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Fish Protein Hydrolysates

- two case studies of potential for utilization of rest raw material as food ingredients in Norway and India

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Food and Technology
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Abstract

An increase in the population of the world calls for decreasing food loss, and better utilization of the available resources. Marine rest raw materials contain valuable nutrients, and the interest in using these as ingredients in products for human consumption is increasing. Studies have shown that the technology of enzymatic hydrolysis of rest raw material can result in products with desirable bioactive properties, like antioxidant activity. Protein hydrolysates with antioxidant activity can be used in food products to prevent oxidation, or as dietary supplements.

Hydrolyzed rest raw material from Atlantic herring (*Clupea harengus*) and Indian surimi production was the base of the analyses conducted in this study. Fish protein hydrolysate from Atlantic herring was received from the company Pelagia, and was fractionated by ultrafiltration, resulting in three different fractions; retentate >150 kDa, retentate >4 kDa and permeate <4 kDa as well as the original hydrolysate. These samples were brought to the research institute CFTRI in India for chemical analyses. Analyses on lipid content, protein content, determination of molecular weight distribution and antioxidant assays were conducted. Antioxidant assays included ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity.

Enzymatic hydrolysis was conducted on rest raw material received from an Indian surimi production plant, and optimum conditions for enzymatic hydrolysis was investigated by the use of response surface methodology. A hydrolysate made under the found optimum conditions was freeze-dried and brought to Norway for analyses where protein content, molecular weight distribution, amino acid composition and antioxidant activity were determined. The results suggested that the protein content in the herring hydrolysate increased by ultrafiltration (4 kDa) and the lipid content decreased. The use of FPLC to investigate molecular weight distribution in the samples showed that the distribution of peptides was fairly similar, and that ultrafiltration did not completely separate the samples. The DPPH radical scavenging activity indicated that the sample with supposedly lower molecular weight exhibited stronger antioxidant activity. As for the ABTS radical activity, the fractionation of the hydrolysate didn't seem to have a significant change in the antioxidant activity.

The most important results in this study was found when conducting the enzymatic hydrolysis on the Indian rest raw material, as a few challenges were met. First of all, maintaining of the cold chain is important in order to get raw materials of good quality as marine rest raw materials are prone to microbiological spoiling. Working with different kinds of raw material, the importance of proper homogenization of the respected raw materials, temperature control and having the proper equipment became clear. Being able to experience and be aware of these challenges will be of importance in future work with enzymatic hydrolysis in India, and is valuable knowledge to both Norwegian and Indian actors.

Due to the challenges met, some of the results from the chemical analyses may be unreliable. However, the molecular weight distribution of the peptides showed that the

hydrolysate consisted of a lot of small size peptides, which are linked to bioactive properties like antioxidant activity. The hydrolysate was found to be of high nutritional value as the amino acid composition showed a high content of essential amino acids.

By the use of a SWOT-analysis as a qualitative analysis tool, with the objective of better utilization of marine rest raw materials in India, it became clear that there are a lot of possibilities and opportunities, but today there are a few constraints in terms of inadequate technology and cold chain, as well as the general knowledge on possibilities for utilization of marine rest raw materials has to improve.

Sammendrag

Med en økende verdensbefolkning øker behovet for å redusere matsvinn og bedre utnyttelse av tilgjengelige ressurser. Marint restråstoff inneholder verdifulle næringsstoffer, og interessen for bruk av disse som ingredienser i produkter for humant konsum øker. Studier har vist at teknologien for enzymatisk hydrolyse av marint restråstoff kan resultere i produkter med ønskede bioaktive egenskaper som antioksidativ aktivitet. Proteiner med antioksidativ aktivitet kan brukes i matvarer for å hindre oksidasjon eller som kosttilskudd.

Hydrolysert restråstoff fra Atlantisk sild (*Clupea harengus*) og indisk surimi produksjon har blitt brukt til analyser i denne studien. Fiskeprotein hydrolysat fra Atlantisk sild ble mottatt fra selskapet Pelagia, og ble fraksjonert ved ultrafiltrering, som resulterte i tre ulike fraksjoner; retentat >150 kDa, retentat > 4 kDa, permeat <4 kDa, samt det originale hydrolysatet. Disse prøvene ble medbrakt til det indiske forskningsinstituttet CFTRI for kjemiske analyser. Det ble utført analyser på lipidinnhold, proteininnhold, molvekylvektfordeling og bestemmelse av antioksidative egenskaper. Analyser på antioksidative egenskaper inkluderte analyse av ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) og DPPH (1,1-diphenyl-2-picrylhydrazyl) radikaler.

Enzymatisk hydrolyse ble utført på restråstoff mottatt fra det indiske surimiproduksjonsanlegget, og optimale betingelser ble undersøkt ved bruk av responsoverflate metodikk. Et hydrolysat produsert under optimale betingelser ble frysetørket og tatt med til Norge for videre analyser av proteininnhold, molekylvektfordeling, aminosyresammensetning og antioksidant aktivitet.

Resultatene antydet at proteininnholdet fra sildehydrolysatet økte ved ultrafiltrering (4 kDa) og reduserte lipidinnholdet. Ved bruk av FPLC til å undersøke molekylvektfordeling i prøvene ble det vist at fordelingen av peptider var relativt lik, og at ultrafiltreringen ikke separerte prøvene helt tilstrekkelig. Den høyeste inhiberingen av DPPH radikaler ble funnet i prøven med antatt lavest molekylvekt. Det ble ikke funnet spesifikke forandringer som følge av ultrafiltrering i antioksidativ aktivitet ved analyse av ABTS radikaler.

De viktigste resultatene i studien ble funnet i forbindelse med enzymatisk hydrolyse på det indiske restråstoffet. Ved å arbeide med forskjellig råmateriale, ble viktigheten av tilstrekkelig homogenisering av råstoffet, kontroll på tid og temperatur, tilgang til nødvendig utstyr samt korrekt håndtering av råmateriale med tanke på kjølekjede tydeliggjort. Det å oppleve og dermed bli klar over disse utfordringene vil være viktig i fremtidig arbeid med enzymatisk hydrolyse i India, og er verdifull kunnskap både for norske og indiske aktører.

På bakgrunn av utfordringene med den enzymatiske hydrolysen, vil noen av resultatene fra de kjemiske analysene være noe usikre. Med dette tatt i betraktning, viste molekylvektfordelingen av peptidene i hydrolysatet at det bestod av mange små peptider som ofte knyttes til bioaktive egenskaper, som antioksidant aktivitet. Hydrolysatet ble funnet til å ha en høy næringsverdi, da aminosyresammensetningen viste et høyt innhold av essensielle aminosyrer.

Ved å utføre en SWOT-analyse som et kvalitativt analyseverktøy, basert på bedre utnyttelse av marint restråstoff i India, ble det klart at her er det store muligheter, men også enkelte utfordringer. Særlig med tanke på teknologi og kjølekjede, samtidig som den generelle kunnskapen rundt utnyttelse av marint restråstoff må bli bedre.

Preface

This master thesis is part of the master program Food and Technology at the Department of Biotechnology and Food Science (IBM) at NTNU Trondheim. The study for this thesis was conducted both at NTNU Trondheim, and at CSIR-CFTRI, Mysore, India. The study was part of the ReFOOD and ReValue project which is funded by the Research Council of Norway. The work with this master thesis has been challenging and frustrating at times, but I am really grateful I was able to be part of these projects which has given me a lot of new knowledge and experiences.

First of all, I would like to thank my supervisors, Eva Falch, Turid Rustad and Kari Helgetun Langfoss at NTNU for guidance and support. At CFTRI I would like to thank my supervisor Dr. N.M. Sachindra and his PhD students for their guidance. I would also like to thank PhD candidate at NTNU, Veronica Hammer Hjellnes for her help with interpreting of results and valuable support throughout the work with this thesis. A big thank you to fellow master students Kristin Brustad and Sara Aakre for good friendship and teamwork before, during and after the exchange in India.

Finally, I wish to thank my family for their support, motivation and encouragement throughout my studies, and these two years in Trondheim.

Trondheim, 31.05.19

Frida Holm Larsen

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Abbreviations

ABTS - 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic aid

BGG – Bovine Gamma Globulin

DH - Degree of Hydrolysis

DPPH – 2,2-diphenyl-1-picrylhydrazyl

EC_{50} – Effective Concentration for 50 % scavenging

FPLC – Fast Protein Liquid Chromatography

HPLC – High Performance Liquid Chromatography

MWCO - Molecular Weight Cut Off

OH – Original Hydrolysate

PG – Propyl Gallate

P4 – Permeat <4 kDa

RRM – Rest Raw Material

RSM – Response Surface Methodology

R4 – Retentate >4 kDa

R150 – Retentate >150 kDa

SD – Standard deviation

SDGs – Sustainable Development Goals

UF – Ultrafiltration

1. Introduction

Reducing food waste and better utilization of our resources has become an aspiration for both Norway and other countries globally, and is included in Sustainable Development Goal number 12 "Ensure sustainable consumption and production patterns" set by the UN (United Nations, 2015). The Norwegian government have expressed their concern regarding food loss and waste and the challenges within the value chain, and has started collaborations within different food sectors in order to get a more sustainable production and value chain (St. meld nr 30 (2008-2009), 2009). India, being the second largest country in the world considering population (Ritchie et al., 2018), was in 2013 considered the 6th largest food producer and exporter (Make in India, 2019). From all of this food produced, approximately 30 % is spoiled due to inadequate technology like food processing sites and management of the cold chain (Widell et al., 2019). With the population of the world growing, utilization of our resources to their fullest, and reducing food loss is of outmost importance.

Every year, 20 million tons rest raw material (RRM) from the worlds fisheries are produced (Caruso, 2016). RRM can be defined as the material that are not the primary product of a raw material (Nofima, 2019). Considering the large amounts of RRM obtained every year, and the possibility to convert this into products of higher value, this could be a good contribution to reduce food loss and improve the utilization. RRM has the ability to be a source of lipids, proteins and peptides with a variety of bioactive properties. Norway is doing a lot of research within this field and since 2014, SINTEF Ocean has had a partnership with India, working on the challenges and opportunities of global bio-economy. The partnership with India has led to the two research projects ReFOOD and ReValue, where NTNU is one of the Norwegian partners.

ReFOOD is an international project and partnership for research and education in energy efficient utilization in food value chains between Norway and India, funded by The Research Council of Norway. The project is focusing on challenges in bio-economy and sustainable utilization of food resources to meet the growing demand for food and feed ingredients around the world. ReFOOD is also focusing on building a cooperation between Norwegian and Indian institutes, industries and governmental stakeholders (SINTEF, 2019a).

The other project, ReValue, focuses on innovative technologies for improving resource utilization in the Indo-European fish value chains. ReValue is working on development of innovative technologies for the surimi industry to reduce food loss by improved cold chain management and efficient conversion of RRM and wash water from food production. The aim is to turn the RRM and wash water into value added protein and oil ingredients for food and feed applications (SINTEF, 2019b).

One of the Indian partners in the projects are Council of Scientific & Industrial Research (CSIR) – Central Food Technological Research Institute (CFTRI). The institute is located in Mysore, Karnataka in Southern India and consists of several research and development departments. CFTRI came into existence during the 1950s with the goal of pursuing in-depth research and development within food science and technology, and is considered one of the leading research institutes in food technology in India. An 11-week long exchange to CFTRI was conducted in connection with the ReFOOD and ReValue project and the master programme food and technology at NTNU.

1.1 Aim of the study

The primary aim of this study was to investigate the relationship between proteins and antioxidant activity in fish protein hydrolysates in two cases, rest raw material from Atlantic herring (*Clupea harengus*) and rest raw material obtained from Indian surimi production. Enzymatic hydrolysis and membrane filtration was used in order to investigate if this could enhance the utilization regarding proteins and antioxidant activity. Secondary aim was to investigate the topic of utilization of marine rest raw materials in Norway and India, with focus on challenges and possibilities.

The study consists of three parts. One part on fieldwork, one part on laboratory work on both cases, and one part which includes the use of qualitative analysis tools. The approach on splitting the study in three parts was decided based on changes and new knowledge and information gained throughout the project.

This study has been part of the two international research projects ReFOOD and ReValue which focuses on strengthening international partnerships, as well as research on challenges and possibilities for the global bio-economy.

2. Background

The world's population keeps increasing, and is estimated to reach 9,1 billion people by 2050, with nearly all of the increasing of the population occurring in developing countries (FAO, 2009). While the world's population is increasing, our natural resources are over exploited, meaning more people to feed with less water and food.

Today, the world waste or loose approximately one third of the food produced. At the same time, 821 million people starve (FAO, 2019). To be able to feed the world's population sustainably in the future, producers need to produce more food while reducing the negative environmental impacts. This includes losses and waste in nutrients, soil and water, as well as reduction of greenhouse gas emissions and degradation of the ecosystem. The people of the world also have to be informed on how to lower their environmental footprint and how to change their dietary habits for the better. Both consumers and the industry also need more information and a better understanding of how to utilize the resources we do have to their fullest potential.

In September 2015, the United Nations General Assembly adopted a set of 17 Sustainable Development Goals (SDGs) as part of the 2030 Agenda for Sustainable Development. This is a shared blueprint for peace and prosperity for people and the planet, now and in the future. The 17 SDGs are an urgent call for action by all member states in the UN, both developed and developing countries, and is a global partnership. This is the world´s common plan to eradicate poverty, fight inequality, improve health and education, take care of life above and below water and fight climate change by 2030 (United Nations, 2015).

Today, we use more of our resources than what is environmentally sustainable. To secure good living conditions for our future generations, it is important to change the way we use these resources. Sustainable development goal 12 "Ensure sustainable consumption and production patterns" focuses on doing more with less resources. Sustainable production involves reduction of resource use, greenhouse gas emissions, and reduction of negative environmental impact when producing a product. In the long run, this can contribute to limitation of the climate changes, increase in life quality to the population of the world, and economic growth (FN-sambandet, 2019). SDG 12 consists of multiple targets and target 12.3 calls for halving per capita global food waste at the retail and consumer levels, and reducing food losses along production and supply chains (including waste after harvesting) within 2030 (Champions 12.3, 2018).

Champions 12.3 is a group of leaders from all over the world dedicated to inspire action towards achieving SDG target 12.3. They publish an annual report on the progress in achieving this target. The group focuses on targets, measurements and action. By setting a target you can quantify food loss and waste and compare to your set target. This makes it easier to understand how much, and why food gets wasted within a specific value chain, which again makes is easier to take action (Champions 12.3, 2018). Exactly what needs to be done to achieve SDG target 12.3 will vary across the world, but common for all countries is that it will require action from multiple big players, as well as each and every single person, from farmer to consumer.

The European Commission are now suggesting and preparing a common methodology for the member states in the EU to measure food waste at the different stages in the food supply chain. This methodology is based on the SDG target 12.3 and an action taken to reduce and fight food waste. According to the European Commission, the Directive on Waste obliges member states to monitor the generation of food waste and take measurements to reduce their generation (European Commission, 2019a). By now, there are no set methodology on how to measure food waste and loss in the EU. This makes it hard for the public authorities to keep track of how much food waste and loss that is generated over time, and its origin. To get a better understanding of the problem, it is important to address the importance of measurement. By measuring the actual amount of waste and loss, it will be easier to set quantitative targets and achieve these. The suggested methodology, as presented in figure 1 includes the stages in the food supply chain and the different methods for measuring the food waste and loss.

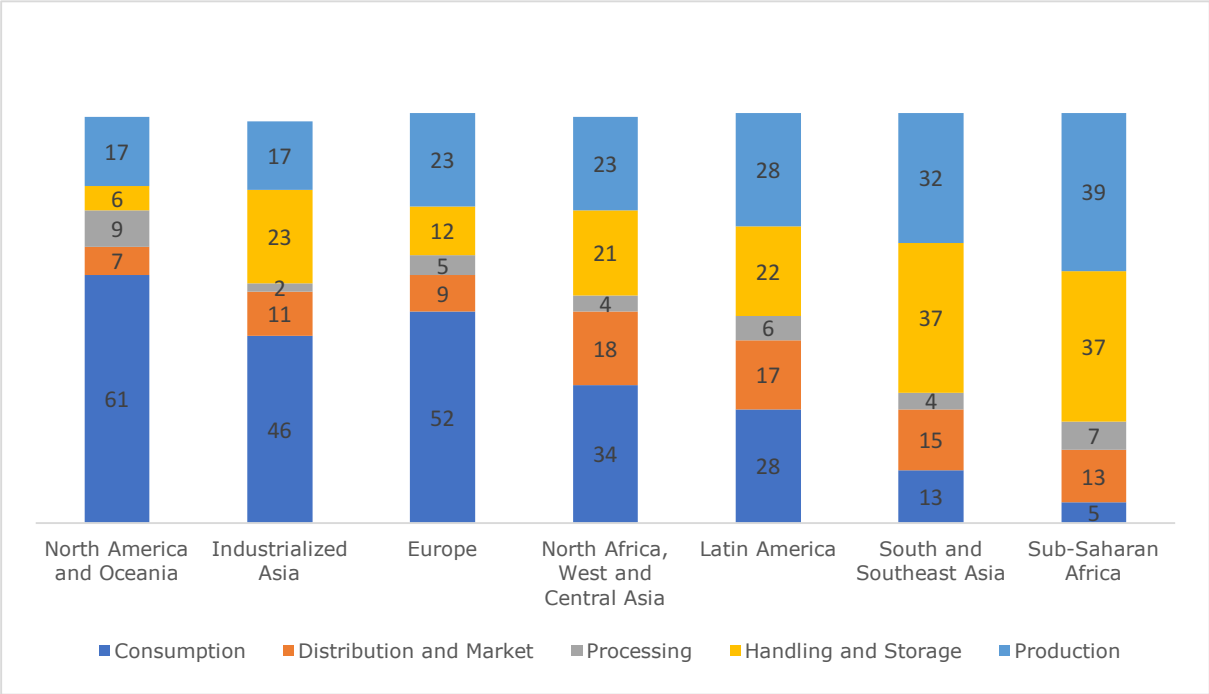
<table>
<tr><td>Stage of the food supply chain</td><td colspan="5">Methods of measurement</td></tr>
<tr><td>Primary production</td><td rowspan="5">Direct measurement</td><td rowspan="4">Mass balance</td><td rowspan="2"></td><td colspan="2">Questionnaires and interviews
Coefficients and production statistics</td></tr>
<tr><td>Processing and manufacturing</td><td colspan="2"></td></tr>
<tr><td>Retail and other distribution of food</td><td rowspan="3">Waste compostal analysis</td><td rowspan="2">Counting/scanning</td><td></td></tr>
<tr><td>Restaurants and food services</td><td></td><td rowspan="2">Diaries</td></tr>
<tr><td>Households</td><td></td><td></td></tr>
</table>

Figure 1. Methodology draft of measuring food waste and loss suggested by the European Commission, modified by the European Commission (2019a).

An article posted by Southey (2019) states that FoodNavigator, an online news source for the food industry, reached out to FoodDrinkEurope (FDE) to investigate the interest for the methodology in the industry. The FDE stated that they are committed to contribute to the implementation of the SDGs, in particular target 12.3 and actively support the development of a common EU food waste measurement methodology (Southey, 2019). To get the food industry on board, starting to measure food waste and loss, is a great start towards achieving SDG target 12.3.

Looking at food loss and waste in the supply chain, food loss and waste in South and Southeast Asia near the production phase are more prevalent compared to Europe where most of the food loss is in the consumption and household phase as presented in figure 2 (Champions 12.3, 2018). Lack of technology, proper equipment and infrastructure are some of the reasons why the majority of the food loss is in the production phase in the supply chain in South and Southeast Asia. The percentage of total food available that are

being lost or wasted are considerably higher in North America and Oceania than in the rest of the world.



42 % 25 % 22 % 19 % 15 % 17 % 23 %
Share of total food available that is lost or wasted

Figure 2. Illustration showing food loss near production being more prevalent in the global south while food waste near consumption are more prevalent in the global north (percent of kcal lost or wasted), modified by Champions 12.3 (2018).

Considering the environment, food that is harvested but lost or wasted generates about 8 % of the greenhouse gas emissions every year. If food loss were a country it would be the world´s third greatest greenhouse gas emitter, with China being the greatest followed by the United States and India on fourth place (Champions 12.3, 2018). In 2015 the population of India was 1.26 billion people, and predicted to reach 1.6 billion by 2050, which means overtaking China as the most populous country in the world (Ritchie et al., 2018).

By utilizing our resources in a better way, and reducing the food loss and waste, we could secure the natural resources for future generations, and greatly reduce the greenhouse gas emissions based on food loss and waste. To be able to accomplish this, information and education about the topic is crucial, as well as technology and infrastructure in both developing and developed countries.

The ReFOOD and ReValue projects have their background in the SDGs, and have a goal to improve the technology and the utilization of our food resources today. Having the cooperation with India, the second largest country in the world considering population, in these two projects is a great start towards exchange of knowledge and new technologies, and can possibly make a big difference in the future considering food loss, waste and greenhouse gas emissions.

2.1 Marine rest raw material

As fish resources across the world are limited, and the population of the world keeps increasing, utilization of our resources to their full potential is important (Rustad et al., 2011). RRM from the fish industry have a high content of valuable nutrients and have a potential for further utilization into higher value-added products. In Norway, marine RRM are defined as the material that are not the primary product of a raw material (Nofima, 2019). RRM can also be referred to as side streams, plus product and co-product. Marine RRM often includes head, skin, bone, viscera and cut-offs (Rustad et al., 2011). By-products are defined as the part of the RRM that are not handled after the hygiene regulations, but are handled after the by-product regulations and differs from RRM in the way that it is not allowed to use for human consumption (Animaliebiproduktforskriften, 2016).

Industrial processing of fish results in a yield of only 30-50 % fish products for human consumption, which means 50-70 % RRM (Dekkers et al., 2011). Marine RRM constitute an important resource in Norwegian aquaculture and fisheries, and today a lot of the RRM are utilized in a good way, but can also be further used in high value-added products.

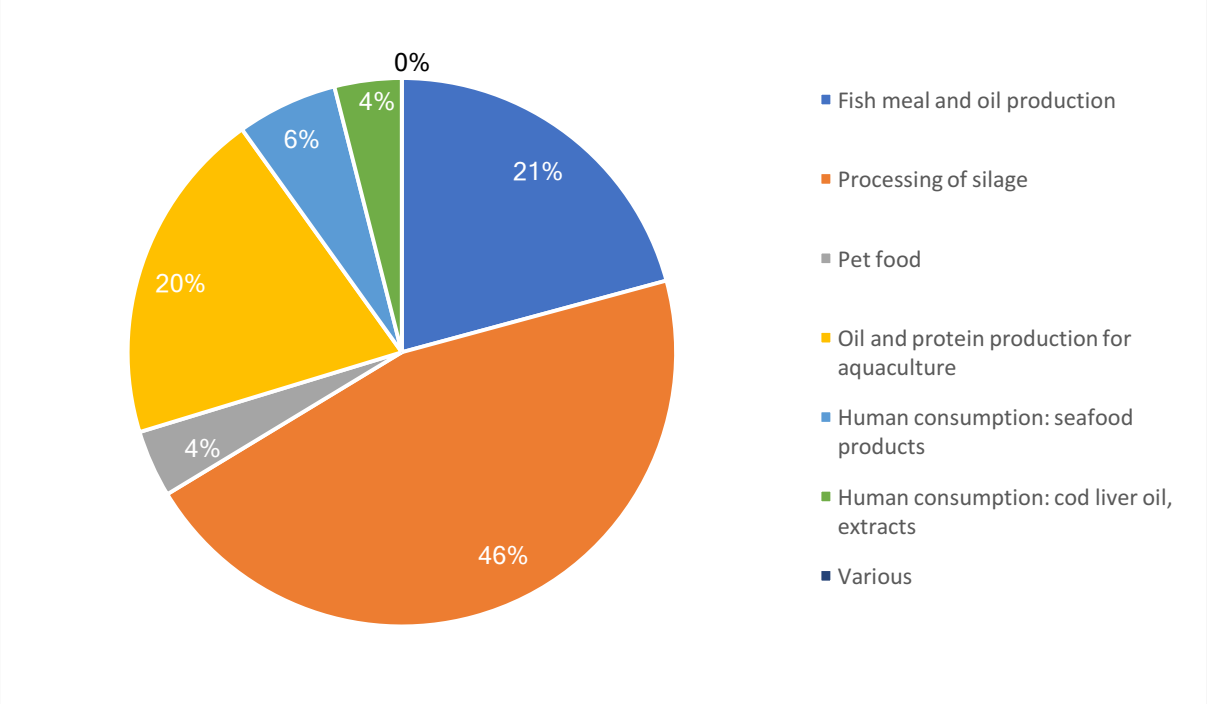


Figure 3. Application of RRM for different productions in Norway in 2016, modified by Richardsen and Nystøyl (2017).

There are many companies in the aquaculture and fisheries industry that are focusing on utilizing the RRM (Richardsen and Nystøyl, 2017). In 2016, a total of 0.91 million tons RRM was produced in Norway, where 76 % was utilized. From this, the majority went to processing of fish silage (46 %), while only 9 % went to human consumption, as presented in figure 3.

Even though a lot of the RRM are utilized in a good way, there are a lot of potential and possibilities for a better utilization and increasing the value of RRM. By making products with a higher content of valuable nutrients for human consumption and as ingredients in foods, dietary supplements and cosmetics, not only in Norway, but on a global basis would create new value to marine RRM.

2.1.1 Rest raw materials from herring

Atlantic herring (*Clupea harengus*) is an oily fish species that can be found in the open seas across the North Atlantic, and is considered a pelagic fish. Herring can reach almost 700 g in weight and 40 cm in length (European Commission, 2019b). The composition of herring is known to vary throughout the season, and the lipid content is usually higher in herring harvested in October-November because the fish feeds between April-September (Slotte, 1999, Aidos et al., 2002).

The pelagic fish sector in Norway mainly consists of herring, mackerel, blue whiting and capelin, where herring is the species giving the most RRM. This is because the majority (75 %) of the herring is being filleted. In 2016, close to 178 000 tons RRM originated from the pelagic fish sector (Richardsen and Nystøyl, 2017).

Pelagia AS is a Norwegian company that is one of the leading producers of pelagic fish products for human consumption, and an important supplier of essential ingredients in fish- and animal feed like protein concentrate, fishmeal and fish oil (Pelagia, 2019).

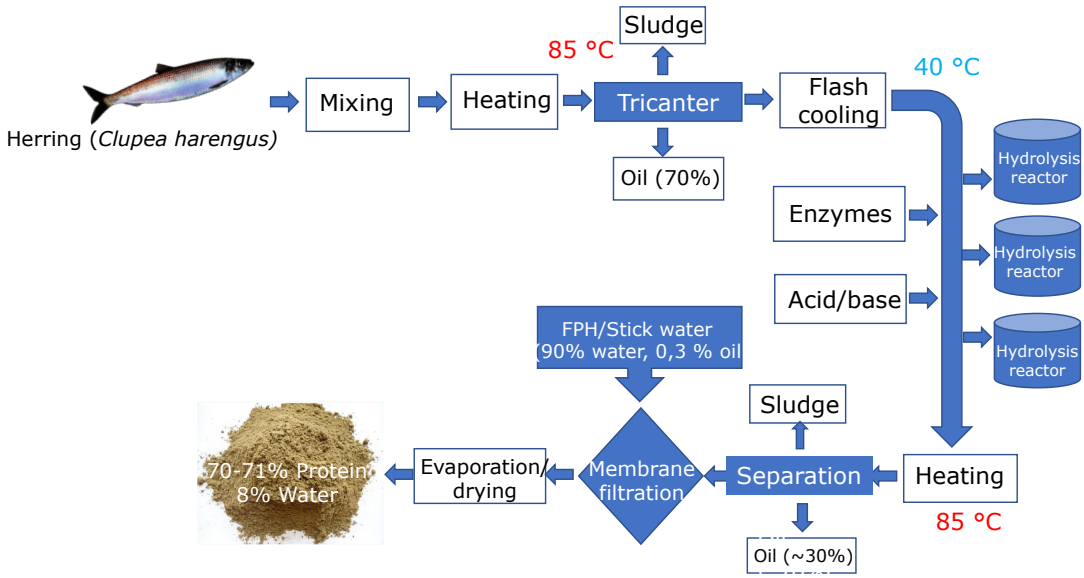


Figure 4. Overview of production of fish protein hydrolysate at Pelagia AS, modified by Hammer Hjellnes (2019).

The production of fish protein hydrolysate at Pelagia AS is presented in figure 4, and starts with the grinding of the RRM from herring. The RRM used at Pelagia AS includes everything except the fish fillets. Heat (85°C) is applied and the mixture goes through a tricanter that separates the sludge and approximately 70 % of the oil from the mixture. The mixture

goes through a cooling step (40°C) and into a hydrolysis reactor before enzymes are added. Acid/base is added to adjust to optimal pH for the respective added enzyme. The mixture is heated (85°C) again for inactivation of the enzymes, and goes through a separation step, which again separates the sludge and most of the remaining oil. What is left after the separation is the fish protein hydrolysate (FPH). The following step including membrane filtration is not part of the present process at Pelagia AS, but is something they want to include in the future.

2.1.2 Rest raw materials from surimi

Surimi is originally a Japanese product, and consists of myofibrillar proteins obtained by mechanically deboning of fish flesh, followed by washing with water and blending with cryoprotectants (Park and Morrissey, 2000). The surimi product is prepared to imitate seafood. The manufacturing of surimi has become a big business in India, and India is one of the biggest suppliers of surimi in Asia (Chateau and Vidal-Giraud, 2007). Part of the ReValue project goal is to reduce food loss and better the utilization of RRM. One of the Indian partners in the project is the Mumbai based company Refrigerated Distributors Private Limited which produces surimi under the name Kaiko surimi. In India, there are 7-8 surimi plants, with Kaiko being one of the smaller ones (Widell et al., 2019). The production of surimi at the Kaiko surimi plant uses a variety of different fish species, presented in table 1.

Table 1. Variety of fish used for surimi production at Kaiko surimi plant, modified by Gupta (2018).

Fish type	Common name	Scientific name
Non-histamine forming	Pink perch	*Nemipterus sp.*
	Big eye	*Priacanthus harmus*
	Ribbon fish	*Lepuracanthus savala/Trichiurus lepturus*
	Lizard fish	*Saurida sp.*
	Croaker fish	*Johnius sp.*
	Barracuda	*Sphyaraena sp.*
	Reef cod	*Eplinephelus sp.*
	Bronze croaker	*Tolithoides biaurituds*
Histamine forming	Sardine	*Sardinella longiceps*
	Mamakari	*Sardinella brachysoma*

At Kaiko surimi production plant, the fresh fish goes through a process of washing, mechanical deboning, and the minced meat gets separated by four washing steps. Finally, cryoprotectants are added, and the surimi gets packed. The process parameters depend on the quality of the raw material and the requirements to the final product (Widell et al., 2019).

At the production plant, 350 kg head waste is produced for every 1 ton of fish. The RRM obtained at the surimi plant are sorted as head waste, skin and bone waste and refined waste. The fish are mechanically beheaded at the surimi production plant, and the viscera are pulled out and included in the head waste. In 2018, 3 000 tons surimi was produced

at the surimi plant, and a total of 900 tons RRM was obtained. Today, the RRM from the surimi production at Kaiko are either used for fish meal production or dried in the sun and used as fertilizer (Naik, 2019).

2.2 Hydrolysis and Fish Protein Hydrolysates

Recovery and utilization of the proteins present in marine RRM, and using these as functional ingredients in foods is an interesting alternative rather than treating the RRM as waste. In the industry, RRM are frequently used in production of fish meal, fish oil and silage (Kristinsson and Rasco, 2000). An alternative way to utilize the proteins in the RRM is production of fish protein hydrolysate (FPH), which is done by hydrolysis. Hydrolysis is a process which involves addition of water and results in a reaction between an organic molecule, in this case a protein and water, and forms two or more substances (Speight, 2017). Protein hydrolysates are defined as the breakdown products of proteins into smaller peptides, and hydrolysis can be performed by either enzymatic or chemical hydrolysis (Chalamaiah et al., 2012, Gamez-Meza et al., 2003).

Chemical hydrolysis of proteins is achieved by cleavage of peptide bonds, either with acid or base. Chemical hydrolysis has previously been the method used in the industry, but parameters like temperature, pressure and pH in the process are often difficult to control. This can lead to products with varying functional properties and chemical composition, along with poor functionality and reduced nutritional qualities (Kristinsson and Rasco, 2000).

In the making of products for human consumptions, enzymatic hydrolysis is often preferred over chemical hydrolysis because enzymatic hydrolysis is carried out under more gentle conditions. Enzymes are substances that catalyzes the chemical processes in living organisms by lowering the activation energy (Hauge et al., 2018). To be able to catalyze a chemical process, the enzyme has to bind to a substrate. The substrate enters the highly specific active site of the enzyme, and creates an enzyme-substrate complex. The substrate is converted into a product, which gives an enzyme-product complex that will release the product (Hauge et al., 2018, UiO, 2011).

2.3 Enzymatic hydrolysis

There are several varieties of enzymatic hydrolysis used within research and in the industry for production of FPH, but the general steps of an enzymatic hydrolysis process are presented in figure 5.

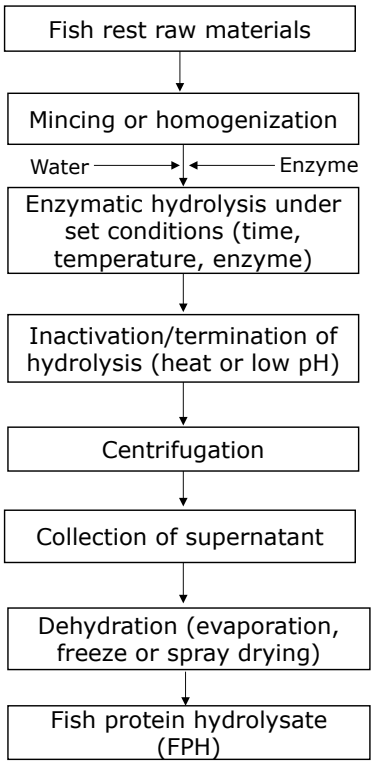


Figure 5. Outline of the general steps in an enzymatic hydrolysis of fish RRM to make FPH, modified by Kristinsson and Rasco (2000).

The RRM gets minced/grinded and homogenized. Desired amount of water, and selected enzyme(s) are added to a reaction vessel. The hydrolysis is carried out under a set temperature for a desired period of time. Termination of the hydrolysis is carried out either by inactivation of the enzymes by high temperature or low pH. The mixture is then centrifuged and will separate into different fractions. Depending on the raw material and its composition, the centrifugation can result in five different fractions. The five different fractions are: sludge on the bottom, fish protein hydrolysate (supernatant), a lipid-protein layer between the fish protein hydrolysate and sludge, sometimes an oil-water emulsion and the oil fraction on top (Slizyte et al., 2004). The supernatant is collected, and is dehydrated or concentrated by evaporation, or freeze dried or spray dried into a powder (Kristinsson and Rasco, 2000).

After hydrolysis, the fish protein hydrolysates will have a higher functional value considering the proteins, compared to the raw material. The hydrolysates consist of a wide range of peptides with different molecular weight, which depends on the degree of hydrolysis (DH). According to Adler-Nissen (1979), DH can be defined as the percentage of peptide bonds cleaved during the hydrolysis. The DH gives an insight in the extent of hydrolysis of the proteins.

2.3.1 Enzymes

Enzymatic hydrolysis can be performed either with proteolytic enzymes that are already naturally present in the fish muscle and viscera (endogenous proteases), or exogenous enzymes that are added to promote hydrolysis (Kristinsson and Rasco, 2000) (Souissi et al., 2007).

Proteolytic enzymes, also called proteases and peptidases, are hydrolytic enzymes that catalyzes the cleavage of peptide bonds in proteins into peptides and amino acids. Proteolytic enzymes can be divided into two different groups depending on their hydrolyzing mechanism; endopeptidases and exopeptidases (Hauge, 2009) (Kristinsson and Rasco, 2000). Endopeptidases cleave or hydrolyze peptide bonds within protein molecules which results in larger peptides. Exopeptidases cleave peptide bonds either at the N-terminus (aminopeptidases) or at the C-terminus (carboxypeptidases). In food protein hydrolysis endopeptidases are always used. Using added commercial enzymes for hydrolysis of food products is important to improve or modify the properties of the product (Kristinsson and Rasco, 2000).

Proteolytic enzymes from plants and microorganisms are the best suited for hydrolysis of fish protein. There are a wide range of commercial enzymes that have been used in protein hydrolysates, and the choice of enzymes often depends on economics and efficacy. Amongst others these include Alcalase, Neutrase, Papain, Flavourozyme, Protamex, Trypsin and Bromelain (Slizyte et al., 2004, Kristinsson and Rasco, 2000, Hoyle and Meritt, 1994, Chalamaiah et al., 2012, Raghavan and Kristinsson, 2008).

2.3.2 Factors affecting hydrolysis

The yield of FPH after hydrolysis depends on multiple factors like type and composition of raw material, amount of water added, temperature, type and amount of enzyme, time and pH (Slizyte et al., 2004) (Kristinsson and Rasco, 2000). The choice of the right enzyme is important if the product is supposed to have specific properties and peptide profiles, and endopeptidases such as Bromelain, Protamex, Alcalase and Papain have shown to be effective in the hydrolysis process.

2.4 Membrane filtration

Membrane filtration can be defined as a series of filtrations where one or multiple components in a liquid gets separated from the others by passing through one or multiple semi permeable membranes with pressure on the feed side of the membrane. The fraction passing through the membrane is called the permeate or filtrate, and contains a reduced concentration of some of the substances dissolved in the feed. The fraction held back by the membrane is called retentate of concentrate, and contains an increased concentration of the retained substances (Nesse, 2017). Ultrafiltration (UF), nanofiltration (NF) and gel filtration (GF) have also been found to refine hydrolysates by separation after the molecular weight of the peptides (Halim et al., 2016). UF membranes with MWCO of 20 to 100 kDa are often used for separation of peptides, while membranes with MWCO of 4 to 8 kDa are often used to fractionate hydrolysates, resulting in a higher concentration of peptides of a specific molecular weight (Bourseau et al., 2009).

2.5 Properties of Fish Protein Hydrolysates

Modification and improvement of the functional properties of fish protein hydrolysates is one of the advantages with enzymatic hydrolysis. Kristinsson and Rasco (2000) defines functional properties as the functional and chemical properties that affect the behavior of proteins in foods during processing, storage, preparation and consumption. Some of the bioactive, nutritional and physiochemical properties of FPH are presented in table 2.

Table 2. Bioactive, nutritional and physiochemical properties of FPH.

FPH properties		
Bioactive	*Nutritional*	*Physiochemical*
Antioxidative	Easily digestible	Solubility
Antihypertensive	Essential amino acids	Water holding capacity
Immunomodulatory	High nutritional value	Emulsifying properties
Obesity modulatory		Foaming properties
Antithrombic		Fat absorption

Bioactive properties

Bioactive properties refer to a compound or a substance that have an effect on a living organism (Guaadaoui et al., 2014). Bioactive properties linked to FPH include antioxidative, antihypertensive, immunomodulatory, obesity modulatory and antithrombic (Kim and Mendis, 2006) (Rustad et al., 2011) (Kristinsson and Rasco, 2000). Antioxidant activity and the possibility to have antihypertensive properties are the most studied bioactive properties. The interest of natural antioxidants that are non-hazardous are increasing, and will be further discussed in chapter 2.8. Angiotensin-I converting enzyme (ACE) plays a central part in the regulation of blood pressure, and turn the inactive angiotensin- I into angiotensin-II , which increases the blood pressure (Vercruysse et al., 2005) (Raghavan and Kristinsson, 2009).

Nutritional properties

Proteins are an important part of the daily diet, and FPH have a relatively high protein content. The nutritional value of foods depends on the amino acid content and how the amino acids are being utilized physiologically after digestion (Friedman, 1996). Since FPH consists of smaller peptides, usually consisting of 2-20 amino acids, it makes it easier to digest than larger peptides (Chalamaiah et al., 2012). Essential amino acids are the ones that are not synthesized in the human body, and has to be constituted through the diet, and FPH are known to contain all of these; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Kristensen, 2018).

Physiochemical properties

The physiochemical properties are important when considering the hydrolysate´s quality when it comes to processing, storage and consumption. The physiochemical properties include solubility, water holding capacity, emulsifying properties, foaming properties and

fat absorption. Solubility is the most important physiochemical property of protein hydrolysates. The solubility of hydrolysates is improved due to the cleavage of the proteins into smaller soluble peptides, increasing the hydrophilicity due to the exposed amino and carboxyl groups at the ends of the peptides (Kristinsson and Rasco, 2000) (Benjakul et al., 2014). Studies have shown that peptides with low molecular weight tend to have higher solubility than peptides with larger molecular weight. The solubility of FPH have also been shown to be good over a wide range of pH and ionic strengths, as well as contributing to the water holding capacity (Halim et al., 2016).

FPH have also been found to have good water holding capacity, which refers to the proteins ability to absorb water and retain it from gravitational force within the protein matrix (Kristinsson and Rasco, 2000). Enzymatic hydrolysis leads to an increase of polar groups like COOH and NH_2 (Taheri et al., 2013). Based on studies by Slizyte et al. (2009) and Balti et al. (2010) the results indicated that FPH gave a higher water holding capacity due to having more hydrophilic polar side chains.

Emulsifying properties rely on the surface properties of the protein. Enzymatically hydrolyzed compounds from fish can reduce the interfacial tension between water soluble hydrophobic and hydrophilic functional groups in foods (Kristinsson and Rasco, 2000). Smaller proteins decrease the emulsion stability due to weak interfacial films around the emulsion droplets, while peptides with larger molecular weight contribute to emulsion stability (Halim et al., 2016). Foaming properties also rely on the surface properties of the protein, by helping to lower the surface and interfacial tension, and are known as air droplets that are enclosed by a liquid that contains soluble surfactants (Kristinsson and Rasco, 2000). Both emulsification and foaming properties are affected by the solubility.

The ability to absorb fat influences the quality and the taste of a product, and is especially important in meat and fish products. Fat absorption is mostly attributed to the physical entrapment of oil, and the hydrophobicity of the surface of peptides (Kristinsson and Rasco, 2000) (Šližytė et al., 2005).

2.6 Challenges in the application of Fish Protein Hydrolysates

FPH has not become commercially available for human consumption yet due to some challenges. As RRM are highly perishable, a reproducible quality of the product is important. The hydrolysis process consists of multiple factors which has to be accurate and reproducible in order to get the same result for every product. Another challenge is that hydrolysis often creates a fish-like smell and a bitter taste to the proteins and peptides. The bitter taste has been found to depend on the average hydrophobicity and the degree of hydrolysis (Rustad et al., 2011).

Other challenges in the application of FPH in both Norway and India includes the fact that the market has to be ready to accept the use of RRM for human consumption. The public has to be aware of the potential of FPH, including its possibility to reduce food loss, and the possible promising health effects. More of the challenges considering the application of FPH in India will be further discussed in conjunction with the chapter on qualitative analysis tools.

2.7 Oxidation

Oxidation is a chemical process where electrons are displaced or released. In aerobic organisms, oxygen is a fundamental part of life, but it can also be very toxic. Oxidative stress and free radicals have been linked to oxidation. Free radicals can be highly unstable

and reactive and can lead to damage to the cells in the human body. Oxidative stress occurs when the balance between the generation of free radicals and antioxidant defense is unfavorable. Adding antioxidants to a diet has been found to may assist and protect the human body from the negative effects of oxidative stress and free radicals (Lobo et al., 2010).

In foods, especially those rich in lipids, oxidation have been related to deterioration of flavor, loss of nutritional value and safety, ageing, biological damage and changes in the functional properties (Ladikos and Lougovois, 1990). Slowing down the oxidation process in foods can be favorable for the preservation and thereby increase the shelf life, for example by addition of antioxidants to the food (Choe and Min, 2009).

2.8 Antioxidants

Antioxidants are defined as any substance that can delay or inhibit oxidation of a substance, and act by different mechanisms. How effective an antioxidant is in a food system depends on multiple factors, for example the actual concentration of the antioxidant. If a too high concentration of some antioxidant is used, then the antioxidant can get the opposite effect and become pro-oxidative (Jacobsen et al., 2014). Antioxidants can be either naturally present in the food, or added to the food product, and can be divided into primary and secondary antioxidants, chelators, quenchers, oxygen scavengers and oxygen regenerators depending on their function. Scavenging of free radicals or oxygen and chelation of metal ions are the most important mechanisms.

Primary antioxidants are radical scavengers that can transfer a hydrogen atom or an electron to a radical, and by this inhibit the initiation of the free radical chain propagation process. The oxidized antioxidant becomes a radical species by transferring a hydrogen atom or an electron. The formed antioxidant radical should be relatively stable, and unable to continue the free radical propagation (Berdahl et al., 2010) (Choe and Min, 2009).

Secondary antioxidants work by converting hydroperoxides into non-radical, non-reactive and thermally stable substances, and are often used in combination with primary antioxidants to yield synergistic stabilization effects (Ambrogi et al., 2017). Hydroperoxides can react with metals and create hydroxyl and alkoxyl radicals. These can extract hydrogen from lipids, which generates carbon-centered radicals that can proceed the propagation cycle (Berdahl et al., 2010).

Chelators prevents initiation of forming radicals by binding to metals, and quenchers can deactivate excited states, like singlet oxygen or free radicals. Oxygen scavengers works by reacting and removing oxygen from the system, and antioxidant regenerators reduce the radicals formed when a primary antioxidant donates an electron or hydrogen atom to a free radical (Berdahl et al., 2010).

Antioxidants have been of interest to biochemists and health professionals because they may help to protect the body from reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) (Shahidi, 2000).

Antioxidants can be either naturally or synthetically present in foods. Natural antioxidants have been found to be present in spices, herbs, teas, fruits and vegetables (Shahidi, 2000) (Brewer, 2011). Phenolic compounds such as tocophenols, polyphenols and phenolic acids, as well as ascorbic acid are the most important natural antioxidants (Choe and Min, 2009). Natural antioxidants found in certain foods can be used in other more complex foods to stabilize them, and are often present in combinations involving many different compounds

(Shahidi, 2000). Proteins, peptides and free amino acids can also serve as antioxidants. Peptides are the preferred ingredient for antioxidant agents in foods over amino acids. This is due to peptides being soluble in both lipid and aqueous foods and soluble across a wider pH range. The antioxidative potency of peptides can also be additive and better than single unit free amino acids as peptides consists of a chain of amino acids. Antioxidant activity in peptides have been found in various animal and plant proteins (Elias et al., 2008) (Aluko, 2015).

Synthetic antioxidants are often added to a product as preservatives to increase the shelf life. Examples of some of the most widely used synthetic antioxidants are butylated hydroxytoluene (BHT), propyl gallate (PG), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and thylenediaminetetra-acetic acid (EDTA) (Shahidi, 2000). PG is primarily added to foods containing oils and fats to prevent oxidation, but can also be used in pharmaceuticals and cosmetics. When using spectrophotometric methods to determine antioxidant activity of other substances, PG is often used as a reference or standard (Gálico et al., 2015). There is a desire to replace synthetic antioxidant with the natural ones, due to concerns about reports that high concentrations of synthetic antioxidants can be carcinogenic, and some countries does not allow use of certain synthetic antioxidants (Shahidi, 2000).

2.9 Fish peptides as antioxidants

Peptides from fish muscle and RRM have been found to be able to serve as a source of antioxidants. In the food industry, enzymatic hydrolysis is the preferred method to generate peptides from food products, because other methods can leave residual solvents or toxic chemicals in the product (Najafian and Babji, 2012). The antioxidant activity of FPH depends on degree of hydrolysis, molecular weight, structure of the peptides and amino acid sequence (Mazorra-Manzano et al., 2018) (Najafian and Babji, 2012). Antioxidative peptides have been found to contain 2-16 amino acids, and FPH with low molecular weight have shown high antioxidative activity (Chalamaiah et al., 2012). In a study by Najafian et al. (2013) patin protein hydrolysate was filtrated by UF into fractions of <10 kDa and <3 kDa. When investigating antioxidative assays like DPPH radical scavenging, ABTS radical scavenging, reducing power and metal chelating, the <3 kDa fraction showed higher radical scavenging activity and reducing power than the <10 kDa fraction. Studies by Ranathunga et al. (2006) gave similar results, and reported that higher antioxidative activity of peptides with lower molecular weight are due to their ability to react with lipid radicals and reduce radical-mediated lipid peroxidation.

Many antioxidant peptides are found to contain hydrophobic amino acids like valine and leucin at the N-terminus, and other amino acids such as proline, histidine and tyrosine in the amino acid sequence (Halim et al., 2016). The aromatic amino acid tyrosine is known to act directly as a radical scavenger due to its possibility to serve as hydrogen donor because of its phenolic groups. Short peptide chains in FPH have higher antioxidative activity than free amino acids, even though free amino acids also possess antioxidative activity (Chalamaiah et al., 2012). Production of fish protein hydrolysates with antioxidant activity, and adding these to other food products can result in products with a higher protein content that are oxidative stable.

3. Materials and Methods

The practical work of this thesis was conducted on two different raw materials, dividing the thesis in two different cases. The materials and methods chapter is built up according to the two different raw materials:

1. Herring protein hydrolysate received from the Norwegian company Pelagia AS
2. Surimi RRM received from an Indian surimi production plant under the name Kaiko

In order to investigate the raw material, get results, and gather information, different techniques were used, and the structure of the thesis is based upon this.

- Fieldwork
- Laboratory work
- Qualitative analysis tools

The fieldwork is based on the approach of gathering information and knowledge for the study. This included workshop with the ReFOOD and ReValue projects in Mumbai, industry visit at the Kaiko surimi production plant, interactive communication with the daily manager of the surimi plant over FaceTime, visit to Amity university which is one of the partners in the projects, conference in New Delhi and the exchange at CFTRI.

The laboratory work was conducted both at NTNU in Norway and at CFTRI in India and was based on quantitative analysis.

The qualitative analysis tools included amongst others a questionnaire. The questionnaire was not conducted following an analytical procedure, but distributed to the partners in the projects. The questionnaire was an act as the laboratory work in India didn't go as planned.

Fieldwork

In connection with the two projects ReFOOD and ReValue, a symposium was held in Mumbai, India, November 28-29th 2018. On November 28th an industry visit to Kaiko surimi processing plant was conducted to see the production and the value chain of the surimi processing. November 29th there was a meeting where the industry visit was discussed in groups. Present at the meeting was a mix of both people from the industry, scientists and research institutes and universities from Norway and India. The goal of the symposium was exchange of knowledge and ideas on technology for sustainable utilization of food resources, and building a cooperation between India and Norway. It was supposed to be a meeting also with Dr. Sachindra at the symposium as he was the representative from CFTRI, but he couldn't attend.

January 7-9th 2019 an Indo-Norwegian business summit was held in New Delhi, India. The summit was part of the official visit by the Norwegian Prime Minister Erna Solberg, for strengthening the bilateral relationship between Norway and India, primarily through increased cooperation to reach the SDGs, with focus on the ocean. Present at the summit were several companies from maritime, seafood, marine, offshore, technology and defense industries. There was a plenary opening session followed by parallel sessions, by others sustainable seafood production. Amongst others, there were speakers from Innovation Norway, The Research Council of Norway, Norwegian seafood Council and some Indian

companies within seafood production. Dr. Souvik Bhattacharyya from the university BITS Pilani spoke on behalf of the ReFOOD and ReValue project. The fact that the Norwegian Prime Minister was present at the summit shows the importance of the topic. Following the session on sustainable seafood production, there was an opening ceremony of the Norwegian Embassy in New Delhi, by the Norwegian Prime Minister. Before the official opening ceremony, the Prime Minister visited different stands, where the ReFOOD and ReValue project was part of the Norwegian Research Council´s stand.

As part of the student exchange, three students from the master programme food and technology had an exchange at CFTRI and three students from the master programme energy and environment at IIT Kharagpur. The exchange was the start of a partnership between Norwegian and Indian educational institutions. The exchange took place as an observational study with both passive and active participation.

Throughout the stay in India, communication with partners in the projects like the surimi production plant, communication with supervisors in Norway and scientists at SINTEF Ocean has been conducted interactively using Skype and email. Table 3 illustrates measures and goals according to increase knowledge regarding the topic RRM and utilization.

Table 3. Measures to gain knowledge related to the study and goals.

Measures to gain knowledge	Goal
Workshop in Mumbai, India	- Gain knowledge on the topic of rest raw materials - Meet Dr. Sachindra - Meet the partners in ReFOOD and ReValue - Group discussions which resulted in a report
Visit to the Kaiko surimi production plant	- Get insight in the process of the production of surimi - Meet the supplier of raw material to the study
Visit at Pelagia AS	- Get insight in the process of the production of protein hydrolysate from herring (conducted by Veronica Hammer Hjellnes and Kristin Brustad) - Meet the supplier of herring protein hydrolysate to the study
Student exchange to CFTRI, India	- Start of a partnership between Norwegian and Indian educational institutions - Observational study - passive and active participation in research on Indian RRM
Active communication with SINTEF Ocean	- Discussions of study and aim of study during and after stay in India - Help with performing analysis and interpreting results

Laboratory work

The laboratory work was conducted at NTNU in Norway and at CFTRI in India on both material from herring and surimi. Some of the analysis were conducted by SINTEF Ocean in Trondheim. The materials and methods are divided according to the analysis conducted on each material.

3.1 Herring

The laboratory work on the herring hydrolysate was conducted in the fall of 2018 at NTNU Kalvskinnet and at CFTRI in the spring of 2019. Some work was also conducted in the spring of 2019 at NTNU Gløshaugen after the return from India. The FPH from herring was fractionated by UF and the different fractions were analyzed on lipid content, protein content, antioxidant assays and molecular weight distribution. Figure 6 illustrates a flow chart over the laboratory work on the herring hydrolysate.

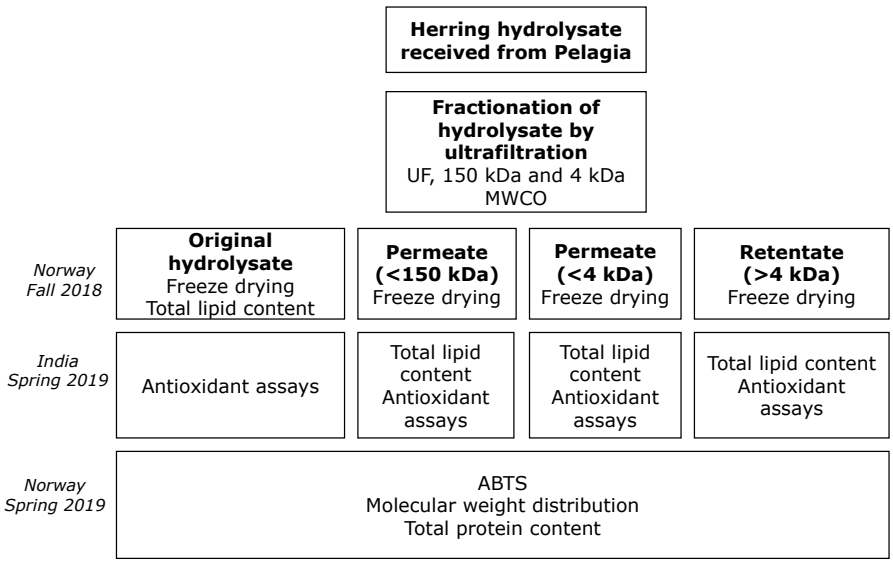


Figure 6. Flow chart of the herring hydrolysate received from Pelagia.

3.1.1 Raw material

The raw material from Atlantic herring (*Clupea harengus*) was received from the company Pelagia Bodø Sildeoljefabrikk AS, which is based in Norway. One batch of FPH was received November 21st 2018, consisting of two 1.5L containers of frozen liquid FPH from herring. The hydrolysate was produced at the factory in Bodø on November 19th 2018. The hydrolysate was produced following the process in figure 4.

3.1.2 Fractionation of hydrolysate by ultrafiltration

Membrane filtration is a technique that can be used to concentrate, refine, extract or fractionate peptides of specific molecular sizes in FPH (Bourseau et al., 2009). The hydrolysate received from Pelagia was fractionated by UF (Triple System, MMS) in collaboration with fellow students Sara Aakre and Kristin Brustad at NTNU Kalvskinnet. The received hydrolysate was stored in a freezer at -20°C and was thawed in a fridge over night before fractionation. The hydrolysate had a high viscosity and was diluted in the ratio 200 g hydrolysate to 400 g distilled water.

First, a ceramic membrane (28 cm^2) with Molecular Weight Cut Off (MWCO) of 150 kDa was used (TAMI Industries) for coarse filtration of the hydrolysate. Hydrolysate solution (600 mL) was poured in the feed tank and filtered through the system. The filtration was carried out until a dead volume of approximately 50 mL was left in the feed tank. This volume was left in the tank to prevent the pumps from drying out. The filtration resulted in two fractions: >150 kDa (retentate) and <150 kDa (permeate). The permeate from the coarse filtration at 150 kDa was then filtrated on a 4 kDa flat sheet membrane (NADIR UH004, Microdyn Nadir) consisting of three cells of 28 cm^2 (total area of 84 cm^2). This filtration resulted again in two fractions: >4 kDa (retentate) and <4 kDa (permeate). The cells in the membrane system was parallel coupled, which means that the permeate is all one equal sample, and the three membranes used had the same MWCO. Fractionation of the hydrolysate is illustrated in figure 7.

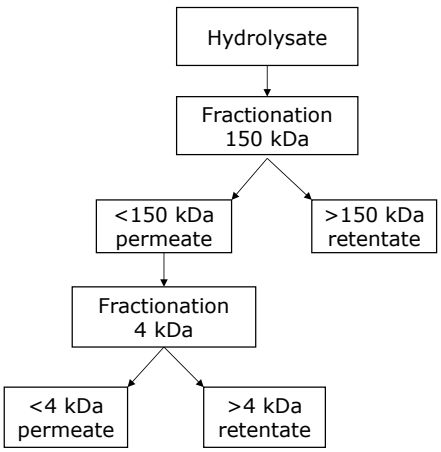


Figure 7. Illustration of UF of the hydrolysate from Pelagia.

The original hydrolysate and the obtained fractions were stored at -80°C for approximately 24 hours before freeze drying (FreeZone 12l Cascade Console Freeze Dry System) for approximately 72 hours. The samples were then transferred to separate zip-lock bags, followed by vacuum packing and stored at -20°C until the samples were brought to India for further analyses.

After UF, the original hydrolysate and the three different fractions were labelled as presented in table 4.

Table 4. Labelling of samples after ultrafiltration.

Sample	Name
Original hydrolysate	OH
Retentate >150 kDa	R150
Retentate > 4 kDa	R4
Permeate < 4 kDa	P4

3.1.3 Determination of protein content

The Bio-Rad method, based on the method described by Bradford (1976) is a rapid and sensitive method for determining the concentration of solubilized protein. The method is based on the principle that maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when the color binds to protein.

Determination of the protein content was carried out in collaboration with fellow master student Sara Aakre at NTNU Gløshaugen. First, all samples were diluted 100 mg/9 mL distilled water in 15 mL test tubes, and the color reagent (BIO-RAD, protein dye reagent concentrate) was prepared by mixing 1 part concentrate with 4 parts distilled water. The standard, Bovine Gamma Globulin (BGG (1,5mg/mL)) were pipetted according to table 5 into 15 mL test tubes.

Table 5. Pipetting for standard curve using BGG

	Blank	1	2	3	4	5
μL standard	0	20	40	60	80	100
μL water	100	80	60	40	20	0

100 μL of the diluted samples were pipetted into 15 mL test tubes. For both the samples and the standards, 5 mL of the diluted color reagent was added to the test tube and mixed well using a vortex mixer. After 5 minutes the absorbance was measured at 595 nm (Genesys 10S UV-Vis, 2L9U235206, Thermo Fischer). 100 μL water mixed with 5 mL diluted color reagent served as blank. The samples and standards were measured in triplicates.

3.1.4 Determination of molecular weight distribution of peptides

For determination of the molecular weight distribution of the peptides in the samples, size exclusion chromatography with an ÄKTA Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare Life Sciences, Uppsala, Sweden) was used, following an internal procedure for NTNU. By the use of FPLC the molecular weight of the different peptides in a sample can be determined based on the retention time through a column. The smaller peptides the longer retention time. This analysis was carried out in collaboration with fellow student Sara Aakre at NTNU Gløshaugen.

All samples (0.1 g) were dissolved in 4 mL sodium acetate buffer (0.05 M, pH 5 and filtered through 2 μm filter). The dissolved samples were then filtered through a sterile 2 μm filter, and approximately 0.8 mL (maximum 1 mL) sample was injected into the FPLC machine using a syringe and the full program was started. For the separation of the samples a Superdex Peptide 10/300 GL column was used. A wavelength of 280 nm, and a flow rate of 0.5 mL/minute was used. All the samples were run separately. The column was able to separate peptides with molecular weights ranging from 100 to 7000 Da.

The results were obtained in Excel as read absorbance at 280 nm (mAU) at different time intervals (min). The raw data were plotted in Excel, resulting in a chromatogram. The peaks in the chromatogram represent peptides in the samples. The standard proteins vitamin B12 (kDa=1.4) and aprotinin (kDa=6.5) were used for comparison.

3.1.5 Determination of total lipid content

The Bligh & Dyer method is a rapid method for extraction and purification of total lipid content from biological materials with the use of methanol, chloroform and water.

The total lipid content in the original hydrolysate received from Pelagia (OH) was analyzed using a modified version of the Bligh & Dyer (1959) method. The analysis of OH was conducted at NTNU Gløshaugen in collaboration with fellow students Sara Aakre and Kristin Brustad, with the assistance of Oskar Speilberg. 5-10 g sample was weighed, and 16 mL H_20, 40 mL methanol (MeOH) and 20 mL chloroform ($CHCl_3$) were added and homogenized with an ultra turrax (IKA T25 digital) for 2 minutes on ice. 20 mL $CHCl_3$ was then added and homogenized for 40 seconds, followed by the addition of 20 mL H_2O and homogenization for 30 seconds. The sample was then centrifuged for 15 minutes.

The bottom phase (chloroform phase) was pipetted out. To determine total lipid content, 2 mL of the chloroform phase was transferred to a test tube, and placed on a heating block (60°C, PIERCE Model 18780 Reacti-Vap) with a steam of N_2 – gas for evaporation. The sample was cooled in a desiccator (1 hour), weighed and % lipid content was calculated by the following equation:

$$\%\,Total\ lipid\ content = \frac{evaporated\ fat\ (g) * added\ chloroform\ (mL)}{evaporated\ chloroform\ (g) * weighed\ sample\ (g)} * 100\ \%$$

The analysis of the rest of the herring samples (R150, R4 and P4) was conducted by Kristin Brustad at CFTRI in India.

3.1.6 Antioxidant activity assays

Before analysis of antioxidant activity assays in India, all the samples were defatted after recommendation by supervisors in India. The freeze-dried samples (2 g) were dissolved in hexane (25 mL) on a shaker over night. The samples were then filtrated (Whatman Qualitative circles, 150 mm, Cat no. 1001-150, China), and the solid sample material was put in a heating cabinet (Heraeus W. C. Heraeus GMBH, Germany) at 50°C for 30 min. The dry samples were then stored in a freezer at -20°C until further analysis. The defatted dry samples were then dissolved 10 mg/mL in distilled water, centrifuged (Eppendorf centrifuge 5804 R, Hamburg) for 20 min at 8 500 x g and the supernatant was transferred

to a new test tube for analysis. The diluted samples were stored in a fridge for up to one week.

ABTS radical scavenging activity

2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is a method to measure radical scavenging activity and can be used for water-soluble and lipid-soluble antioxidants, pure compounds and food extracts (Re et al., 1999). The most common colorimetric method used for ABTS is based on the reduced ABTS being oxidized to a blue/green, positively charged ABTS. The blue/green ABTS is reduced to its original colorless state when it gets mixed with another substance that can be oxidized (Erel, 2004). The decolorization of ABTS is measured which makes it possible to quantify the concentration of the reduced radicals by the antioxidant.

The ABTS was performed both at CFTRI and at NTNU on all the samples. The ABTS performed in India was done following the method described by Sowmya, R. and Sachindra, N.M. (2012). The decolorization of the ABTS radical solution was started by mixing 10 μL sample and 15 μL methanol with 250 μL ABTS solution in a 96-well microplate. The ABTS solution was prepared by mixing 25 mL Acetate buffer (pH 4.5, 0.05 M), 1.25 mL ready ABTS solution and 4 μL peroxidase and kept at 37°C for 15 hours in the dark. For the sample blank, 10 μL sample and 15 μL methanol was mixed with 250 μL acetate buffer (pH 4.5, 0.05 M). 25 μL methanol mixed with 250 μL ABTS solution served as control, and 25μL methanol mixed with 250 μL acetate buffer served as blank. The samples were then left for incubation (Pisces Reevo SL 3000, India) for 1 hour at 37°C before the absorbance was measured (Tecan Spark 10M) at 405 nm. The analysis was conducted in triplicates. The following equation was used to calculate the scavenging activity:

$$\%\,Scavenging\ activity = \frac{control - (sample\ test - sample\ blank)}{control} * 100$$

The ABTS radical scavenging activity was supposed to be presented as TBHQ equivalents, using a premade TBHQ standard curve, however this was never received.

The same samples were analyzed at NTNU after the return from India, following the method described by Re et al. (1999), Nenadis et al. (2004) and Nenadis et al. (2007). For preparation of the ABTS solution, 25 mL 7 mM ABTS solution and 440 μL 140 mM potassium persulfate ($K_2S_2O_8$) were mixed, covered with foil and left to react in room temperature over night. The reaction mixture was diluted with 80 % methanol until the absorbance was 0.75 ± 0.05 at 734 nm, with water as reference. For preparation of the Propyl Gallate (PG) stock solution, 0.53 g of PG powder was dissolved in 250 mL 80 % methanol. A series of 5 dilutions from the PG solution and methanol was made with a concentration of 10-50 μM and used as standards. For analysis of the standards and the samples, 2 mL ABTS was mixed with 200 μL PG or sample (0.1 g/4.5 mL distilled water) and incubated for 6 minutes at room temperature before the absorbance was measured (Genesys 10S UV-Vis, 2L9U235206, Thermo Fischer) at 734 nm using water as reference. 80 % methanol instead of PG/sample served as blank. The standards and samples were measured in triplicates. The radical scavenging activity was represented as PG equivalents.

DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a well-known method to measure antioxidant activity. DPPH is a free radical due to its spare electron delocalization. With a maximum absorbance at 520 nm, the delocalization causes a deep violet color. When DPPH solution is mixed with a substrate that donates a hydrogen atom, a stable non-radical form of DPPH (1,1-diphenyl-2-picrylhydrazine) is obtained. This can be seen by a change of color from violet to yellow (Szabo et al., 2007) (Molyneux, 2004).

For determination of DPPH radical scavenging activity, the method described by Duan et al. (2006) was used. All the samples were analyzed at CFTRI. For the sample test 100, 200, 300, 400 and 500 μL of each sample was mixed with methanol (Merck, India) to a total volume of 2 mL, and further mixed with 2 mL premade DPPH solution. Sample blank was prepared by replacing the DPPH solution with methanol to a total volume of 4 mL. 2 mL methanol mixed with 2 mL DPPH solution served as control. 4 mL methanol served as blank. The samples were then incubated at 37°C for 1 hour in the dark before absorbance was measured (Hitachi U-2900 spectrophotometer) at 517 nm. The samples were measured in duplicates. The following equation was used to calculate the scavenging activity:

$$\%\,Scavenging\ activity\ = \frac{control - (sample\ test - sample\ blank)}{control} * 100$$

The calculated % scavenging activity for the samples were plotted against values of a standard protein given by one of the PhD students at CFTRI and the Effective Concentration of protein for 50 % scavenging (EC_{50}) was calculated.

The DPPH radical scavenging activity was supposed to be presented as TBHQ equivalents, using a premade TBHQ standard curve, however this was never received.

Nitric oxide scavenging activity

The nitric oxide scavenging activity was performed in a modified method as described by Tsai et al. (2007). All samples were analyzed. 50 μL sample, 450 μL Phosphate buffer saline (PBS, pH 7.4) and 500 μL Sodium Nitroprusside (SNP, 10mM) were added to a test tube and put for incubation (Pisces Reevo SL 3000, India) at 32°C for 1 hour under light. After incubation, 1 mL Griess reagent was added to measure nitric oxide scavenged in terms of nitrite present. Griess reagent consisted of Griess reagent A (1% sulphanilamide (5 g) in 5% phosphoric acid (25 mL)) + Griess reagent B (0.1% napthalamine dihydrochloride) (1:1). Reagent A and B were prepared fresh and mixed right after incubation to get the Griess reagent. The samples were measured (Hitachi U-2900 spectrophotometer) in triplicates at 540 nm. The following equation was used to calculate the scavenging activity:

$$\%\,Scavenging\ activity\ = \frac{control - (sample\ test - sample\ blank)}{control} * 100$$

The nitric oxide scavenging activity was supposed to be presented as α-tocopherol equivalent, using a premade α-tocopherol standard curve, however this was never received.

3.2 Surimi

The laboratory work was conducted at CFTRI in the spring of 2019. Some work was also conducted in the spring 2019 at NTNU Gløshaugen after the return from India. The RRM received from the surimi factory was characterized, enzymatic hydrolysis was carried out and degree of hydrolysis, protein content, lipid content, molecular weight distribution, amino acid composition and antioxidant activity assays were investigated. Response surface methodology was also used. A flow chart over the surimi RRM is presented in figure 8.

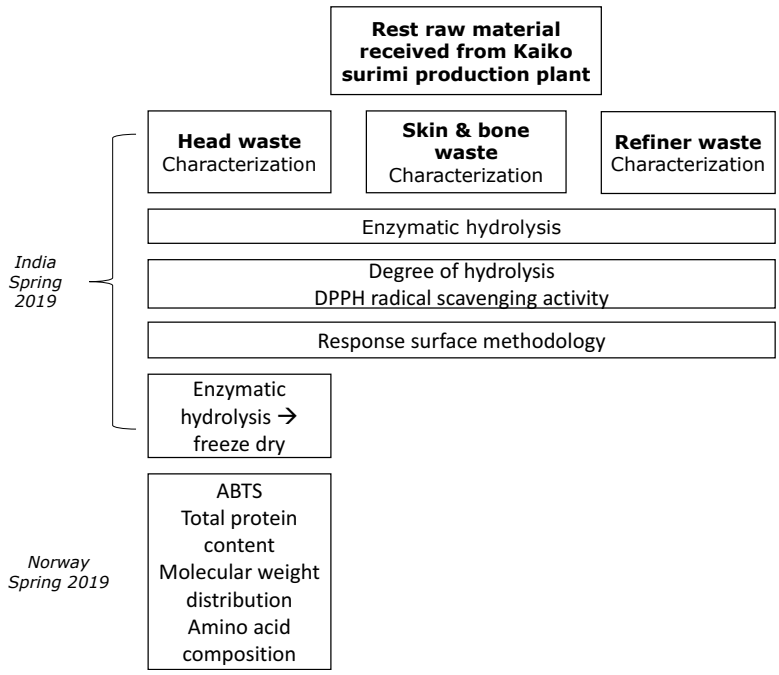


Figure 8. Flow chart of the rest raw material received from Kaiko surimi production plant.

3.2.1 Raw material

The RRM from production of surimi was received January 23rd 2019 from Kaiko surimi production plant which is based in Mumbai, India. The material was transported by plane from Mumbai to Bangalore, and then transported from Bangalore to Mysore by truck for approximately 4 hours. The material was received in frozen condition, but considering the known challenges with the cold chain in India, the raw material might have been exposed to heat and thawing somewhere along the cold chain. When received at the lab at CFTRI, the material was put in a -20°C freezer. Three different fractions were received; 1) head waste (3 kg), which included head and viscera, 2) skin and bone (3 kg) and 3) refined waste (2 kg) which consisted of connective tissue of the fish, small scales, small bones and some meat, illustrated in figure 9.

Figure 9. Thawed raw material received from Kaiko surimi production plant. Head waste, skin and bone waste and refined waste. 3 kg head waste, 3 kg skin and bone waste and 2 kg refined waste was received.

Labelling of the different fractions are presented in table 6. The raw material mainly consisted of RRM from the lean fish species commonly known as pink perch (*Nemipterus sp.).*

Table 6. Labelling of samples received from Kaiko surimi production plant.

Sample	Name
Head waste	HW
Skin and bone waste	SBW
Refined waste	RW

3.2.2 Characterization of the raw material

The moisture, fat, protein and ash content was determined for the different fractions of the raw material received from Kaiko surimi plant. The different fractions were thawed in water bath for approximately 90 minutes and minced separately using a vertical cutter (robot coupe R 10 serie 5) followed by a blender (Prestige PRO 250) to get the masses as homogenous as possible prior to the analysis.

Moisture

Moisture content was determined by the AOAC (1990) method. First, a glass petri dish was weighed, then 5-15 g sample from each fraction was weighed and placed on the petri dish before incubation at 105°C over night. The weight of the petri dish and the dried sample was measured the next day. The measurements were conducted in duplicates. The moisture content was calculated by the following equation:

$$\%\,Moisture = \frac{total\ weight - final\ weight}{sample\ weight} * 100$$

The calculated moisture content was used to calculate dry matter of the sample.

Fat

To determine the fat content, the Soxhlet method following AOAC (1990) was used. First the empty round bottom flask was weighed, followed by weighing of the sample on a filter paper. The sample was wrapped in the filter paper and placed in the extraction tube. The tube was then filled with petroleum benzene boiling range 60-80°C (Merck, India) until the solvent had circulated for one round in the Soxhlet extraction unit (Quest International). The extraction tube was then filled until the sample was covered with solvent, and plugged loosely with cotton. The temperature was set at 60°C and the reaction was left for 6 hours. After 6 hours, the round bottom flask was left for incubation at 105°C overnight. The round bottom flask was cooled before weighing, and the fat content was calculated using the following equation:

$$\% \, Fat = \frac{weight\ flask\ after\ drying - weight\ empty\ flask}{sample\ weight} * 100$$

N-protein

For determining the total protein content, the Dumas (1831) method was used, which is a method that measures total nitrogen in the sample. The dried sample from measuring moisture content was crushed into a powder and 50 mg powder was weighed accurately and wrapped in aluminum cups. The rest of the analysis was carried out by a staff member at Central Instruments Facility & Services (CFS) at CFTRI. The detected nitrogen content was then multiplied with a nitrogen to protein conversion factor of 6.25.

Ash

Determination of ash was conducted following the AOAC method (1990). To determine the ash content in the raw material, the crucible was first weighed, followed by addition of 0.5-1 g sample and the crucible was placed in a desiccator. The samples were charred on a gas burner until only ash was left in the crucible. The samples were then placed in a muffle furnace at 550°C for 5 hours. The furnace was turned off and the samples were left in the furnace over night to cool off. The analysis was performed by Tharak Trivans. The analysis was performed in duplicates, and the ash content was calculated using the following equation:

$$\% \, Ash = \frac{final\ weight\ crucible - empty\ weight\ crucible}{sample\ weight} * 100$$

3.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis was conducted on the RRM received from Kaiko surimi production plant. The different fractions (HW, SBW and RW) were thawed in water bath for approximately 90 minutes and minced separately using a vertical cutter (robot coupe R 10 serie 5) followed by a blender (Prestige PRO 250) to get the masses as homogenous as possible. The minced masses were then transferred to three smaller plastic bags of 50 g for each fraction and stored at -20°C. The remaining masses were stored in bigger plastic bags at -20°C.

An experimental design of the hydrolysis, presented in table 7, was designed by Dr. Sachindra at CFTRI. The experimental design was made with the idea of first conducting a small-scale hydrolysis according to the experimental design, on all three raw materials.

When finishing the hydrolysis, all samples were to be analyzed for degree of hydrolysis (DH) and DPPH radical scavenging activity and the results plotted into a software called STATISTIKA in order to use Response Surface Methodology (RSM) to find optimum conditions for enzymatic hydrolysis for the respective raw materials. The optimum conditions were then to be used on a large-scale hydrolysis under the optimum conditions followed by further analyses.

Table 7. Experimental design enzymatic hydrolysis

	Temp. (℃)	Time (min)	Enzyme (% of raw material)
1	30	30	0.5
2			1.5
3			2.5
4		90	0.5
5			1.5
6			2.5
7		150	0.5
8			1.5
9			2.5
10	45	30	0.5
11			1.5
12			2.5
13		90	0.5
14			1.5
15			2.5
16		150	0.5
17			1.5
18			2.5
19	60	30	0.5
20			1.5
21			2.5
22		90	0.5
23			1.5
24			2.5
25		150	0.5
26			1.5
27			2.5

The small-scale hydrolysis was performed at one temperature at a time, meaning 10 different conical flasks for each hydrolysis. One bag of 50 g sample was first thawed in water bath for approximately 30 minutes. 40 g sample and 120 mL distilled water was added to a beaker and stirred. 10 mL of the mixed sample was then transferred to 10 different conical flasks each. 0.5, 1.5 and 2.5 % Alcalase enzyme, *Bacillus licheniformis*

(LOT: 2920868, Merck, Germany) was then added to the different flasks. One conical flask was left without enzyme. The conical flasks were then incubated at 30, 45 and 60°C for 30, 90 and 150 min.

After the respected incubation times, 1 mL hydrolyzed sample and 1 mL 5 % TCA were transferred to a test tube and centrifuged (Sigma 1-14 K, Germany) for 20 min at 4 500 x g. The supernatant was transferred to a new test tube and put in the freezer at -20°C. 1 mL of the hydrolyzed sample without enzyme was pipetted out of the conical flask into a test tube, and 1 mL 5 % TCA was added. The conical flask was left for incubation for the remaining incubation times. The sample was then treated the same way as the samples with added enzyme. The remaining hydrolysate in the conical flask was transferred to a 15 mL test tube and placed in water bath for inactivation at 90°C for 15 min. The test tube was then centrifuged (Eppendorf 5805 R, Hamburg) for 10 min at 8 800 x g. The supernatant was transferred to a new test tube and put in the freezer at -80°C until further analyses.

The samples inactivated with 5 % TCA was later used for determination of DH and DPPH radical scavenging activity. The samples inactivated by heat was used by fellow master student Sara Aakre for analysis of ACE inhibitory activity.

The large-scale hydrolysis was conducted following the same procedure, under the found optimum conditions (30°C, 30 min, 0.5 % enzyme) based on RSM, and was only conducted on the HW fraction due to lack of time and suitable equipment at the lab at CFTRI. When conducting the large-scale hydrolysis, it was decided to use 500 g raw material to 500 g distilled water, instead of 1:3 like the small-scale hydrolysis. The hydrolysate from the large-scale hydrolysis was freeze-dried (Christ Gamma 2-16 LSC plus) and brought to Norway for further analyses.

3.2.4 Degree of hydrolysis

The trinitrobenzenesulfonic acid (TNBS) method determines the concentration of primary amino groups in the hydrolysate. This is a spectrophotometric method where a chromophore is formed by the reaction of TNBS and primary amines (Adler-Nissen, 1979).

Degree of hydrolysis (DH) was measured on the samples from the small-scale hydrolysis at CFTRI using a modified version of the TNBS method described by Benjakul and Morrissey (1997). 50 μL hydrolyzed sample with added 5 % TCA was transferred to a 96-well plate with 50 μL TNBS reagent and 100 μL of phosphate buffer (pH 8.2). The 96-well plate was covered with foil and incubated at 50°C for 30 min before addition of 100 μL sodium sulphite. Absorbance was then measured at 420 nm (Tecan Spark 10M). The blank sample was prepared using water instead of sample. The same procedure was followed to measure the absorbance of the samples without added enzyme. A premade standard curve for the amino acid leucin was used in the calculations. The DH was calculated using the following equation:

$$\%DH = \frac{L_t - L_0}{L_{max}} * 100$$

Where L_t corresponded to amount leucine at time (30,90,150), L_0 corresponded to amount of leucin in original sample (samples without enzyme) and L_{max} corresponded to the maximum amount of leucin in original sample after hydrolysis (found by oil bath digestion).

To find the L_{max} value, 1 gram of each raw material sample was put in a test tube with 5 mL 6N HCl. The test tubes were capped and kept in oil bath for 24 hours at 105°C. After the oil bath digestion, sample OH and R4 was dried out, so 5 mL HCl was added to each test tube, and the samples were put in water bath at 60°C for 2 hours to dissolve. After the samples had dissolved, they were filtered and the filtrate was measured at 420 nm using the same method as for the rest of the samples.

The calculated %DH was plotted in to STATISTIKA software to determine the optimum conditions for hydrolysis using the Response Surface Methodology (RSM).

3.2.5 Response Surface Methodology

Response Surface Methodology (RSM) is a technique of mathematics and statistics based on how the polynomial equation fits the experimental data. This must describe the behavior of the data set, considering it is supposed to make statistical previsions. RSM can be used when a response or a set of responses are influenced by multiple variables. The goal is to find the levels of these variables that will give the best system performance (Bezerra et al., 2008).

The experimental design of the small-scale enzymatic hydrolysis was plotted in the STATISTIKA software, as well as the results from the analysis of DPPH scavenging activity and %DH after the enzymatic hydrolysis of the different fractions received from Kaiko surimi production plant. This part was conducted by Dr. Sachindra at CFTRI.

Based on the results from DPPH and DH from the small-scale hydrolysis, the software calculated the optimum conditions for enzymatic hydrolysis based on the combinations in the experimental design (table 7) of the enzymatic hydrolysis. The independent factors considered for optimization included time, temperature and enzyme concentration, while the DPPH radical scavenging activity and %DH was the dependent factors.

The results in terms of optimum conditions for enzymatic hydrolysis was later used to make a bigger batch of hydrolysate, under the given conditions. It was only made a big batch of hydrolysate from HW, due to limited time and equipment.

3.2.6 Determination of protein content

Determination of the protein content was only performed on the fish protein hydrolysate from HW from the large-scale hydrolysis. This was performed at NTNU together with the herring samples in collaboration with fellow master student Sara Aakre. See chapter 3.1.3 for procedure.

3.2.7 Determination of molecular weight distribution of peptides

Determination of the molecular weight distribution in the freeze-dried hydrolysate from HW was carried out in collaboration with fellow master student Sara Aakre at NTNU Gløshaugen together with the herring samples. See chapter 3.1.4 for procedure.

3.2.8 Determination of amino acid composition

The amino acid composition of dried raw material, dried sediments from the hydrolysis and freeze dried FPH from HW was analyzed by a High Performance Liquid Chromatography (HPLC) system (Agilent Infinity 1260, Agilent Technologies). The HPLC system was coupled to an on-line-post-column derivatization module (Pinnacle PCX, Pickering laboratories, Mountain View, CA, USA) using a Na^+-ion exchange column (4.6 x 110 mm, 5 mm) and nynhydrin (Trione) as derivatising reagent. Standard curves measured with amino acid standards were used to quantify standard amino acids. The analysis was conducted at SINTEF Ocean Trondheim by Rasa Slizyte.

3.2.9 Antioxidant activity assays

ABTS radical scavenging activity

The ABTS radical scavenging activity was only performed on the freeze-dried fish protein hydrolysate from HW from the large-scale hydrolysis. This was performed at NTNU together with the herring samples. See chapter 3.1.6 for procedure.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined for the three different fractions received from Kaiko surimi factory, and was determined for all the 27 different conditions in the experimental design from the small-scale enzymatic hydrolysis.

For analysis of DPPH radical scavenging activity a modified version of the method described by Duan et al. (2006) was used. This modified method was used due to the large amount of samples, and was modified for the use of a 96-well plate. Sample material used was the hydrolysate that had been added 5 % TCA to inactivate the enzymes after hydrolysis. For sample test, 10 μL sample, 90 μL methanol and 100 μL DPPH solution was added to the well. Sample blank was prepared by replacing the DPPH solution with methanol to a total volume of 200 μL in the well. 100 μL methanol and 100 μL DPPH solution served as control, and 200 μL methanol served as blank. The samples were then incubated at 37°C for 30 min in the dark. After incubation the absorbance was measured (Tecan Spark 10M) at 517 nm. To calculate the scavenging activity the following equation was used:

$$Scavenging\ activity\ (\%) = \frac{control - (sample\ test - sample\ blank)}{control} * 100$$

The calculated % scavenging activity was plotted in to STATISTIKA software to determine the optimum conditions for hydrolysis using RSM.

Qualitative analytical tools

Guidelines on Health, Security and Environment (HSE) differs in Norway and India, and as a result to this and the fact that the laboratory work didn't go as planned in India, the course of the thesis had to be readjusted, and it was decided to make a questionnaire on the topic of utilization of marine RRM in India. The questionnaire was made together with fellow student Kristin Brustad and the supervisors in Norway.

A questionnaire is a qualitative analysis, and can in this situation be seen as an exploratory study. An exploratory study basically means that along the way in a project, new insight is gained, and the project may change (De nasjonale forskningsetiske komiteene, 2010). Issues and strategy can adjust the project and its progress, and may lead to reduced validity and reliability in the results, which is basically what happened during the work with this project. The questionnaire was distributed to Dr. Sachindra, Dr. Ajay, Dr. Kudre and Dr. Rathina at CFTRI, and to Dr. Nutan and her PhD students at Amity university, as well as daily manager Asif Naik at Kaiko surimi production plant, which are all partners in the ReFOOD and ReValue projects. The questionnaire was also distributed to the researchers at SINTEF Ocean that are part of the projects.

4. Results and discussion

Fieldwork

Attending the workshop in Mumbai, visiting the surimi factory, attending the conference in New Delhi, the exchange at CFTRI and constant communication with partners and supervisors in the ReFOOD and ReValue projects has been good tools to gain knowledge and information to contribute to this study. The fact that there are a lot of money put into research projects like these, and the Norwegian Prime Minister participating on conferences in India on the topic shows the importance of exchange of knowledge and research in this field.

The exchange at CFTRI in India took place as an observational study with both passive and active participation. The passive participation involved the experience of observing how the students and system at CFTRI works, while the active participation included working at the laboratory, going to the fish market, talk to the locals and student to get their perspective on RRM and laboratory work. By conducting laboratory work in India, it became clear that there is a big difference on how the practice works. In Norway, students are used to following procedures based on research literature describing exactly how to perform the analysis to get the strongest scientific results. For most of the analyses conducted in India there was internal procedures that was followed. Most of the procedures were notes in the PhD students' lab journal, with own modifications from other procedures.

Most of the analyses were conducted without the use of parallels, which was perceived as not regarded as a means to obtain most of the accurate and scientifically correct results. Regarding procedures with HSE, it became clear that there are some differences between Norway and India, as Norway is really strict considering HSE. In example, In Norway the use of fume hoods when conducting analyses or handling potentially dangerous chemicals is a matter of course, but as far as experienced these procedures were not followed in India.

The challenges faced in the laboratory at CFTRI became a barrier to conducting the analyses that was originally part of the experimental plan. However, it also led to a lot of new knowledge and experiences about how environmental factors and cultural differences influences the possibility for global cooperation, research and knowledge exchange. This led to the realization that qualitative analyses could be used as a tool to better understand the observed differences.

Laboratory work

4.1 Herring

4.1.1 Fractionation of hydrolysate by ultrafiltration

At least two batches of FPH from Pelagia were supposed to be received in order to compare the eventual variation in the batches, but only one batch was received. As the other batches were not received, the laboratory work was only conducted on one batch.

Fractionation of the herring hydrolysate by UF resulted in 3 different fractions, with water soluble peptides in all fractions, as described in chapter 4.1.3. The decision of using a ceramic membrane first for coarse filtration was done as this has shown to work on seafood streams containing fat, mainly due to hydrophobicity (Torp, 2019). Removing substances in the hydrolysate with larger molecular weight, could also improve the separation using membranes with lower MWCO. The different fractions after filtration with the ceramic membrane (150 kDa) followed by the flat sheet membrane (4 kDa) included retentate (>150 kDa), permeate (<4 kDa) and retentate (>4 kDa). The different fractions, including the original hydrolysate was freeze dried, which resulted in the different products presented in figure 21 in appendix A. OH, R150 and R4 had a completely dry consistency, while P4 were slightly more sticky.

Using a 4 kDa membrane would theoretically yield a retentate containing peptides larger than 4 kDa and a permeate containing peptides smaller than 4 kDa. The cut-off value can however not always dictate the purity of the obtained fractions. A study by Picot et al. (2010) investigated the use of UF and NF on FPH from prolastin, a commercial elastin hydrolysate obtained from North Atlantic lean fish. The study used UF with a MWCO of 4 kDa and NF with a MWCO of 300 Da. The results from the study showed that the UF retentate contained a large amount of peptides below 4 kDa, and the NF retentate contained approximately 22 % of peptides below 300 Da. These results demonstrated that membranes do not separate good enough, as peptides with varying molecular weight was found in several of the fractions. A study by Sabeena Farvin et al. (2014) using UF on FPH from cod got similar results, and found that it may be a result of retention of the small molecules as a result of fouling of the membrane. The small molecules may also have affinity for some of the larger proteins and bind to these during the filtration, resulting in fractions with proteins with a variety of molecular weights. It can be assumed that the concentration of peptides below 4 kDa is higher in the permeate compared to the original hydrolysate after membrane filtration. The molecular weight distribution of peptides of the different fractions was analyzed using FPLC, and will be discussed in chapter 4.1.4.

When evaluating the results of the membrane filtration it was seen that the flux, concentration factor (CF) and exact volumes of the different fractions after the membrane filtration in this study should have been noted in order to amongst other be able to investigate the yield.

4.1.2 Protein content

The Biorad method was used to determine the concentration of water soluble protein in the freeze-dried herring samples. The samples had been fractionated by UF, resulting in slightly different protein content in the different fractions. The protein content in the freeze-dried samples were found to range from 74.44-90.16 %, as presented in table 8.

Table 8. Protein content in freeze-dried herring samples. OH: original hydrolysate, R150: retentate >150 kDa, R4: retentate >4 kDa, P4: permeate <4 kDa.

Sample	Protein content (%)
OH	78.52
R150	74.44
R4	90.16
P4	00.00

R4 was found to have the highest protein content of 90.16 % suggesting that most protein is water soluble. The values of OH and R150 are also fairly high (78.52 and 74.44 %). For sample P4, protein content was not detected. This might be due to the size of these peptides, which in theory should be below 4 kDa, and the sensitivity of the method used. The fact that the content of water soluble protein increases by the use of membrane filtration, especially when using the 4 kDa membrane, the use of membrane filtration could be a good technology for the industry to implement in their process in order to obtain a product of higher value.

The Biorad method is known to be simpler, faster, more sensitive and subject to less interference than the Lowry method (Kruger, 2009). However, Seevaratnam et al. (2009) conducted a comparative study of total protein concentration in skeletal muscle using the Biorad/Bradford and the Lowry method. The study found that the Biorad method is unable to detect peptides with a molecular weight lower than 3-5 kDa, but is subject to less interference than the Lowry method. The Biorad method has the disadvantage that binding of the dye to the proteins depends on the size of the protein and the amino acid composition, and will thereby not bind to smaller peptides and amino acids (Lucarini and Kilikian, 1999). This might explain why the protein content in P4 was not found. It would possibly be better to use the Lowry method for determination of protein in FPH.

4.1.3 Molecular weight distribution of peptides

To determine the molecular weight distribution of the peptides in the samples, the SDS-PAGE method was first attempted at CFTRI. The samples didn't separate in the gel, and didn't give any useful results. It was then decided to determine the molecular weight distribution of the herring samples by gel filtration using the FPLC system at NTNU Gløshaugen. Determination of the molecular weight distribution of the samples were performed in order to characterize the size of the peptides in the samples. It was also of interest to study the molecular weight distribution of the herring samples as these had been separated by UF.

The size of the peptides in a hydrolysate will affect the sensory properties, as well as its bioactive, nutritional and physiochemical properties. Smaller peptides are known to have increased solubility, bitterness and bioactive properties, while larger peptides are necessary for the emulsifying and foaming properties (Gbogouri et al., 2004) (Halim et al., 2016).

The column used for gel filtration has pores of specific sizes, and peptides of small molecular sizes can fit in these pores. Peptides of larger molecular size will go right through the column, and be eluted before the smaller peptides. The Superdex Peptide 10/300 GL column that was used separates peptides of molecular weights in the range of 100-7000 Da. The UV detector then detects the peptides at 280 nm, as presented in figure 10.

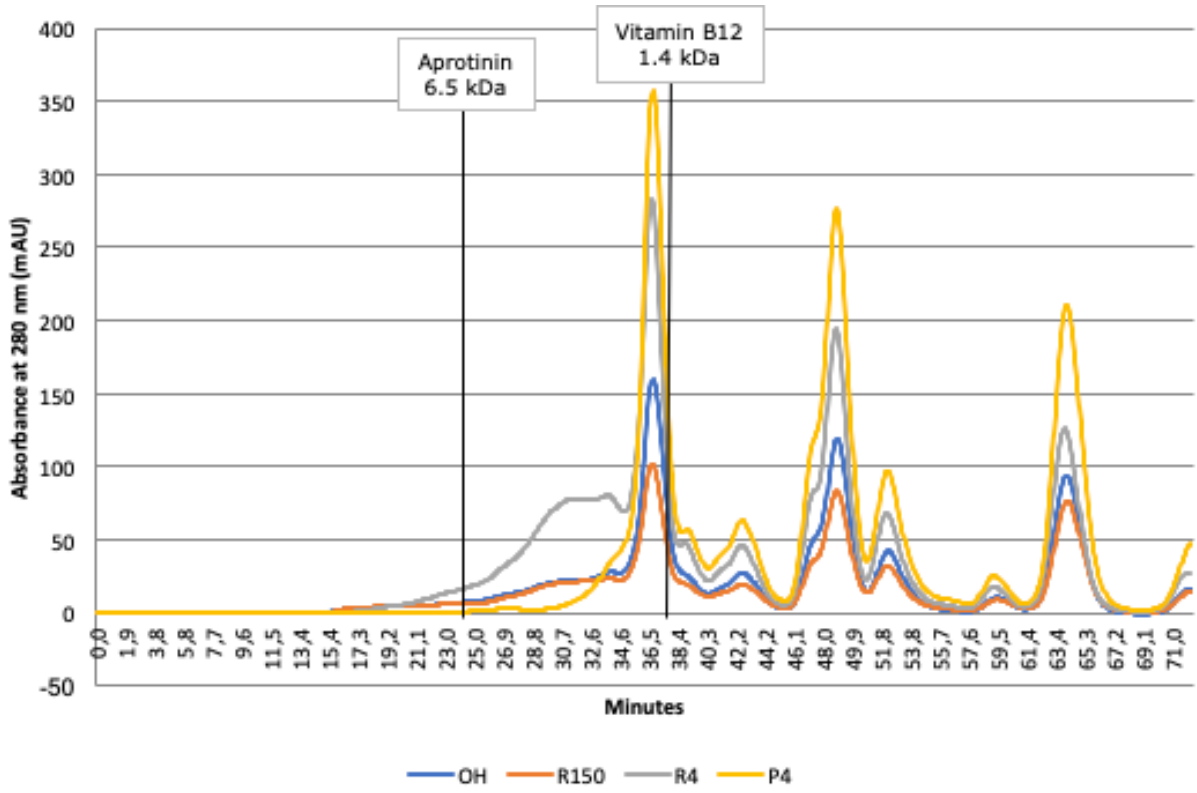


Figure 10. Chromatogram showing the distribution of molecular weight in peptides in the herring samples compared to known standard proteins.

In order to compare the size distribution profiles of the samples, the profiles were compared to two known standard proteins with known molecular weight. The standard proteins used were vitamin B12 (1.4 kDa) and aprotinin (6.5 kDa). The standard proteins were plotted in Excel separately, and the peaks were marked as vertical lines in the chromatogram in figure 11.

The highest absorbance for all samples are at approximately 35 minutes into the gel filtration in the FPLC system. This peak is between aprotinin and vitamin B12, and pretty close to vitamin B12 which has a molecular weight of 1.4 kDa. This indicates that all the samples contain peptides with a molecular weight slightly larger than 1.4 kDa. There is also a broad distribution of peptides smaller than 1.4 kDa, which may indicate efficient hydrolysis of the raw material, and the possibility of the hydrolysate to possess desirable bioactive properties. Even though smaller peptides are known to possess desirable

bioactive properties like antioxidant activity, they are also known to have a bitter taste, which is not desirable if the product is supposed to be used for human consumption (Cho et al., 2004) (Hevia and Olcott, 1977).

The distribution of peptides in the different samples are fairly similar, which suggests that the samples consists of pretty much peptides of the same molecular weights, and the fact that all samples contains water soluble peptides, as discussed in chapter 4.1.2. There is a trend of R150 having the lowest absorbance and P4 having the highest absorbance at all peaks. The reason R150 has the lowest absorbance may be because the fact that this fraction was supposed to contain peptides >150 kDa. P4 on the other hand, had the highest absorbance which indicates the fact that this fraction was supposed to contain peptides <4 kDa. Considering all the samples having the same peaks supports the literature (Picot et al., 2010) (Sabeena Farvin et al., 2014) that membrane filtration does not separate peptides sharp enough.

4.1.4 Lipid content

The lipid content in the samples (dry weight) were analyzed using the Bligh & Dyer method. OH sample was analyzed at NTNU Gløshaugen, while the remaining samples were analyzed by fellow master student Kristin Brustad at CFTRI. The samples were found to range from 0.48 – 20.54 %, and are presented in table 9.

Table 9. Lipid content (dw %) in the herring samples. OH: original hydrolysate (n=6, ± SD), R150: retentate >150 kDa, R4: retentate >4 kDa, P4: permeate <4 kDa

Sample	Lipid content (dw %)
OH	13.80 ± 0.29
R150	20.54
R4	1.13
P4	0.48

When evaluating the results, it has to be taken into consideration that the results for R150, R4 and P4 are very uncertain, as the analysis was not conducted on ice, which can lead to evaporation of the chloroform. This will affect the measured volume of the solution, and may lead to an inaccurate result from the calculation. The analysis of R150, R4 and P4 were not conducted in parallels, which also reduces the reliability of the results.

The results showed that OH had a fairly high lipid content of 13.80 % in dry weight. According to Pelagia, the received hydrolysate was only supposed to contain approximately 0.3 % lipid in wet weight. Considering the result showing a lipid content of 13.80 % in dry weight it may indicate that the processing step with the tricanter and separation at the Pelagia factory isn't good enough. It is generally accepted that FPH should have a lipid content lower than 0.5 % (dry weight) in order to be stable and prevent lipid alteration during storage (SPINELLI et al., 1972). Stability is an essential property if the FPH is supposed to be used as functional ingredients in foods.

Taking the uncertainties with sample R150, R4 and P4 into consideration, R150 has a lipid content of 20.54 %. The lipid content is higher in R150 than OH due to being more concentrated as a result of the UF. R150 is the retentate >150 kDa and it is reasonable to

believe that most of the lipids got separated into this fraction by the UF. With lipid contents of 13.80 and 20.54 %, OH and R150 are unfit considering production of high quality FPH. R4 and P4 contained a much lower lipid content, 1.13 and 0.48 % respectively, than OH and R150 and it is clear that most of the lipids ended up in R150 after UF. In a study by Slizyte et al. (2005) it was found that the higher lipid content in the raw material for the hydrolysis, the higher lipid content in the FPH. As herring is a fatty fish, this may support the results of the lipid content. Based on the results in this study, the use of UF at 4 kDa separated most of the lipids from the FPH, and membrane filtration may be a good tool for separation of lipids in FPH.

4.1.5 Antioxidant assays

ABTS radical scavenging activity

The ABTS results from India are only presented as calculated % scavenging activity due to lack of the premade TBHQ standard curve. The calculated % scavenging are presented in table 10. % scavenging activity was found to range from 9.85 – 25.16 % with R150 having the lowest, and R4 having the highest scavenging activity.

Table 10. Calculated % scavenging activity of the herring samples (n=3, ± SD). OH: original hydrolysate, R150: retentate >150 kDa, R4: retentate >4 kDa, P4: permeate <4 kDa.

Sample	% Scavenging activity
OH	19.10 ± 0.00
R150	9.85 ± 0.02
R4	25.16 ± 0.01
P4	17.45 ± 0.00

The expected results would be an increase in % scavenging activity from OH to P4. R4 and P4 are of smaller molecular weight than OH and R150, and peptides of small molecular weight are shown to possess antioxidative properties (Chalamaiah et al., 2012). One explanation for the high value of scavenging activity obtained for OH could be the inherent color of the hydrolysate. Since ABTS radical scavenging activity is determined spectrophotometrically, the color components of OH could have interacted and contributed to incorrect values.

The samples were reanalyzed at NTNU Gløshaugen after the return from India, and the ABTS radical scavenging activity is presented as PG equivalents in table 11.

Table 11. Calculated antioxidant content (μmol/g) in the herring samples as PG equivalents (n=3, ± SD). OH: original hydrolysate, R150: retentate >150 kDa, R4: retentate >4 kDa, P4: permeate <4 kDa.

Sample	Antioxidant content (μmol/g)
OH	-0.03 ± 0.00
R150	-0.70 ± 0.03
R4	23.85 ± 0.01
P4	18.28 ± 0.01

The absorbance values of OH and R150 were outside of the standard curve, and no antioxidative activity were detected by the ABTS method for these two samples. The scavenging activity and antioxidant content was found to be highest in R4 in both the analysis conducted in India and in Norway.

DPPH Radical scavenging activity

The DPPH radical scavenging activity of the herring samples were analyzed at CFTRI, and determined as an EC_{50} value (effective concentration for 50 % scavenging). The % scavenging activity was calculated and plotted against values of a standard protein given by one of the PhD students at CFTRI in order to get a standard curve and an equation for calculating how much protein is necessary to get 50 % scavenging. Results are presented in table 12.

Table 12. Protein concentration (μg) necessary for 50 % scavenging. OH: original hydrolysate, R150: retentate >150 kDa, R4: retentate >4 kDa, P4: permeate <4 kDa.

Sample	Protein concentration (μg) for 50 % scavenging
OH	14.18
R150	12.97
R4	18.03
P4	11.78

In a study by Picot et al. (2010) antioxidant activity in fractionated FPH were studied. DPPH radical scavenging activity was investigated, and the results showed that the FPH had a high DPPH radical scavenging activity, with an EC_{50} value of 24.7 mg/mL in the unfractionated hydrolysate. After fractionation of the FPH, the antioxidant and DPPH radical

scavenging activity got slightly reduced. Another study by Sonklin et al. (2018) on mungbean meal protein hydrolysate found that the fractions after UF had lower EC_{50} value than the original hydrolysate, which indicate that purified peptides of low molecular weight exhibit stronger antioxidant activity. This supports the results obtained in this master study. P4 was found to contain peptides which has the highest radical scavenging activity, with a protein concentration of 11.78 μg necessary for 50 % scavenging.

According to Pihlanto (2006), the antioxidant activity of hydrolysates seems to be correlated to the characteristic amino acid sequences of the peptides, depending on the protease specificity.

Nitric oxide scavenging activity

Nitric oxide scavenging activity were also analyzed at CFTRI, but due to lack of sample blank and the premade α-tocopherol standard curve the scavenging activity could not be calculated.

4.2 Surimi

4.2.1 Characterization of the raw material

To characterize the different raw material received from Kaiko surimi production plant, moisture, fat, protein and ash content was determined. The composition of the raw material is presented in table 13. Dry matter and ash content was determined in duplicates while fat and protein content only had one parallel.

Table 13. Composition of the raw material received from Kaiko surimi production plant. Results are presented in wet weight. Dry matter and ash are presented as n=2 ± SD, fat and protein as n=1. HW: head waste, SBW: skin and bone waste, RW: refined waste.

	Dry matter (%)	Fat (%)	Protein (%)	Ash (%)
HW	21.42 ± 0.14	1.44	12.48	7.04 ± 0.44
SBW	17.33 ± 0.94	0.78	9.46	7.29 ± 0.73
RW	15.41 ± 0.37	0.54	12.07	2.96 ± 0.37

The samples had been frozen prior to the characterization of the raw material, and after thawing in water bath it was clear that it was quite a lot of water in the bags. The plastic bags had not been completely sealed at the surimi production plant, which was not discovered until after thawing of the samples.

The fat content was found to be highest in HW (1.44 %) and lowest in RW (0.54 %). Considering lean fish species, like pink perch store fat in the liver, it makes sense that the HW fraction, which includes viscera, had the highest fat content. It was found a higher protein content in RW (12.07 %) than the two other fractions (9.46 – 12.48 %). These results are reasonable considering RW contained minor pieces of connective tissue and some meat which contains a lot of protein. Ash content was found to be higher in SBW (7.29 %) and HW (7.04 %) than RW (2.96 %) as these fractions had a high content of bones and bone like substances.

4.2.2 Enzymatic hydrolysis

The enzymatic hydrolysis was not conducted under optimal conditions, considering equipment and the circumstances at the lab in India. The raw material was frozen at the time of arrival at CFTRI, but it is likely to believe that the cold chain had been broken somewhere during the transport from Kaiko surimi production plant to Mysore, due to the known challenges with technology and infrastructure in India.

Before homogenization of the raw material it had to be thawed. This was done in water bath with no temperature control. It also appeared to be holes in the plastic bags containing the raw material, which resulted in water sieving into the bag. When homogenizing the raw material, the available equipment didn't homogenize the material sufficiently. This resulted in uneven distribution of raw material and water in the different conical flasks at the beginning of the small-scale. Some flasks got bigger chunks, while others were more homogenized. Considering it was only used an amount of 10 mL in each conical flask for the small-scale hydrolysis, it was hard to get an even distribution, see figure 11. The uneven distribution of raw material in the conical flasks possibly lead to differences in how the enzymes work according to differences in enzyme - substrate ratio. As for the large-

scale hydrolysis, the homogenization was a bit easier due to a larger sample volume, but still not optimal.

Figure 11. Illustration of the uneven distribution of raw material and water in the small–scale hydrolysis, as well as the large-scale hydrolysis of HW. The two conical flasks are from the small-scale hydrolysis of RW, where the flask on the right has a big chunk of raw material while the flask on the left is more homogenous. The big beaker was used for the large-scale hydrolysis of HW.

The amount of water used in the small-scale hydrolysis was a bit questionable. Raw material and water had a ratio of 1:3, as recommended by Dr. Sachindra, but according to studies by Slizyte et al. (2004), Aspmo et al. (2005) and Slizyte et al. (2005) a ratio of 1:1 or 1:0.75 is more optimal as the amount of water should be as low as possible in order to reduce expenses for heating, transportation, evaporation and drying of the hydrolysate in an eventually industrial process. The enzyme concentrations (0.5, 1.5 and 2.5 %) used in the small-scale hydrolysis was also a bit questionable. Enzymes are expensive, and a concentration as low as possible is desirable from an economic point of view. Studies by Slizyte et al. (2004) and Opheim et al. (2015) have used an enzyme concentration of 0.1 % for enzymatic hydrolysis of fish protein. Implementation of enzymatic hydrolysis in the industry will mean large volumes, and a low concentration of added enzymes will be advantageous, taking the economy into account.

During the hydrolysis at CFTRI, the conical flasks were placed on a shaker for continuous stirring. For the small-scale hydrolysis, this shaker was good enough, but for the large-scale hydrolysis the shaker stopped working, and the hydrolysate had to be stirred manually. The fact that the hydrolysate had to be stirred manually also led to fluctuations in the temperature as the shaker was an enclosed instrument with temperature control, and the lid had to be open. However, the shaker it didn't seem to have the best quality as the temperature kept varying throughout the process, even when the lid was closed.

Some challenges concerning separation of the hydrolysate into its different fractions were also met. For the large-scale hydrolysis, a separator (figure 12) was recommended to use due to the large sample volume. After trying the separator, it was decided to use the smaller centrifuge at the lab, both from a hygienic point of view, and the fact that the separator didn't separate the hydrolysate into the desired fractions.

Figure 12. Attempt of separation of the hydrolysate by a separator.

Because the separator didn't work, and the hydrolysate was considered contaminated, a new hydrolysis on HW had to be conducted. It was then decided to use the regular centrifuge for separation of the different fractions of the hydrolysate, although it was a large sample volume and time consuming to centrifuge the hydrolysate. After centrifugation, the oil didn't separate from the protein hydrolysate fraction, which resulted in only sediments and the protein hydrolysate fraction, as illustrated in figure 13.

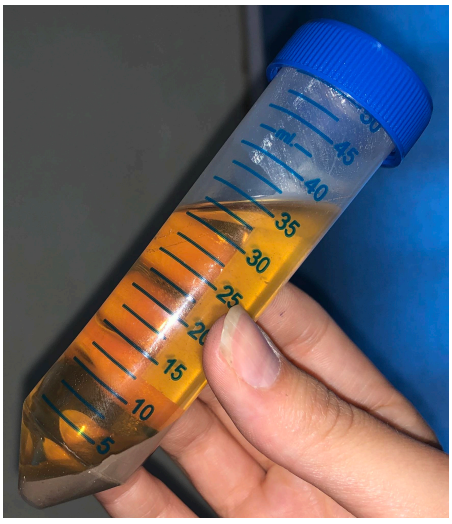


Figure 13. Tube after centrifugation of hydrolysate of HW (large-scale hydrolysis). Separated into sediment and hydrolysate fraction, but no oil fraction.

The reason why the centrifugation didn't result in an oil fraction, may be due to the composition of content in the HW raw material. The HW fraction consisted of heads and viscera, but when the raw material was received, it was observed that it was mostly heads, see figure 9. It may be reasonable to believe that there wasn't enough lipid in the HW raw material to get a good separation of the oil by centrifugation.

The hydrolysate from the large-scale hydrolysis was freeze-dried. Only one liter hydrolysate was able to be freeze-dried, as you needed a slot in order use the freeze-drier, which was booked up to one month ahead by other students. The sediments from the hydrolysis were dried at 70°C over night and brought back to Norway for possible analysis.

After multiple attempts on enzymatic hydrolysis at the lab at CFTRI there are a few points that stand out in terms of an ideal process and succeeding with enzymatic hydrolysis. First of all, the raw material has to be of good quality. This is one of the major threats in India, concerning the infrastructure not being sufficiently developed, which makes the maintenance of the cold chain difficult. By not having a well-functioning cold chain, the raw material will deteriorate fast, as marine RRM are prone to microbiological spoiling.

Once the raw material is of good quality, the technology and equipment necessary for conducting enzymatic hydrolysis has to be in place. This includes equipment for proper homogenization, being able to keep temperature under control during the hydrolysis and proper separation of the hydrolysate after hydrolysis. Knowledge about the raw material, enzymes and under what conditions the respected enzymes work the best is important before starting an enzymatic hydrolysis in order to obtain the best result of the FPH. When conducting the hydrolysis on the RRM received from Kaiko surimi production plant, all fractions contained quite a lot of bones and scales. By not having any experience with these raw materials, and the proper equipment for homogenization of these, it turned out to be a challenge. By experiencing these challenges with enzymatic hydrolysis, it may be easier to work with similar raw material later, as the challenges now are known.

4.2.3 Degree of hydrolysis

Degree of hydrolysis gives an insight in the number of cleaved peptide bonds during hydrolysis, and can be used to get information about the average size of the peptides in the hydrolysate. % DH was determined for all the fractions (HW, SBW and RW) received from Kaiko after the small-scale enzymatic hydrolysis following the experimental design (27 x 3), and the results are presented in table 14.

Table 14. Calculated degree of hydrolysis (%) from enzymatic hydrolysis of the different fractions from surimi rest raw materials. HW: head waste, SBW: skin and bone waste, RW: refined waste.

Temp (°C)	Time (min)	Enzyme (%)	HW DH (%)	SBW DH (%)	RW DH (%)
30	30	0.50	17.94	5.15	2.99
		1.50	20.58	28.18	4.66
		2.50	20.43	23.73	9.78
	90	0.50	14.90	2.49	53.77
		1.50	15.69	30.69	57.10
		2.50	15.11	5.01	56.59
	150	0.50	2.03	40.93	0.68
		1.50	10.83	42.19	5.15
		2.50	5.52	59.48	10.51
45	30	0.50	9.10	45.76	55.94
		1.50	12.36	43.16	55.73
		2.50	11.30	43.99	55.45
	90	0.50	7.27	0.28	53.15
		1.50	7.51	4.23	56.77
		2.50	8.25	12.62	53.45
	150	0.50	0.54	48.34	44.57
		1.50	3.29	52.37	45.12
		2.50	4.06	63.29	48.22
60	30	0.50	8.26	51.11	45.56
		1.50	8.00	49.55	46.66
		2.50	5.72	55.82	46.48
	90	0.50	2.30	46.67	34.46
		1.50	3.20	95.67	35.12
		2.50	8.61	52.29	31.68
	150	0.50	2.32	3.62	42.82
		1.50	0.77	25.17	47.34
		2.50	1.10	19.29	38.12

The % DH was found to have a wide range, and it was hard to find any logic in the results. According to supervisors in India these were good results, and were therefore used in order to obtain RSM graphs. Later on, looking at the results, it is reasonable to believe that the varying results may be due to the multiple possible sources of error in the experimental

phase of the hydrolysis as discussed in chapter 4.2.2. In example, the method for determination of L_{max}, which was used in the calculation of % DH was a bit questionable, as illustrated in figure 14.

Figure 14. Setup of oil bath digestion to find L_{max} when calculating % DH.

The standard curve that was used for the calculations of the % DH was a premade standard curve based on μg leucine. Based on this, the results can not be compared to other studies on DH, especially studies where other methods have been used, and it was chosen to not put as much emphasis on DH in the discussion.

4.2.4 Response Surface Methodology

Response Surface Methodology was used in order to find the optimum conditions for conducting hydrolysis on the respective raw materials, considering DH and DPPH. The calculated % DH and % DPPH (presented in chapter 4.2.3 and 4.2.8) for all the fractions and hydrolysates from the small-scale hydrolysis were plotted into the STATISTIKA software by Dr. Sachindra at CFTRI. The independent factors considering optimum conditions for hydrolysis were time, temperature and enzyme concentration, while % DH and % DPPH were dependent factors.

Only the results from HW are presented as only this fraction was decided to proceed with for the large-scale hydrolysis due to time-limitations. The results are presented as response surface plots obtained from the STATISTIKA software.

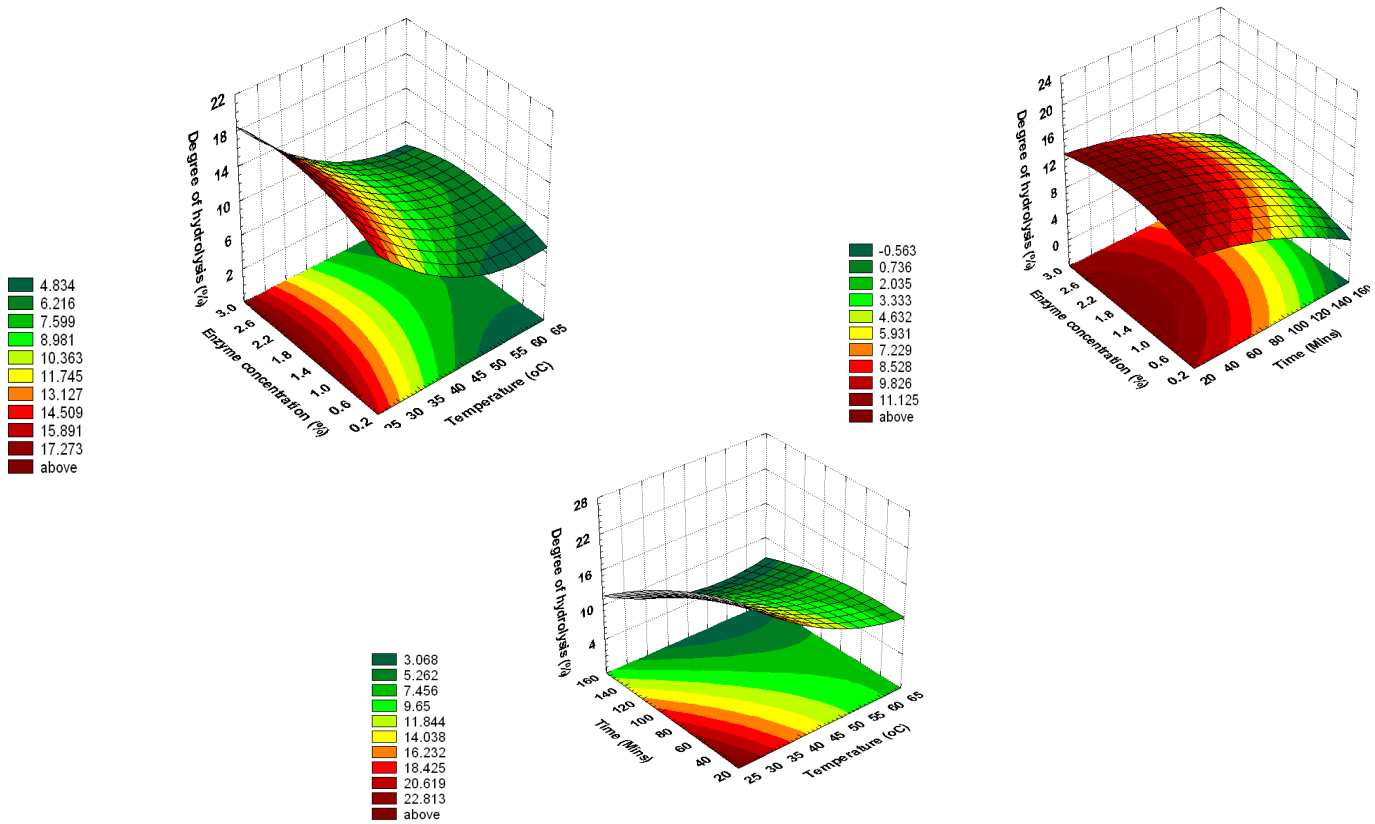


Figure 15. Response surface plots of effect of enzyme concentration, temperature and time on % DH.

The response surface plots illustrate the effect of enzyme concentration, temperature and time based on % DH. The red areas indicate where the response of % DH is highest based on the different independent factors. It is clear that the response declines as time and temperature increases. The enzyme concentration gives a good response over a broad range.

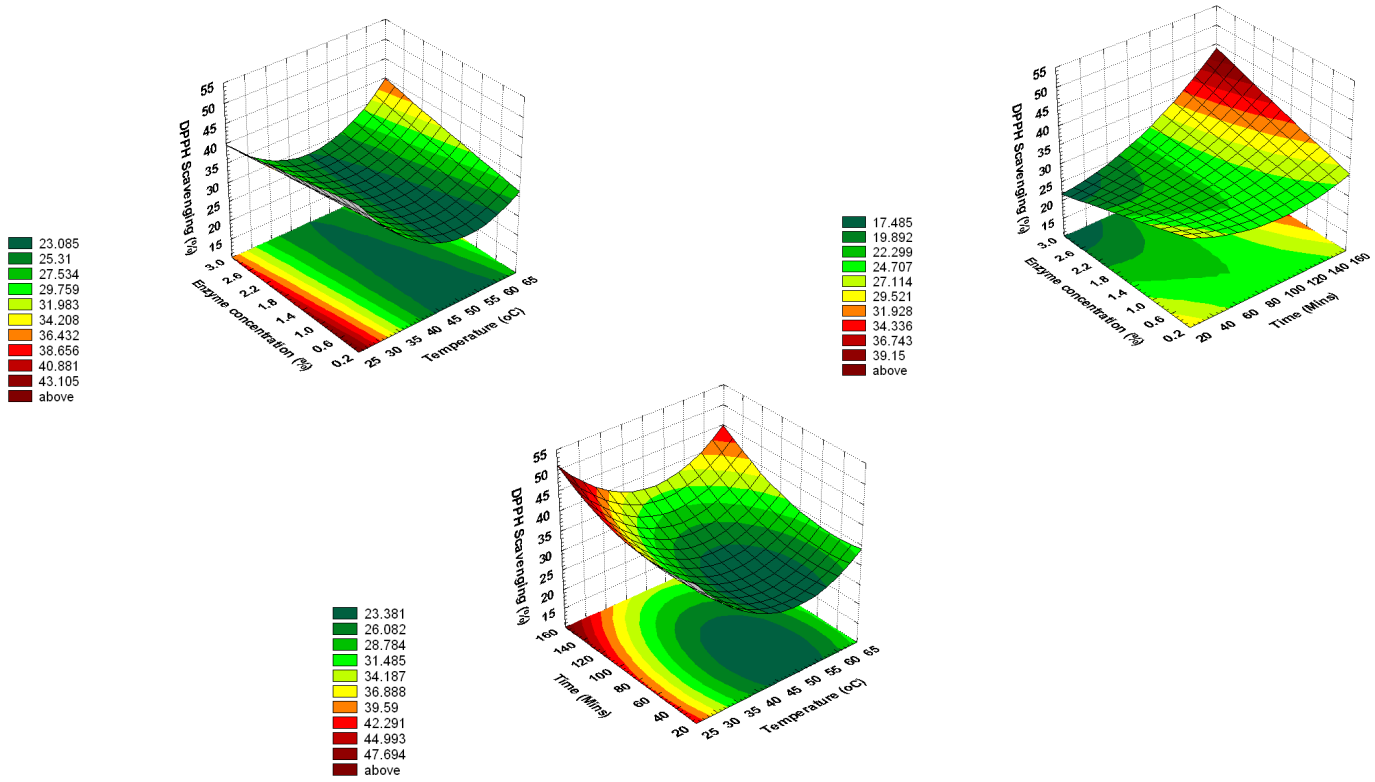


Figure 16. Response surface plots of effect of enzyme concentration, temperature and time on % DPPH.

The response surface plots illustrate the effect of enzyme concentration, temperature and time based on % DPPH. The red areas indicate where the response of % DPPH is highest based on the different independent factors. A low enzyme concentration and low temperature gives a good response of % DPPH, while time seems to give a good response over a broad range.

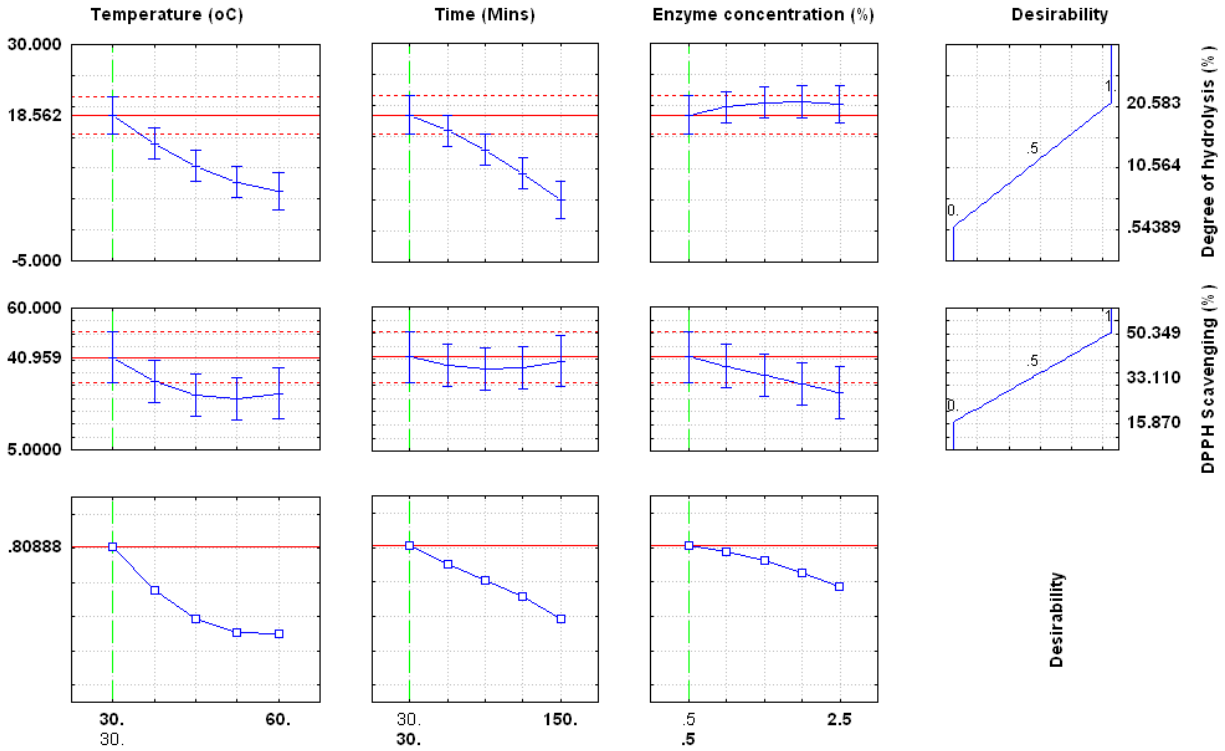


Figure 17. Summary of response surface plots showing optimum conditions for hydrolysis on HW.

A summary of the response surface plots is illustrated in figure 17. The figure illustrates the effect temperature, time and enzyme concentration has on % DH and DPPH scavenging (%). Based on this figure, a temperature of 30°C, 30 min hydrolysis and an enzyme concentration of 0.5 % was found to be the optimum conditions for hydrolysis of HW. By following these conditions, a predicted response of 20.58 % DH, and 50.35 % DPPH scavenging activity should be obtained. The found optimum conditions was used for the large-scale hydrolysis.

A previous study by See et al. (2011) on optimization of enzymatic hydrolysis on salmon skin by the use of Alcalase used the response surface methodology, and found that the optimum temperature for Alcalase was 55.3°C. Another study by Normah et al. (2005) on threadfish bream hydrolysate found the optimum temperature of Alcalase to be at 60°C. Benjakul and Morrissey (1997) did a study on protein hydrolysates from Pacific whiting solid wastes with the use of Alcalase, and found an optimum temperature at 60°C and optimum time of one hour. Looking at these previous studies, the found optimum conditions in this study are a bit questionable. This can be linked to the some odd results from % DH and % DPPH which was used in order to find the optimum conditions for the enzymatic hydrolysis. The odd results can be linked to the challenges met in the experimental phase of the enzymatic hydrolysis, as discussed in chapter 4.2.2.

4.2.5 Protein content

The protein content in the freeze-dried protein hydrolysate from the large-scale hydrolysis of HW was determined together with the herring samples, and the result is presented in table 15.

Table 15. Protein content in the Kaiko HW hydrolysate. HW: head waste.

Sample	Protein content (%)
Kaiko HW	18.84

The protein content in the freeze-dried Kaiko HW hydrolysate was found to be 18.48 %, which is much lower than the herring samples. This indicates that Kaiko HW contains lower amounts of water soluble peptides compared to OH, R150 and R4 from herring (74.44-90.16 %).

4.2.6 Molecular weight distribution of peptides

To determine the molecular weight distribution of the peptides in the samples, the SDS-PAGE method was first performed at CFTRI. The samples didn't separate in the gel, and didn't give any useful results. It was then decided to determine the molecular weight distribution of the freeze-dried hydrolysate from Kaiko HW by gel filtration using the FPLC system at NTNU Gløshaugen. This was performed together with the herring samples. Determination of the molecular weight distribution of the sample was performed in order to characterize the size of the peptides in the sample.

In order to compare the size distribution profile of the sample, the profile was compared to two known standard proteins with known molecular weight. The proteins used were vitamin B12 (1.4 kDa) and aprotinin (6.5 kDa). The standard proteins were plotted in Excel separately, and the peaks are marked as vertical lines in figure 18.

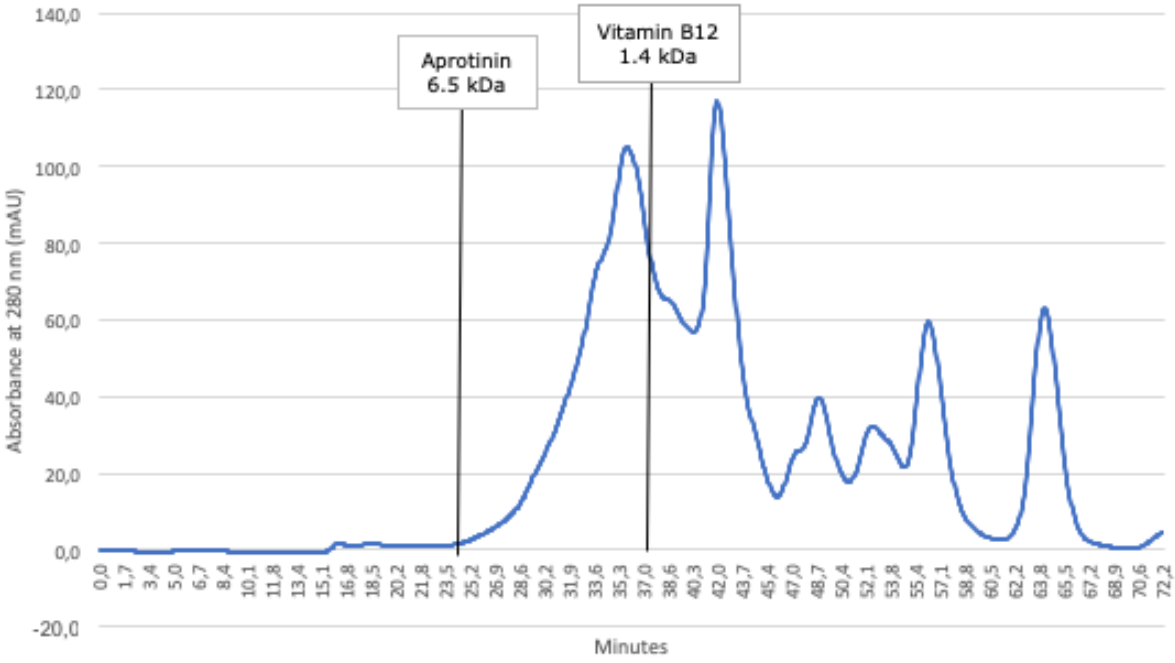


Figure 18. Chromatogram showing the distribution of molecular weight in peptides in the freeze-dried HW hydrolysate sample compared to known standard proteins.

The freeze-dried HW hydrolysate sample has one peak slightly larger than vitamin B12 with a molecular weight of 1.4 kDa, similar to the herring samples. The rest of the peaks have a broad distribution which all are smaller than 1.4 kDa. This can indicate that the hydrolysate has a high degree of hydrolysis as the content are of small peptide sizes, and the hydrolysate may possess desirable bioactive properties, like the herring samples. Approximately 42 minutes into the gel filtration in the FPLC system is the peak with the highest absorbance.

4.2.7 Amino acid composition

In order to characterize and find the nutritional value of the dried raw material, the dried sediment from the hydrolysis and the FPH from head waste, the total amino acid composition was investigated. The analysis was conducted at SINTEF Ocean by Rasa Slizyte.

The samples were analyzed and the results showed total amino acids (protein) as percentage of the samples, where dried raw material was found to contain 46.7 %, dried sediments 28.7 % and freeze dried FPH from HW 71.5 % amino acids (protein). See appendix H for results, as given by Rasa Slizyte. The composition of amino acids was determined by evaluating each amino acid in the samples, as presented in figure 19. Tryptophan is not included because it is not adapted to the method used. Most of the tryptophan will be damaged by processing and will not be detected by the HPLC system.

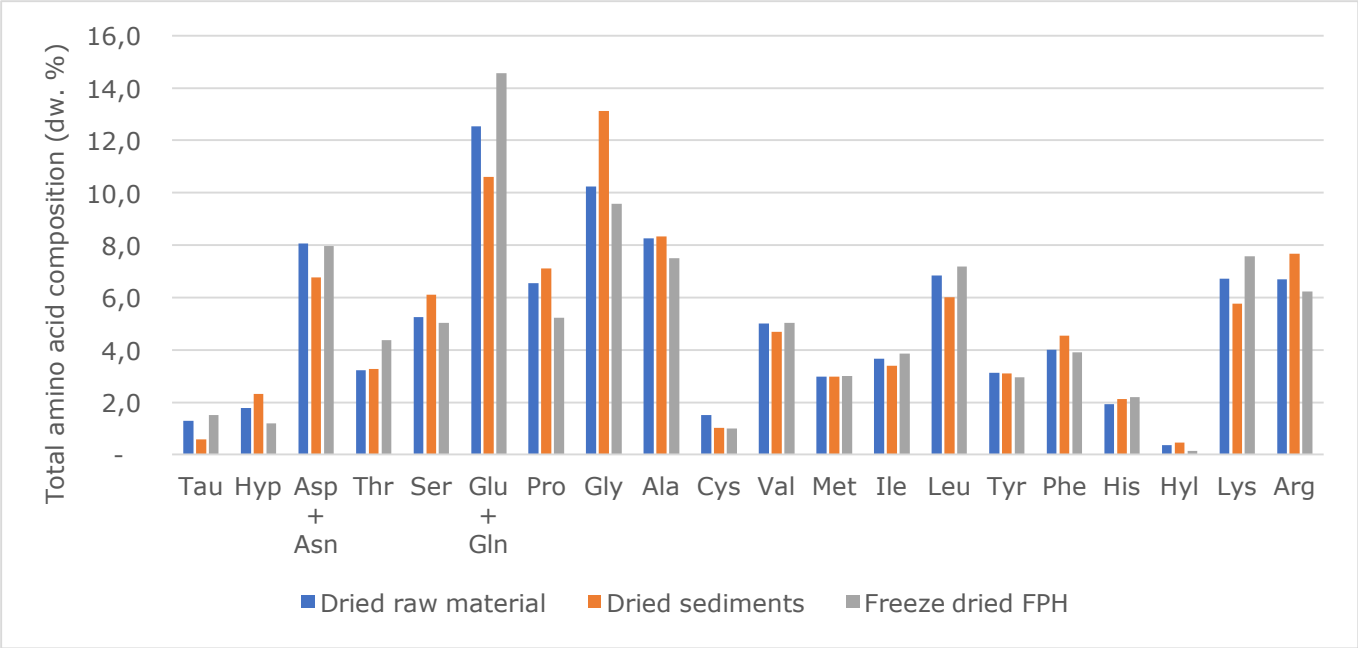


Figure 19. Amino acid composition (dw %) in dried raw material, dried sediments and freeze dried FPH from head waste.

The distribution of amino acids is pretty similar for all the samples, with a high content of Glu+Gln, Gly, Asp+Asn, Ala, Leu, Lys and Arg. In a study on FPH from herring by Liceaga-Gesualdo and Li-Chan (1999) the same amino acids were found to be the ones dominating. The amount of Glu+Gln ranged from 10.6-14.6 % with the highest amount found in FPH from HW. The amount of Gly was also found to be high, with the highest content of 13.1 % in dried sediments.

The amount (dw %) essential (His, Thr, Met, Val, Phe, Ile, Leu and Lys), hydrophobic (Ala, Tyr, Met, Val, Phe, Ile and Leu) and aromatic (Phe, Tyr and His) amino acids in the samples are presented in table 16.

Table 16. Essential, hydrophobic and aromatic amino acids in dried raw material, dried sediments and freeze dried FPH from head waste.

Dw %	Dried raw material	Dried sediments	FPH HW
Essential AA	34.38	32.78	37.12
Hydrophobic AA	33.88	33.06	33.44
Aromatic AA	9.05	9.76	9.05

Studies by Mendis et al. (2005) and Aluko (2015) have found that hydrophobic and aromatic amino acids can be linked to antioxidative activity in fish peptides. The samples were found to contain a high amount of hydrophobic amino acids (33.06-33.88 %) which can be linked to antioxidative properties of the samples. All of the essential amino acids were present in the samples, except Tryptophan, whereas Lys and Leu had the highest content (5.76–7.58 % and 6.01–7.17 %). The content of essential amino acids is above the suggested content of 32 % essential amino acids of total amino acids in a product. This may indicate that the samples are of high nutritional value (Lee et al., 1978). However, hydrophobic amino acids are known to have a bitter taste which is not desirable in products

for human consumption (Kirimura et al., 1969) (Lalasidis and Sjoberg, 1978). A study by Kim et al. (1999) found that the most bitter-tasting peptides are peptides with molecular weights lower than 1.7 kDa. In the study, they also analyzed the amino acid sequence, which indicated that many smaller peptides are composed of uncharged polar amino acids as well as hydrophobic amino acids. By looking at the results from molecular weight distribution in chapter 4.1.3, it was found to contain a lot of peptides below 1.4 kDa, which may indicate that it may have a bitter taste.

4.2.8 Antioxidant activity assays

ABTS radical scavenging activity

The ABTS radical scavenging activity of the Freeze-dried Kaiko HW sample was analyzed together with the herring samples at NTNU Gløshaugen. The result is presented as PG equivalents in table 17.

Table 17. Antioxidant content (μmol/g) in hydrolysate from Kaiko head waste (n=3, ± SD).

Sample	Antioxidant content (μmol/g)
Kaiko HW	110.64 ± 0.01

The ABTS radical scavenging activity in the hydrolysate from Kaiko HW was found to be 110.64 μmol/g, which is a lot higher than the herring samples (18.28 – 23.85 μmol/g). Again, this might be due to the inherent color of, and maybe also the size and nature of the components in the hydrolysate. Both might affect the measured absorbance, and thus yield inaccurate values.

DPPH radical scavenging activity

The DPPH radical scavenging activity of HW, SBW and RW from the small-scale hydrolysis was analyzed in order to plot into the STATISTIKA software to obtain RMS graphs showing the optimum conditions for hydrolysis.

Table 18. Calculated DPPH radical scavenging activity (%) from enzymatic hydrolysis of the different fractions from surimi rest raw materials. HW: head waste, SBW: skin and bone waste, RW: refined waste.

Temp (°C)	Time (min)	Enzyme (%)	HW DPPH (%)	SBW DPPH (%)	RW DPPH (%)
30	30	0.50	50.35	30.13	10.48
		1.50	28.27	32.52	15.22
		2.50	24.61	35.24	22.14
	90	0.50	32.07	31.37	15.99
		1.50	37.45	21.40	13.68
		2.50	21.92	31.19	22.18
	150	0.50	39.49	25.93	25.96
		1.50	45.11	30.58	30.55
		2.50	49.42	22.77	17.30
45	30	0.50	18.11	28.84	27.77
		1.50	24.48	29.56	27.94
		2.50	25.80	34.21	30.17
	90	0.50	26.00	38.51	18.82
		1.50	25.79	35.53	29.66
		2.50	27.24	35.19	29.83
	150	0.50	15.87	18.07	6.80
		1.50	30.82	29.51	4.01
		2.50	32.29	29.16	14.66
60	30	0.50	25.36	28.93	20.38
		1.50	20.77	22.44	19.66
		2.50	25.35	28.79	18.66
	90	0.50	30.02	22.96	13.25
		1.50	24.40	23.00	15.08
		2.50	24.04	22.28	9.23
	150	0.50	33.39	32.04	29.97
		1.50	34.39	40.22	33.69
		2.50	43.63	37.41	28.35

For HW a hydrolysis at 30°C, 30 minutes and an enzyme concentration of 0.5 % gives the highest DPPH value of 50.35 %. For SB and RW a hydrolysis at 60°C, 150 minutes and an enzyme concentration of 1.5 % gives the highest DPPH value of 40.22 % and 33.69 %. As with the DH % results discussed in chapter 4.2.3, these were good results according to supervisors in India, and was used to obtain RSM graphs. Later on, looking at the results, it is reasonable to believe that also these results are affected by the multiple possible sources of error in the experimental phase of the hydrolysis as discussed in chapter 4.2.2.

Qualitative analytical tools

As the laboratory work didn't go quite as planned, it was decided to do a questionnaire as a qualitative analytical tool by using the knowledge and experiences gained by the exchange in India, and the valuable networks that had been established. However, the questionnaire only resulted in a few people responding, and it has to be taken into account that the questionnaire only represents personal opinions and knowledge on the topic, and not the population of India or Norway as a whole. The questionnaire can be found in appendix F. Based on the answers from the questionnaire on utilization of RRM in India, as well as personal experiences from the fieldwork like the exchange and knowledge gained by participating in workshops and industry visits, it was decided to represent the results as a SWOT analysis, as presented in figure 20. SWOT is a tool to help businesses, organizations or people to identify the strengths, weaknesses, opportunities and threats to an objective. The objective in this case was better utilization of marine RRM in India.

	Helpful to achieving the objective	**Harmful** to achieving the objective
Internal origin	*Strengths* - Large amounts of rest raw material - Big market	*Weaknesses* - Inadequate technology - No maintenance of cold chain - Not sufficiently developed infrastructure - Majority of the Indian population don't see the value and potential of rest raw materials
External origin	*Opportunities* - Better utilization of rest raw materials - Reduce food loss and waste - Growing economy - Growing market - Exchange of knowledge in research projects	*Threats* - International framework has to be followed in order to be accepted on the global market

Figure 20. SWOT analysis based on the issue of utilization of marine rest raw materials in India.

Harmful issues to achieving the objective

India has a long coastline and many inland lakes for fishing, which results in large amounts of RRM and a big potential for utilization of these, however there are a few constraints. The biggest weakness considering utilization of RRM in India concerns inadequate technology and infrastructure. By not having the necessary technology and processing techniques, the result is under-utilization of RRM and development of a product with lower

value and quality. RRM in India today are used as ingredients in fish meal, or dried and used as fertilizer.

By own experience, some challenges with technology were met. Interactive communication with the daily manager of the Kaiko surimi plant took place with the use of FaceTime on mobile phones. This was done while in India, and was an attempt to see if interactive communication from inside the surimi plant to partners in Norway could be a possible way of communication and exchange of knowledge in the future. However, this had to take place while the daily manager was at home, as the surimi plant is placed a bit outside of the city, and the mobile online network wasn't established.

As the basic and necessary infrastructure is not in place, it makes the handling of RRM much harder and inefficient. Some places it can be a distance of up to 600 km from where the RRM origins till a large RRM processing site (Widell et al., 2019). Development of infrastructure for fishing harbors, landing centers, transportation and processing sites following international standards would be a big step towards the ability to utilize RRM much better and in an efficient way. Due to the current lack of infrastructure, maintaining the cold chain is also a major problem. With RRM being so prone to microbiological spoiling, it is of outmost importance that the cold chain is maintained at all times in order to get a product of good quality. By following international standards and framework it would also make it easier to be accepted on the global market, which would be of economic interest.

Based on the responses on the questionnaire and by own experience, it seems like the majority of the Indian population don't really have much knowledge about RRM, hence they don't see the value and potential of it. The last couple of years, there has globally been more public information and knowledge about SDGs and food loss and waste, as the environment and climate changes has become a hot topic. Still, India is far behind Norway when it comes to public knowledge on utilization of RRM. In order to get a change, it is important to get the Indian State fisheries department to see the importance and possibilities of RRM (Widell et al., 2019).

Helpful issues to achieving the objective

On the other hand, India has big opportunities for utilization of RRM of higher quality and value. Most of the fish in India ends up as a whole fish in the home of Indian families or gets exported. Still a considerable amount of fish goes through processing, in example for the production of surimi where a considerable amount of RRM are obtained. Establishment of local small-scale RRM processing sites, could possibly contribute to utilization of RRM into value added products, instead of being discarded as it often is today. By establishing local RRM processing sites, it would lead to more employment as well. With the growing economy in India, the infrastructure will also probably improve over the years.

Participating in international research projects contributes to exchange of knowledge and global partnerships. In the questionnaire, one of the partners in the projects expressed that by being part of these projects he had gained a lot of new knowledge by being able to attend workshops where partners from different actors participate, meeting students and industry visits. This shows the importance of international collaborations like the two projects ReFOOD and ReValue. Also, by doing student exchanges, new insight to the topic is gained. From the exchange to CFTRI and being part of this observational study, with both passive and active participation, it has been interesting to see differences and similarities in the way of thoughts and work with RRM.

As explained earlier, this SWOT analysis is based on just a limited amount of responses to the questionnaire, as well as personal knowledge and experiences. Nevertheless, it gives a certain insight in how the situation regarding utilization of RRM in India are today, and possibilities for the future. And personally, by being part of the exchange and ReFOOD and ReValue, I have acquired a lot of new knowledge I never would have learned otherwise, which again shows the importance of international partnerships.

5. Conclusions

The results suggested that all samples exhibited antioxidant activity. From the herring hydrolysate that was fractionated by UF, the permeate<4 kDa was found to have the highest antioxidant activity in the DPPH radical scavenging assay. This supports the theory that purified peptides of low molecular weight exhibit stronger antioxidant activity. However, the molecular weight distribution showed that the distribution of peptides was fairly similar, and that the UF didn't separate the peptides sharp enough. Even though the peptides in the samples weren't separated sharp enough, they consisted of peptides of smaller sizes that are known to possess desirable bioactive properties like antioxidant activity.

The results on the Indian raw material are a bit questionable and not reliable considering the challenges with the enzymatic hydrolysis. Challenges in homogenizing the raw material, temperature control, proper equipment and proper handling of the raw material were met. Maintaining and keeping the cold chain are of outmost importance when it comes to marine rest raw material as these are prone to microbiological spoiling. Good quality of the raw material is important in order to get a satisfactory quality of the fish protein hydrolysate. With these challenges taken into account, and the uncertainty in the results this led to, the hydrolysate from Indian RRM exhibited antioxidant activity, showed a content of peptides of small molecular sizes and was found to have a good nutritional value with a high content of essential amino acids.

The importance of having the technology and infrastructure in place is essential in order to utilize marine RRM, both in Norway and in India. These challenges are a bit more prominent in India than in Norway, but India has a lot of opportunities and possibilities to increase the utilization of RRM. But firstly, education and technology around the topic has to be applied.

In the future, the use of FPH may be applied in products for human consumption as they have been shown to possess antioxidant activity and have a high nutritional value, but there are a few obstacles along the way, and more research is necessary.

5.1 Further work

By taking part in this exchange to India a lot of new knowledge and insight in the work with utilization of marine RRM in India has been gained. Challenges considering enzymatic hydrolysis and chemical analyses in the lab that were not known in advance, and not thought of as challenging have been uncovered. This wouldn't have been uncovered if it wasn't for this exchange and is of great importance for the future work on marine RRM in India.

References

ADLER-NISSEN, J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of agricultural and food chemistry,* 27**,** 1256-1262.

AIDOS, I., VAN DER PADT, A., LUTEN, J. B. & BOOM, R. M. 2002. Seasonal changes in crude and lipid composition of herring fillets, byproducts, and respective produced oils. *Journal of Agricultural and Food Chemistry,* 50**,** 4589-4599.

ALUKO, R. 2015. Amino acids, peptides, and proteins as antioxidants for food preservation. *Handbook of antioxidants for food preservation.* Elsevier.

AMBROGI, V., CARFAGNA, C., CERRUTI, P. & MARTURANO, V. 2017. Additives in polymers. *Modification of Polymer Properties.* Elsevier.

ANIMALIEBIPRODUKTFORSKRIFTEN. 2016. *Forskrift om animalske biprodukter som ikke er beregnet på konsum (animaliebiproduktforskriften)* [Online]. Available: https://lovdata.no/dokument/SF/forskrift/2016-09-14-1064/ [Accessed 25.04 2019].

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

ASPMO, S. I., HORN, S. J. & H. EIJSINK, V. G. 2005. Enzymatic hydrolysis of Atlantic cod (Gadus morhua L.) viscera. *Process Biochemistry,* 40**,** 1957-1966.

BALTI, R., BOUGATEF, A., ALI, N. E. H., ZEKRI, D., BARKIA, A. & NASRI, M. 2010. Influence of degree of hydrolysis on functional properties and angiotensin I-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (Sepia officinalis) by-products. *Journal of the Science of Food and Agriculture,* 90**,** 2006-2014.

BENJAKUL, S. & MORRISSEY, M. T. 1997. Protein Hydrolysates from Pacific Whiting Solid Wastes. *Journal of Agricultural and Food Chemistry,* 45**,** 3423-3430.

BENJAKUL, S., YARNPAKDEE, S., SENPHAN, T., HALLDORSDOTTIR, S. M. & KRISTINSSON, H. G. 2014. Fish protein hydrolysates: production, bioactivities and applications. *Antioxidants and functional components in aquatic foods, 1st ed. Reykjavik, Iceland: Matil Ltd***,** 237Á83.

BERDAHL, D., NAHAS, R. & BARREN, J. 2010. Synthetic and natural antioxidant additives in food stabilization: current applications and future research. *Oxidation in foods and beverages and antioxidant applications.* Elsevier.

BEZERRA, M. A., SANTELLI, R. E., OLIVEIRA, E. P., VILLAR, L. S. & ESCALEIRA, L. A. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta,* 76**,** 965-977.

BLIGH, E. G. & DYER, W. J. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology,* 37**,** 911-917.
BOURSEAU, P., VANDANJON, L., JAOUEN, P., CHAPLAIN-DEROUINIOT, M., MASSÉ, A., GUÉRARD, F., CHABEAUD, A., FOUCHEREAU-PERON, M., LE GAL, Y. & RAVALLEC-PLÉ, R. 2009. Fractionation of fish protein hydrolysates by ultrafiltration and nanofiltration: impact on peptidic populations. *Desalination,* 244**,** 303-320.
BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72**,** 248-254.
BREWER, M. 2011. Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive reviews in food science and food safety,* 10**,** 221-247.
BRUSTAD, K. 2019. Fractions ultrafiltration.
CARUSO, G. 2016. Fishery wastes and by-products: a resource to be valorised. *Journal of FisheriesSciences. com,* 10**,** 12.
CHALAMAIAH, M., DINESH KUMAR, B., HEMALATHA, R. & JYOTHIRMAYI, T. 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry,* 135**,** 3020-3038.
CHAMPIONS 12.3 2018. SDG target 12.3 on food loss and waste: 2018 progress report.
CHATEAU, D. & VIDAL-GIRAUD, B. 2007. World surimi market. *Globefish Research Programme.* Rome: FAO.
CHO, M. J., UNKLESBAY, N., HSIEH, F.-H. & CLARKE, A. D. 2004. Hydrophobicity of bitter peptides from soy protein hydrolysates. *Journal of Agricultural and Food Chemistry,* 52**,** 5895-5901.
CHOE, E. & MIN, D. B. 2009. Mechanisms of antioxidants in the oxidation of foods. *Comprehensive Reviews in Food Science and Food Safety,* 8**,** 345-358.
DE NASJONALE FORSKNINGSETISKE KOMITEENE. 2010. *Kvalitative og kvantitative forskningsmetoder - likheter og forskjeller* [Online]. Available: https://www.etikkom.no/forskningsetiske-retningslinjer/medisin-og-helse/kvalitativ-forskning/1-kvalitative-og-kvantitative-forskningsmetoder--likheter-og-forskjeller/ [Accessed 03.04 2019].
DEKKERS, E., RAGHAVAN, S., KRISTINSSON, H. G. & MARSHALL, M. R. 2011. Oxidative stability of mahi mahi red muscle dipped in tilapia protein hydrolysates. *Food Chemistry,* 124**,** 640-645.
DUAN, X.-J., ZHANG, W.-W., LI, X.-M. & WANG, B.-G. 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga, Polysiphonia urceolata. *Food chemistry,* 95**,** 37-43.
DUMAS, J. 1831. Procedes de I'analyse organique. *Ann. Chim. Phys,* 47**,** 198-205.
ELIAS, R. J., KELLERBY, S. S. & DECKER, E. A. 2008. Antioxidant Activity of Proteins and Peptides. *Critical Reviews in Food Science and Nutrition,* 48**,** 430-441.

EREL, O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical biochemistry,* 37**,** 277-285.
EUROPEAN COMMISSION. 2019a. *Food waste measurement - the common EU methodology* [Online]. European Commission,

. Available: https://ec.europa.eu/info/law/better-regulation/initiatives/ares-2018-705329_en [Accessed 29.03 2019].
EUROPEAN COMMISSION. 2019b. *Herring (Clupea harengus)* [Online]. Available: https://ec.europa.eu/fisheries/marine_species/wild_species/herring_en [Accessed 17.02 2019].
FAO. 2009. *How to feed the world in 2015* [Online]. Available: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf [Accessed 18.02 2019].
FAO. 2019. *Ensure sustainable consumption and production patterns* [Online]. Available: http://www.fao.org/sustainable-development-goals/goals/goal-12/en/ [Accessed 18.02 2019].
FN-SAMBANDET. 2019. *FNs bærekraftsmål* [Online]. Available: https://www.fn.no/Om-FN/FNs-baerekraftsmaal [Accessed 21.02 2019].
FRIEDMAN, M. 1996. Nutritional value of proteins from different food sources. A review. *Journal of Agricultural and Food Chemistry,* 44**,** 6-29.
GÁLICO, D., NOVA, C., GUERRA, R. & BANNACH, G. 2015. Thermal and spectroscopic studies of the antioxidant food additive propyl gallate. *Food chemistry,* 182**,** 89-94.
GAMEZ-MEZA, N., NORIEGA-RODRIGUEZ, J., MEDINA-JUÁREZ, L., ORTEGA-GARCIA, J., MONROY-RIVERA, J., TORO-VÁZQUEZ, F., GARCIA, H. & ANGULO-GUERRERO, O. 2003. Concentration of eicosapentaenoic acid and docosahexaenoic acid from fish oil by hydrolysis and urea complexation. *Food research international,* 36**,** 721-727.
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**,** C615-C622.
GUPTA, K. 2018. Report of visit to Kaiko Surimi producer. Amity University, Noida: Amity Food and Agriculture Foundation.
GUAADAOUI, A., BENAICHA, S., ELMAJDOUB, N., BELLAOUI, M. & HAMAL, A. 2014. What is a bioactive compound? A combined definition for a preliminary consensus. *International Journal of Nutrition and Food Sciences,* 3**,** 174-179.
HALIM, N. R. A., YUSOF, H. M. & SARBON, N. M. 2016. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology,* 51**,** 24-33.
HAMMER HJELLNES, V. 10.04 2019. *RE: Production of hydrolysate at Pelagia AS.* Type to LARSEN, F. H.

HAUGE, J. G. 2009. *Proteolytiske enzymer* [Online]. Store norske leksikon. Available: https://snl.no/proteolytiske_enzymer [Accessed 27.03 2019].
HAUGE, J. G., VAAJE-KOLSTAD, G. & KIERULF, P. 2018. *Enzymer* [Online]. Store norske leksikon. Available: https://snl.no/enzymer [Accessed 25.03 2019].
HEVIA, P. & OLCOTT, H. S. 1977. Flavor of enzyme-solubilized fish protein concentrate fractions. *Journal of Agricultural and Food Chemistry,* 25**,** 772-775.
HOYLE, N. T. & MERITT, J. H. 1994. Quality of fish protein hydrolysates from herring (Clupea harengus). *Journal of food Science,* 59**,** 76-79.
JACOBSEN, C., HORN, A. F., SØRENSEN, A.-D. M., FARVIN, K. S. & NIELSEN, N. S. 2014. Antioxidative strategies to minimize oxidation in formulated food systems containing fish oils and omega-3 fatty acids. *Antioxidants and Functional Components in Aquatic Foods.* Wiley-Blackwell.
KIM, M.-R., CHOI, S.-Y., KIM, C.-S., KIM, C.-W., UTSUMI, S. & LEE, C.-H. 1999. Amino acid sequence analysis of bitter peptides from a soybean proglycinin subunit synthesized in Escherichia coli. *Bioscience, biotechnology, and biochemistry,* 63**,** 2069-2074.
KIM, S.-K. & MENDIS, E. 2006. Bioactive compounds from marine processing byproducts – A review. *Food Research International,* 39**,** 383-393.
KIRIMURA, J., SHIMIZU, A., KIMIZUKA, A., NINOMIYA, T. & KATSUYA, N. 1969. Contribution of peptides and amino acids to the taste of foods. *Journal of Agricultural and Food Chemistry,* 17**,** 689-695.
KRISTENSEN, T. 2018. *Essensielle aminosyrer* [Online]. Available: https://snl.no/essensielle_aminosyrer [Accessed 26.04 2019].
KRISTINSSON, H. G. & RASCO, B. A. 2000. Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties. Boca Raton, Fla.
KRUGER, N. J. 2009. The Bradford method for protein quantitation. *The protein protocols handbook.* Springer.
LADIKOS, D. & LOUGOVOIS, V. 1990. Lipid oxidation in muscle foods: A review. *Food chemistry,* 35**,** 295-314.
LALASIDIS, G. & SJOBERG, L. B. 1978. Two new methods of debittering protein hydrolysates and a fraction of hydrolysates with exceptionally high content of essential amino acids. *Journal of Agricultural and Food Chemistry,* 26**,** 742-749.
LEE, Y. B., ELLIOTT, J. G., RICKANSRUD, D. A. & HAGBERG, E. Y. C. 1978. Predicting protein efficiency ratio by the chemical determination of connective tissue content in meat. *Journal of Food Science,* 43**,** 1359-1362.
LICEAGA-GESUALDO, A. & LI-CHAN, E. 1999. Functional properties of fish protein hydrolysate from herring (Clupea harengus). *Journal of Food Science,* 64**,** 1000-1004.

LOBO, V., PATIL, A., PHATAK, A. & CHANDRA, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews,* 4**,** 118.
LUCARINI, A. & KILIKIAN, B. 1999. Comparative study of Lowry and Bradford methods: interfering substances. *Biotechnology techniques,* 13**,** 149-154.
MAKE IN INDIA. 2019. *Food processing* [Online]. Available: http://www.makeinindia.com/sector/food-processing [Accessed 29.05 2019].
MAZORRA-MANZANO, M., RAMÍREZ-SUÁREZ, J., MORENO-HERNÁNDEZ, J. & PACHECO-AGUILAR, R. 2018. Seafood proteins. *Proteins in food processing.* Elsevier.
MENDIS, E., RAJAPAKSE, N. & KIM, S.-K. 2005. Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of agricultural and food chemistry,* 53**,** 581-587.
MOLYNEUX, P. 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol,* 26**,** 211-219.
NAIK, A. 07.05 2019. *RE: Rest raw material from Kaiko.* Type to LARSEN, F. H. & BRUSTAD, K.
NAJAFIAN, L. & BABJI, A. S. 2012. A review of fish-derived antioxidant and antimicrobial peptides: their production, assessment, and applications. *Peptides,* 33**,** 178-185.
NAJAFIAN, L., JAFARZADE, M., SAID, M. & BABJI, A. S. 2013. Biochemical properties and antioxidant activity of myofibrillar protein hydrolysates obtained from patin (Pangasius sutchi). *International Journal of Food Science & Technology,* 48**,** 2014-2022.
NENADIS, N., LAZARIDOU, O. & TSIMIDOU, M. Z. 2007. Use of reference compounds in antioxidant activity assessment. *Journal of Agricultural and Food Chemistry,* 55**,** 5452-5460.
NENADIS, N., WANG, L.-F., TSIMIDOU, M. & ZHANG, H.-Y. 2004. Estimation of scavenging activity of phenolic compounds using the ABTS•+ assay. *Journal of agricultural and food chemistry,* 52**,** 4669-4674.
NESSE, N. 2017. *Membranseparasjon* [Online]. Available: https://snl.no/membranseparasjon [Accessed 21.05 2019].
NOFIMA. 2019. *Rest raw material* [Online]. Available: https://nofima.no/en/forskningsomrade/marine-biotechnology/rest-raw-material/ [Accessed 25.04 2019].
NORMAH, I., JAMILAH, B., SAARI, N. & YAAKOB, B. C. M. 2005. Optimization of hydrolysis conditions for the production of threadfin bream (Nemipterus japonicus) hydrolysate by alcalase. *Journal of Muscle Foods,* 16**,** 87-102.
OPHEIM, M., ŠLIŽYTĖ, R., STERTEN, H., PROVAN, F., LARSSEN, E. & KJOS, N. P. 2015. Hydrolysis of Atlantic salmon (Salmo salar) rest raw materials—Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. *Process Biochemistry,* 50**,** 1247-1257.

PARK, J. W. & MORRISSEY, M. T. 2000. Manufacturing of surimi from light muscle fish. *FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-*, 23-58.
PELAGIA. 2019. *About us* [Online]. Available: https://pelagia.com/about/ [Accessed 18.02.19 2019].
PICOT, L., RAVALLEC, R., FOUCHEREAU-PÉRON, M., VANDANJON, L., JAOUEN, P., CHAPLAIN-DEROUINIOT, M., GUÉRARD, F., CHABEAUD, A., LEGAL, Y. & ALVAREZ, O. M. 2010. Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture,* 90, 1819-1826.
PIHLANTO, A. 2006. Antioxidative peptides derived from milk proteins. *International Dairy Journal,* 16, 1306-1314.
RAGHAVAN, S. & KRISTINSSON, H. G. 2008. Antioxidative efficacy of alkali-treated tilapia protein hydrolysates: A comparative study of five enzymes. *Journal of Agricultural and Food Chemistry,* 56, 1434-1441.
RAGHAVAN, S. & KRISTINSSON, H. G. 2009. ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chemistry,* 117, 582-588.
RANATHUNGA, S., RAJAPAKSE, N. & KIM, S.-K. 2006. Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster). *European Food Research and Technology,* 222, 310-315.
RE, R., PELLEGRINI, N., PROTEGGENTE, A., PANNALA, A., YANG, M. & RICE-EVANS, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine,* 26, 1231-1237.
RICHARDSEN, R. & NYSTØYL, R. 2017. Analyse marint restråstoff 2016. *SINTEF Ocean, Kontali Analyse AS*.
RITCHIE, H., REAY, D. & HIGGINS, P. 2018. Sustainable food security in India—Domestic production and macronutrient availability. *PloS one,* 13, e0193766.
RUSTAD, T., STORRØ, I. & SLIZYTE, R. 2011. Possibilities for the utilisation of marine by-products. *International Journal of Food Science & Technology,* 46, 2001-2014.
SABEENA FARVIN, K. H., ANDERSEN, L. L., NIELSEN, H. H., JACOBSEN, C., JAKOBSEN, G., JOHANSSON, I. & JESSEN, F. 2014. Antioxidant activity of Cod (Gadus morhua) protein hydrolysates: In vitro assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry,* 149, 326-334.
SEE, S., HOO, L. & BABJI, A. S. 2011. Optimization of enzymatic hydrolysis of Salmon (Salmo salar) skin by Alcalase. *International Food Research Journal,* 18.
SEEVARATNAM, R., PATEL, B. P. & HAMADEH, M. J. 2009. Comparison of Total Protein Concentration in Skeletal Muscle as Measured by the Bradford and Lowry Assays. *The Journal of Biochemistry,* 145, 791-797.

SHAHIDI, F. 2000. Antioxidants in food and food antioxidants. *Food/nahrung,* 44**,** 158-163.
SINTEF. 2019a. *Re-FOOD* [Online]. Available: https://www.sintef.no/prosjekter/re-food/ [Accessed 30.01 2019].
SINTEF. 2019b. *ReValue* [Online]. Available: https://www.sintef.no/prosjekter/revalue/ [Accessed 30.01 2019].
ŠLIŽYTE, R., DAUKŠAS, E., FALCH, E., STORRØ, I. & RUSTAD, T. 2005. Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (Gadus morhua) by-products. *Process Biochemistry,* 40**,** 1415-1424.
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**,** 2021-2033.
ŠLIŽYTĖ, R., MOZURAITYTĖ, R., MARTÍNEZ-ALVAREZ, O., FALCH, E., FOUCHEREAU-PERON, M. & RUSTAD, T. 2009. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (Gadus morhua) backbones. *Process Biochemistry,* 44**,** 668-677.
SLIZYTE, R., NGUYEN, J. V., RUSTAD, T. & STORRO, I. 2004. Hydrolysis of Cod (Gadus morhud) By-Products: Influence of Initial Heat Inactivation, Concentration and Separation Conditions. *Journal of aquatic food product technology,* 13**,** 31-48.
SLOTTE, A. 1999. Differential utilization of energy during wintering and spawning migration in Norwegian spring-spawning herring. *Journal of Fish Biology,* 54**,** 338-355.
SONKLIN, C., LAOHAKUNJIT, N. & KERDCHOECHUEN, O. 2018. Assessment of antioxidant properties of membrane ultrafiltration peptides from mungbean meal protein hydrolysates. *PeerJ,* 6**,** e5337.
SOUISSI, N., BOUGATEF, A., TRIKI-ELLOUZ, Y. & NASRI, M. 2007. Biochemical and functional properties of sardinella (Sardinella aurita) by-product hydrolysates. *Food technology and biotechnology,* 45**,** 187.
SOUTHEY, F. 2019. *From primary production to household: EU quantifies food waste across the bloc* [Online]. Food navigator. Available: https://www.foodnavigator.com/News/Policy/From-primary-production-to-household-EU-quantifies-food-waste-across-the-bloc?utm_source=newsletter_daily&utm_medium=email&utm_campaign=27-Mar-2019&c=YZG1O%2FCKoRt8aO31mvnEMkPCrpf2fx92&p2= [Accessed 29.03 2019].
SOWMYA, R. & SACHINDRA, N. 2012. Evaluation of antioxidant activity of carotenoid extract from shrimp processing byproducts by in vitro assays and in membrane model system. *Food Chemistry,* 134**,** 308-314.
SPEIGHT, J. G. 2017. Environmental Organic Chemistry for Engineers. *Chapter 3 - Industrial Organic Chemistry.* Butterworth Heinemann.
SPINELLI, J., KOURY, B. & MILLER, R. 1972. Approaches to the utilization of fish for the preparation of protein isolates enzymic modifications of myofibrillar fish proteins. *Journal of Food Science,* 37**,** 604-608.

ST. MELD NR 30 (2008-2009) 2009. Klima for forskning. Oslo: Kunnskapsdepartementet.
SZABO, M., IDIŢOIU, C., CHAMBRE, D. & LUPEA, A. 2007. Improved DPPH determination for antioxidant activity spectrophotometric assay. *Chemical Papers,* 61**,** 214-216.
TAHERI, A., ANVAR, S., AHARI, H. & FOGLIANO, V. 2013. Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (Onchorhynchus mykiss) viscera. *Iranian journal of fisheries sciences,* 12**,** 154-169.
TORP, E. 24.05 2019. *RE: Ultrafiltration.* Type to LARSEN, F. H.
TSAI, P.-J., TSAI, T.-H., YU, C.-H. & HO, S.-C. 2007. Comparison of NO-scavenging and NO-suppressing activities of different herbal teas with those of green tea. *Food Chemistry,* 103**,** 181-187.
UIO. 2011. *Enzym* [Online]. Universitetet i Oslo: Institutt for biovitenskap. Available: https://www.mn.uio.no/ibv/tjenester/kunnskap/plantefys/leksikon/e/enzym.html [Accessed 25.03.19].
UNITED NATIONS. 2015. *Sustainable Development Goals* [Online]. Available: https://sustainabledevelopment.un.org/?menu=1300 [Accessed 18.02 2019].
VERCRUYSSE, L., VAN CAMP, J. & SMAGGHE, G. 2005. ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. *Journal of agricultural and food chemistry,* 53**,** 8106-8115.
WIDELL, K. N., TVEIT, G. M., THAKUR, M. & AURSAND, M. 2019. Summary of Workshop and project meetings in Mumbai 27-30 Nov 2018. SINTEF Ocean.

Appendix A

Raw material

A rough description of the original and the different fractions of the hydrolysate received from Pelagia after ultrafiltration are listed below.

- OH: fine powder, some bigger lumps, brown color
- R150: fine powder, some lumps, brown color
- R4: fine, even powder, no lumps, light color
- P4: very sticky consistency, all material stuck together, yellow-orange color

Figure 21. Freeze-dried hydrolysate from Pelagia, and the different fractions after fractionation by ultrafiltration (Brustad, 2019). From left: original hydrolysate, retentate >150 kDa, retentate >4 kDa and permeate <4 kDa.

Appendix B

Chromatograms standard compounds for molecular weight distribution

Table 19. Molecular weight and time at peak of the standards used for determination of molecular weight distribution.

Standard	Molecular weight (kDa)	Peak (min)
Vitamin B12	1.3	~ 37
Aprotinin	6.5	~ 24

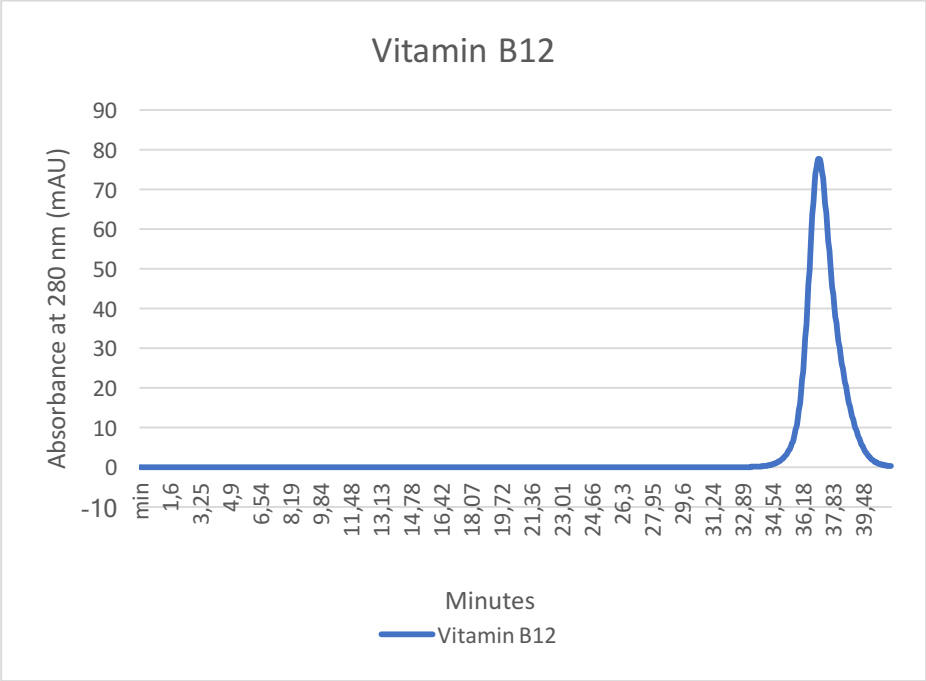


Figure 22. Chromatogram of vitamin B12, used as standard. Retention time (min) is plotted against absorbance at 280 nm (mAU).

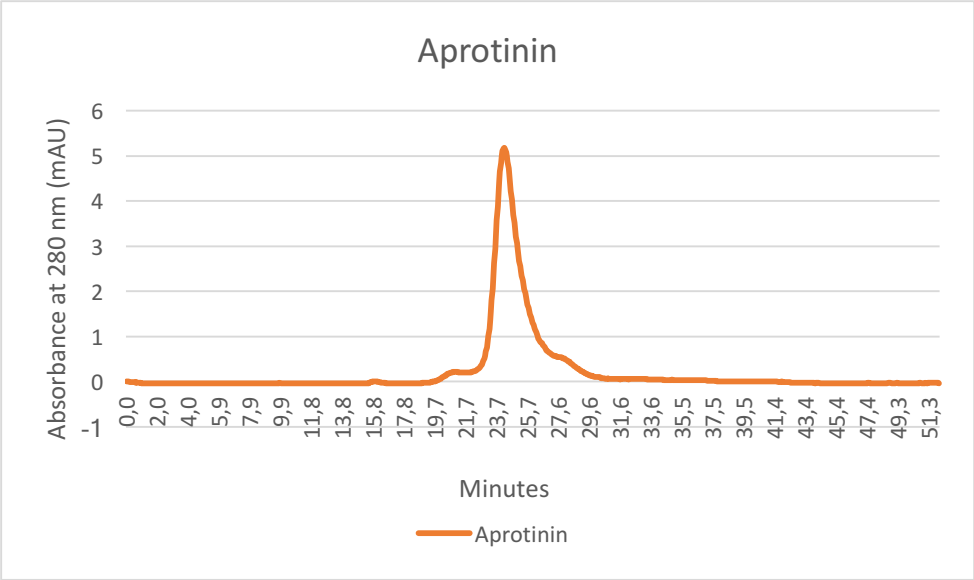


Figure 23. Chromatogram of aprotinin used as standard. Retention time (min) is plotted against absorbance at 280 nm (mAU).

Appendix C

Amino acid composition

Results of the amino acid composition of dried raw material, dried sediments and freeze dried FPH heads as received after analysis at SINTEF Ocean.

	Tørket råstoff - head		Tørket sediments		FPH heads	
	per dry weight		*per dry wei*	0	*per dry weight*	
	AA % (dw)		AA % (dw)		AA % (dw)	
Amino acid	Average	St,dev,p	Average	St,dev,p	Average	St,dev,p
Taurin	0,6	0,1	0,2	0,0	1,1	0,4
Methionine sulfoxide	-	-	-	-	-	-
Hydroxyproline	0,8	0,2	0,7	0,0	0,9	0,1
Aspartic acid + Asparagine	3,8	0,0	1,9	0,1	5,7	0,3
Threonine	1,5	0,0	0,9	0,1	3,1	0,2
Serine	2,5	0,1	1,7	0,2	3,6	0,2
Glutamic acid + Glutamine	5,9	0,2	3,0	0,2	10,4	0,4
Proline	3,1	0,1	2,0	0,3	3,7	0,5
Glycine	4,8	0,1	3,8	0,0	6,8	0,2
Alanine	3,9	0,2	2,4	0,1	5,4	0,5
Cystine (Cys-Cys)	0,7	0,2	0,3	0,3	0,7	0,5
Valine	2,3	0,0	1,3	0,0	3,6	0,1
Methionine	1,4	0,0	0,9	0,0	2,1	0,1
Isoleucine	1,7	0,0	1,0	0,0	2,8	0,0
Leucine	3,2	0,0	1,7	0,0	5,1	0,1
Tyrosine	1,5	0,0	0,9	0,0	2,1	0,2
Phenylalanine	1,9	0,0	1,3	0,0	2,8	0,1
Histidine	0,9	0,0	0,6	0,0	1,6	0,1
Hydroxylysine	0,2	0,0	0,1	0,0	0,1	0,1
Lysine	3,1	0,0	1,7	0,0	5,4	0,1
Tryptophan	-	-	-	-	-	-
Ammonia	0,7	0,0	0,5	0,0	1,2	0,0
Arginine	3,1	0,0	2,2	0,0	4,5	0,1
SUM (AA only)	46,7	0,4	28,7	0,0	71,5	1,9
SUM (AA + ammonia)	47,4	0,4	29,2	0,0	72,7	1,9

	Dried raw material		Dried sediments		FPH head	
	AA % (dw)		AA % (dw)		AA % (dw)	
Amino acid	Average	St,dev,p	Average	St,dev,p	Average	St,dev,p
Taurin	1,30		0,59		1,51	
Methionine sulfoxide	-		-		-	
Hydroxyproline	1,78		2,33		1,20	
Aspartic acid + Asparagine	8,06		6,78		7,97	
Threonine	3,23		3,28		4,37	
Serine	5,25		6,10		5,02	
Glutamic acid + Glutamine	12,53		10,60		14,57	
Proline	6,54		7,12		5,22	
Glycine	10,23		13,13		9,57	
Alanine	8,26		8,34		7,51	
Cystine (Cys-Cys)	1,51		1,02		0,99	
Valine	5,01		4,69		5,04	
Methionine	2,98		2,97		3,00	
Isoleucine	3,67		3,40		3,85	
Leucine	6,84		6,01		7,17	
Tyrosine	3,12		3,10		2,95	
Phenylalanine	4,00		4,54		3,92	
Histidine	1,93		2,12		2,19	
Hydroxylysine	0,36		0,46		0,15	
Lysine	6,72		5,76		7,58	
Tryptophan	-		-		-	
Ammonia	1,54		1,74		1,62	
Arginine	6,69		7,66		6,23	
	101,5		101,7		101,6	
	1,5		1,7		1,6	

Figure 24. Results as total amino acids (protein) in the samples, and percentage of the specific amino acids as a total.

Appendix D

Determination of protein content by the Bio-Rad method

Protein content in the samples was found by the Bio-Rad method. The absorbance of the BGG standard measured at 595 nm, presented in table 20, was plotted against the BGG concentration to get the standard curve presented in figure 25 below.

Table 20. Absorbance of the BGG standards at 595 nm.

BGG (mg/mL)	Parallel 1	Parallel 2	Parallel 3	Average	SD
0.3	0.21	0.21	0.22	0.21	0.01
0.6	0.40	0.40	0.40	0.40	0.00
0.9	0.56	0.56	0.57	0.56	0.00
1.2	0.71	0.73	0.69	0.71	0.03
1.5	0.85	0.87	0.88	0.87	0.02

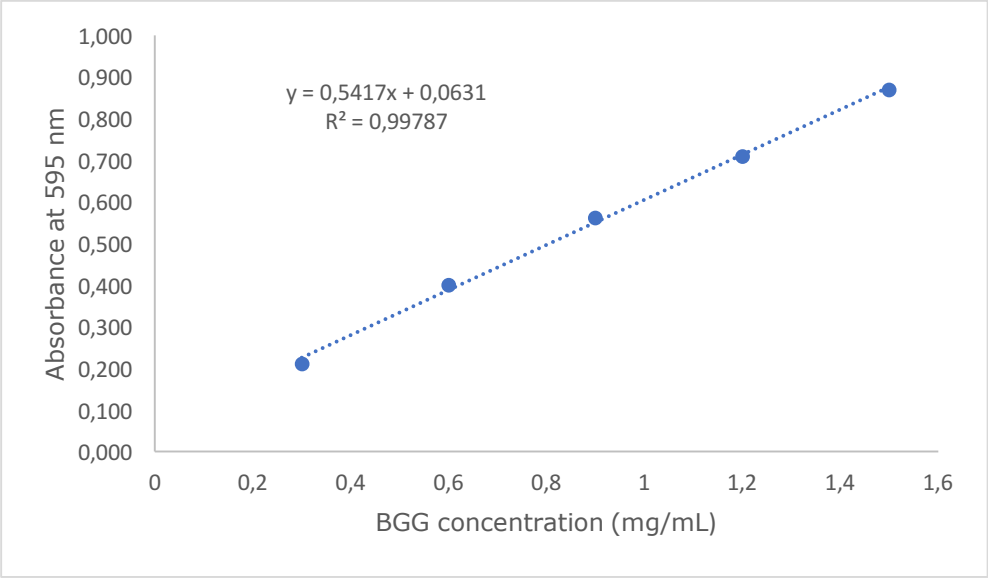


Figure 25. Standard curve generated from plotting the BGG concentrations against the absorbance at 595 nm.

By linear regression the equation y=0.5417x+0.0631 was found, and was used to calculate the protein content in the samples.

Table 21. Absorbance of the samples at 595 nm and the determined protein concentration.

	Parallel 1	Parallel 2	Parallel 3	Average	SD	Protein conc. (mg/mL)
OH	0.52	0.54	0.53	0.53	0.01	0.86
R150	0.51	0.52	0.50	0.51	0.01	0.82
R4	0.61	0.61	0.58	0.60	0.02	0.99
P4	0.00	0.00	0.01	0.00	0.00	0.00
Kaiko HW	0.18	0.18	0.17	0.18	0.01	0.21

Appendix E

Determination of lipid content

Only results from OH fraction of the herring hydrolysate analyzed at NTNU Gløshaugen, as determination of lipid content on the remaining fractions were conducted by fellow student Kristin Brustad at CFTRI.

Table 22. Raw data for calculation of lipid content (%) in OH.

Sample	Weight test tubes (g)	Weighed fat after evaporation (g)	Differance (g)	Weight of sample (g)	Lipid content (%)	SD
S1 I	13,98	14,01	0,03	5,02	13,36	0,29
S1 II	14,21	14,25	0,04	5,02	13,99	
S2 I	14,29	14,32	0,04	5,05	14,16	
S2 II	14,11	14,15	0,03	5,05	13,77	
S3 I	14,40	14,44	0,04	5,04	13,88	
S3 II	14,32	14,35	0,03	5,04	13,61	

Appendix F

Questionnaire

1. How is rest raw materials from the marine sector utilized in India today? Do you have any numbers of how much rest raw materials gets utilized and what goes to waste during one year?
2. What technologies and processes could be used for utilization of rest raw materials for value added products, especially in regard to lipids and proteins?
3. Has India historically traditions for utilization of rest raw materials? What knowledge do you think the general public have about rest raw material? and how it can be used as a source to proteins and lipids? How can you increase the awareness?
4. Local fish markets generate a big amount of rest raw material. Is this utilized in any way or just discarded? In what ways could it be possible to make it profitable for the sellers to take care of this?
5. Rest raw material can be used to produce different valuable products, like feed, food ingredients, nutraceuticals and similar. Do you think there is a market for this in India? What laws and framework has to be followed?
6. What are the major challenges in utilization of rest raw materials? Transportation, hygiene, inadequate technology, quality, consumer acceptance, others? Which aspects needs to be improved?
7. The UN has set a number of sustainable development goals they want to achieve by 2030. Goal 12 talks about responsible production and consumption. What needs to be done to achieve this goal?
8. Do you think value added products from marine rest raw materials would be accepted/consumed by vegetarians?
9. In regard to pollution, what are common threats found in Indian fish species? When utilizing rest raw material for human consumption these have to be taken in consideration.
10. In what area does India benefit with cooperation with Norway, and vice versa?

Appendix G

Degree of hydrolysis

Example of calculation of % DH of HW fraction in the small-scale hydrolysis. The standard curve used in order to calculate the % DH was premade and received by one of the PhD students at CFTRI.

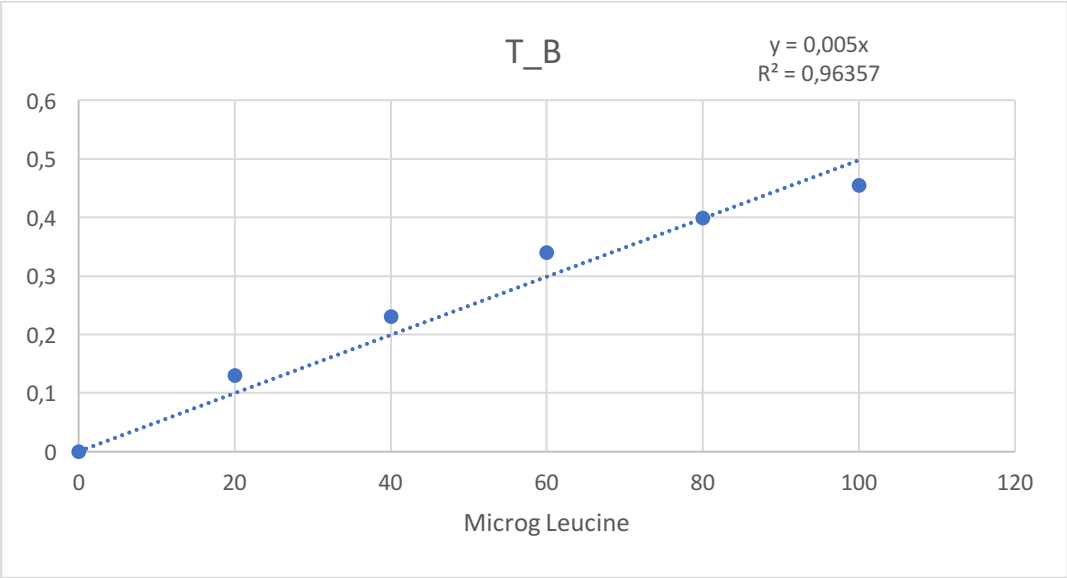


Figure 26. Premade standard curve received in order to calculate % DH.

	Aver	protein (ug	Sample volu	Total leucine equivalent (microg)				
Head	0,71	142,19	0,05	2843,73	Lmax			
HS 30°c 3	0,27	53,86	0,05	1077,20	LT - L0	LMAX-L0	LT - L0/LMAX	DH(%)
H 0.5	0,40	79,37	0,05	1587,47	510,27	1766,53	0,18	17,94
H1.5	0,42	83,13	0,05	1662,53	585,33	1766,53	0,21	20,58
H2.5	0,41	82,91	0,05	1658,27	581,07	1766,53	0,20	20,43
HS 90M	0,28	55,95	0,05	1119,07			0,00	
H 0.5	0,39	77,13	0,05	1542,67	423,60	1724,67	0,15	14,90
H1.5	0,39	78,27	0,05	1565,33	446,27	1724,67	0,16	15,69
H2.5	0,39	77,43	0,05	1548,67	429,60	1724,67	0,15	15,11
HS 150M	0,35	70,32	0,05	1406,40			0,00	
H0.5	0,37	73,20	0,05	1464,00	57,60	1437,33	0,02	2,03
H1.5	0,43	85,72	0,05	1714,40	308,00	1437,33	0,11	10,83
H2.5	0,39	78,17	0,05	1563,47	157,07	1437,33	0,06	5,52
HS 45°c 3	0,33	65,73	0,05	1314,67			0,00	
H 0.5	0,39	78,67	0,05	1573,33	258,67	1529,07	0,09	9,10
H1.5	0,42	83,31	0,05	1666,13	351,47	1529,07	0,12	12,36
H2.5	0,41	81,82	0,05	1636,40	321,73	1529,07	0,11	11,31
HS 90M	0,34	68,35	0,05	1367,07			0,00	
H 0.5	0,39	78,69	0,05	1573,73	206,67	1476,67	0,07	7,27
H1.5	0,40	79,03	0,05	1580,67	213,60	1476,67	0,08	7,51
H2.5	0,40	80,09	0,05	1601,73	234,67	1476,67	0,08	8,25
HS 150M	0,38	75,83	0,05	1516,67			0,00	
H0.5	0,38	76,61	0,05	1532,13	15,47	1327,07	0,01	0,54
H1.5	0,40	80,51	0,05	1610,13	93,47	1327,07	0,03	3,29
H2.5	0,41	81,60	0,05	1632,00	115,33	1327,07	0,04	4,06
HS 60°c 3	0,35	69,44	0,05	1388,80			0,00	
H 0.5	0,41	81,18	0,05	1623,60	234,80	1454,93	0,08	8,26
H1.5	0,40	80,81	0,05	1616,20	227,40	1454,93	0,08	8,00
H2.5	0,39	77,58	0,05	1551,60	162,80	1454,93	0,06	5,72
HS 90M	0,37	73,41	0,05	1468,27			0,00	
H 0.5	0,38	76,69	0,05	1533,80	65,53	1375,47	0,02	2,30
H1.5	0,39	78,10	0,05	1562,00	93,73	1375,47	0,03	3,30
H2.5	0,43	85,66	0,05	1713,20	244,93	1375,47	0,09	8,61
HS 150M	0,39	78,93	0,05	1578,53			0,00	
H0.5	0,41	82,23	0,05	1644,53	66,00	1265,20	0,02	2,32
H1.5	0,40	80,02	0,05	1600,40	21,87	1265,20	0,01	0,77
H2.5	0,40	80,49	0,05	1609,73	31,20	1265,20	0,01	1,10

Figure 27. Example calculation % DH for Kaiko HW fraction from the small-scale hydrolysis

Appendix H

Antioxidant assays

ABTS radical scavenging activity

Results from ABTS assay performed at CFTRI.

Table 23. Absorbance of samples for ABTS assay performed at CFTRI. SB: sample blank, ST: sample test.

	Parallel 1	Parallel 2	Parallel 3	Average	SD	Test-blank	% scavenging
Blank	0.06	0.06	0.06	0.06	0.00		
Control	0.33	0.35	0.33	0.34	0.01		
SB OH	0.08	0.08	0.10	0.08	0.01		
ST OH	0.36	0.35	0.36	0.36	0.00	0.27	19.10
SB R150	0.11	0.11	0.14	0.12	0.02		
ST R150	0.42	0.40	0.46	0.42	0.03	0.30	9.85
SB R4	0.09	0.06	0.07	0.07	0.01		
ST R4	0.33	0.32	0.31	0.32	0.00	0.25	25.16
SB P4	0.07	0.06	0.07	0.06	0.00		
ST P4	0.34	0.35	0.34	0.34	0.00	0.28	17.45

Following, the results from the ABTS assay performed at NTNU Gløshaugen are presented.

Table 24. Absorbance of PG standard at 734 nm

Standard	Parallel 1	Parallel 2	Parallel 3	Average	STD
10 uM	0,55	0,57	0,57	0,56	0,01
20 uM	0,47	0,47	0,46	0,47	0,01
30 uM	0,40	0,39	0,39	0,39	0,00
40 uM	0,35	0,34	0,34	0,35	0,01
50 uM	0,27	0,28	0,27	0,27	0,00

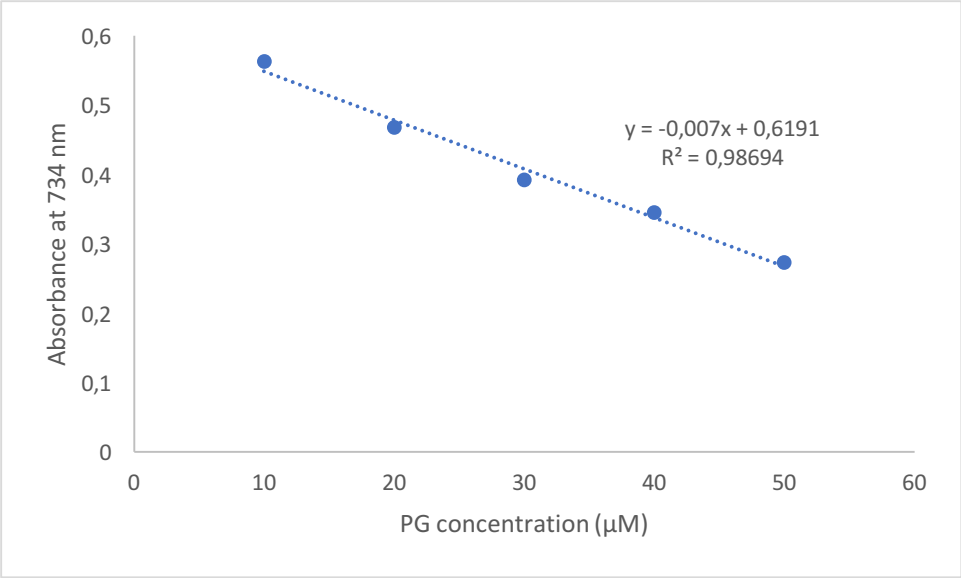


Figure 28. Standard curve of PG used for ABTS assay. Absorbance at 734 nm plotted against concentration (µM) of PG. The equation y=0.007x+0.6191 was found by linear regression.

Table 25. ABTS analyzed at Gløshaugen.

	OH	R150	R4	P4	Kaiko HW
Dilution factor	-	-	100	100	100
Parallel 1	0.62	0.76	0.59	0.58	0.44
Parallel 2	062	0.71	0.59	0.60	0.45
Parallel 3	0.62	0.72	0.57	0.59	0.45
Average	0.62	0.73	0.58	0.59	0.45
SD	0.00	0.03	0.01	0.01	0.01
Antioxidant content (µM)	-0.56	-15.56	5.30	4.06	24.59
Antioxidant content in undiluted sample (µM)	-0.56	-15.56	530.00	406.19	2458.57
Antioxidant content (µmol/g)	-0.03	-0.70	23.85	18.28	110.64

DPPH radical scavenging activity

Herring samples

Figure 29 shows an example of calculation of EC_{50} value of OH.

	Abs 517	Average	SD	Test-Blank	% Scavenging
Control	0,781				
SB	0,005				
100a	0,677	0,685	0,011	0,680	12,932
100b	0,693				
SB	0,006				
200a	0,618	0,625	0,009	0,619	20,807
200b	0,631				
SB	0,004				
300a	0,563	0,567	0,005	0,563	27,977
300b	0,570				
SB	0,003				
400a	0,514	0,514	0,000	0,511	34,571
400b	0,514				
SB	0,004				
500a	0,466	0,469	0,004	0,465	40,525
500b	0,471				

Figure 29. Read absorbance at 517 nm, with the average, SD and calculated % scavenging.

The calculated % scavenging was plotted against values of a standard protein given by a PhD student at CFTRI in order to obtain the standard curve.

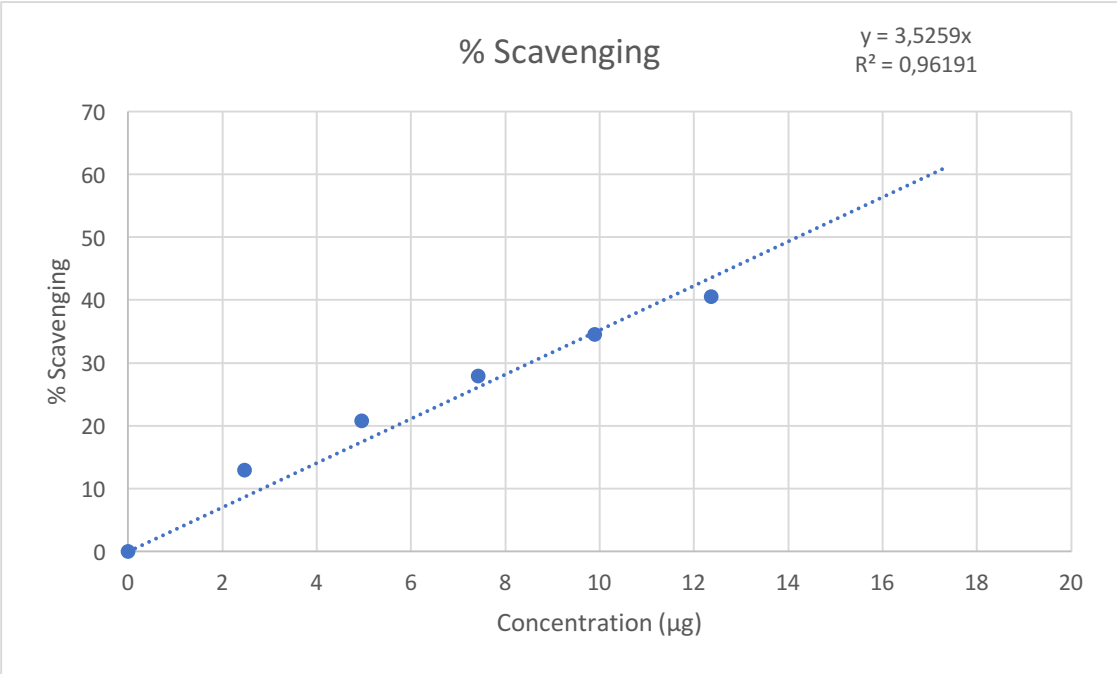


Figure 30. Standard curve used in order to calculate EC_{50} value of OH.

The equation y=3.5259x was used to calculate the EC_{50} value of OH.

Kaiko samples

Example of calculation of % DPPH radical scavenging of HW in the small-scale hydrolysis. In order to get the correct % scavenging, the value of the samples without added enzyme had to be subtracted from the sample with added enzyme.

	Paralell 1	Paralell2	Paralell 3	Average	SD	Test-blank	% scavenging	Enz - no enz
Control	0,48	0,44	0,46	0,46	0,02			
Blank	0,06	0,05	0,06	0,06	0,00			
SB A30 30 0,5	0,05	0,06	0,05	0,06	0,00			
A30 30 0,5	0,19	0,11	0,11	0,14	0,05	0,08	82,31	50,35
SB A30 30 1,5	0,07	0,06	0,06	0,06	0,01			
A30 30 1,5	0,27	0,24	0,23	0,24	0,02	0,18	60,22	28,27
SB A30 30 2,5	0,06	0,07	0,05	0,06	0,01			
A30 30 2,5	0,28	0,26	0,24	0,26	0,02	0,20	56,57	24,61
SB A30 90 0,5	0,13	0,12	0,10	0,12	0,01			
A30 90 0,5	0,29	0,29	0,28	0,29	0,00	0,17	62,51	32,07
SB A30 90 1,5	0,06	0,06	0,06	0,06	0,00			
A30 90 1,5	0,25	0,17	0,20	0,21	0,04	0,15	67,89	37,45
SB A30 90 2,5	0,06	0,06	0,06	0,06	0,00			
A30 90 2,5	0,28	0,28	0,27	0,28	0,01	0,22	52,36	21,92
SB A30 150 0,	0,06	0,06	0,06	0,06	0,00			
A30 150 0,5	0,26	0,26	0,28	0,27	0,01	0,21	54,62	39,49
SB A30 150 1,	0,09	0,13	0,09	0,10	0,02			
A30 150 1,5	0,29	0,28	0,29	0,29	0,00	0,18	60,23	45,11
SB A30 150 2,	0,06	0,06	0,06	0,06	0,00			
A30 150 2,5	0,19	0,22	0,26	0,22	0,03	0,16	64,54	49,42
SB A45 30 0,5	0,05	0,06	0,05	0,06	0,01			
A45 30 0,5	0,28	0,30	0,28	0,29	0,01	0,23	49,97	18,11
SB A45 30 1,5	0,05	0,06	0,07	0,06	0,01			
A45 30 1,5	0,26	0,27	0,25	0,26	0,01	0,20	56,34	24,48
SB A45 30 2,5	0,08	0,07	0,06	0,07	0,01			
A45 30 2,5	0,27	0,26	0,27	0,27	0,00	0,20	57,66	25,80
SB A45 90 0,5	0,05	0,07	0,06	0,06	0,01			
A45 90 0,5	0,26	0,27	0,27	0,26	0,01	0,20	55,96	26,00
SB A45 90 1,5	0,06	0,06	0,06	0,06	0,00			
A45 90 1,5	0,26	0,26	0,26	0,26	0,00	0,20	55,75	25,79
SB A45 90 2,5	0,06	0,06	0,06	0,06	0,00			
A45 90 2,5	0,24	0,27	0,26	0,26	0,02	0,20	57,20	27,24
SB A45 150	0,07	0,07	0,07	0,07	0,00			
A45 150 0,5	0,35	0,24	0,30	0,30	0,06	0,23	51,08	15,87
SB A45 150	0,10	0,06	0,11	0,09	0,03			
A45 150 1,5	0,25	0,24	0,25	0,25	0,01	0,16	66,04	30,82
SB A45 150	0,07	0,10	0,06	0,08	0,02			
A45 150 2,5	0,24	0,22	0,22	0,23	0,01	0,15	67,50	32,29
SB A60 30 0	0,06	0,06	0,09	0,07	0,02			
A60 30 0,5	0,27	0,27	0,27	0,27	0,00	0,20	57,02	25,36
SB A60 30 1	0,07	0,06	0,06	0,06	0,00			
A60 30 1,5	0,29	0,28	0,27	0,28	0,01	0,22	52,43	20,77
SB A60 30 2	0,08	0,07	0,06	0,07	0,01			
A60 30 2,5	0,26	0,28	0,27	0,27	0,01	0,20	57,01	25,35
SB A60 90 0	0,05	0,07	0,07	0,06	0,01			
A60 90 0,5	0,23	0,27	0,22	0,24	0,03	0,17	62,30	30,02
SB A60 90 1	0,06	0,06	0,07	0,06	0,00			
A60 90 1,5	0,25	0,29	0,25	0,26	0,02	0,20	56,68	24,40
SB A60 90 2	0,06	0,06	0,06	0,06	0,00			
A60 90 2,5	0,26	0,28	0,25	0,26	0,02	0,20	56,32	24,04
SB A60 150	0,06	0,06	0,07	0,07	0,01			
A60 150 0,5	0,23	0,23	0,23	0,23	0,00	0,16	64,40	33,39
SB A60 150	0,07	0,07	0,07	0,07	0,00			
A60 150 1,5	0,21	0,22	0,26	0,23	0,03	0,16	65,41	34,39
SB A60 150	0,08	0,15	0,06	0,10	0,05			
A60 150 2,5	0,23	0,21	0,21	0,21	0,01	0,12	74,65	43,63

Figure 31. Calculation of % DPPH scavenging activity of HW from the small-scale hydrolysis.