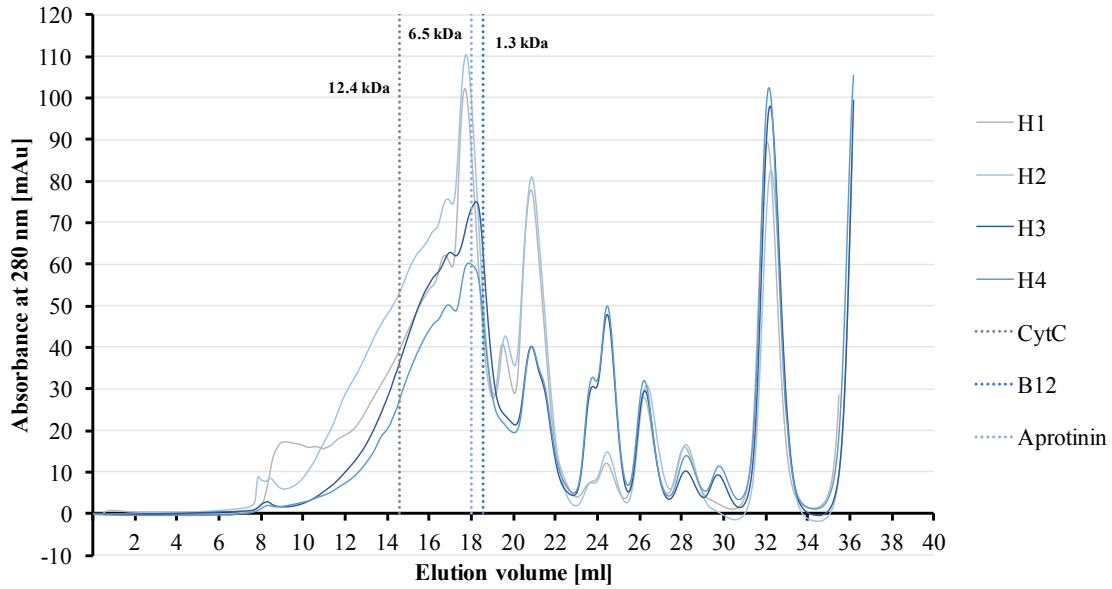


Figure 4.2: Gel filtration chromatogram showing the molecular weight distributions of H1, H2, H3 and H4 compared to the peaks of known standards.

The ultramembrane filtration of the hydrolysates lead to a separation of the retentate (> 4 kDa) and the permeate (< 4 kDa). As Figure 4.3 illustrates, the four retentates of the hydrolysates show a high absorbance at a molecular weight larger than 6.5 kDa. Whereas the elution volume of the permeates are distinctly higher, indicating a lower molecular weight and smaller peptides. The retentates show a higher intensity in their peaks, than the permeates. This is probably due to the difference in their protein concentration, compared to the permeates as can be seen in the results of the Lowry method in Table 4.5.
Furthermore, it can be observed, that the retentates show peaks at a molecular weight smaller than 4 kDa. Therefore, implying that the ultramembrane filtration did not show a clear cut in the separation of a (> 4 kDa) and (< 4 kDa) fraction. This can be due to the dead volume of 50 ml, thas has to be kept in the filtration system. This dead volume accounts to approximately 1/3 of the initial volume that was put into the feed tank.
As Figure 4.3 illustrates, the peptide profile of the retentates and permeates differ, but the fractions were not 100 % separated as the retentates show peaks in a molecular weight range > 4 kDa. Whereas the permeates show no peaks higher than approximately 4 kDa.

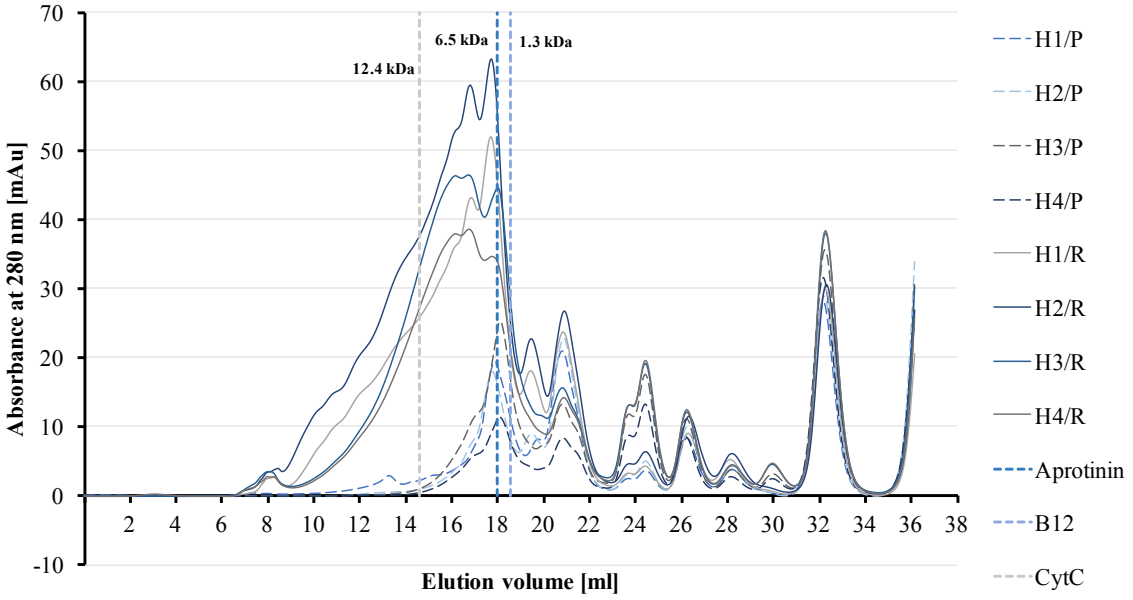


Figure 4.3: Gel filtration chromatogram showing the molecular weight distributions from permeates (H1-4/P) and retentates (H1-4/R) compared to the peaks of known standards.

4.4 Oxidation indicators

4.4.1 Carbonyl groups

Cellular proteins are often exposed to oxidative stress. The present reactive oxygen species (ROS) can lead to a damage in the protein composition. ROS can affect amino acid residues by initiating nitration or oxidation. Furthermore, depending on the present ROS, they may lead to the formation of advanced glycation end products (AGE) or advanced oxidation protein products (AOPP). These generated products give strong evidence of oxidative stress (Cell Biolabs, Inc., 2019). The higher the carbonyl concentration in the samples, the more oxidised they are.

The difference in the carbonyl contents of the raw material samples and the hydrolysate samples can be observed in Table 4.10. The results are shown in nmol carbonyls per mg soluble protein per sample. The difference in the concentration is further illustrated in Figure 4.4. The raw material samples show low values in their carbonyl concentration. RRM3 shows the highest concentration with 10.3 nmol/mg. RRM3 is the oxidised raw material sample and therefore the significantly higher concentration is reasonable. RRM4 is also oxidised, but BHT was added as antioxidant and the result (8.86 nmol/mg) is slightly lower than for RRM3, but noticeable higher than RRM1 (2.40 nmol/mg). It can be seen that the oxidation process was inhibited by the antioxidant.

Concerning the hydrolysate samples, a difference between the samples is noticeable. H3 shows the highest carbonyl concentration with 5.143 nmol/mg, which is approximately 2 nmol/mg higher than the rest of the hydrolysates. It seems that the oxidised raw material has an effect on the oxidation level of the produced hydrolysate. It can also be noticed, that the carbonyl concentration of H4 is only slightly higher than the one in H1 (2.751 nmol/mg versus 2.273 nmol/mg), leading to the assumption, that the added antioxidant to the raw material has a positive effect

on the hydrolysate as well. As BHT was added as antioxidant into the hydrolysis tank of H2, it was not expected that H2 shows the second highest content of carbonyls within the hydrolysate samples. It seems that the time at which the antioxidant is added, does make a difference for the oxidation of the hydrolysates. For H4, the BHT was already added to the raw material at the same time as the oxidants were added. Whereas in sample H2, BHT was added directly into the hydrolysis tank, at the starting point of the hydrolysis. As Table 4.10 and Figure 4.4 show, the raw material has significantly higher concentrations of carbonyls. It can be assumed, that the removal of the lipid fraction and the further dehydration of the aqueous protein fraction lowered the ability to generate carbonyl groups.

Table 4.10: Amount of Carbonyl groups in RRM1, RRM3 and RRM4 [nmol Carbonyls/mg protein]. Standard deviation given.

Sample	Carbonyl Concentration [nmol/mg]
RRM1	2.40 $\pm$0.59
RRM3	10.3 $\pm$0.86
RRM4	8.86 $\pm$2.44
H1	2.27 $\pm$0.17
H2	3.67 $\pm$0.90
H3	5.14 $\pm$0.81
H4	2.75 $\pm$1.51

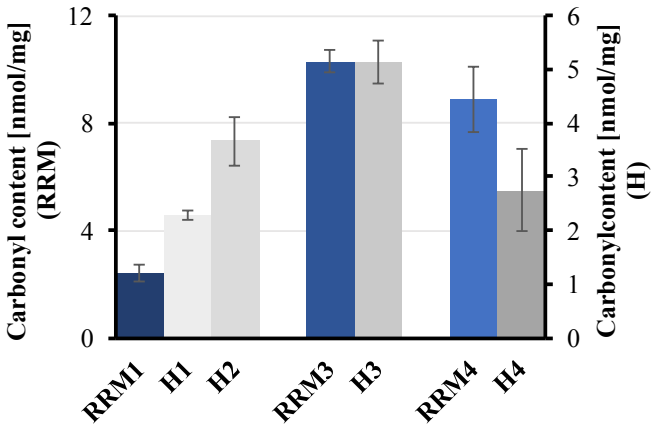


Figure 4.4: Comparison of the carbonyl concentration of the raw material samples (RRM1, RRM3, RRM4) and hydrolysates (H1-4). Values are given as means with error bars showing standard deviation.

4.4.2 Thiol groups

Oxidative stress leads to the formation of disulphide bonds. Thiol groups are vulnerable to oxidative stress and therefore to the formation of these unwanted formations (Von Wachenfeldt, 2018). The loss of thiol groups in the samples was determined due to the reaction with the Ellmann reagent DTNB. The thiols react with the DTNB and lead to the formation of disulfide and 2-nitro-5-thiobenzoic acid (TNB). These reactions are stoichiometric, meaning 1 TNB accounts

for 1 protein thiol. Therefore, the formation of TNB can be used to determine the number of free tiols.

A significant difference between the thiol contents of the raw material samples and the hydrolysate samples can be observed in Table 4.11. The results are shown in nmol thiols per mg soluble protein per sample. The difference in the concentration is further illustrated in Figure 4.5. The raw material samples show noticeable higher concentrations than the hydrolysates. RRM1 has the highest thiol concentration with approximately 64.3 nmol/mg, whereas the oxidised RRM3 shows a 18.3 nmol/mg less thiol concentration. The hydrolysates do not vary a lot between their thiol concentration. H2 shows the lowest thiols with a concentration of 1.084 nmol/mg. As H2 shows the lowest thiol and the second highest carbonyl concentration it is very high in oxidation indicators, thus assuming a great onlset of oxidation.

Table 4.11: Amount of Thiol groups in the RRM1, RRM3 and RRM4 [nmol Thiols/mg protein]. Values are given as means with error bars showing standard deviation.

Sample	Thiol Concentration [nmol/mg]
RRM1	64.3 ±5.3
RRM3	46.1 ±2.6
RRM4	52.3 ±3.8
H1	2.997 ±0.30
H2	1.084 ±0.63
H3	2.895 ±1.67
H4	3.265 ±1.89

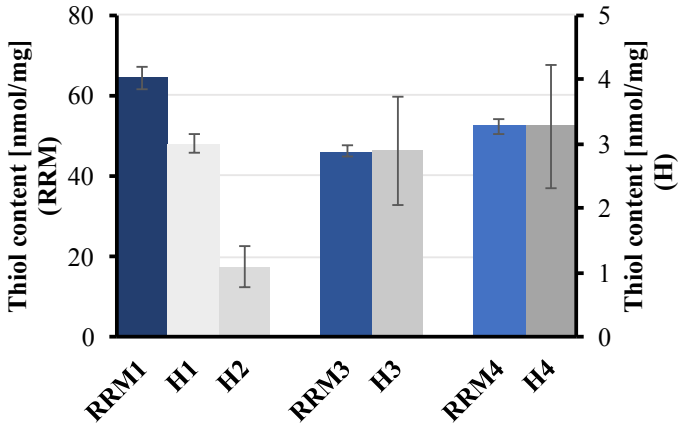


Figure 4.5: Comparison of the thiol concentration of the raw material samples (RRM1, RRM3, RRM4) and hydrolysates (H1-4). Standard deviation given.

4.5 Amino acid composition

The amino acid composition gives information about the nutritional as well as the functional properties of the hydrolysates. Marine peptides constituting of 3 to 20 amino acid residues are suggested be able to exhibit bioactive properties (Lee et al., 2012). To characterize the nutritional value of the raw material and the hydrolysates, the total amino acid composition, as well as the free amino acid composition was determined.

4.5.1 Total amino acid composition

The total amino acid (TAA) content of the raw material and the hydrolysates can be seen in Table 4.12. The results from the raw material samples refer to their wet weight. The values are given as mean with a standard deviation of 3 for the hydrolysates and 2 for the raw material. The standard deviations are low, except for the phenylalanine values of sample H3.
The TAA of the raw material samples range from 43.5 to 76.9 mg/g raw material, with the highest values measured in sample RRM3. Concerning the hydrolysates, a range of a TAA composition of 476.0 to 711.5 mg/g hydrolysate could be observed, whereas H2 shows the lowest and H3 the highest values. In the study of Sathivel et al. (2005) about salmon head hydrolysates, values of about 988 mg/g protein were measured. The results given in Table 4.12 are in gram per raw material wet weight or hydrolysate dry weight, which are not constituted of 100 % protein.

Table 4.12: Total amino acid content in RRM1, RRM3 and RRM4 [mg/g] and average of TAA/sample [wt.%]. Standard deviation given (n=3 for hydrolysates and n=2 for raw material).

Sample	TAA [mg/g]	TAA [wt.%]
RRM1	43.5 $\pm$6.2	4.3
RRM3	76.9 $\pm$8.9	7.7
RRM4	71.4 $\pm$12.8	7.1
H1	516.2 $\pm$127	51.6
H2	476.0 $\pm$51	47.6
H3	711.5 $\pm$7.0	71.1
H4	556.2 $\pm$155	55.6

Figure 4.6 illustrate the total amino acid composition of the different raw material samples in weight percentages. The amino acid asparagine (Asn) was excluded in the graphics, as it was only detected in very low traces. A table of the amino acid compositions can be seen in Appendix D.

In the raw material samples, the highest values were detected for the amino acids glycine/arginine, glutamic acid, aspartic acid, alanine, lysine and leucine. The amino acid composition in the raw material samples are mostly similar, with only one exception for the glycine/arginine content. The values range from 14.84 % to 17.34 %, with the highest amount found in RRM3 and the lowest in RRM1. The lowest values, are asparagine, glutamine (Gln), Histidine (His) and Methionin (Met). Glutamine and asparagine get converted into glutamic and aspartic acid, due to the acid hydrolysis. This explains the high values for glutamic/ aspartic acid and the low values for asparagine and glutamine.

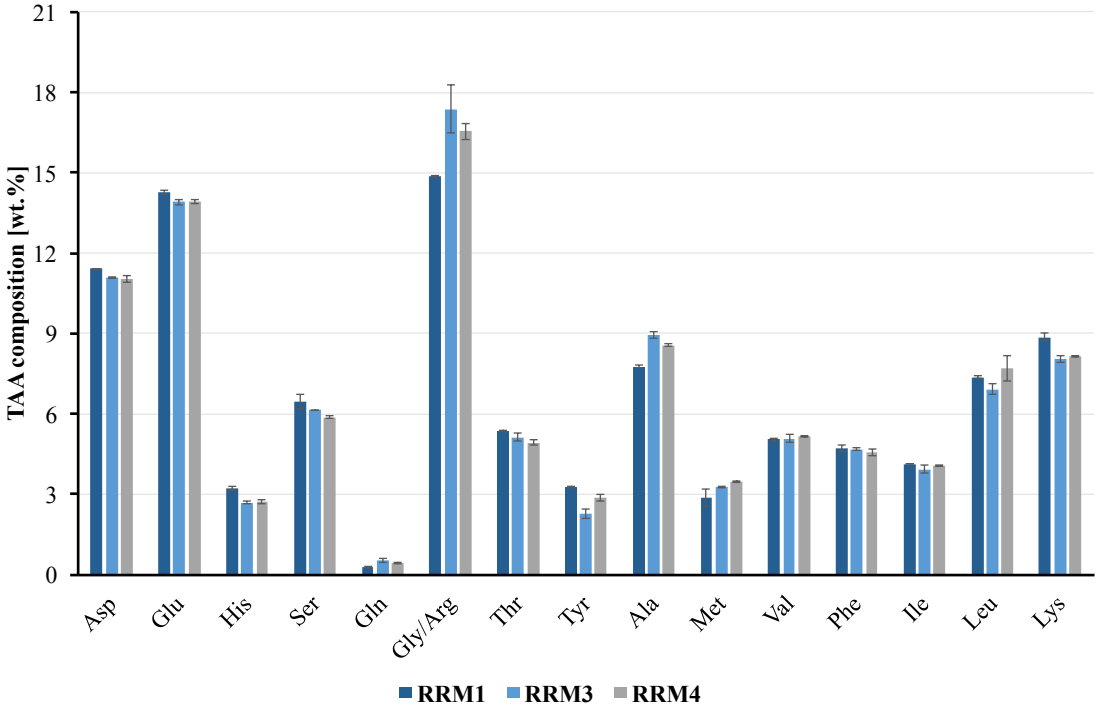


Figure 4.6: Weight fractions (%) of TAA for raw material samples RRM1, RRM3 and RRM4 as found by HPLC. Values are given as means with error bars showing standard deviation; (n=2)

Figure 4.7 demonstrates the total amino acid composition for H1-H4. In the hydrolysates, glycine/arginine, glutamic acid, alanine, aspartic acid, lycine and leucine were also predominant. In the study of Sathivel et al. (2005), the salmon head hydrolysates showed similarities in the abundance of the predominant amino acids, where glutamic acid was the most abundant amino acid next to glycine and aspartic acid.
Furthermore, the lowest values in the hydrolysates are found for asparagine and glutamine. This is the same for the raw material samples, as they get converted to aspartic and glutamic acid due to acid hydrolysis. Tyrosine shows noticeable lower values compared to the raw material. The amino acid composition within the hydrolysate samples is generally similar, with an exception for phenylalanine. H1, H2 and H4 show quite similar contents (3.52 %, 3.62 % and 3.59 %), whereas H3 exceeds these contents (5.58 %). Even though H3 shows a higher value, it has to be mentioned, that the standard deviation is significantly higher than for the other hydrolysates with 2.8.

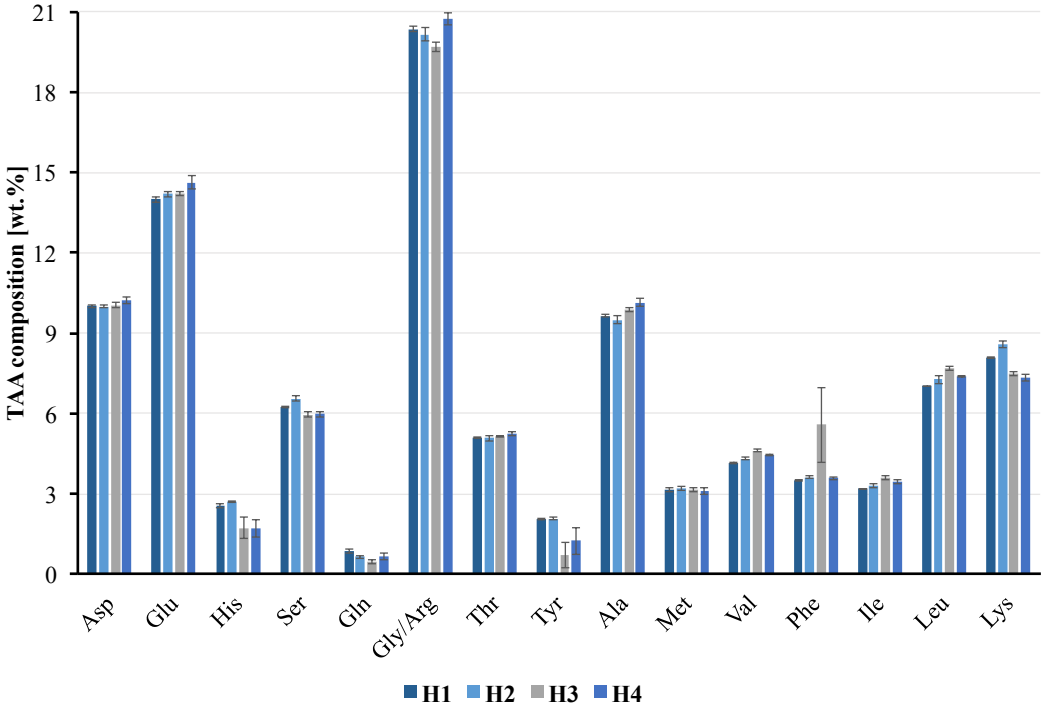


Figure 4.7: Weight fractions (%) of TAA for hydrolysate samples H1, H2, H3, and H4 as found by HPLC. Values are given as means with error bars showing standard deviation; (n=3).

Table 4.13 gives an overview of the hydrophobic AA (Ala, Ile, Leu, Met, Phe, Tyr and Val), aromatic AA (His, Phe, Tyr) and essential AA (His, Ile, Leu, Lys, Met, Phe, Thr and Val) fractions in the raw material and hydrolysate samples. Hydrophobic and aromatic amino acids are associated with the ability to exhibit antioxidant activities in marine peptides (Samaranayaka and Li-Chan, 2011; Ren et al., 2008). The antioxidant properties of peptides is strongly related to the amino acid sequence and their hydrophobicity. The raw material as well as in the hydrolysate samples were found to contain high amounts of HAA fractions. The hydrolysates show slightly lower values (32.77 to 35.24 %) compared to the raw material samples (35.05 to 36.35 %).

According to Ren et al. (2008) tyrosine and phenylalanine are associated to scavenge radicals directly. Based on their special structural properties, histidine, methionine and cysteine enhance the radical scavenging activity of marine peptides (Chalamaiah et al., 2012). Samaranayaka and Li-Chan (2011) write that cysteine, methionine, histidine as well as the aromatic amino acids tryptophan, tyrosine and phenylalanine belong to the most reactive amino acids. Histidines strong radical scavenging activity is associated with its imidazole ring (Samaranayaka and Li-Chan, 2011) and the phenolic groups of tyrosine function as hydrogen donors in directly scavenging radicals (Ren et al., 2008; Jun et al., 2004). The AAA fractions of both the raw material samples and the hydrolysates show similar values within their group: 9.62 to 11.22 % in the raw materials, 6.55 to 8.40 % in the hydrolysates.

Both the raw material samples and the hydrolysates show high EAA values. Compared to the suggestion of FAO (1985), all samples exceed the required value of essential amino acids and exhibit a high nutritional value. The samples show similar values to the ones of the study

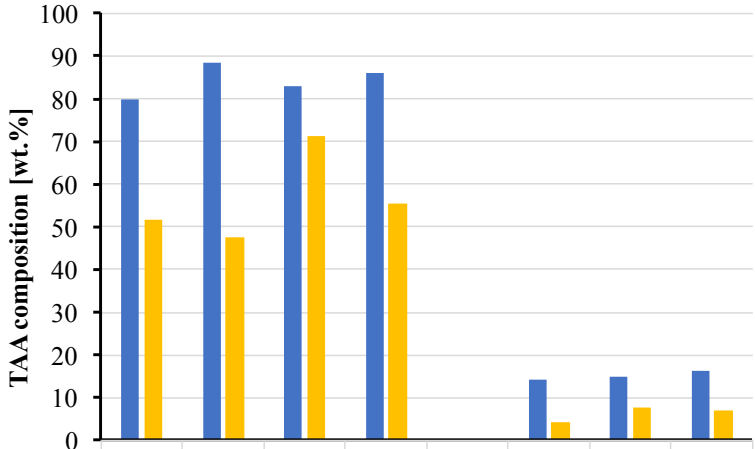
from Sathivel et al. (2005), where the salmon head hydrolysates were found to have EAA values ranging from 35.8 to 40.8 % out of the TAA content, excluding tryptophan and cystein. The raw material samples are higher in their results (39.68 % in RRM3 up to 41.55 % in RRM1) compared to the hydrolysates (36.32 % in H4 up to 39.02 % in H3). The results from HPLC exclude tryptophan and cystein, as they are destroyed during acid hydrolysis (Bartolomeo and Maisano, 2006). Furthermore, Bartolomeo and Maisano (2006) state that serine and threonine undergo losses during HPLC and that there is a probability that methionine undergoes oxidation.

Table 4.13: HAA (hydrophobic amino acids), AAA (aromatic amino acids) and EAA (essential amino acids) in raw material and hydrolysate samples.

Sample	HAA [wt.%]	AAA [wt.%]	EAA [wt.%]
RRM1	35.19	11.22	41.55
RRM3	35.05	9.62	39.68
RRM4	36.35	10.15	40.70
H1	32.77	8.13	36.80
H2	33.33	8.40	38.10
H3	35.24	8.02	39.02
H4	33.39	6.55	36.32

The analysis of the total amino acid composition in the raw material and hydrolysates showed a noticeable difference to the results from the Kjeldahl method. The Kjeldahl method showed higher contents of total protein in both the raw material and the hydrolysates than the received ones through HPLC. Both HPLC and Kjeldahl method are known to be precice methods with high accuracy in the results. Nevertheless, the results in the total protein/TAA from HPLC are mostly approximately 50 % lower than the ones from the Kjeldahl method as can be seen in Figure 4.8. An exception is sample H3, where the results only varies in about 11 %.

One possible reason for the huge difference might be the acid hydrolysis, a crucial step in the preparation of samples for HPLC. The preparation of the samples, including the acid hydrolysis involves many steps. It is possible that some proteins got destroyed due to the acid hydrolysis. Another reason could be that some of the proteins got lost during the neutralization or filtration. Therefore, the gathered TAA contents should be seen as reference for the amino acid composition and indicator for the amino acid distribution. The values received from the Kjeldahl method should be seen as the absolute protein values.



	H1	H2	H3	H4		RRM1	RRM3	RRM4
Results Kjeldahl	79.70	88.53	83.09	85.90		14.28	14.95	16.28
Results HPLC	51.76	47.60	71.10	55.60		4.30	7.70	7.10

Figure 4.8: Comparison of the total amino acid/ protein results received from the Kjeldahl method and HPLC.

4.5.2 Free amino acid composition

The total free amino acid (FAA) contents in the raw material samples and the hydrolysates are given in Table 4.14. The results from the raw material samples refer to their wet weight. The values are given as mean with a standard deviation of 2 for the hydrolysates and the raw material. RRM1 (1.78 mg/g) shows noticeable lower values for FAA compared to RRM3 (3.29 mg/g) and RRM4 (2.88 mg/g). The FAA results in the hydrolysates are noticeable higher, which is due to hydrolysis. H2 showing the highest FAA content with 36.77 mg/g and H1 showing the lowest with 29.49 mg/g. Šližytė et al. (2016) reports FAA contents of 24 to 170 mg/100g in hydrolysates derived from salmon backbone. The FAA results from the hydrolysates are within this range.

Table 4.14: Free amino acid content in RRM1, RRM3 and RRM4 [mg/g] and average of FAA/sample [wt.%]. Standard deviation given (n=2).

Sample	FAA [mg/g]	FAA [wt.%]
RRM1	1.78 ±0.24	0.18
RRM3	3.29 ±0.00	0.33
RRM4	2.88 ±0.14	0.29
H1	29.49 ±1.00	2.95
H2	36.77 ±4.41	3.75
H3	30.83 ±0.42	3.08
H4	31.49 ±0.55	3.15

According to Chalamaiah et al. (2012) the FAA content correlates with the degree of hydrolysis. Chalamaiah et al. (2012) states, that the more free amino acids, the higher the degree of hydrolysis. The samples from Šližytė et al. (2016) that show the same range of FAA contents as the analysed samples, have similar degrees of hydrolysis. Šližytė et al. (2016) states, that most of the samples that show a high amount of FAA, show a higher DH as well (higher than

20 %). A similarity was observed for the studied samples. It was seen that H1 shows the least FAA and the lowest DH, whereas H2 shows the highest FAA but the second lowest DH. As the DH and the FAA contents of H1-H4 show great similarities, the DH within the samples show great similarity as well. Furthermore, the oxidation status of the raw material does not seem to affect the FAA content in the hydrolysates. Šližytė et al. (2016) produced salmon backbone hydrolysates using different enzymes. The enzymes papain and bromelain, which were also used in this study, showed higher contents of FAA compared to other hydrolysates like seabzyme hydrolysates Šližytė et al. (2016). Therefore, the choice of enzymes, used for hydrolysis, is crucial for the outcome of the FAA content in the hydrolysates.

The free amino acid composition of the raw material samples can be seen in Figure 4.9, excluding asparagine, as it was only detected in traces. The total FAA content of RRM1 was the lowest within the raw material samples. But RRM1 shows higher values in lysine, glutamine, glutamic acid and serine. Moreover RRM1 has a histidine value of 9.5 %, whereas RRM3 and RRM4 only contain histidine in traces (less than 0.06 %). Furthermore, the difference in lysine (RRM1 - 9.2 %, RRM3 and RRM4 - less than 0.7 %), glutamine (RRM1 - 19.7 % RRM3 and RRM4 - less than 15.0 %), glutamic acid (RRM1 - 12.8 %, RRM3 and RRM4 - less than 9.0 %) and serine (RRM1 5.7 %, RRM3 and RRM4 - less than 1.2 %) is significant. Both RRM3 and RRM4 have noticeable higher contents of tyrosine, alanine and valine. Especially the high contents of alanine are outstanding with 18.9 % (RRM3) and 19.4 % (RRM4), compared to RRM1 (9.6 %).

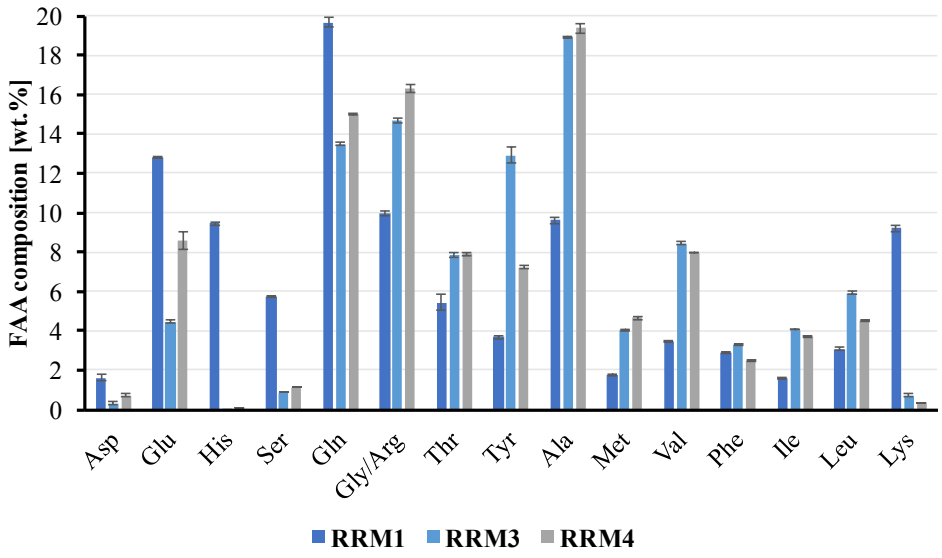


Figure 4.9: Weight fractions (%) of FAA for raw material samples RRM1, RRM3 and RRM4 as found by HPLC. Values are given as means with error bars showing standard deviation; (n=2)

Figure 4.10 shows the free amino acid composition of the hydrolysates. The total FAA content does not vary a lot within the hydrolysates. Nevertheless, a difference in the composition of FAA can be seen. H1 and H2 show significant higher values in lysine compared to H3 and H4 (9.4 % and 9.7 %, compared to 1.9 % and 1.5 %). Furthermore, the values in histidine and serine show high variances: 5.0 %, 5.7 % and 5.7 %, 6.5 % for H1 and H2, 1.5 %, 3.1 % and

1.5 %, 2.9 % for H3 and H4.

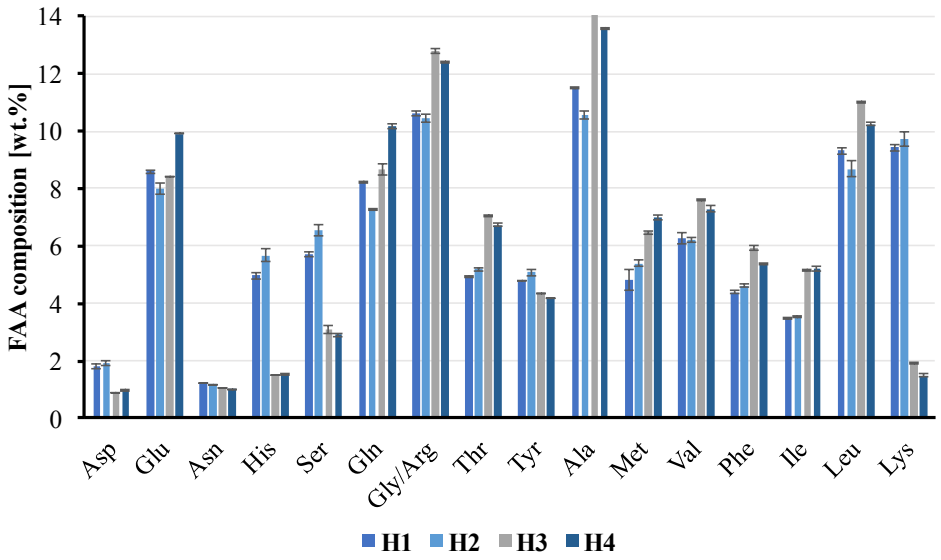


Figure 4.10: Weight fractions (%) of FAA for hydrolysate samples H1, H2, H3, and H4 as found by HPLC. Values are given as means with error bars showing standard deviation; (n=2)

4.6 Correlations

Figure 4.11 illustrates the correlation of the free amino acid content and the degree of hydrolysis for the hydrolysate samples. As no correlation and linear regression was found between all four hydrolysate samples, only H1, H3 and H4 were used and a correlation for these samples was found. A strong linear correlation with a regression of $R^2 = 0.931$ can be seen in Figure 4.11 for the samples H1, H3 and H4. A Pearson-Correlation coefficient of $r = 0.965$ was determined, indicating a strong positive correlation between the DH and FAA for the samples H1, H3 and H4. This implicates, that a higher degree of hydrolysis is connected with a higher amount of free amino acids in these samples. Moreover, this suggests a greater cleavage of peptide bonds. The hydrolysis was conducted the same way for H1, H3 and H4, but differently for H2. In the hydrolysis process of H2, BHT as antioxidant was added directly into the hydrolysis tank before hydrolysis was started. This difference in the production step might be the reason why H2 does not correlate and show no linear regression with the other three samples.

Furthermore, no correlation was found between the DH and acid soluble peptides or between ASP and FAA between the samples. Indicating that there is no significant difference in the ASP content with higher or lower DH ($r = -0.273$). Furthermore, it seems that the content of ASP does not correlate with the content of FAA in the hydrolysates, as only a low correlation coefficient was found ($r = 0.305$). Nevertheless the results of the determination of DH, ASP and FAA indicate, that the oxidation of the raw material does not affect the ASP, FAA and DH results, as the FAA, DH and ASP concentrations of the hydrolysates only show minor variations.

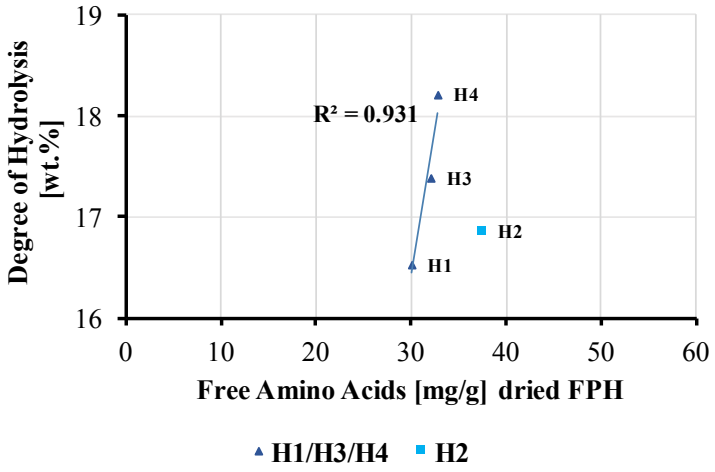


Figure 4.11: Comparison of the free amino acid content [mg/g] to the DH [wt.%] of the hydrolysate samples H1, H2, H3, H4.

Figure 4.12 illustrates the correlation between the protein solubility and the thiol concentration as well as the correlation between protein solubility and carbonyl concentration of the hydrolysates. As no correlation and linear regression was found between all four hydrolysate samples, only H1, H2 and H4 were used and H3 was left out for the calculation. A correlation for these samples was found. The solubility shows a strong negative correlation with a regression of $R^2 = 0.989$ with the thiol concentrations of H1, H2 and H4. A strong negative Pearson-Correlation coefficient of $r = -0.995$ was found, implicating a connection between high solubility and low thiol concentrations in H1, H2 and H4. Sample H3, produced through hydrolysis of oxidised raw material shows no correlation between solubility and thiol concentration and no linear regression when compared with the other three samples. H3 shows about the same thiol concentration as H1. Sample H2 shows the highest amount of water soluble proteins and the lowest thiol content.

The relationship between the protein solubility and the carbonyl content of H1, H2 and H4 shows a positive correlation with a regression of $R^2 = 0.715$. A correlation coefficient of $r = 0.846$ was detected for H1, H2 and H4. The calculation of the correlation and linear regression only includes H1, H2 and H3 and excluds H3, as no relationship between all four samples was found. It seems that greater protein solubility goes along with a higher oxidation. It can be seen that sample H2 has the second highest carbonyl content after H3 and the highest solubility. The high carbonyl concentration and the significant low thiol concentration implicates the onset of oxidation in this sample. Compared with the other hydrolysates, H3 shows a noticeable higher carbonyl concentration, but does not significantly differ in the thiol concentration. Furthermore, the solubility of H3 does neither correlate with thiol nor carbonyl concentration, as it shows the least water soluble proteins in the hydrolysates.

Even though the oxidation indicators of H3 did not correlate with the protein solubility, it shows a noticeable lower concentration of water soluble protein compared to the other hydrolysates. It seems that the oxidation status of the raw material affects the solubility, but does not necessarily mean a high oxidation in the hydrolysate. This can also be seen in sample H4, where BHT was added to the oxidised raw material before storage and hydrolysis. The solubility is lower compared to H1 and H2, but the indicators for oxidation are lower as well. Figure 4.13

points out the correlation between the oxidation status of the raw material and the solubility of the hydrolysates.

The observations made through illustrating the correlation between DH and FAA as well as solubility and thiols/carbonyls show, that sample H2 behaves differently compared to the other hydrolysates. This leads to the assumption that the addition of BHT as antioxidant influences the hydrolysis process and the quality of the hydrolysate. The required effect of the antioxidant seems to not appear and therefore H2 shows a relatively higher onset of oxidation compared to H1 and H4.
Furthermore, it can be seen, that sample H4 shows lower carbonyl and higher thiol concentrations than H2 and H3. This implicates, that the antioxidant was effective in retarding the onset of the raw material's oxidation. Therefore it is suggested to add the BHT already to the stored raw material to effectively postpone oxidation, instead of adding it into the hydrolysis tank as was done for H2.

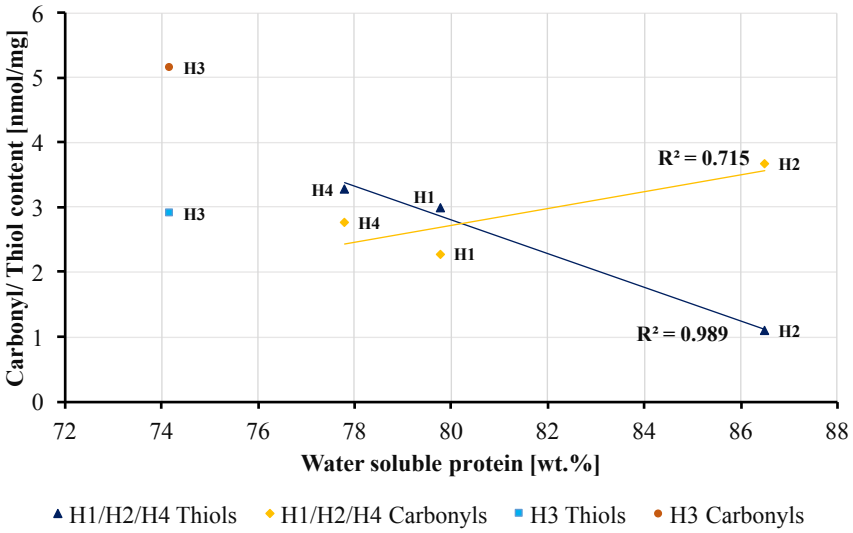


Figure 4.12: Correlation of the oxidation status [nmol Thiols/Carbonyls per mg protein] and protein solubility [wt.%] for H1, H2, H3 and H4.

Figure 4.13 proves the assumption of the possible correlation between the oxidation status of the raw material and the protein solubility of the hydrolysates. A positive correlation with a regression of $R^2 = 0.716$ can be seen for the solubility of the hydrolysates compared to the thiol concentration in the raw material. A strong correlation coefficient of $r = 0.846$ supports the result. Furthermore, a negative correlation between the solubility of the hydrolysates and the carbonyl concentration of the raw material was detected. The samples show a regression of $R^2 = 0.689$ and a correlation coefficient of $r = -0.830$. Therefore, high solubility can be associated with low carbonyl concentrations. This suggests, that raw material that has little oxidation leads to a high solubility in the hydrolysates.

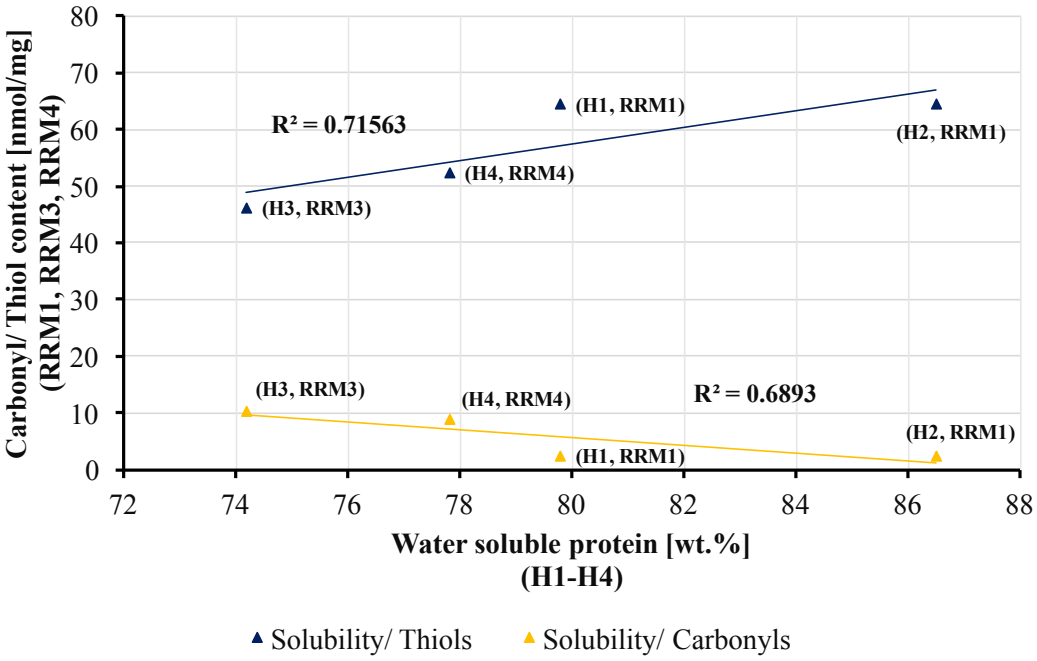


Figure 4.13: Correlation of the oxidation status [nmol Thiols/Carbonyls per mg protein] of the raw material to the protein solubility [wt.%] of the hydrolysate samples.

CHAPTER 5

Conclusion

The results showed that the hydrolysates have a high protein content with good solubility in their proteins, where almost all of the total proteins were detected to be soluble. The hydrolysis lead to an increase of cleaved peptide bonds and increased acid soluble proteins as well as content of free amino acids. A great similarity in the ACP content, FAA content and the degree of hydrolysis between the hydrolysate samples was found.

The investigation of the molecular weight distribution of the raw materials and hydrolysates showed a noticeable shift to smaller peptide fractions for the hydrolysates. The hydrolysates had similar molecular weight distributions, with only little variance.
A greater variance could be seen when comparing the molecular weight distributions of the raw materials. The fresh raw material sample RRM1, showed larger peaks in the high molecular weight range. Furthermore, a higher density of smaller peptides for RRM1 and RRM3 was found in a lower molecular weight range compared to sample RRM4. This indicates, that the oxidation of RRM3 does not negatively affect the molecular weight distribution of the peptides. Furthermore, the added antioxidant BHT in RRM4 might be the reason for a different peptide distribution compared to the other samples.

The hydrolysates were found to exhibit high nutritional values, as all samples contain high amounts of essential amino acids. Little variance was seen in the composition between the hydrolysates, indicating that the status of the raw material does not affect the total amino acid composition. Furthermore, the high protein content as well as the high solubility of the proteins in combination with the good nutritional value, leads to the conclusion that they are a good source of dietary proteins. The determination of free amino acids showed no significant difference between the contents of the hydrolysates. But a noticeable difference could be seen in the free amino acid composition. H1 and H2 showed significant higher results in histidine, serine and lysine. Moreover, the status of the raw material seems to not influence the concentration of free amino acids in the hydrolysates, as no significant difference could be found between oxidised and fresh samples.

The overall quality of the hydrolysates are influenced by the oxidation status of the raw material. No linear correlation was found between oxidative status and free amino acid concentrations, but a linear correlation between degree of hydrolysis and free amino acid content of three out of the four hydrolysate samples was found. The correlation was found in the three samples, that were hydrolysed using the same hydrolysis condition. The hydrolysate where BHT was added directly into the hydrolysis tank, did not fit in the correlation. This indicates that the time of adding the antioxidant plays a crucial role for the outcome and quality of the hydrolysate.
Furthermore, a linear correlation between oxidation indicators and protein solubility of three of the four hydrolysates was observed. It was found that a higher oxidation in the raw material lead to a decreased solubility in the hydrolysates, but does not necessarily mean a high oxidation in

the hydrolysates. This implicates, that the ongoing oxidation process of not correctly stored rest raw material does not have a negative effect on the nutritional value of the hydrolysates, but changes the digestibility of the proteins.

Bibliography

AAT Bioquest, Inc., 2018. PBS (Phosphate Buffered Saline) (1X, pH 7.4) recipe and preparation.
URL https://www.aatbio.com/resources/buffer-preparations-and-recipes/pbs-phosphate-buffered-saline

Adler-Nissen, J., 2019/05/14 1984. Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. Journal of Chemical Technology and Biotechnology. Biotechnology 34 (3), 215–222.
URL https://doi.org/10.1002/jctb.280340311

AOAC, 1990. Official methods of analysis. Journal of AOAC International Washington DC, USA.

Bartolomeo, M. P., Maisano, F., 04 2006. Validation of a reversed-phase hplc method for quantitative amino acid analysis. Journal of biomolecular techniques : JBT 17 (2), 131–137.
URL https://www.ncbi.nlm.nih.gov/pubmed/16741240

Bekkevold, S., Olafsen, T., 2007. Råvarer med muligheter.

Bernardi, D. M., Bertol, T. M., Pflanzer, S. B., Sgarbieri, V. C., Pollonio, M. A. R., 2016a. ÏL'-3 in meat products: benefits and effects on lipid oxidative stability 96 (8), 2620–2634.
URL https://doi.org/10.1002/jsfa.7559

Bernardi, D. M., Paris, L. D. d., Dietrich, F., Siva, F. G. A. D. e., Boscolo, W. R. A., Sary, C., Signor, A., Bertol, T. M., Sgarbieri, V. C., 12 2016b. Production of hydrolysate from processed Nile tilapia (Oreochromis niloticus) residues and assessment of its antioxidant activity. Food Science and Technology 36, 709 – 716.

Bimal Mohanty, Arabinda Mahanty, S. G. e. a., 2014. Amino acid compositions of 27 food fishes and their importance in clinical nutrition. Journal of Amino Acids 2014, 7.

Blackburn, S., 1978. Sample preparation and hydrolytic methods. Amino acid determination methods and techniques, 7–37.

Büchi Switzerland, 07 2013. Application note no. 114/2013: Nitrogen and protein determination in meat products.

Cell Biolabs, Inc., 2018. OxiSelect™ Protein Carbonyl ELISA Kit.

Cell Biolabs, Inc., 2019. Protein Oxidation / Nitration.
URL https://www.cellbiolabs.com/protein-oxidation?gclid=Cj0KCQjwn8_mBRCLARIsAKxi0GInIBOkBiY0AGCMNRPvFZ0SDwF_Bc26oCiW6QbhsSidvtpHTQLYd6kaAl0IEALw_wcB

Chalamaiah, D. M., Dinesh Kumar, B., Hemalatha, R., Tummala, J., 12 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Vol. 135.

Chen, X., Xie, N., Fang, X., Yu, H., Ya-mei, J., Zhen-da, L., 2010. Antioxidant activity and molecular weight distribution of in vitro gastrointestinal digestive hydrolysate from flying squid (ommastrephes batramii) skin-gelatin. Food Sci 31, 123–130.

Cheung, R. C. F., Ng, T. B., Wong, J. H., 06 2015. Marine peptides: Bioactivities and applications. Marine drugs 13 (7), 4006–4043.
URL https://www.ncbi.nlm.nih.gov/pubmed/26132844

Cold Spring Harbor Laboratory Press, 2006. Sodium phosphate.
URL http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8303.full

Cundy, T., Reid, I. R., Grey, A., Marshall, W. J., Lapsley, M., Day, A. P., Ayling, R. M., 2014. CHAPTER 31 - Metabolic bone disease. Churchill Livingstone, pp. 604–635.
URL http://www.sciencedirect.com/science/article/pii/B9780702051401000316

Damodaran, S., Parkin, K. L., 2017. Fennema's food chemistry.

Dauksas, E., Šližytė, R., Rustad, T., Storro, I., 07 2004. Bitterness in fish protein hydrolysates and methods for removal. Journal of Aquatic Food Product Technology 13 (2), 101–114.
URL https://doi.org/10.1300/J030v13n02_09

Diniz, F., Martin, A., 1997. Fish protein hydrolysates by enzymatic processing. Agro Food Industry Hi-Tech (8), 9–13.

Dong, F. M., Fairgrieve, W. T., Skonberg, D. I., Rasco, B. A., 1993. Preparation and nutrient analyses of lactic acid bacterial ensiled salmon viscera. Aquaculture 109 (3), 351–366.

Drotningsvik, A., Vikøren, L. A., Mjøs, S. A., Oterhals, Å., Pampanin, D., Flesland, O., Gudbrandsen, O. A., 2018. Water-soluble fish protein intake led to lower serum and liver cholesterol concentrations in obese zucker fa/fa rats. Marine Drugs 16 (5).
URL http://www.mdpi.com/1660-3397/16/5/149

Ellman, G. L., 1959. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82 (1), 70 – 77.
URL http://www.sciencedirect.com/science/article/pii/0003986159900906

Erdmann, K., Cheung, B. W., Schröder, H., 2008. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. The Journal of Nutritional Biochemistry 19 (10), 643 – 654.
URL http://www.sciencedirect.com/science/article/pii/S0955286307002756

Estévez, M., 2011. Protein carbonyls in meat systems: A review. Meat Science 89 (3), 259 – 279, 57th International Congress of Meat Science and Technology (57th ICoMST), 7-12 August 2011, Ghent, Belgium.
URL http://www.sciencedirect.com/science/article/pii/S0309174011001616

FAO, 2018. FAO yearbook. Fishery and Aquaculture Statistics 2016/FAO annuaire. FAO, Rome.

FAO, WHO, U., 1985. Energy and protein requirements: report of a joint fao/who/unu expert consultation (1981: Rome, italy.

FAO, Fisheries and Aquaculture Department, 2014. World fish trade to set new records.
URL http://www.fao.org/news/story/en/item/214442/icode/

Fernàndez-Musoles, R., Manzanares, P., Burguete, M. C., Alborch, E., Salom, J. B., 2013. In vivo angiotensin i-converting enzyme inhibition by long-term intake of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats. Food Research International 54 (1), 627 – 632.
URL http://www.sciencedirect.com/science/article/pii/S0963996913004298

García-Tejedor, A., Sánchez-Rivera, L., Castelló-Ruiz, M., Recio, I., Salom, J. B., Manzanares, P., 02 2014. Novel antihypertensive lactoferrin-derived peptides produced by kluyveromyces marxianus: Gastrointestinal stability profile and in vivo angiotensin i-converting enzyme (ace) inhibition. Journal of Agricultural and Food Chemistry 62 (7), 1609–1616.
URL https://doi.org/10.1021/jf4053868

Gbogouri, G., Linder, M., Fanni, J., Parmentier, M., 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. Journal of Food Science 69 (8), C615–C622.

GE Healthcare, 09 2010. Gel filtration, principles and methods.

Guerard, F., Shahidi, F., 2007. 6 - Enzymatic methods for marine by-products recovery. Woodhead Publishing, pp. 107–143.

Hayes, M., 2013. Biological activities of proteins and marine-derived peptides from byproducts and seaweeds. In: Marine Proteins and Peptides: Biological Activities and Applications. John Wiley and Sons, pp. 139–165.

Hoyle, N. T., Merritt, J. H., 1994. Quality of fish protein hydrolysates from herring (clupea harengus). Journal of Food Science 59 (1), 76–79.
URL https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2621.1994.tb06901.x

Hussain, B., Mahboob, S., Hassan, M., Liaqat, F., Sultana, T., Tariq, H., 01 2011. Comparitive analysis of proximate composition of head from wild and farmed catla catla. The Journal of Animal & Plant Sciences 21, 207–210.

Jenkelunas, P. J., Li-Chan, E. C. Y., 2018. Production and assessment of pacific hake (merluccius productus) hydrolysates as cryoprotectants for frozen fish mince. Food Chemistry 239, 535–543.

Jeon, Y., Byun, H.-G., Kim, S., 01 2000. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Vol. 35.

Jun, S.-Y., Park, P.-J., Jung, W.-K., Kim, S.-K., Jun 2004. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (limanda aspera) frame protein. European Food Research and Technology 219 (1), 20–26.
URL https://doi.org/10.1007/s00217-004-0882-9

Kim, J. S., Park, J. W., Shahidi, F., 2007. 9 - Mince from seafood processing by-product and surimi as food ingredients in Maximizing the Value of Marine By-products. Woodhead Publishing, pp. 196–228.

Kim, S.-K., Mendis, E., 2006. Bioactive compounds from marine processing byproducts –a review. Food Research International 39 (4), 383–393.

Klomklao, S., Benjakul, S., Kishimura, H., 2013. Functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with viscera extract from hybrid catfish. International Journal of Food Science & Technology 48 (7), 1483–1489.

Klompong, V., Benjakul, S., Kantachote, D., Shahidi, F., 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. Food Chemistry 102 (4), 1317–1327.

Kousoulaki, K., Albrektsen, S., Langmyhr, E., Olsen, H. J., Campbell, P., Aksnes, A., 2009. The water soluble fraction in fish meal (stickwater) stimulates growth in atlantic salmon (salmo salar l.) given high plant protein diets. Aquaculture 289 (1), 74 – 83.
URL http://www.sciencedirect.com/science/article/pii/S0044848609000556

Kousoulaki, K., Olsen, H. J., Albrektsen, S., Langmyhr, E., Mjøs, S. A., Campbell, P., Aksnes, A., 2012. High growth rates in atlantic salmon (salmo salar l.) fed 7.5% fish meal in the diet. micro-, ultra- and nano-filtration of stickwater and effects of different fractions and compounds on pellet quality and fish performance. Aquaculture 338-341, 134 – 146.
URL http://www.sciencedirect.com/science/article/pii/S0044848612000403

Kristinsson, H. G., 2007. 10 - Aquatic food protein hydrolysates in Maximising the Value of Marine By-Products. Woodhead Publishing, pp. 229–248.

Kristinsson, H. G., Rasco, B. A., 03 2000a. Biochemical and functional properties of atlantic salmon (salmo salar) muscle proteins hydrolyzed with various alkaline proteases. Journal of Agricultural and Food Chemistry 48 (3), 657–666.
URL https://doi.org/10.1021/jf990447v

Kristinsson, H. G., Rasco, B. A., 2000b. Fish protein hydrolysates: Production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition 40 (1), 43–81, pMID: 10674201.

Laidmäe, I., McCormick, M. E., Herod, J. L., Pastore, J. J., Salum, T., Sawyer, E. S., Janmey, P. A., Uibo, R., 2006. Stability, sterility, coagulation, and immunologic studies of salmon coagulation proteins with potential use for mammalian wound healing and cell engineering. Biomaterials 27 (34), 5771–5779.

Laidmäe, I., Salum, T., Sawyer, E. S., Janmey, P. A., Uibo, R., 04 2010. Characterization of the biological effect of fish fibrin glue in experiments on rats: immunological and coagulation studies. Journal of biomedical materials research. Part A 93 (1), 29–36.

Leach, A., 1960. Notes on a modification of the neuman & logan method for the determination of the hydroxyproline. Biochemical Journal 74 (1), 70.

Lee, C. M., 2007. 14 - Seafood flavor from processing by-products in Maximising the Value of Marine By-Products. Woodhead Publishing, pp. 304–327.

Lee, J. K., Jeon, J.-K., Kim, S.-K., Byun, H.-G., 2012. Chapter 4 - characterization of bioactive peptides obtained from marine invertebrates. In: Kim, S.-K. (Ed.), Marine Medicinal Foods. Vol. 65 of Advances in Food and Nutrition Research. Academic Press, pp. 47 – 72.
URL http://www.sciencedirect.com/science/article/pii/B9780124160033000044

Lee, J. K., Kang, S. I., Kim, Y. J., Kim, M. J., Heu, M. S., Choi, B. D., Kim, J.-S., Feb 2016. Comparison of collagen characteristics of sea- and freshwater-rainbow trout skin. Food Science and Biotechnology 25 (1), 131–136.
URL https://doi.org/10.1007/s10068-016-0020-z

Liaset, B., Julshamn, K., Espe, M., 07 2003. Chemical composition and theorical nutrition evaluation of produced fraction from enzymic hydrolysis of salmon frames with Protamex™. Vol. 38.

Lowry, O., Rosebrough, N., Farr, A., Randall, R., 01 2017. Protein measurement with the folin phenol reagent. J. biol. Chemistry 193, 256–275.

Lund, M. N., Heinonen, M., Baron, C. P., EstÃl'vez, M., 2011. Protein oxidation in muscle foods: A review. Molecular Nutrition & Food Research 55 (1), 83–95.

Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., Huang, L., 01 2008. Bitter peptides activate htas2rs, the human bitter receptors. Biochemical and biophysical research communications 365 (4), 851–855.
URL https://www.ncbi.nlm.nih.gov/pubmed/18037373

Mæhre, H. K., Dalheim, L., Edvinsen, G. K., Elvevoll, E. O., Jensen, I.-J., 2018. Protein determination—method matters. Foods 7 (1).
URL http://www.mdpi.com/2304-8158/7/1/5

Nikoo, M., Benjakul, S., Rahmanifarah, K., 2016. Hydrolysates from marine sources as cryoprotective substances in seafoods and seafood products. Trends in Food Science & Technology 57, 40–51.

Nurdiani, R., Dissanayake, M., Street, W. E., Donkor, O. N., Singh, T. K., Vasiljevic, T., 2019/05/10 2015. Sustainable use of marine resources –turning waste into food ingredients. International Journal of Food Science & Technology 50 (11), 2329–2339.

Osnes, K. K., Mohr, V., 1985. Peptide hydrolases of antartic krill, euphausia superba. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 82 (4), 599–606.

Ovissipour, M., Safari, R., Motamedzadegan, A., Shabanpour, B., 02 2012. Chemical and Biochemical Hydrolysis of Persian Sturgeon (Acipenser persicus) Visceral Protein. Vol. 5.

Parvathy U., Nizam K. M., Z. A. A. N. G. P. S. K. R. C. N., 2018. Characterization of fish protein hydrolysate from red meat of euthynnus affinis and its application as an antioxidant in iced sardine. Journal of Scientific and Industrial Research 77, 111–119.

Pedersen, B., 10 1994. Removing bitterness from protein hydrolysates. Vol. 48.
URL https://www.researchgate.net/publication/292268029_Removing_bitterness_from_protein_hydrolysates

Petrova, I., Tolstorebrov, I., Eikevik, T. M., Sep 2018. Production of fish protein hydrolysates step by step: technological aspects, equipment used, major energy costs and methods of their minimizing. International Aquatic Research 10 (3), 223–241.
URL https://doi.org/10.1007/s40071-018-0207-4

Rajakumar, K., 2003. Vitamin d, cod-liver oil, sunlight, and rickets: A historical perspective. Pediatrics 112 (2), e132.

Rajapakse, N., Mendis, E., Byun, H.-G., Kim, S.-K., 2005. Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. The Journal of Nutritional Biochemistry 16 (9), 562–569.

Ramis-Ramos, G., Caballero, B., 2003. ANTIOXIDANTS | Synthetic Antioxidants. Academic Press, pp. 265–275.
URL http://www.sciencedirect.com/science/article/pii/B012227055X000547

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., Xue, S. J., 2008. Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. Food Chemistry 108 (2), 727–736.
URL http://www.sciencedirect.com/science/article/pii/S0308814607011521

Richardsen, R., Nystøyl, R., Strandheim, G., Marthinussen, A., 2017. Analyse marint restrÃěstoff, 2016 - tilgang og anvendelse av marint restrÃěstoff i norge.

Ruiz-Ruiz, J., Dàvila-Ort´iz, G., Chel-Guerrero, L., Betancur-Ancona, D., 2013. Angiotensin i-converting enzyme inhibitory and antioxidant peptide fractions from hard-to-cook bean enzymatic hydrolysates. Journal of Food Biochemistry 37 (1), 26–35.
URL https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1745-4514.2011.00594.x

Rustad, T., Storrø, I., Šližytė, R., 2019/05/10 2011. Possibilities for the utilisation of marine by-products. International Journal of Food Science & Technology 46 (10), 2001–2014.

Rutherfurd, S. M., 2010. Methodology for determining degree of hydrolysis of proteins in hydrolysates: a review. Journal of AOAC International 93 (5).

Ryu, B., Kim, S. K., 2013. Potential beneficial effects of marine peptide on human neuron health. Current Protein & Peptide Science 14 (3), 173–176.

Sabetian, M., Delshad, S., Moini, S., Rajabi Islami, H., Motallebi, A., 01 2012. Identification of Fatty Acid Content, Amino Acid Profile and Proximate Composition in Rainbow Trout (Oncorhynchus mykiss). Vol. 8.

Sáez-Plaza, P., Michałowski, T., Navas, M. J., Asuero, A. G., Wybraniec, S., 2013. An overview of the kjeldahl method of nitrogen determination. part i. early history, chemistry of the procedure, and titrimetric finish. Critical Reviews in Analytical Chemistry 43 (4), 178–223.
URL https://doi.org/10.1080/10408347.2012.751786

Samaranayaka, A. G. P., Li-Chan, E. C. Y., 2011. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. Journal of Functional Foods 3 (4), 229–254.

Sathivel, S., Smiley, S., Prinyawiwatkul, W., Bechtel, P. J., 2005. Functional and nutritional properties of red salmon (oncorhynchus nerka) enzymatic hydrolysates. Journal of Food Science 70 (6), c401–c406.

Sato, K., Ohashi, C., Ohtsuki, K., Kawabata, M., 07 1991. Type v collagen in trout (salmo gairdneri) muscle and its solubility change during chilled storage of muscle. Journal of Agricultural and Food Chemistry 39 (7), 1222–1225.
URL https://doi.org/10.1021/jf00007a005

Shahidi, F., Han, X.-Q., Synowiecki, J., 1995. Production and characteristics of protein hydrolysates from capelin (mallotus villosus). Food Chemistry 53 (3), 285 – 293.
URL http://www.sciencedirect.com/science/article/pii/030881469593934J

Šližytė, R., Daukšas, E., Falch, E., Storrø, I., Rustad, T., 2005. Characteristics of protein fractions generated from hydrolysed cod (gadus morhua) by-products. Process Biochemistry 40 (6), 2021–2033.

Šližytė, R., Grimsmo, L., Storrø, I., 2010. Prosessering av biråstoff fra sild til olje og proteinhydrolysat. laboratorieforsøk med ulike proteaser og pilotforsøk med ultraferskt råstoff.

Šližytė, R., Rommi, K., Mozuraityte, R., Eck, P., Five, K., Rustad, T., 08 2016. Bioactivities of fish protein hydrolysates from defatted salmon backbones. Biotechnology reports (Amsterdam, Netherlands) 11, 99–109.

Suarez-Jimenez, G.-M., Burgos-Hernandez, A., Ezquerra-Brauer, J.-M., 04 2012. Bioactive peptides and depsipeptides with anticancer potential: sources from marine animals. Marine drugs 10 (5), 963–986.

Taylor, W. H., 1957. Formol titration: an evaluation of its various modifications. Analyst 82, 488–498.
URL http://dx.doi.org/10.1039/AN9578200488

Thiansilakul, Y., Benjakul, S., Shahidi, F., 12 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (Decapterus maruadsi). Vol. 103.

Toppe, J.and Olsen, R. P. O., James, D., 2018. Production and utilization of fish silage. a manual on how to turn fish waste into profit and a valuable feed ingredient or fertilizer. FAO, Rome, 28 pp.

Tylingo, R., Mania, S., Panek, A., Piątek, R., Pawłowicz, R., 2016. Isolation and characterization of acid soluble collagen from the skin of african catfish (*Clarias gariepinus*), salmon (*Salmo salar*) and baltic cod (*Gadus morhua*). Journal of Biotechnology and Biomaterials 6 (2), 1–6.

Venugopal, V., 2016. Chapter three - enzymes from seafood processing waste and their applications in seafood processing. In: Kim, S.-K., Toldrá, F. (Eds.), Marine Enzymes Biotechnology: Production and Industrial Applications, Part I - Production of Enzymes. Vol. 78 of Advances in Food and Nutrition Research. Academic Press, pp. 47 – 69.

Von Wachenfeldt, C., 2018. Protein thiol oxidative stress.
URL https://www.biology.lu.se/research/research-groups/microbiology-group/research-projects/bacterial-stress-responses/protein-thiol-oxidative-stress

Wang, L. Z., Gorlin, J., Michaud, S. E., Janmey, P. A., Goddeau, R. P., Kuuse, R., Uibo, R., Adams, D., Sawyer, E. S., 2000. Purification of salmon clotting factors and their use as tissue sealants. Thrombosis Research 100 (6), 537–548.

Wattanasiritham, L., Theerakulkait, C., Wickramasekara, S., Maier, C. S., Stevens, J. F., 2016. Isolation and identification of antioxidant peptides from enzymatically hydrolyzed rice bran protein. Food Chemistry 192, 156–162.
URL http://www.sciencedirect.com/science/article/pii/S0308814615009498

Wilson, R., Cowey, C., 1985. Amino acid composition of whole body tissue of rainbow trout and atlantic salmon. Aquaculture 48 (3), 373 – 376.
URL http://www.sciencedirect.com/science/article/pii/0044848685901401

Xiong, Y., Zhang, Q., Sun, X., Tan, W., Xin, X., Ji, W., Jan 2000. Nonlinear optical response of inorganic metal cluster mocu3se4(pph3)3cl solution. Applied Physics A 70 (1), 85–88.
URL https://doi.org/10.1007/s003390050016

Yang, R., Zhang, Z., Pei, X., Han, X., Wang, J., Wang, L., Long, Z., Shen, X., li, Y., 03 2009. Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (Oncorhynchus keta) in mice. Vol. 113.

APPENDIX A

Sample Characteristics

Physical characteristics of the samples are described below. A.1 shows the rest raw material samples RRM1, RRM3 and RRM4. A.2 shows the four hydrolysates H1, H2, H3 and H4.

Rest raw material samples:

- **RRM1**: crude raw material; fresh red colour of the meat; fishy odour; evenly minced but with visible bony parts
- **RRM3**: oxidised raw material; visible difference to RRM1; grey colour; fishy and rotted odour; evenly minced but with visible bony parts
- **RRM4**: oxidised raw material with added antioxidant; visible difference to the other samples; brown, slightly grey colour; fishy and rotted odour; evenly minced but with visible bony parts

Hydrolysate samples:

- **H1**: hydrolysate from crude raw material; light, yellow colour; slightly fishy odour; fine and even powder; dissolves good in water
- **H2**: hydrolysate with added antioxidant in the hydrolysis tank; very light in colour and lightest of all samples; slightly fishy odour; fine and even powder; dissolves good in water
- **H3**: hydrolyste from oxidised raw material; light brown colour; slightly fishy odour; coarse and also fine parts in the powder; difficult to dissolve in water
- **H4**: hydrolysate from oxidised raw material with added antioxidant; light sandy brown colour; slightly fishy odour; some coarse parts and also even parts in the powder

Figure A.1: Rest raw material, from the left to the right sample RRM1, RRM3 and RRM4.

Figure A.2: Hydrolysates, from the left to the right sample H1, H2, H3 and H4.

APPENDIX B

Calculations

Calculations for Degree of Hydrolysis

The calculation of degree of hydrolysis was done according to Taylor (1957). With the results received from formoltitration, equation (B.1) can be calculated. In order to get the DH (%), the received total nitrogen content from Kjeldahl analysis was used (B.2).

$$\frac{\mathrm{A} \cdot 14.007 \cdot 100}{\mathrm{C} \cdot 1000} = \% \text{ amino groups (D)} \tag{B.1}$$

A = ml NaOH used
B = the concentration of the solution used for titration (0.1 M NaOH)
C = amount of sample (g)

$$\frac{\mathrm{D} \cdot 100}{\mathrm{E}} = \text{degree of hydrolysis (\%)} \tag{B.2}$$

D = % free amino groups
E = % nitrogen

Calculations for total protein content

The results are calculated as a percentage of nitrogen. In order to calculate the protein content of the sample, the nitrogen content is multiplied with a sample-specific protein factor, which is 6.25 for meat products (Büchi Switzerland, 2013). The calculations can be seen in (B.3) - (B.6).

$$\mathrm{w_N} = \frac{(\mathrm{V_{Sample}} - \mathrm{V_{Blank}}) \cdot \mathrm{z} \cdot \mathrm{c} \cdot \mathrm{f} \cdot \mathrm{M_N}}{\mathrm{m_{Sample}} \cdot 1000} \tag{B.3}$$

$$\%\mathrm{N} = \mathrm{w_N} \cdot 100\,\% \tag{B.4}$$

$$\%\mathrm{P} = \mathrm{w_N} \cdot \mathrm{PF} \cdot 100\,\% \tag{B.5}$$

$$\%\mathrm{N_{Gly}} = \frac{\%\mathrm{N} \cdot 100}{\mathrm{P}} \tag{B.6}$$

w_N = weight fraction in nitrogen
V_{Sample} = amount of titrant for the sample [ml]
V_{Blank} = mean amount of titrant for the blank [ml]
z = molar valence factor
c = titrant concentration [mol/l]
f = titrant factor (for commercial solutions normally 1000)
M_N = molecular weight of nitrogen (14.007 g/mol)
m_{Sample} = sample weight [g]
1000 = conversion [ml/l
%N = percentage of weight of nitrogen
$\%N_{Gly}$ = percentage of weight of nitrogen corrected for the purity of reference substance glycine [%]
%P = percentage of weight of protein
P = purity of the reference substance glycine [%]
PF = sample-specific protein factor (6.25 for meat products)

APPENDIX C

Chromatograms of standard compounds

Table C.1: Molecular weight [kDa] and peaks at elution volume [ml] of known standards used in FPLC.

Standard	Molecular weight [kDa]	peak [ml]
Cytochrome C	12.4	14.6
Aprotinin	6.5	18.0
Vitamin B12	1.3	18.5

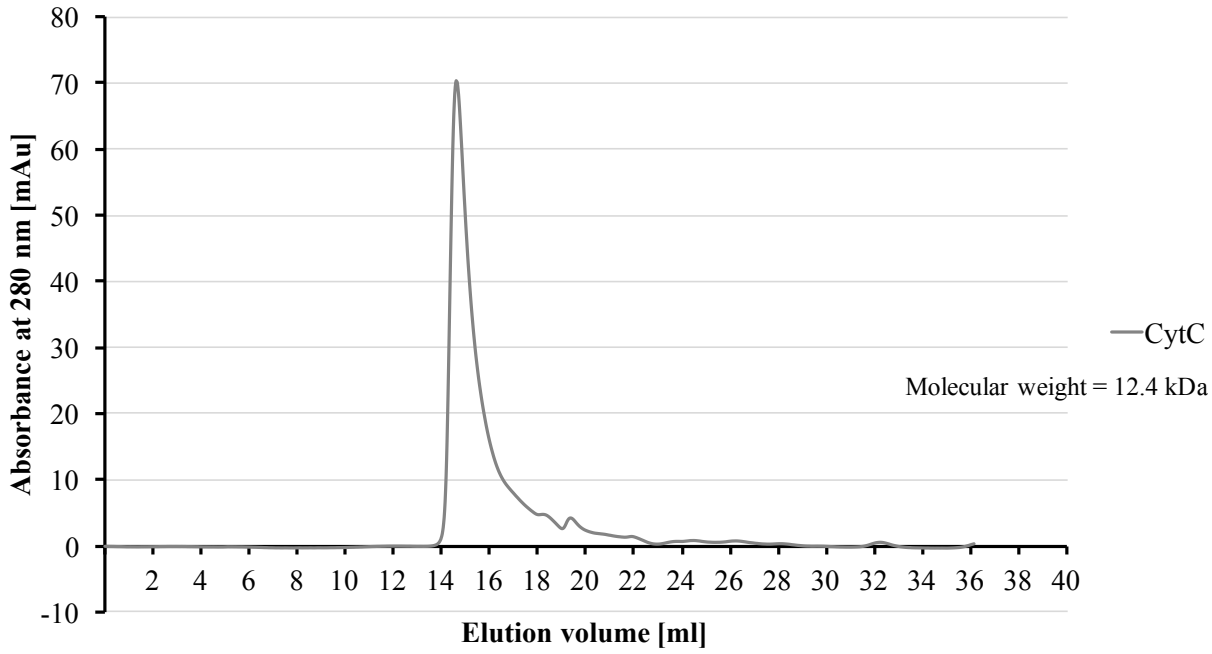


Figure C.1: Gel filtration chromatogram showing Cytochrome C, used as a standard in FPLC. The elution volume [ml] is plotted against the absorbance [mAu]. Molecular weight = 12.4 kDa

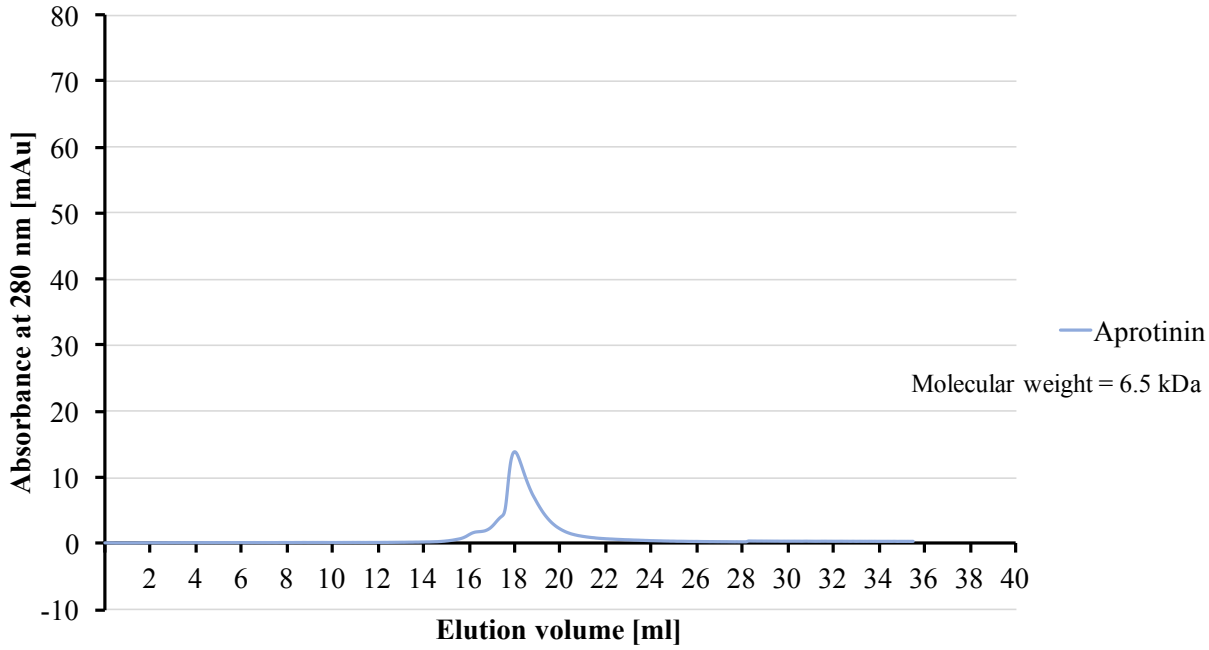


Figure C.2: Gel filtration chromatogram showing Aprotinin, used as a standard in FPLC. The elution volume [ml] is plotted against the absorbance [mAu]. Molecular weight = 6.5 kDa

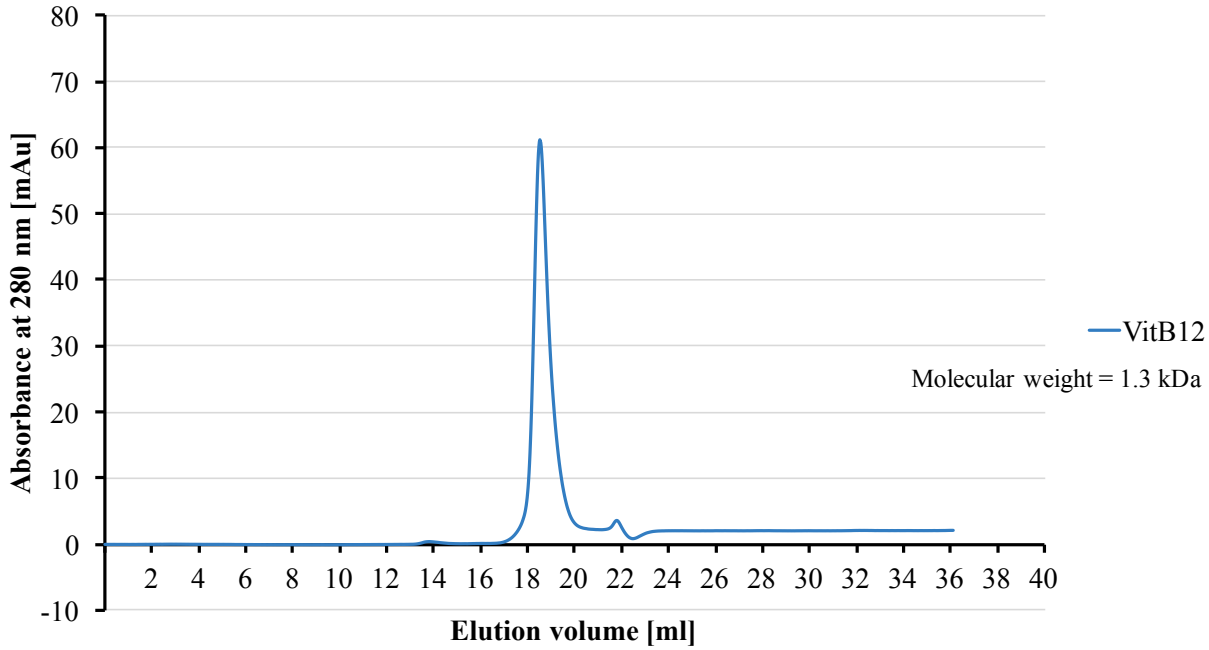


Figure C.3: Gel filtration chromatogram showing Vitamin B12, used as a standard in FPLC. The elution volume [ml] is plotted against the absorbance [mAu]. Molecular weight = 1.3 kDa

APPENDIX D

HPLC - TAA and FAA

Following tables show the total amino acid (TAA) as well as the free amino acid (FAA) compositions in percentages for the raw material and hydrolysate samples.

Total amino acid compositions

Amino acid	H1/1	H1/2	H1/3	AV	STD
Asp	10,114416	10,0487951	9,90326322	10,0221581	0,10806722
Glu	14,0771513	14,1773876	13,7544988	14,0030126	0,22097766
His	2,54385798	2,71878711	2,40125879	2,55463463	0,15903824
Ser	6,15304774	6,25271612	6,30301282	6,23625889	0,07632504
Gln	0,88221299	0,67016166	1,04766906	0,86668123	0,18923236
Gly/Arg	20,2496672	20,2762084	20,560928	20,3622679	0,17255574
Thr	5,13242394	5,06867489	5,09612114	5,09907332	0,0319769
Tyr	1,98749985	2,10544327	2,05341124	2,04878479	0,05910766
Ala	9,68652388	9,53694233	9,71291027	9,64545883	0,09489959
Met	3,01921294	3,24855752	3,26327378	3,17701475	0,13685832
Val	4,20461551	4,16048498	4,1181771	4,16109253	0,04322241
Phe	3,55540751	3,53768844	3,4795464	3,52421412	0,03968495
Ile	3,21770781	3,18717807	3,16690274	3,19059621	0,02557443
Leu	7,05895927	7,01058159	7,0041068	7,02454922	0,02997531
Lys	8,1116192	7,99486112	8,12924382	8,07857472	0,07303171

Figure D.1: Total amino acid composition [wt.%] of sample H1, including average and standard deviation.

Amino acid	H2/1	H2/2	H2/3	AV	STD
Asp	9,96013522	10,1678141	9,8954542	10,0078012	0,14229901
Glu	14,2722707	14,383185	13,9201403	14,1918653	0,24176719
His	2,68015504	2,75828079	2,65722956	2,69855513	0,05297887
Ser	6,33137262	6,6825059	6,68402811	6,56596888	0,20316774
Gln	0,64718756	0,53441753	0,78062841	0,65407783	0,12324997
Gly/Arg	19,9161233	20,7409382	19,8439648	20,1670088	0,49834524
Thr	4,92261339	5,24922562	5,07045889	5,08076597	0,16354989
Tyr	1,99009072	2,18956273	2,08861838	2,08942394	0,09973844
Ala	9,38127096	9,81403274	9,32360788	9,50630386	0,26805607
Met	3,0748335	3,3528355	3,23822835	3,22196578	0,13971267
Val	4,27407742	4,44194372	4,2341284	4,31671651	0,11027407
Phe	3,58758169	3,71230864	3,55345567	3,617782	0,08362183
Ile	3,29351032	3,43319511	3,19470446	3,30713663	0,11982781
Leu	7,24195132	7,55151325	7,02614034	7,27320164	0,2640769
Lys	8,40600773	8,8975415	8,48022069	8,59458997	0,26497474

Figure D.2: Total amino acid composition [wt.%] of sample H2, including average and standard deviation.

Amino acid	H3/1	H3/2	H3/3	AV	STD
Asp	9,88161017	10,2688448	9,97413834	10,0415311	0,20222268
Glu	14,0625914	14,3445558	14,1820317	14,1963929	0,14152974
His	0,78574376	2,17898517	2,21333483	1,72602125	0,8144853
Ser	5,73159937	6,13284331	5,99378993	5,9527442	0,20374675
Gln	0,31024407	0,50923569	0,62280313	0,48076096	0,15821315
Gly/Arg	19,3564181	20,0252093	19,66169	19,6811058	0,33481806
Thr	5,07766539	5,18927116	5,2107986	5,15924505	0,07146529
Tyr	0,18337463	0,15522952	1,78942393	0,70934269	0,93548364
Ala	9,82584646	10,0725018	9,72893367	9,87576064	0,17713931
Met	3,00390409	3,19161107	3,25963785	3,15171767	0,13245207
Val	4,52921575	4,71915694	4,5972222	4,6151983	0,09623809
Phe	8,8392161	4,00803639	3,90788999	5,58504749	2,81863749
Ile	3,46867179	3,71639615	3,62426277	3,60311024	0,12520947
Leu	7,52773617	7,85739802	7,72463309	7,70325576	0,16586735
Lys	7,36632342	7,61889284	7,50364083	7,4962857	0,12644525

Figure D.3: Total amino acid composition [wt.%] of sample H3, including average and standard deviation.

Amino acid	H4/1	H4/2	H4/3	AV	STD
Asp	10,4387224	10,30	9,94383397	10,2287369	0,25580874
Glu	14,9706442	14,86	14,0473688	14,6272632	0,50503789
His	0,97933642	2,12	2,04726192	1,71627366	0,63930599
Ser	6,16958875	5,87	5,88229685	5,97503096	0,16855327
Gln	0,406937	0,75	0,9023589	0,68562928	0,25345659
Gly/Arg	21,1150518	20,28	20,8013994	20,7337816	0,41918951
Thr	5,28178251	5,13	5,41167364	5,27321691	0,14293216
Tyr	0,14102828	1,68	1,93124952	1,24919149	0,96819277
Ala	10,51316	9,97	9,95200659	10,14537	0,31865621
Met	3,27965322	2,84	3,18245318	3,10040273	0,23145324
Val	4,52155014	4,40	4,46120199	4,46205532	0,05907278
Phe	3,47343961	3,64	3,63436449	3,58365764	0,09555308
Ile	3,57812856	3,43	3,37316163	3,46025902	0,10589171
Leu	7,46356426	7,41294229	7,29227709	7,38959455	0,08799807
Lys	7,63681797	7,28662485	7,13380636	7,3524164	0,25787898

Figure D.4: Total amino acid composition [wt.%] of sample H4, including average and standard deviation.

Amino acid	RRM1/1	RRM1/2	AV	STD
Asp	11,4305745	11,41	11,420875	0,01371716
Glu	14,1855239	14,38	14,2830049	0,137859
His	3,32119065	3,12	3,2200824	0,14298866
Ser	6,82393941	6,09	6,45470231	0,52218012
Gln	0,29073154	0,25	0,26952034	0,02999717
Gly/Arg	14,9194852	14,83	14,8765075	0,06077959
Thr	5,35585577	5,36	5,35958519	0,00527419
Tyr	3,25152274	3,32	3,28780012	0,05130396
Ala	7,81543915	7,73	7,77191249	0,061556
Met	2,41464126	3,33	2,87162141	0,64626753
Val	5,11357666	4,99	5,05224429	0,08673707
Phe	4,8803763	4,54	4,71023651	0,24061399
Ile	4,15357071	4,12	4,13678151	0,02374351
Leu	7,30557977	7,41764211	7,36161094	0,07924004
Lys	8,64446845	9,07191151	8,85818998	0,30224789

Figure D.5: Total amino acid composition [wt.%] of sample RRM1, including average and standard deviation.

Amino acid	RRM3/1	RRM3/2	AV	STD
Asp	11,0376442	11,1023281	11,0699861	0,04573846
Glu	14,0252977	13,7805176	13,9029077	0,17308569
His	2,75112318	2,59764579	2,67438449	0,1085249
Ser	6,15024435	6,13930829	6,14477632	0,00773296
Gln	0,43312131	0,6539669	0,5435441	0,15616141
Gly/Arg	16,0960262	18,6645469	17,3802866	1,81621838
Thr	5,31884099	4,9288509	5,12384594	0,27576464
Tyr	2,49095723	2,03971417	2,2653357	0,31907703
Ala	8,74159047	9,1288138	8,93520213	0,27380824
Met	3,28560552	3,22432839	3,25496695	0,04332947
Val	5,28697524	4,85974457	5,0733599	0,3020977
Phe	4,76601112	4,59017761	4,67809436	0,12433307
Ile	4,12414163	3,73286531	3,92850347	0,27667414
Leu	7,18091378	6,64949123	6,91520251	0,37577249
Lys	8,25236538	7,85196405	8,05216472	0,2831265

Figure D.6: Total amino acid composition [wt.%] of sample RRM3, including average and standard deviation.

Amino acid	RRM4/1	RRM4/2	AV	STD
Asp	10,8546339	11,2173173	11,0359756	0,25645589
Glu	13,8142943	14,0590453	13,9366698	0,17306506
His	2,60043691	2,83295187	2,71669439	0,1644129
Ser	5,80418444	5,9302458	5,86721512	0,08913885
Gln	0,40991719	0,4559821	0,43294964	0,03257281
Gly/Arg	16,984469	16,1324541	16,5584616	0,60246551
Thr	4,76045048	5,10671968	4,93358508	0,2448493
Tyr	2,722304	3,01722867	2,86976633	0,20854323
Ala	8,61377252	8,49138103	8,55257678	0,08654385
Met	3,42410905	3,51274423	3,46842664	0,06267454
Val	5,16848398	5,14872941	5,15860669	0,01396859
Phe	4,37796973	4,74607025	4,56201999	0,26028637
Ile	4,00817153	4,07713999	4,04265576	0,04876806
Leu	8,35656131	7,03938496	7,69797313	0,93138433
Lys	8,08895986	8,19341776	8,14118881	0,07386289

Figure D.7: Total amino acid composition [wt.%] of sample RRM4, including average and standard deviation.

Free amino acid composition

Amino acid	H1/1	H1/2	AV	STD
Asp	1,66995694	1,93969151	1,80482422	0,19073115
Glu	8,52919109	8,67499332	8,6020922	0,10309774
Asn	1,19819248	1,21709679	1,20764464	0,01336737
His	5,0790176	4,83686164	4,95793962	0,17123012
Ser	5,61455455	5,8255498	5,72005217	0,14919617
Gln	8,29364566	8,15064214	8,2221439	0,10111876
Gly/Arg	10,7463156	10,5205292	10,6334224	0,1596551
Thr	5,00550463	4,86713171	4,93631817	0,09784443
Tyr	4,76388101	4,79111576	4,77749839	0,01925787
Ala	11,5223618	11,4428007	11,4825812	0,0562582
Met	4,28898187	5,30905719	4,79901953	0,72130218
Val	6,56939805	5,95965821	6,26452813	0,43115118
Phe	4,46055291	4,31337573	4,38696432	0,10406999
Ile	3,5007006	3,43480356	3,46775208	0,04659625
Leu	9,47653148	9,13801772	9,3072746	0,23936537
Lys	9,28121374	9,57867506	9,4299444	0,21033692

Figure D.8: Free amino acid composition [wt.%] of sample H1, including average and standard deviation.

Amino acid	H2/1	H2/2	AV	STD
Asp	2,04515102	1,82820842	1,93667972	0,15340158
Glu	8,26009656	7,74321645	8,00165651	0,36548943
Asn	1,19580348	1,1806178	1,18821064	0,0107379
His	5,9914036	5,36048934	5,67594647	0,44612375
Ser	6,80465417	6,26986056	6,53725737	0,37815619
Gln	7,23392499	7,28810429	7,26101464	0,03831055
Gly/Arg	10,2158478	10,6541466	10,4349972	0,30992405
Thr	5,23695088	5,13238026	5,18466557	0,07394259
Tyr	5,19941732	4,94535949	5,0723884	0,17964601
Ala	10,3771578	10,7796975	10,5784277	0,28463855
Met	5,53521905	5,23589658	5,38555782	0,21165295
Val	6,06227368	6,33843317	6,20035343	0,19527425
Phe	4,68204083	4,54001875	4,61102979	0,10042477
Ile	3,55351953	3,50813301	3,53082627	0,03209312
Leu	8,26904348	9,094351	8,68169724	0,58358054
Lys	9,33749581	10,1010868	9,71929129	0,53994035

Figure D.9: Free amino acid composition [wt.%] of sample H2, including average and standard deviation.

Amino acid	H3/1	H3/2	AV	STD
Asp	0,88006969	0,86505365	0,87256167	0,01061794
Glu	8,39859265	8,41213147	8,40536206	0,00957339
Asn	1,03377976	1,03829856	1,03603916	0,00319527
His	1,53134572	1,4788494	1,50509756	0,03712051
Ser	3,31172576	2,90424776	3,10798676	0,28813046
Gln	8,37840866	8,92108154	8,6497451	0,38372767
Gly/Arg	12,8794206	12,676046	12,7777333	0,14380758
Thr	7,02719572	7,11106114	7,06912843	0,05930181
Tyr	4,34012126	4,37433328	4,35722727	0,02419155
Ala	14,1788002	14,0367257	14,107763	0,10046181
Met	6,56086796	6,37224487	6,46655642	0,13337667
Val	7,6424383	7,53984895	7,59114363	0,07254163
Phe	5,8327288	6,05903364	5,94588122	0,16002169
Ile	5,11313223	5,21326797	5,1632001	0,07080666
Leu	10,9903056	11,0595297	11,0249177	0,04894887
Lys	1,90106705	1,93824628	1,91965667	0,02628968

Figure D.10: Free amino acid composition [wt.%] of sample H3, including average and standard deviation.

Amino acid	H4/1	H4/2	AV	STD
Asp	0,99526499	0,95681763	0,97604131	0,02718639
Glu	9,89899173	9,90323116	9,90111144	0,00299773
Asn	1,0215444	0,9929794	1,0072619	0,02019851
His	1,50834556	1,54425843	1,52630199	0,02539424
Ser	2,97851809	2,84114471	2,9098314	0,09713765
Gln	10,0268233	10,3156508	10,171237	0,20423192
Gly/Arg	12,4371756	12,3621676	12,3996716	0,05303867
Thr	6,82660111	6,64834437	6,73747274	0,12604655
Tyr	4,19451109	4,19867048	4,19659079	0,00294113
Ala	13,5998105	13,5445929	13,5722017	0,03904474
Met	6,90197518	7,07999526	6,99098522	0,1258792
Val	7,43729215	7,14357912	7,29043563	0,20768647
Phe	5,32047425	5,42056743	5,37052084	0,07077657
Ile	5,12050988	5,30864599	5,21457793	0,13303232
Leu	10,30272	10,1721278	10,2374239	0,09234262
Lys	1,42944231	1,56722698	1,49833464	0,09742848

Figure D.11: Free amino acid composition [wt.%] of sample H4, including average and standard deviation.

Amino acid	RM1/1	RM1/2	AV	STD
Asp	1,81440175	1,41934305	1,6168724	0,27934869
Glu	12,8220602	12,7774072	12,7997337	0,03157444
His	9,35326912	9,58281187	9,46804049	0,16231123
Ser	5,80892841	5,69048906	5,74970873	0,08374927
Gln	20,0313564	19,3159279	19,6736422	0,50588437
Gly/Arg	10,1517132	9,78494112	9,96832716	0,25934703
Thr	4,84726392	5,99670507	5,42198449	0,81277763
Tyr	3,56569874	3,78731535	3,67650705	0,15670661
Ala	9,39425377	9,82427787	9,60926582	0,30407296
Met	1,8513622	1,6802227	1,76579245	0,1210139
Val	3,48798561	3,35915148	3,42356854	0,09109948
Phe	2,90291219	2,85149498	2,87720359	0,03635745
Ile	1,5979781	1,52840886	1,56319348	0,04919288
Leu	3,19964449	2,95448052	3,0770625	0,1733571
Lys	9,00306217	9,42615494	9,21460855	0,29917176

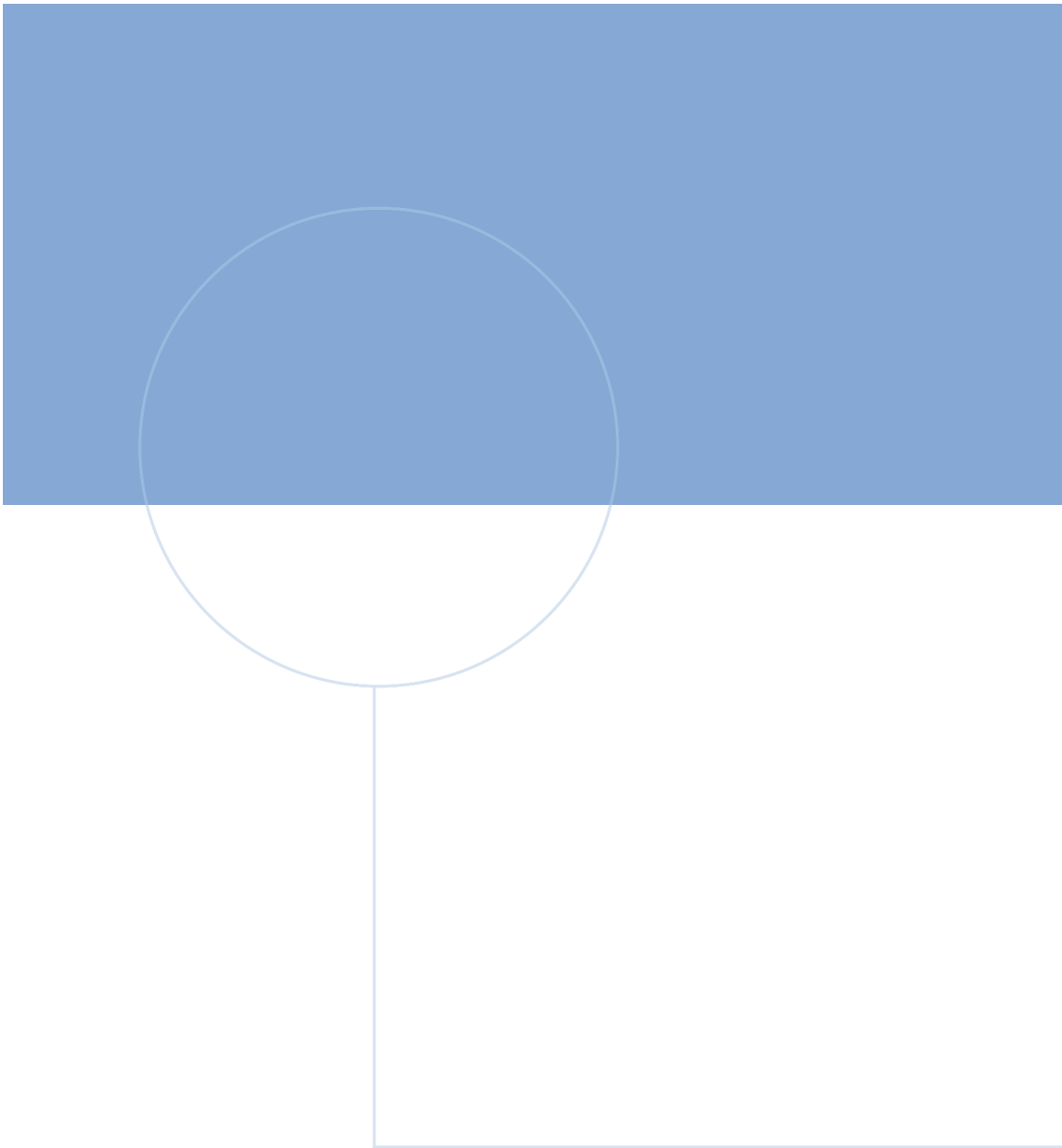
Figure D.12: Free amino acid composition [wt.%] of sample RRM1, including average and standard deviation.

Amino acid	RM3/1	RM3/2	AV	STD
Asp	0,39266456	0,23468502	0,31367479	0,11170841
Glu	4,35946754	4,55651292	4,45799023	0,13933213
His	0,02087877	0,01318698	0,01703288	0,00543892
Ser	0,87041513	0,91119822	0,89080667	0,02883799
Gln	13,6138707	13,3733639	13,4936173	0,17006397
Gly/Arg	14,8746301	14,5148169	14,6947235	0,25442638
Thr	8,026725	7,65583291	7,84127895	0,26226031
Tyr	12,3264686	13,5319547	12,9292116	0,85240743
Ala	18,8740861	18,9556723	18,9148792	0,05769012
Met	3,98762251	4,07845119	4,03303685	0,06422558
Val	8,55554787	8,31192725	8,43373756	0,17226579
Phe	3,39069854	3,20241791	3,29655822	0,13313451
Ile	4,03087944	4,05432974	4,04260459	0,01658186
Leu	6,00897678	5,80618701	5,9075819	0,14339402
Lys	0,64071988	0,79733848	0,71902918	0,11074608

Figure D.13: Free amino acid composition [wt.%] of sample RRM3, including average and standard deviation.

Amino acid	RM4/1	RM4/2	AV	STD
Asp	0,65272534	0,79703335	0,72487935	0,10204118
Glu	7,9284737	9,26002811	8,59425091	0,94155116
His	0,07390094	0,05051839	0,06220966	0,01653396
Ser	1,15628938	1,10202116	1,12915527	0,03837343
Gln	15,0182286	14,9280015	14,9731151	0,06380025
Gly/Arg	16,6365435	16,0230568	16,3298002	0,43380058
Thr	7,74727185	8,03496405	7,89111795	0,2034291
Tyr	7,08098083	7,35368551	7,21733317	0,19283132
Ala	19,7418964	19,0202382	19,3810673	0,51028943
Met	4,77580461	4,55042013	4,66311237	0,1593709
Val	7,93539916	7,98111705	7,95825811	0,03232743
Phe	2,51724311	2,44800292	2,48262302	0,0489602
Ile	3,80606472	3,6398788	3,72297176	0,11751119
Leu	4,54639733	4,49808977	4,52224355	0,0341586
Lys	0,37359736	0,30716165	0,3403795	0,04697714

Figure D.14: Free amino acid composition [wt.%] of sample RRM4, including average and standard deviation.



NTNU
Norwegian University of
Science and Technology