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Hydrolysis of Marine Cod (Gadus Morhua) Head

Utilization of rest raw material from cod for production of ingredients for human consumption

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Aquatic Food Production - Safety and Quality
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Preface

This master thesis project is a part of the international M.Sc. programme *Aquatic Food Production - Safety & Quality* (AQFood). It was carried out at the Norwegian University of Science and Technology (NTNU) in collaboration with SINTEF Fisheries and Aquaculture. The hydrolysis part of the project was performed in laboratories of SINTEF Fisheries and Aquaculture and the laboratory work was carried out partly at the department of biotechnology at NTNU and partly at SINTEF, in the period of March to June 2015.

I would like to express my deepest appreciation to those who made this thesis possible.

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Summary

A large number of wild cod caught in Norwegian waters is landed without head, while there is a great potential for increased utilization and profitability for this protein rich marine byproduct. The aim of this study was to investigate the possibilities of freezing and thawing of cod heads before hydrolysis and to study the effect of freezing and thawing of cod heads on yield and properties of cod head hydrolysates.

The parameters that were investigated included storage conditions: frozen storage of minced versus whole head for 20-21 days, and thawing methods: air thawing (4ºC for 20 hours) versus water immersion (6-10ºC for 3 hours); in addition to comparison between two hydrolysis time of 30 and 60 minutes by Protamex^{TM} enzyme.

The biochemical characteristics and changes in the raw material and the final product, fish protein hydrolysates were determined. The analyses were mostly performed on the 60 minutes hydrolysis due to limited time available. The analysis methods used in the project included determination of composition (protein, lipid, ash and dry matter content of raw material, FPH and sediment fractions), degree of hydrolysis, TCA-solubility, and amount of free amino acids.

Results showed that the yield of the dry matter in hydrolysate fractions ranged between 10.6% and 12.3% compared to the FPH produced from fresh raw material with 11.0% yield. The lipid content in freeze-dried FPH ranged from 0.37-0.71%, and the protein content was 88.4% for day one and 67.7-71.6% in the other samples. The degree of hydrolysis varied more significantly and showed higher values for the whole head thawed in water and minced head thawed in air and rather low for the day one sample.

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1 Introduction

1.1 Fish Byproducts

The byproducts from fish processing are very important for the fish industry, not only because of the nutritional value, but also due to the economic, political, and environmental parameters that are associated with bioconversion technologies of fish byproducts (Venugopal & Shahidi, 1998). However, the most common use of byproducts from the fish-filleting industry in Norway is in fish silage production, where it is concentrated or dried and used for feed (Bekkevold & Olafsen, 2007).

The marine raw materials have become more expensive globally due to the quota system. In Europe, the total allowable catches (TACs) for most commercial fish stocks (including cod) is set annually by the Council of fisheries ministers (European Commission, 2014). In Norway, the TAC is enforced by the Norwegian Ministry of Fisheries and Coastal Affairs to ensure long-term, optimal exploitation of living marine resources. Therefore, it is of great importance for the fish processing

companies to utilize the whole catch including the valuable fractions of the marine processing byproducts, such as: fish oil, proteins, collagen and gelatine, enzymes, minerals, and bioactive compounds. This amount should be maximized for human consumption and other value added ingredients.

Enzymatic hydrolysis is one of the effective methods to recover different compounds from fish rest raw material. The resulting fish protein hydrolysate can be applied to improve the functional, nutritional and nutraceutical properties of different food products.

1.1.1 Fish Byproducts – Definition

According to The Marine Ingredients Organisation (IFFO), normally, less than half of a fish is used for human consumption. The rest of the fish is called byproducts and is not consumed directly by humans (IFFO, 2013). According to Shahidi (1994), the discards and byproducts of seafood processing account for approximately three-quarters of the total weight of the catch. Furthermore, Falch, et al. (2006) estimated that the byproduct (including viscera) represents up to two-third of the weight of a round fish.

Considering the high value of the fish proteins and the oil content, the so-called byproducts can be transformed into highly valuable products that in some cases can be worth even more than the fish fillets (Rustad, et al., 2011).

In Norway, the definition distinguishes between marine byproducts and wastes, where byproducts include *"products that are not regarded as ordinary saleable products (fillet, round, eviscerated, or beheaded fish), but which can be recycled after treatment; while, wastes are products that cannot be used for feed or value-added products, but which have to be composted, burned, or destroyed"* (Bekkevold & Olafsen, 2007).

There are different definitions for fish byproducts, and different phrases and terms used to name them, particularly with recent developments in understanding the value of the fractions that can be obtained from the fish byproducts. Therefore, the term has gradually changed from "*waste*" and "*waste via co-products*" or "*co-streams*" to "*byproducts*" and "*rest raw material*" (Rustad, et al., 2011).

1.1.2 Fish Byproducts – Generation

Fish byproducts are generated during fish processing, which can be divided into two major stages: the handling of the raw fish after catching (wild fish) or harvesting (farmed fish), and the processing of the fish to fish products.

Wild fish is caught using different methods, such as different kinds of net, hook, pot, etc. In spite of the method used, struggling and stress might be the consequence of the capturing that can affect the fish quality and increase the amount of waste. Right after the fish is caught, some processing steps can be taken, in order to keep them fresh and undamaged during storage and transportation. For instance, if a fish is not bled within 30 minutes of its death, it will not bleed properly. Therefore, quality defects such as discoloration might occur in the product (Digre, 2013).

The typical on-board processes can consist of operations such as: transferring of the catch from the fishing gear (e.g. trawl, net, etc.) to the vessel, sorting and grading of the catch based on the type and size, bleeding, gutting, washing, chilling, storage and finally landing the fish at the port and transportation to the next part in the supply chain, either the distributer or a production site.

The number and order of the operations that are applied on the catch on board and on shore (e.g. plant) depends on the type of the fishing gear, the size and the equipment of the vessel, the time between catching and landing, the type and demand of the market, and the fish species (Ababouch, 2005). The primary operation of the fish on the fishing vessel can be either manual or automated. The latter is supposed to reduce wastes by avoiding rough handling of the catch.

One of the first steps in fish processing after slaughtering is gutting, either on board or in the processing plant and it is done to prevent autolytic spoilage rather than bacterial spoilage (Shewan, 1961) and also to eliminate the possibility of parasite migration from the intestines into the muscle (Borderıas & Sanchez-Alonso, 2011).

The residual raw materials that are obtained from processing of fish include head, bones, trimmings and visceral fractions, such as: liver, stomach, roe, and gonads. Based on the activity level of endogenous enzymes in the different organs, the byproduct fractions have different degrees of degradation potential, namely: easily

degradable byproducts (e.g. viscera, or blood), and relatively stable byproducts (e.g. heads, skin and bones) (Rustad, et al., 2011). Therefore, the treatment, preservation, and processing method used for each fraction should be chosen according to its susceptibility.

Biochemical deterioration of the raw materials (e.g. autolysis and lipid oxidation) leads to formation of degradation products, such as fatty acids and lipid oxidation products that has a negative influence on organoleptic and nutritional properties of the fish and shortens the shelf life of the product. To prevent the problem, the enzymatic activity should be reduced or the enzymes (lipases and proteases) should be inactivated soon after slaughtering. The enzyme inactivation can be achieved by heating or freezing. In addition, the enzyme activity can be minimized or slowed down as long as the fish is frozen or stored at very low temperature.

There is also an alternative, which is to keep the fish alive and fresh by maintaining suitable conditions, for instance keeping the fish in cold clean water to reduce the metabolic rate (Ababouch, 2005). Different methods of preservation of fish on-board are discussed further in Chapter 1.1.3.

These rest raw materials can add value to the total production by further processing and utilization. Different applications for marine rest raw material are described in Chapter 1.1.6.

1.1.3 Fish Byproducts – Preservation methods

Marine-based raw materials are highly perishable and unstable (Chapter 1.1.4). The spoilage of rest raw material can result in off flavors and odors that develop due to protein degradation and lipid oxidation. In order to produce FPH with desired characteristics, fresh or well-preserved raw material should be used. Therefore, storage and transportation of the raw material to the manufacturing sites should be carried out under controlled conditions, such as temperature, oxygen, etc.

Different preservation methods can be used to prolong the period of storage between catch and landing, such as acidification by inorganic acids, chilling and freezing. In this project, different freezing and thawing methods are investigated in

order to find practical solutions for storage conditions on board and maximizing the yield of the resulting fish protein hydrolysate.

Stable low temperature is mainly used to reduce lipolysis and proteolysis, while acidification has antimicrobial effect (Rustad, et al., 2011), and therefore, the preservation method should be selected based on the characteristics of the raw material and its vulnerability. For instance, because cod is a lean fish, the lipid is concentrated in the liver that makes the byproduct containing liver susceptible to lipid oxidation, and these byproducts should be treated differently than the other fractions. However, in this project cod heads were the only raw material used and the lipid content in the heads is reported to be rather low (Chapter 1.2.1).

As mentioned earlier, one of the ways to keep the fresh marine rest raw material for the time period between catching and landing without perceptible changes in the quality is rapid freezing soon after catching and filleting, and then storing at a suitable constant low temperature. The product should be worth the space, energy and cost that the on-board freezing requires. For instance, the value of cod liver is well known for its oil content and the fishermen know that it is worth saving on board the catching vessel (World Fishing and Aquaculture, 2015). The growing knowledge on the health beneficial effects of marine protein hydrolysates and their great potential as ingredients in functional foods increases the market demand for the other fractions of the fish rest raw material that have so far been discarded at sea.

1.1.4 Fish Byproducts – Factors affecting quality of raw materials

The most important biochemical changes that can occur during storage and affect the quality of marine rest raw material include: lipid and protein oxidation, enzymatic reactions, reactions with lipid oxidation products, and changes in pH, which can lead to protein aggregation, denaturation, cross-linking and breakdown of polypeptide chains. Such changes in protein structure can reduce the nutritional value of the product (e.g. reduced digestibility), decrease the yield of fish protein hydrolysate (FPH), change the organoleptic properties (e.g. color and odor), and alter protein solubility and other functional properties (Thorkelsson, et al., 2009). Some of the most important factors that can influence the quality of marine raw material are discussed in this section.

Protein and lipid oxidation

Both proteins and lipids are targeted for oxidative reactions in marine rest raw material during processing and storage, which is generally initiated by reactive oxygen species (ROS), such as hydroxyl radicals and singlet oxygen (Choe & Min, 2006). Singlet oxygen reacts with aromatic amino acids, while oxidation with hydroxyl radical (OH^-) leads to formation of cross-linked products and carbonyl compounds (Choe & Min, 2006; Davies, 1987). Partial protein denaturation induced by OH^- oxidation can result in unfolding of the protein structure and therefore, the accessibility of peptide bonds is increased for the enzymes (Davies, 1987); while excessive denaturation have an adverse effect on enzyme activity.

The lipid content of marine raw material is highly susceptible to oxidation. Lipid oxidation products can influence the production, yield, quality, composition and functionality of fish protein hydrolysate. Interaction between lipid oxidation products and proteins can lead to Schiff base formation (Yong & Karel, 1978), cross linking (Tironi, et al., 2002), polypeptide chain scission, denaturation and destruction of some amino acids (Soyer & Hultin, 2000; Refsgaard, et al., 2000; Thorkelsson, et al., 2009). The contribution of lipid oxidation products in protein functionality depends on the extent of oxidation, for instance gelling properties of FPH can be improved (or unchanged) by mild oxidation, while highly oxidized lipids can reduce gel forming capacity of FPH (Tunhun, et al., 2002; Thorkelsson, et al., 2009).

Furthermore, the covalent bond between secondary lipid oxidation products (formed by hydroperoxide decomposition) and proteins can damage the protein and amino acids and reduce the yield of FPH in hydrolysis process. The lipid-protein interaction depends on the level of oxygen in the system, the extent of lipid oxidation and formation of secondary products, the temperature and time at which the raw material is stored, the degree of unsaturation of lipid, the contact surface area between proteins and lipids, and the concentration of prooxidants and antioxidants (Aubourg & Medina, 1999; Thorkelsson, et al., 2009).

The oxidative activities are not completely inhibited by frozen storage conditions, particularly in the presence of catalysts such as, light, transition metals, heme groups, etc. Therefore, a variety of oxidation products are formed in the raw material before

the hydrolysis process takes place, which are normally small molecules that interact with the other components and reduce the nutritional and sensory properties of the product (Aubourg, 2001; Thorkelsson, et al., 2009). For example, lipid oxidation may be one of the factors contributing to the loss of protein solubility during frozen storage (Saeed & Howell, 2002).

Endogenous enzymes (autolysis)

The endogenous enzymes that are present in different organs of the fish and the bacterial enzymes are responsible for autolytic process and changes in the properties of the raw material that leads to alterations in texture, flavor and odor. The concentration of autolytic enzymes is the highest in the internal organs, which makes the marine rest raw material containing viscera more vulnerable towards autolysis (Thorkelsson, et al., 2009). There are also proteolytic enzymes in fish muscles and other tissues that are activated by pH drop due to post mortem glycolysis and leads to softening of the muscle and formation of peptides and free amino acids (Thorkelsson, et al., 2009).

Autolysis of lipids and phospholipids can lead to formation of degradation products, such as fatty acids, which reduce sensory quality and oxidative stability of fat (i.e. promoting oxidation); while, proteolytic degradation results in reduced molecular weight of proteins that affects their functional properties (Falch, et al., 2007).

1.1.5 Fish Byproducts – Statistics

On-board processing of fish can lead to a decrease in the utilization of the total catch, since a significant part of the rest raw material is discarded into the sea (Rustad, et al., 2011). The amount of the marine byproduct and the discarded processing leftovers depends on the fish species, size, season and fishing grounds (Falch, et al., 2006).

According to SINTEF Fisheries and Aquaculture report, in 2013 there was a total production of 3.07 million tons fish and crustaceans, including live catch and farmed seafood in Norway, from which 0.87 million tons (28% of total catch) of byproducts were produced (Olafsen, et al., 2014). This means that a significant share of the total

fish production is considered byproducts, which is a valuable resource for further economic exploitation.

The report also states that in 2013, 600,000 tons of the produced byproducts were utilized, which is only 69% of the total. In addition, statistics shows that in 2013, a sum of up to 260-280,000 metric tons of demersal fisheries' byproducts was not used for further processing, mainly due to on-board processing that was performed on trawlers and other types of long distance fishing fleets (Olafsen, et al., 2014). The main reason for discarding the byproducts in the sea can be lack of technical solutions and perceived economic intensives to bring byproducts ashore.

The fish head, guts and livers from demersal species have made up most of the non-used parts of marine byproducts (Olafsen, et al., 2014). Figure 1 shows that a sum of 87% of the total available byproducts ends up as various types of feed ingredients, and only 13% goes directly or indirectly to human consumption.

The different applications, in which the available byproduct was used for in Norway in 2013 includes: direct human consumption (as fresh, frozen seafood products, only 8% of the total byproduct in 2013), indirect human consumption (e.g. fish protein hydrolysate (FPH) and cod liver oil, 5% of the total produced byproduct in 2013), fishmeal and fish-oil, and silage refinement (i.e. further processing of silage into oils and fish protein concentrate) (Figure 1).

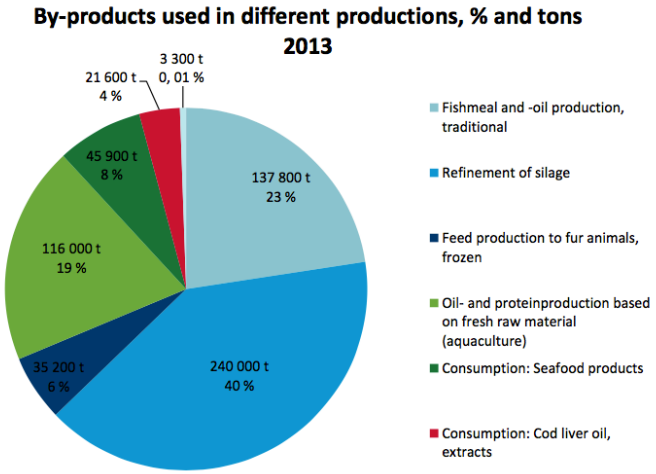

Figure 1. Byproducts used in different productions (% and tons), 2013, Norway, source: (Olafsen, et al., 2014)

1.1.6 Fish Byproducts – Applications

The main part of the fish byproduct from the fish-filleting industry in Norway is used in fish silage production, where it is concentrated or dried and used for feed (Bekkevold & Olafsen, 2007).

Nevertheless, fish residual raw materials can be used directly for human consumption, for instance, dried cod head is utilized in Africa; fish liver can be canned or used for its oil content; the trimmings can be turned to minced fish products, and the roe/milt of some species is a valuable product that can be processed in different ways. The most common preservation methods used for these products include salting, drying, smoking and freezing.

The fish byproducts are rich in nutrients such as proteins, lipids, etc., and there have been numerous investigations to develop different methods for the separation and purification of these nutrients.

The traditional extraction of fish oil consists of cooking and pressing followed by centrifugal separation. Due to the high temperatures used, this method may not only damage the lipids (i.e. faster degradation and oxidation), but also have a negative effect on the protein fraction. Therefore, biological methods such as enzymatic hydrolysis are the preferred technology to produce lipid and protein fractions of high quality (Rustad, et al., 2011). Since cod is a lean fish with a low concentration of lipid, lipid extraction will not be discussed in this report.

Fish is also a rich source of protein and different types of protein fractions can be produced from fish rest raw material, such as surimi (washed fish mince), fish protein isolates (FPI), fish protein concentrates (FPC), and fish protein hydrolysates (FPH). The difference between the three is as follows: FPI is the fish protein that is purified by the pH shift process to achieve a protein content of more than 90% of the dry matter; FPC is a product of chemical hydrolysis; and FPH is produced using enzymatic hydrolysis (Arason, et al., 2009).

FPH is used in this project to determine the influence of freezing storage and thawing methods on the quality of the product. The processing methods and properties of FPH will be discussed in more details in Chapter 1.3.

1.2 Atlantic Cod (Gadus morhua)

Atlantic cod (Gadus morhua) is a benthopelagic fish that belongs to the family Gadidae. It has a dark green or brown skin with a long, lateral line and a barbel or whisker that protrudes from its lower jaw (Figure 2). Cod is a lean fish with firm, white flesh that readily flakes when cooked.

Figure 2. Atlantic Cod (Gadus morhua)

1.3.1. Biology and distribution

The Atlantic cod can live for 25 years, grow to 2 meters in length, and weigh up to 96 kg. The growth rate and sexual maturity age varies between years and areas, for example, the sexual maturity age can be between two and up to eight years.

The largest cod stock in the world belongs to the North-East Arctic cod, which is managed by Norway and Russia, and the stock is in a good condition. The joint Norwegian-Russian Fisheries Commission sets the annual total allowable catch for the stock (The Norwegian Ministry of Trade, Industry and Fisheries, 2015). Different fishing methods, such as trawling, long-line, gill net, Danish seine and hook and line gears are used to catch cod. The other Norwegian cod stock is the North Sea cod, which is managed together with the EU and unfortunately is in a very poor condition.

The main spawning period is March-April, when female cod releases the eggs in batches and the male cod compete to fertilize them. When the eggs are fertilized, they are carried slowly with ocean currents and grow into larvae.

The main nursery and feeding areas for Northeast Arctic cod are in the Barents Sea, where the water temperature is above 0ºC (south of the polar front), and the main spawning areas are along the Norwegian coast north of 67°N (The Norwegian

Ministry of Trade, Industry and Fisheries, 2015). Figure 3 shows the spawning, migration, and feeding areas for the Northeast Arctic cod.

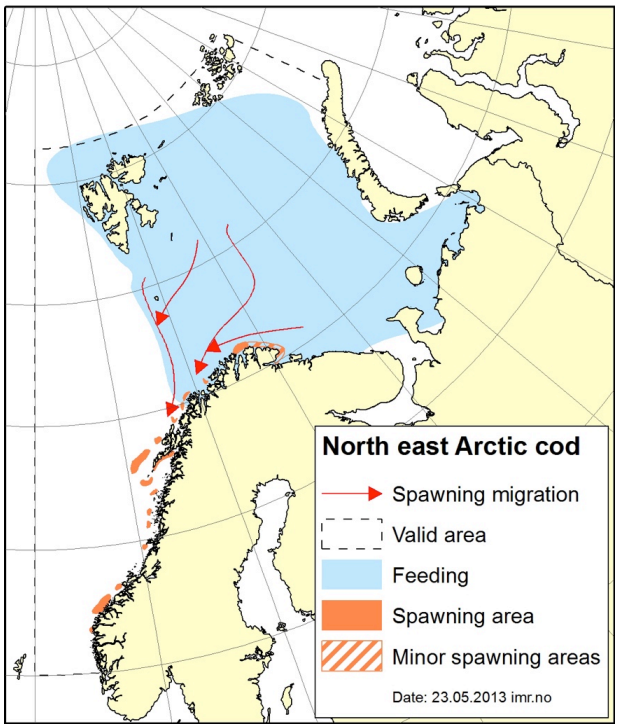

Figure 3 The North east Arctic cod, spawning area, spawning migration and feeding area source: (The Norwegian Ministry of Trade, Industry and Fisheries, 2015)

1.3.2. Cod rest raw material

As mentioned in Chapter 1.1, the rest raw material of a round fish such as cod includes what is left after filleting, such as the head, backbones, trimmings, skin, roe, viscera, etc., and it can represent up to two-third of the fish (Gildberg, 2002; Slizyte, et al., 2009; Rustad, et al., 2011).

Some of the fresh and high-quality cod byproducts are directly used for human consumption, such as cod roe (sugar salted, cooked and chilled, smoked, frozen or canned), head (including cheeks, chin medallions, and tongues), backbone (to obtain muscle strips and swim bladders), liver (oil production), milt, stomach, and trimmings (minced fish products) (Rustad, et al., 2011). Different ingredients, such as proteins, lipids, creatine, taurine, collagen, calcium, and phosphorous can also be extracted from cod byproducts.

As mentioned earlier, the main challenge with cod rest raw material is the long fishing trips (i.e. up to two weeks), during which, the cod is processed and stored on

board and the rest raw material is often discarded at sea. In a study by Akse, et al. (2002), a storage time of 12 hours after catch for gutted and un-gutted fish is suggested. They found that the quality of the fish and the byproducts is not reduced during this period. There is also a difference between spawning cod and heavily feeding cod (i.e. capelin cod), where the former could be stored for up to 48 hours versus 12 hours for the latter (Rustad, et al., 2011).

1.3.3. Cod head

In this project, cod heads were removed from caught wild fish and used to produce fish protein hydrolysate, and therefore a brief description of this raw material is given in this section.

Approximately 20% of the live weight of cod consists of heads, but the percentage will vary depending on the type of machine that is used to remove the head, for example whether or not collar is included in the removed head (Falch & Slizyte, 2008).

According to Stoknes & Økland (2002), farmed cod has smaller head ratio to the round weight (13-15% of body weight) compared to wild fish (about 18%). Cod head consists of about 55% muscle, 20% bone, 15% gills, 5% skin and 4% eyes, with an average protein content of about 14-15% (Stoknes & Økland, 2002; Arnesen & Gildberg, 2006). In a report published by Møreforsking (Økland & Kjerstad, 2002), chemical composition of cod head is determined (Table 1):

Table 1. Chemical composition of cod head (% weight), source: (Økland & Kjerstad, 2002)

Sample	Water (%)	Protein (%)	Lipids (%)	Ash (%)
Cod head	79.3	14.2	4.1	6.4

Stoknes and Økland (2002) described micro and macro mineral composition in cod heads. In addition, Stoknes et al. (2004) described Fatty acid and fat class composition of the eyes and brain. Different authors suggest several applications for the fractions that can be extracted from fish head in health food, cosmetics, ingredient and infant formula (Stoknes et al., 2004; Stoknes & Økland 2002; Stoknes & Hellevik, 2000). However, there has not been any evidence of using cod heads to the recovery of protein and gelatin in Norway (Aas & Kjerstad, 2008).

In Norway, a large proportion of the catch is landed without head. While in other fishing nations like Iceland, Denmark, the Faroe Islands and Canada, cod is normally landed with head on and the head can be utilized either for direct human consumption or as ingredients in food and animal feed industries (Bekkevold & Olafsen, 2007). Drying, salting and freezing are some of the methods used to preserve whole heads.

In Norway, heads from wild caught cod have traditionally been underutilized (Aas & Kjerstad, 2008). While, the Icelandic statistics shows that they have developed an appropriate utilization and market for cod head, including exportation of salted and fermented cod heads to Nigeria and Asian countries such as Korea and China and frozen head to Portugal and Spain (Aas & Kjerstad, 2008). There is a great potential for Norway to achieve a better utilization of cod heads in general and wild cod heads in particular, due to its larger size compared to the farmed cod.

Due to the large size of wild cod head, a large space is required to store them, and therefore mincing is an option that should be considered to minimize the required space. If the heads are supposed to be stored in frozen state, there is an extra processing cost of freezing and frozen-storage space as well. In this project, the possibility of frozen storage of whole versus minced cod heads is investigated. The main purpose is to determine if it is profitable to spend the space and energy to store cod head and use it as raw material for production of value added FPH, despite the changes that might take place during freezing and thawing.

1.3 Fish protein hydrolysates (FPH)

Kristinsson and Rasco (2000) defined protein hydrolysates as chemically or enzymatically broken down proteins into free amino acids and/or peptides, which can present a large range of molecular weight distribution depending on the greater or lesser degree of hydrolysis.

The protein hydrolysates can be used to change the functional characteristics (e.g., water-holding, texture, gelling, foaming and emulsification properties) of different food systems; in addition, the resulting proteins are less allergenic and easier to digest. Several bioactive properties are also attributed to protein hydrolysates that will be discussed in section 1.2.6.

1.3.1 Chemical hydrolysis

Chemical hydrolysis of fish byproducts is used in some industrial practices, and it is done by using strong chemicals and solvents (acid or alkaline) at extreme temperature (up to 118ºC) and pH conditions. Hydrochloric and sulphuric acid are the most common acids used for chemical hydrolysis of fish proteins, which result in an extensively hydrolysed product with high solubility (Slizyte, 2004).

Although the process is relatively inexpensive and the method is rather simple, it is difficult to control the process, and the chemical and compositional properties of the resulting hydrolysate might be variable from batch to batch (Kristinsson & Rasco, 2000). In addition, the extreme conditions of production may reduce the nutritional value of the products, for instance destruction of susceptible amino acids (e.g. tryptophan), which would limit their use in food applications.

The other issue that arises when acid is used for hydrolysis is that it should be neutralized by a base (e.g. sodium hydroxide), which leads to formation of a large amount of salt. The salt decreases the desirability of the product, and interferes with functional properties of the protein. Therefore, the acid hydrolysis is mainly used for production of fertilizers (Kristinsson & Rasco, 2000).

The alkaline reactants (e.g. sodium hydroxide) are also used to hydrolyse fish proteins, primarily by using fish protein concentrate (FPC) as the starting substrate (Slizyte, 2004). However, alkaline hydrolysis can also cause some defects in the product, including an adverse effect on the nutritive value of the product and several unpleasant reactions. For example, by removal of hydrogen from the alpha carbon of an amino acid, racemization of L-amino-acid to D-amino-acid might happen. D-amino-acid cannot be absorbed in human gastrointestinal system. In addition, cleavage of disulfide bonds in some essential amino acids (e.g. cysteine, serine and threonine) not only destroys and eliminates them from the product, but also may lead to formation of toxic and highly reactive substances such as dehydroalanine (DHA) (Kristinsson & Rasco, 2000).

1.3.2 Enzymatic hydrolysis

Enzymatic hydrolysis of fish proteins is performed either by endogenous enzymes that are mainly originated from viscera and liver (i.e. autolysis), or/and exogenous enzymes that are added to promote the hydrolysis process. Many enzymes of microbial, plant and animal origin have been used in hydrolysis of fish proteins, including: papain, Alcalase®, bromelain, Protamex™, etc. (Souissi, et al., 2007).

When commercial proteases are used, the rate of enzymatic hydrolysis is accelerated compared with autolysis. The process can be controlled by the choice of added enzyme(s), process conditions and the time of the hydrolysis reaction. Hydrolysis process causes a decrease in the peptide size, which can improve the functional properties of the proteins, quality, and yield. The functional properties of the fish protein hydrolysate (FPH) depend on many different factors, including type of processing method used, characteristics of the raw material, and the extent of the hydrolysation.

However, there are some disadvantages in using enzymatic hydrolysis that can restrict the application and production of fish protein hydrolysates on an industrial scale. One of the disadvantages of enzymatic hydrolysis of fish rest raw material is the relatively high price of commercial enzymes that are used as exogenous enzymes in the process. The other problem is the challenges in controlling the hydrolysis process to obtain uniform results and products with the same quality and characteristics (Kristinsson & Rasco, 2000). The protein yield is also variable in this method and depends on different factors (Chapter 1.3.4).

The enzymatic hydrolysis of fish protein (cod head) is the method used in the current project. There are more details about properties and applications of FPH in Chapter 1.3.

Enzymes

Enzymes are proteins consisting of long chains of amino acids, which are bound together by peptide bonds, and are present in every living cell. The main function of enzymes in biological systems is to control the metabolic processes, by which energy and fresh cell materials are produced.

The proteases can be divided into groups based on, mechanism of action and three-dimensional structure (Neurath, 1989). For example, based on their active site, peptidases can be divided into two classes: the exopeptidases and endopeptidases. Exopeptidases (exoproteases or aminopeptidases) can cleave the proteins only near the N- or C-terminus, and endoproteases (endoproteases or proteinases) preferentially act away from the termini and cleave the internal peptide bonds within the protein molecule. Therefore, the hydrolysis products of endopeptidases are usually smaller peptides, while exopeptidases produce free amino acids, di- or tri-peptides (Slizyte, 2004) Endopeptidases have complex specificities with regard to which peptide bonds they can hydrolyse. The specificity of endopeptidase determines the number of cleavages in a peptide chain, and thereby the size distribution and characteristics of the hydrolysis product, such as hydrophobicity and aromatic properties. In some processes, both endogenous and exogenous enzymes are used.

The proteases can also be classified based on their pH requirements into acidic (mainly produced by molds and yeasts), neutral or alkaline. The other way to characterize proteases is based on their mechanism of action and sensitivities towards different inhibitors due to the principal functional groups in their active sites, namely: serine, thiol, metallo and aspartic proteases, which have serine, thiol, metal ion or aspartic acid, respectively, in their active sites (Venugopal, 1994; Kristinsson & Rasco, 2000).

The desirability of a proteinase for industrial purposes depends on the hydrolysis product that is required. In other words, the choice of enzyme can be based on the raw material characteristics, the desired chemical and functional properties (e.g. gelation, foaming, emulsifying), and on the bioactive properties of the product. For instance, usually bioactive peptides are smaller than peptides contributing to functional properties.

The extent of hydrolysis can be controlled either by choosing site-specific enzymes (e.g. trypsin or chymotrypsin) versus non-specific proteases (e.g. papain) or by maintaining the hydrolysis process conditions, namely hydrolysis time.

Hydrolysis process

The hydrolysis process using one or more commercial proteases generally involves the preparation of raw material (e.g. collection and mincing of the fish rest raw material), addition of water (the amount of water could vary in different processes), addition of enzyme and providing constant optimum temperature for the enzyme, followed by deactivation of the enzyme at a particular time of hydrolysis and separation of the fractions. The inactivation of the enzyme can be achieved by heating to 75-100°C for 5-30 minutes or by chemical inactivation (e.g. extreme pH values).

After hydrolysis, a suspension of different particles (depending on the raw material) is obtained, such as lipids, scales, bones, and insoluble proteins. The mixture is separated into fractions using centrifugation, ultrafiltration, nanofiltration, or sieving to recover the hydrolysates as a soluble phase and separate it from the lipid phase and insoluble sludge phase. Centrifugation is the most common practice to separate these fractions. It uses the action of centrifugal force to promote accelerated settling of particles with different densities in a solid-liquid mixture.

Five distinct major phases might be formed in the vessel during centrifugation depending on the composition of raw material, namely: sludge on the bottom, aqueous phase in the middle (containing protein hydrolysate), a lipid-protein fraction between aqueous layer and sludge, an oil-water emulsion, and the oil layer on the top (Slizyte, 2004).

The aim of the fractionation step in the hydrolysis process is to obtain the desirable compounds with maximum purity. Optimum centrifugal separation depends on the size, shape and density of the particles in the mixture and the viscosity of the hydrolysis suspension, and on the speed of rotation in the centrifuge (Slizyte, 2004).

Eventually, the aqueous supernatant is either concentrated or dehydrated using a spray-dryer or freeze-dryer (Kristinsson & Rasco, 2000). The concentration or drying method is chosen based on different factors, such as: the cost of operation, the changes in the properties of the product due to high temperature (e.g. coagulation and denaturation of proteins), the appearance of the end product (e.g. color), and the length of the operation time. For example, longer operation can cause quality defects due to oxidation and increase the total cost of operation.

1.3.3 Degree of hydrolysis

Degree of hydrolysis (DH) indicates the percentage of the peptide bonds that are cleaved by the hydrolysis, and can be calculated as follow:

$$DH\% = h/h_{tot} \times 100$$

Where:

h - The number of hydrolysed peptide bonds

h_{tot} - The total number of peptide bonds present

DH is used to describe the extent of the hydrolysis process, which determines the characteristics of the FPH (Slizyte, et al., 2005). For example, the size of the peptides in the FPH defines the interfacial activities and properties of the protein, and therefore the degree of hydrolysis is of great significance (Slizyte, et al., 2009).

There are different direct and indirect methods to determine DH of protein hydrolysates, including the pH-stat method, amount of soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), o- phthaldialdehyde (OPA), amino acid nitrogen, and formol titration methods (Rutherfurd, 2010). Formol titration and soluble nitrogen after TCA precipitation (which is an indirect method) are used in this project to determine the DH.

Generally, hydrolysates with lower degrees of hydrolysis (i.e. partial hydrolysis) have larger molecular weight and higher surface hydrophobicity. Hydrophobicity plays an important role in determining functional properties of hydrolysates, including solubility, foaming, interfacial properties (e.g. emulsification), and antioxidant effects. Under normal conditions, peptides with larger molecular weight (i.e. minimum length of >20 residues) increase emulsifying stability and activity, and decrease solubility and gelling properties (Kristinsson & Rasco, 2000).

The most important drawback of the low molecular weight peptides (i.e. molecular weight from 1000-6000 Da) is their bitter flavor. When proteins are hydrolysed, the buried hydrophobic peptides are released, which can interact with the taste buds causing a bitter taste (Hou, et al., 2011). According to Damodaran, et al., (2007), bitter peptides have a mean residue hydrophobicity of more than 1.4 kcal/mol.

Adler-Nissen (1984) indicated that a DH value of 3-5% could usually reduce bitterness, and according to Daukas, et al. (2004), the highest risk of bitterness is in the DH range between 4 and 40%.

The bitterness of FPH can be eliminated by employing a mixture of endo- and exopeptidases that can further break the bitter peptides down into free amino acids. However, formation of free amino acids can lead to decreased functional properties of fish protein hydrolysates. Therefore, in order to achieve the most appropriate %DH and to ensure the desirable functional and bioactive properties and preventing the development of bitter taste, strict control of hydrolysis process and terminating the process at that point is of great importance (Kristinsson & Rasco, 2000).

An alternative method to avoid or reduce formation of bitter peptides and to have a better control over the process is to inactivate the endogenous enzymes prior to the hydrolysis. Different enzyme inactivation methods can be used, such as heat treatment, pressure induced inactivation, and mechanical (i.e. shearing) inactivation, of which the heating process is the most common method. One disadvantage of heat inactivation of the endogenous enzymes is the possible decrease in the yield and nutritional value of FPH (Chapter 1.3.4).

1.3.4 Yield

Hydrolytic process yield determines the profitability of hydrolysis process, which is one of the most important factors in production of FPH. The yield of the FPH as the soluble fraction in enzymatic hydrolysis depends on the type, properties and concentration of the substrate, the type and properties of the enzyme, the enzyme-substrate compatibility, the hydrolysis conditions (e.g. pH, temperature and the time of enzymatic reaction, concentration of enzyme and degree of dilution), and the fractionation conditions after hydrolysis process (Slizyte, 2004). Some of the most important factors and the way they influence the yield of the FPH are described here.

The substrate

The substrate is the raw material used for hydrolysis, which is a determining factor for both the hydrolysis process and properties of the final product. Some of the most important properties of the substrate that influence the yield and characteristics of the final product include: the conformation of protein molecules (e.g. denaturation),

lipid content (e.g. lipid oxidation, protein-lipid complexes), and the composition of the raw material (e.g. bones, head, skin, viscera) (Slizyte, 2004).

Protein denaturation, whether it happens before or after hydrolysis step, has a negative effect on the yield of FPH fraction. The heat treatment might be applied on the raw material prior to hydrolysis (in order to deactivate the endogenous enzyme), which can cause protein denaturation. The denatured proteins in raw material can increase the resistance to hydrolysis by enzymes (Mohr, 1980). The hydrolysate is heated after hydrolysis to inactivate the enzyme(s), resulting in protein denaturation after hydrolysis. Protein denaturation occurs when the hydrophobic interactions between peptides occurs, which leads to self-association of larger peptides, possibly precipitation of proteins and eventually decreased yield of hydrolysate fraction (Mohr, 1980; Slizyte, et al., 2005).

The amount of lipid in raw material is the other factor that has a significant effect on the protein recovery and the percentage of solubilized protein. In addition, if the lipid content consists mainly of phospholipids and other polar lipids, initial heating of raw material can lead to formation of protein-lipid complexes (Slizyte, et al., 2005). The formation of protein-lipid complexes increases the resistance to enzymatic breakdown, resulting in reduction in the yield of both oil and FPH fractions (Slizyte, et al., 2005).

The enzyme

The second factor, commercial enzymes, as explained in Chapter 1.3.2, is usually a combination of endo- end exopeptidases that are used to produce FPH with different properties. The enzyme for hydrolysis should be selected based on its specificity and efficiency, the enzyme stability in the substrate system, the chemical and functional properties of the desired product, and the economic cost/benefit (Kristinsson & Rasco, 2000). It is suggested in literature that using broad-spectrum endopeptidases (e.g. pepsin) are more effective in solubilizing fish proteins (i.e. increasing yield) than when highly specific enzymes (e.g. tripsin) are used for enzymatic hydrolysis process (Mohr, 1980; Gildberg, 2002).

The concentration of enzyme & substrate

Another significant factor in enzymatic hydrolysis and the yield of FPH is the concentration of both enzyme(s) and substrate (i.e. protein content) in the system. An increase in the concentration of total fish solids can reduce the yield of FPH in the solubilized phase, due to the product inhibition (Mohr, 1980). Therefore, by adding proper amount of water to the raw material prior to hydrolysis, the access of enzymes to the proteins is enhanced and therefore the yield of the FPH can be significantly improved (Kristinsson & Rasco, 2000; Gildberg *et al.*, 2002). In a project by Slizyte, et al. (2005), different amounts of water were added to the raw material prior to hydrolysis and its influence on yield of different fractions was investigated. The authors suggested that the higher moisture content makes proteins more available for enzymes, and therefore the yield of the fractions increases. The results showed that the added water was more important regarding the yield of all fractions than type of the enzyme used. However, the yield of the lipid fraction was the highest in the samples without addition of water, while the yield of FPH in these samples was lower than the samples with added water (Slizyte, et al., 2005).

The hydrolysis conditions

Eventually, the conditions, at which the process takes place, such as pH and temperature should be adjusted to the optimal conditions for both activity and stability of the selected enzymes (Mohr, 1980). Since it is rather expensive to adjust the pH, the temperature adjustment is used to improve the conditions for enzyme activity and stability.

1.3.5 Properties and applications of FPH

There is a great potential in the food industries (e.g. bakery products, appetizers, cookies and soups) for FPH as protein supplement, because of its effective characteristics. These positive aspects include good nutritional value with well-defined chemical composition, quick intestinal absorption/utilization in the body, flavor enhancing properties, and functional, antioxidative and bioactive properties. FPH is particularly of interest for individuals with special nutritional and physiological needs, for instance it can be utilized in formula for infants who have allergies to intact proteins or are born with inborn errors of metabolism. According to

Aas & Elsayed (1969), an extensive hydrolysis with proteolytic enzymes can eliminate the allergenic and antigenic properties of cod products.

Functional properties

According to Hall & Ahmad (1992), the functional properties can be defined as: *"The overall physicochemical behavior of performance of proteins in food systems during processing, storage, and consumption"*. In addition, a more general definition given by Pour-El (1976) is: *"Any property of food or food ingredients except its nutritional ones that affects its utilization"*.

Functional properties of proteins can be categorized into three main groups based on the mechanism of action (Damodaran, et al., 2007):

1. Hydration-related properties, such as: absorption of water/oil, solubility, thickening, wettability
2. Structural-related and rheological characteristics, such as: viscosity, elasticity, adhesiveness, aggregation, and gelation)
3. Interface-related properties, such as: emulsifying and foaming activities

The functional properties of FPH is determined with regard to different compositional and processing parameters, such as: physiochemical properties of the original protein (i.e. raw material, fish), enzyme specificity, hydrolysis conditions and reaction time, composition of the FPH, the amino acid sequences, molecular size, the electrostatic changes in the protein structure, water affinity, etc. (Pires & Batista, 2013; Souissi, et al., 2007).

Some of the functional properties that make them suitable for food applications are briefly explained in this section. However, the details are left out, since this project is not focused on functional properties of FPH.

- *Water holding capacity*

Since the functional properties of proteins in food matrices depends greatly on the water-protein interactions, the water holding capacity (WHC) is one of the factors that are of significance in protein hydrolysates. WHC is defined as the ability of the

protein to absorb water and retain it against gravitational force within a protein matrix, such as protein gels or beef and fish muscle (Feng & Hultin, 1997). FPH can contribute to increased water holding capacity in food matrices. In a study by Slizyte, et al. (2009), the WHC of cod mince increased by addition of FPH powders by approximately 12% for FPH obtained from frozen backbones and 16% for FPH obtained from fresh backbones.

The increased water holding capacity in hydrolysed protein compared to native proteins is due to the increased surface area to mass ratio with exposure of some hydrophobic groups that are buried in the intact protein structure (Damodaran, et al., 2007). However, according to Slizyte, et al. (2009), excessive hydrolysis decreases water-holding capacity of the hydrolysates. The sludge fraction of enzymatic hydrolysis of cod byproduct have shown even higher water holding capacity, fat absorption and emulsifying properties than FPH, which can inspire increased utilization of marine byproducts via enzymatic hydrolysis (Slizyte, et al., 2004).

- *Interfacial properties*

The other functional property of protein that can be maintained by hydrolysis is their interfacial properties, including emulsification and foaming characteristics, which is useful when FPH is used as surfactants in food products, such as sausages and dressings.

An emulsion is a mixture of two or more immiscible liquids, such as oil and water; and proteins are used to improve emulsion formation and increase the stability of the emulsion. FPH can reduce the interfacial tension at oil-water interface and form an interfacial film to prevent coalescence of the droplets (Damodaran, et al., 2007). The good emulsification property of FPH is due to the interfacial features, such as solubility in water and surface activity that is determined by their hydrophilic and hydrophobic functional groups (Slizyte, et al., 2009). For instance, they can adsorb to the surface of the oil droplets that are formed during homogenization, and by forming a protective membrane prevent the droplets from coalescing and therefore, promote the oil-in-water emulsion (Slizyte, et al., 2005). However, according to Kristinsson & Rasco (2000), the emulsifying properties of the hydrolysate decreases with an excess increase in the degree of hydrolysis, due to formation of small peptides, that have lower surface activity and less hydrophobicity.

Foaming properties of FPH is related to their film-forming capacity at the air-water interface. Hydrogen bonding, electrostatic and hydrophobic interactions are the intermolecular interactions that are involved at the interface. There is a difference between rapid foam formation ability of a protein and the stability of the formed foam. Foam is formed more rapidly in presence of low molecular weight and amphipathic (i.e. a compound with both hydrophilic and hydrophobic properties) proteins, which is not necessarily ideal for the protein-protein interactions required to form stable foams (Damodaran, et al., 2007).

- *Solubility*

Solubility is another important functional property that is determined by the degree of hydrolysis (DH). Hydrolysis of proteins increases their solubility and if a moderate heating is applied, aggregation and precipitation of protein content could be avoided and the FPH can remain soluble in a wide pH range (Rustad & Hayes, 2012).

Antioxidant properties

Antioxidant properties of FPH are of particular interest in foods, since lipid oxidation is one of the major challenges in food industries. The lipid oxidation compounds may cause undesirable changes in flavor, color, texture and loss of nutritional value of food products. In addition, some reactive oxygen species (ROS), such as superoxide anion ($O2^{-}$), singlet oxygen ($O2^{*}$) and hydroxyl radicals ($^{\cdot}HO$), together with free radicals and peroxides are produced in human body during cellular respiration. ROS formation have to be prevented either by endogenous antioxidants, like enzymes, tocopherol, ascorbic acid and peptides, or by consumption of antioxidants in food or dietary supplements (Zahara, 2007; Batista, 2013).

Antioxidant properties of FPH are related to its inhibitory effect on several different oxidation pathways, such as inactivation of ROS, scavenging free radicals, chelating of pro-active transition metal ions (e.g. Fe^{2+} and Cu^{2+}), and non-radical reduction of hydroperoxydes to nonreactive hydroxides (Jensen, et al., 2013; Shahidi, et al., 1995; Slizyte, et al., 2009). The antioxidant property of FPH is important in production of oxidatively stable food products that are fortified with proteins (Slizyte, et al., 2009).

Bioactive properties

There is an increasing interest in the positive impact of food-derived bioactive peptides on human health, such as: antimicrobial, antihypertensive, cholesterol-lowering, antithrombotic, mineral absorption enhancement, antioxidative, and immune-modulatory properties (Rutherfurd-Markwick & Moughan, 2005; Kim & Mendis, 2006).

Bioactive peptides are physiologically active peptides within the intact protein molecule sequence consisting of generally 3-20 amino acid residues in length. They can be released by enzymatic hydrolysis during fermentation, enzymatic hydrolysis with selective protease, or gastrointestinal digestion (Korhonen & Pihlanto, 2003).

Hypertension is an important health issue causing cardiovascular diseases, which is regulated by Angiotensin I Converting Enzyme (ACE). ACE influences blood pressure by converting the inactive angiotensin 1 into the potent vasoconstrictor angiotensin 2 (Jensen, et al., 2013). One of the most important bioactivity aspects of fish protein hydrolysates is their ability to inhibit the action of the ACE (Gálvez, et al., 2013).

2 Aim of the work

The main aim of this project was to investigate the possibility of utilizing marine cod heads, and whether it would be profitable to freeze-store and thaw them to produce high quality FPH with high yield. Furthermore, since the minced head requires less storage space, the raw material, cod head, was frozen either as whole head or in minced state.

Therefore, the parameters that were investigated in order to find practical solutions for storage conditions on board and further utilization of this byproduct to produce fish protein hydrolysates included: the state of raw material (minced or whole), the effect of freezing, and two different thawing methods.

Other than determining the composition of raw materials and FPHs, other methods were employed to study influence of the applied conditions on the product. The degree of hydrolysis was investigated to determine the degree of protein degradation, and the amino acid content and composition was examined to differentiate between the FPH produced from fresh and frozen raw material with regard to the total amino acid content and the dominating amino acid in FPH.

In addition, it is important to obtain the highest yield for FPH compared to the other fractions regarding the concentration of protein in the dry matter. Therefore, the yield of protein content in FPH obtained by two different hydrolysis time, 30 and 60 minutes, was measured for the hydrolysates produced from fresh raw material, and frozen and thawed samples.

3 Materials and methods

3.1 Preparation of the cod heads

The raw material used in this project was cod heads that were removed from live fish caught by two fishermen and delivered at SINTEF Fisheries and Aquaculture AS. Approximately 40 cod were caught in different areas of Trondheimsfjorden in week 10 (2015) and kept alive onboard the vessels in plastic tubes (600 L) of fresh seawater that was pumped and circulated. The fish were stored in small fish pens (ventemerd) in the sea outside Ila Fiskemottak for a maximum of 2.5 weeks and a minimum of a couple of days before the experiment.

The fish was delivered alive to SINTEF Fisheries and Aquaculture AS and gutting and removing of the head was performed the day before the first hydrolysis (day zero). The heads were covered with ice and stored at 4ºC overnight until further processing. The removal of heads was carried out manually, and some of the heads contained neck meat as well (Figure 4). In addition, the ice that was used to cover the heads during storage time was melted and some of the heads were soaked in the melted water, which caused some physical changes such as deformation and discoloration (Figure 4).

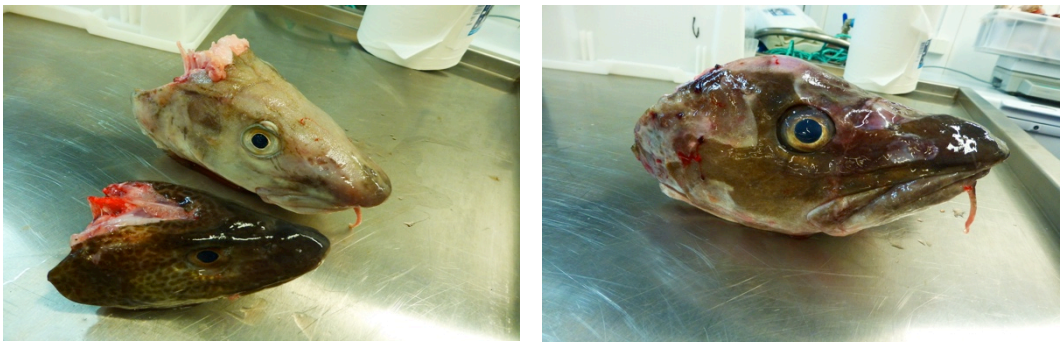

Figure 4. Examples of the cod heads used for the project, demonstrating inclusion of neck meat in some samples and deformation and discoloration in the heads that were soaked in water during storage time.

Upon separation of the heads for mincing and storage, we tried to make a blend of the heads with neck meat and without, the samples that were soaked in water and the dried ones, and regarding the size of the heads.

Figure 5 shows a summary of the main hydrolysis steps and the process conditions used in this project.

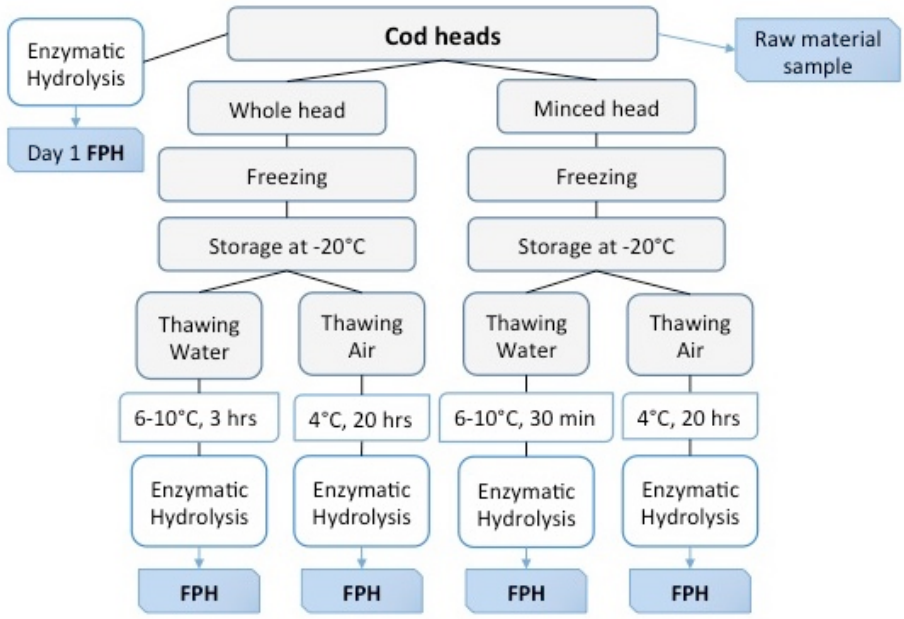

Figure 5. Flow diagram illustrating an overview of the whole project and handling of the cod head as the raw material prior to hydrolysis. The samples for analysis are shown in blue boxes.

Three kg of the total heads were used for hydrolysis on day one in two parallels and the rest were divided into two parts. Half of them were frozen as whole heads and the other half were minced and vacuum packed in plastic bags (0.5–0.6 kg per unit) and used in 1.5 kg batches (Figure 6). The mincing was carried out in a Hobart mincer (10 mm holes) to produce a homogenous substrate.

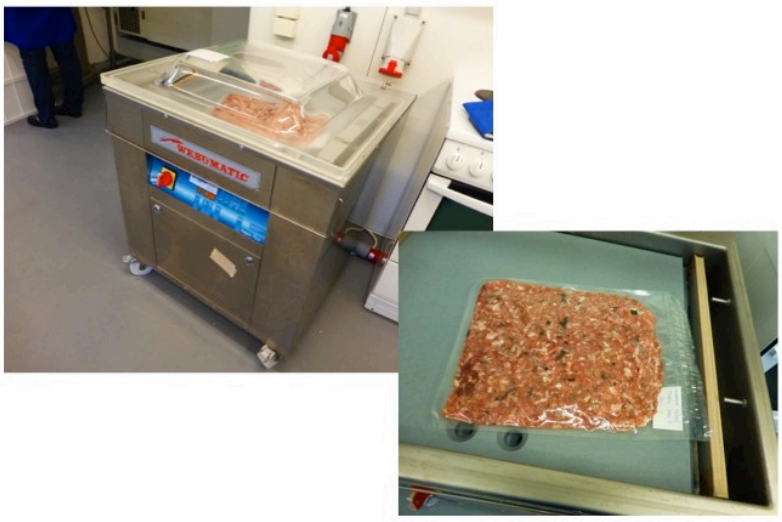

Figure 6. Minced cod head; the vacuum packing machine on the left and the vacuum-packed minced cod head on the right

To investigate the influence of freezing and thawing, whole and minced cod heads were frozen using an impingement freezer (Figure 7) with air temperature of -37ºC for about 25 min and stored at -20ºC.

Figure 7. The impingement freezer used to freeze the raw material as the intact or vacuum-packed minced cod head at -37ºC

After 20 days, half of the whole heads and half of the minced heads were removed from the freezer and placed under running tap water (6 to 10ºC) to thaw and be prepared for hydrolysis process. The minced heads were packed as a thin layer (0.8-1.3 cm), and therefore only 30 minutes was enough for them to thaw; while for the whole heads, it took 3 hours until the core temperature of -2ºC was achieved (Appendix I). In addition, since the whole heads were directly in touch with water, and the water was running for three hours, some particles and soluble compounds were removed from the head and into the water that was discarded.

The rest of the stored cod heads were moved to a cold storage room on the 21st day from delivery date (Figure 8), and after about 20 hours, the core temperature of the whole heads reached 0ºC. It took much shorter time for the minced head to thaw at 4ºC, but they were left in cold room until the whole heads were thawed.

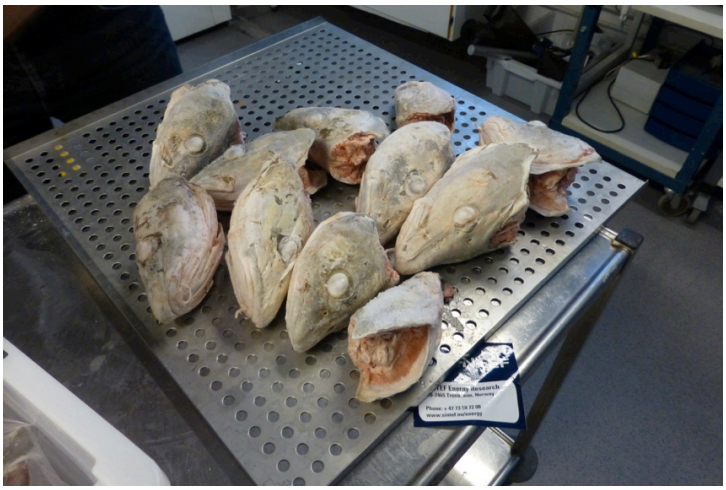

Figure 8. The frozen cod heads removed from the freezer and ready for thawing in either water or air

3.2 Hydrolysis process

The hydrolysis processing steps to produce fish protein hydrolysate from cod heads are summarized in Figure 9. The thawed cod heads were either already minced (on the day of delivery) or were grinded using a Hobart mincer with 10 mm holes and mixed prior to hydrolysis to produce a homogenous batch.

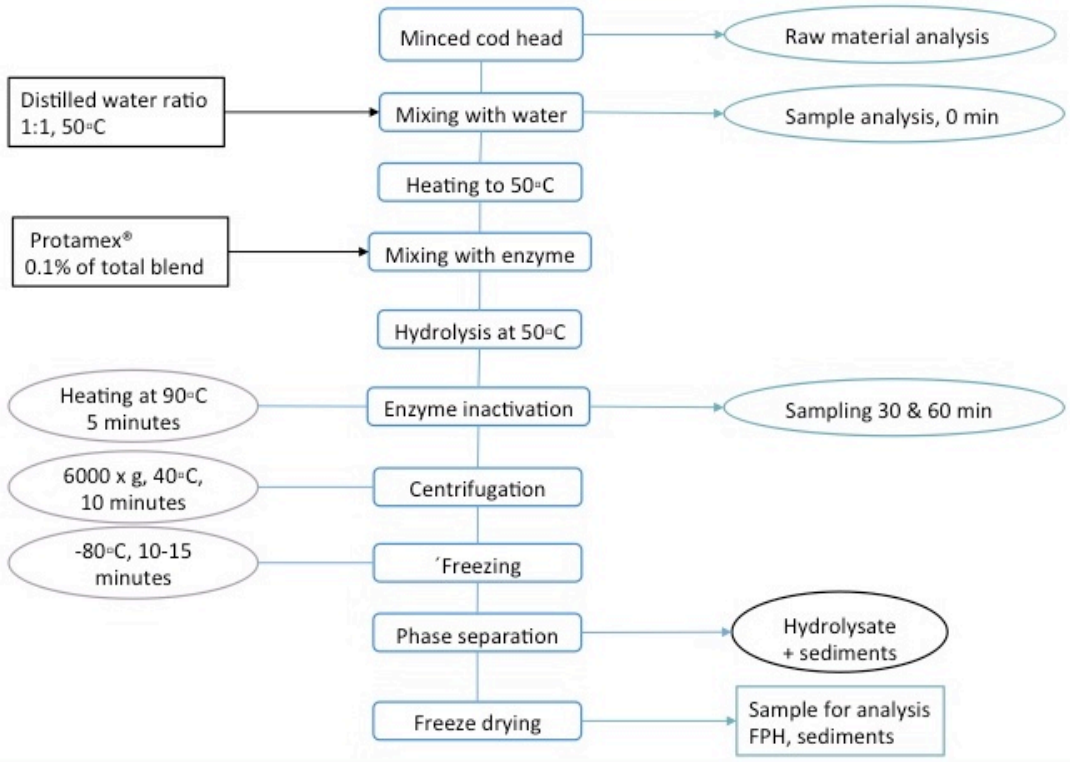

Figure 9. Flow diagram illustrating the main steps in hydrolysis of cod rest raw material (heads)

One kg of the raw material (approx. 3-5 cod heads) was mixed with 1 kg of 50ºC distilled water, and put into two parallel hydrolysis reactors (with 3 liter capacity) that were placed in the same preheated water bath with controlled temperature to provide a constant temperature of 50ºC the entire time. An electrical impeller was mixing the blend constantly during hydrolysis, in order to ensure uniform temperature and condition in the entire mixture (Figure 10).

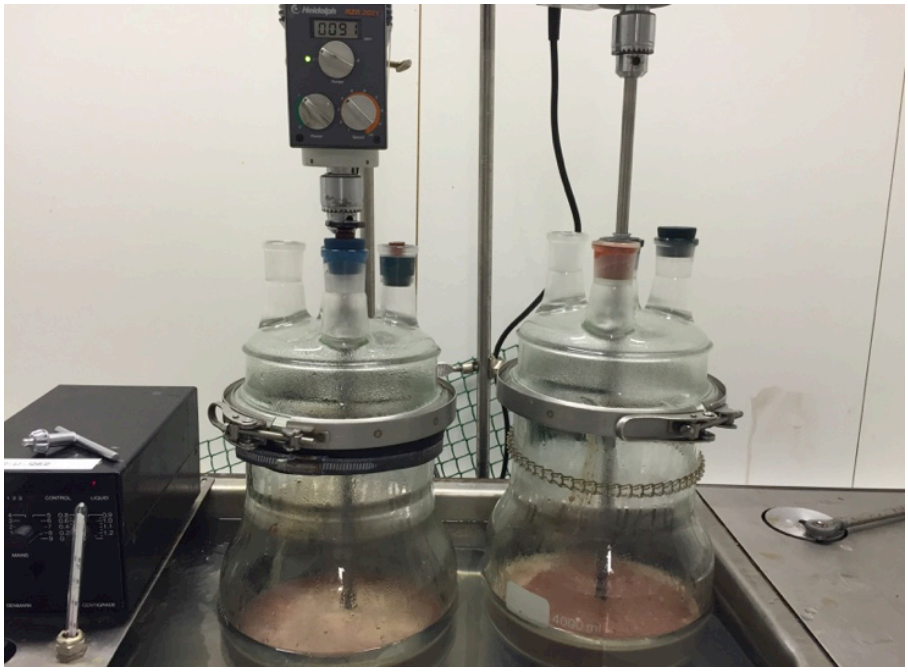

Figure 10. The reactor equipped with mechanical stirring placed in a water bath used for hydrolysis process of cod heads.

The temperature of the raw material was monitored using a digital thermometer, and when the blend reached a temperature of 50ºC, the enzyme was added. Right before addition of the enzyme, 500 g of the raw material was removed from each reactor to be analyzed as the zero-sample of the batch. For this project, the enzyme Protamex^{TM} (Novozymes AS - Denmark) was used with a concentration of 0.1% dry weight of enzyme per wet weight of the raw material (i.e. total weight of fish and water). The enzyme was diluted with a few drops of distilled water and mixed with the mixture of minced cod head and water. A constant temperature of 50ºC and the rotation in the reactor provided optimal condition for the enzyme activity in the reactors.

After 30 minutes of hydrolysis, another 500 grams of the cod head mixture was removed from each reactor and the remaining mixture was left in the reactors for 30

more minutes. The samples after 0 and 30 minutes hydrolysis were only analyzed to measure the dry weight, acid vs. total soluble peptides, and free amino acid composition; but 60 minutes FPH samples were in addition analyzed for lipid and protein content, degree of hydrolysis and yield.

Right after removal from the water batch, the enzymes were inactivated by heating the hydrolysates at 90ºC in a microwave oven for 5 minutes. After heat-treatment and deactivation of the enzymes, the samples were mixed and divided into 50 ml graduated centrifuge tubes, and centrifuged for 10 minutes using a Sigma 3K- 2 for 6000×g at 40°C. In the centrifugation of the mixture, only two fractions were separated, namely the fish protein hydrolysate (the aqueous phase), and the sediments in the bottom of the tube (Figure 11).

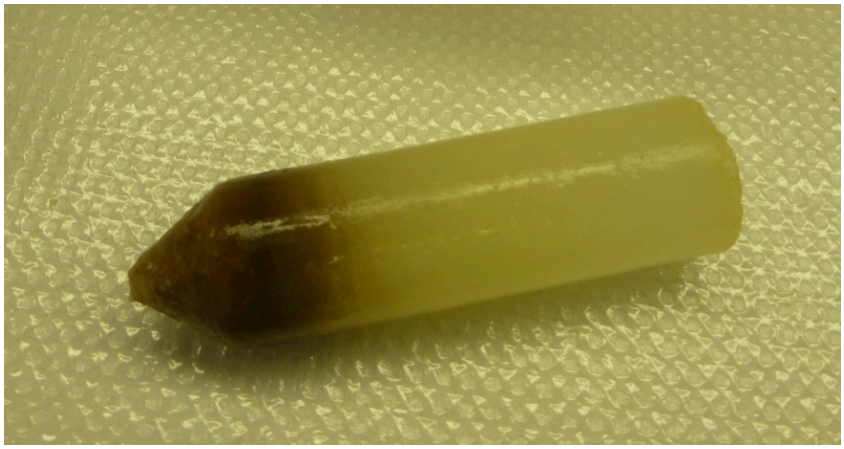

Figure 11. Two phases in one of the hydrolysed products (frozen sample) separated by centrifugation (6000×g at 40°C); the aqueous light color phase on the right (hydrolysate) and the dark solid phase in the left of the picture (sediment)

Since the concentration of lipid is very low (maximum 3.91% of raw material according to our results, Chapter 4.2), the lipid and emulsion phases were not separated in this project. However, some traces of narrow yellow lines were observed in the liquid phase of 30 and 60 minutes hydrolysate samples, which were not separable from the hydrolysate phase.

After separation, the aqueous phase containing the fish protein hydrolysate was thawed at room temperature and filtered using glass wool in a funnel and was freeze-dried after freezing at -80ºC to obtain the FPH in a powder state for further analysis. The sediment fraction was also thawed in room temperature, mixed, frozen at -80ºC and freeze-dried.

Table 2. The samples used for analysis including: A. day zero raw material: B. day one with no frozen storage and three different hydrolysis times (0, 30 and 60 minutes), C. Whole (WH) and minced cod heads (MH) stored in frozen state for 20 days and thawed in 6-10ºC running water (W) and three different hydrolysis times (0, 30 and 60 minutes), and D. Whole and minced cod heads stored frozen for 21 days and thawed in air (A) at 4ºC cold room for 20 hours and three different hydrolysis times (0, 30 and 60 minutes),

A. Day zero: raw material

Sample	Hydrolysis time (min)
1	-

B. Day one

Reactor	Hydrolysis time (min)
1 & 2	0
1 & 2	30
1 & 2	60

C. Whole and minced cod heads thawed in water (W)

Reactor	Whole/Minced Head	Hydrolysis time (min)
1 & 2	WH	0
1 & 2	WH	30
1 & 2	WH	60
1 & 2	MH	0
1 & 2	MH	30
1 & 2	MH	60

D. Whole and minced heads thawed in air (A)

Reactor	Whole/Minced Head	Hydrolysis time (min)
1 & 2	WH	0
1 & 2	WH	30
1 & 2	WH	60
1 & 2	MH	0
1 & 2	MH	30
1 & 2	MH	60

The total number of samples used for analysis was 61, including the hydrolysates, sediments and raw material sample taken right after receiving the cod heads on day

zero, which was also freeze dried. As explained earlier, two thawing methods and two parallels per raw material and three hydrolysis times of zero, 30 and 60 minutes were used, providing 30 fish protein hydrolysate and 30 sediment samples (Table 1).

3.3 Analysis of raw material, sediments and FPH

The following analysis was performed on all samples including raw materials for each batch, wet and dry (i.e. freeze dried) sediments, and wet and dry (i.e. freeze dried) fish protein hydrolysates for all three hydrolysis times of zero, 30 and 60 minutes.

3.3.1 Total dry matter content

The dry and empty glass container was weighed and filled with 1-2 g of the thoroughly mixed sample and weighed again immediately. The container with the sample was placed in the drying oven at 105 ± 2°C for 20-24 hours and weighed again immediately. The procedure was carried out in triplicate per sample. The difference in the mass represents the amount of water that was removed, and the dry matter content can be calculated using the following equation:

$$\text{DM\%} = \frac{\text{weight of dry sample(g)}}{\text{weight of wet sample(g)}} \times 100$$

Due to inhomogeneous composition, it proved very difficult to obtain representative samples of the sediment fraction, and the dry matter analysis and results were not reliable.

3.3.2 Yield and Mass balance

In order to estimate the yield of the fractions based on dry matter, the mass balance for the hydrolysates was calculated based on the dry weight of the fraction and the raw material, and the corresponding DM% using the calculation shown below:

$$\text{Yield} = \frac{\text{dry weight of fraction (g)}}{\text{dry weight of 100 g raw material (g)}}$$

$$= \frac{\text{wet weight of fraction (g)}}{\text{dry weight of 100 g raw material (g)}} \times \text{DM\%}$$

The mass balance for the sum of dry weight (DW) of all fractions per 100 g raw material was calculated as follows:

$$\frac{\text{DW of raw material}}{\text{DW of 100 g raw material}} = \frac{\text{DW of hydrolysate}}{\text{DW of 100 g raw material}} + \frac{\text{DW of sediment}}{\text{DW of 100 g raw material}}$$

Since only two fractions are obtained in hydrolysis of cod head, the sum of dry weight of hydrolysate and sediment fraction should be (more or less) equal to the total dry weight of raw material per 100 g raw material.

3.3.3 Total ash content

Ash is the inorganic part of the sample that has low volatility and remains after the water and organic matter have been removed by heating in the presence of oxygen. The ash content provides a measure of the total amount of minerals within a product.

A known amount of sample (≈ 1g) was placed into a dried pre-weighed porcelain crucible, burning the organic matter off the sample in an air atmosphere at temperature above 550ºC overnight. The crucible was weighed after it was cooled to room temperature in a desiccator. The total ash content was calculated as follows:

$$\text{Ash content\%} = \frac{\text{weight of sample after ashing (g)}}{\text{weight of wet sample (g)}} \times 100$$

3.3.4 Total lipid content

The lipid content in the raw material, sediments and hydrolysates were determined using the Bligh and Dyer method, which is based on the insolubility of lipids in polar solvents versus their solubility in non-polar solvents (Bligh & Dyer, 1959). Merethe Selnes performed the analysis at SINTEF Fisheries and Aquaculture.

Exactly 10 grams of the sample was mixed with distilled water (10 ml), chloroform (20 ml) and methanol (40 ml) in a chloroform resistant centrifuge tube and homogenized for 2 minutes using an Ultra Torrax homogenizer. Another 20 ml chloroform was added and homogenized for 30 seconds, followed by addition of 20 ml distilled water and 30 more second homogenization. The sample was centrifuged for 10 minutes at 9000 rpm, and 1 ml of the chloroform phase was transferred to a pre weighed glass tube using a pipette. The chloroform was removed by evaporation at

70ºC under a stream of N_2 gas, and the tubes were placed in a desiccator overnight and weighed afterwards to determine the lipid content of the sample using following calculation:

$$\text{Total lipid content \%} = \frac{\text{lipid after evaporation (g)} \times \text{added chloroform (ml)}}{\text{evaporated chloroform (ml)} \times \text{sample (g)}} \times 100$$

3.3.5 Total protein content

The total amount of nitrogen in the samples was determined using a C/N-analyzer (Elemental Combustion System 4010, CHNS-O) by Marte Schei at SINTEF Fisheries and Aquaculture. The samples were prepared by weighing about 1-2 mg of the samples for each parallel; six parallels per sample were analyzed. The total amount of protein in the fish protein hydrolysate and the sediments was calculated by multiplying the nitrogen content by 6.25 (Adler-Nissen, 1986). The nitrogen content was also used to determine the degree of hydrolysis by formol titration method that will be described later (Chapter 3.4.3).

3.4 Analysis of FPH

3.4.1 Protein recovery

Protein recovery represents the amount of protein in the raw material that is found in the hydrolysate fraction after 60 minutes hydrolysis regarding the dry weight of FPH, which is calculated using following equation:

$$\text{Protein recovery (\%)} = \frac{\text{protein in FPH from 100 g raw material}}{\text{protein in 100 g raw material}} \times 100$$

$$= \frac{\text{yiled of FPH from 100 g raw material} \times \text{protein content in FPH}}{\text{protein in 100 g raw material}} \times 100$$

$$= \frac{\frac{\text{DW of FPH (g)}}{\text{DW of 100 g raw material (g)}} \times \frac{\text{protein content in FPH (\%)}}{\text{dry matter in FPH (\%)}}}{\text{protein in 100 g raw material (g)}} \times 100$$

3.4.2 Degradation and solubilization of proteins

The trichloroacetic acid soluble nitrogen (SN-TCA) method is used in this project, which does not determine the number of peptide bonds broken, and therefore is not

considered a direct method to determine DH. However, in this method the TCA-soluble nitrogen is quantified, which is assumed to consist only of amino acids and small size peptides resulted from degradation of proteins. Non-hydrolysed protein is precipitated by TCA and separated by centrifugation, and the nitrogen content of the supernatant is determined by Lowry method, and compared with the total content of soluble peptides (Rutherfurd, 2010).

Determination of total soluble peptides:

Amount of total soluble proteins in all cod head protein hydrolysate samples were determined using the Lowry method (Lowry, et al., 1951), where bovine serum albumin (BSA) at different concentrations was utilized as standard. A stock solution of BSA (1000 µg/ml) was used to make 10 ml dilutions with 12.5, 25, 50, 100, 150, 200, and 300 µg/ml concentrations. 0.1 g of each FPH and raw material sample was dissolved in 10 ml doubly distilled water, filtered using filter paper in a funnel and diluted to a ratio of 1:50 with doubly distilled water.

0.5 ml of the following solutions was added to separate test tubes: One blank sample containing only water, triplicates of the filtered total soluble peptides for the raw material and each FPH sample, and triplicates for each dilution of BSA standard solutions.

Alkaline copper agent was prepared by adding 1 ml of 1% CuSO4 and 1 ml of 2% potassium sodium tartrate to 100 ml of 2% Na2CO3 in 0.1 M NaOH. 2.5 ml of the alkaline copper reagent was added to each test tube and mixed with whirlmixer right away. After 10 minutes at room temperature, 0.25 ml Folin-Ciocalteu reagent diluted in a ratio of 1:2 with doubly distilled water was added to the test tubes, mixed and left for 30 minutes at room temperature. The absorbance at 750 nm was read in a spectrophotometer (Ultrospec 2000, UV/Visible spectrophotometer, Pharmacia Biotech). The blank sample was held as the reference and the BSA solutions were used to plot the standard curve to determine the percentage of acid soluble peptides in the dry matter.

Determination of acid soluble peptides:

The TCA soluble peptides were separated from the sample using Hoyle & Merritt (1994) method, and the Lowry method was used to determine the amount of acid soluble peptide in percentage of dry matter (Lowry, et al., 1951).

0.1 g of the FPH was dissolved in 10 ml doubly distilled water, of which 2 ml was added to 2 ml TCA 10% and mixed well using whirlmixer. The sample in the test tubes were left for 30 minutes in ambient temperature, and then filtered by filter paper placed in a funnel. The transparent filtered solution contained acid soluble peptides of the sample and was diluted to a ratio of 1:20 with doubly distilled water. The Lowry method, as explained for the total soluble peptides was applied to determine the amount of acid soluble peptides. The analysis was run in duplicate for all fish protein hydrolysates that were hydrolysed for zero, 30 and 60 minutes.

3.4.3 Determination of degree of hydrolysis (DH)

The DH can be evaluated as the proportion (%) of α-amino group nitrogen with respect to the total N in the sample (Taylor, 1957). The amount of free amino groups was determined using the formol titration method, which is based on the reaction between formaldehyde and the amino group of an amino acid at neutral or alkaline pH that leads to liberating a proton and lowering the pKa of the amino acid-formaldehyde complex. NaOH is used to titrate the protons in order to increase the pH of the reaction mixture to the pKa of the amino acid, providing a direct measurement of the amount of free amino groups present. Formol titration is a rapid method that needs real-time monitoring and the results can be variable (Rutherfurd, 2010).

A hydrolysate solution was prepared by weighing FPH (0.25 g) in a beaker and addition of distilled water to a total of 50 g. The solution was mixed with enough 0.1 M NaOH to reach pH 7.0. Formaldehyde (10 ml) was added and the solution was left for 5 minutes in room temperature. The mixture was titrated by 0.1 M NaOH to pH 8.5, and the amount of NaOH consumed for titration was used to calculate the amount of free amino groups in the FPH using the following equation:

$$\%\ \text{free amino groups} = \frac{\text{NaOH used (ml)} \times \text{concentration of NaOH} \times 14.007 \times 100}{\text{FPH sample (g)} \times 1000}$$

The degree of hydrolysis (DH) was determined by dividing the amount of free amino groups by the total amount of nitrogen, calculated from the protein concentration:

$$\%\ \text{Degree of hydrolysis} = \frac{\%\ \text{free amino groups} \times 100}{\%\ \text{nitrogen content}}$$

3.4.4 Determination of amount of free amino acids

The method described by Osnes & Mohr (1985) was used to remove the proteins and separate the free amino acids for HPLC analysis. A concentration of 0.05 g of the powder FPH was dissolved in 5 ml distilled water. Two 1 ml parallels of this solution were precipitated with addition of 0.25 ml of 10% sulphosalisylic acid to each sample. The mixture was mixed vigorously, left at 4ºC for 30 minutes, and then centrifuged for 10 minutes with 10,000-rpm speed at 4ºC. In order to ensure that all proteins are precipitated, only 1 ml of the top supernatant of one parallel was transferred into a new eppendorf tube and the same process was repeated. Lack of precipitation in the second process indicates that all the protein was removed in the first round and the first sample could be used. After precipitation of all the protein, the supernatant was filtered using a filter with a pore size of 0.2 µm, and 0.205 ml of each filtered sample was sent for HPLC analysis.

4 Results and Discussion

In order to determine the influence of the state of the substrate on the characteristics of the fish protein hydrolysate, the cod heads were frozen as either intact whole heads or minced, and stored for up to 21 days. Two thawing methods were used to defrost the cod heads, namely by soaking the frozen raw material in running water of 6-10ºC or placing in the cold room of 4ºC overnight. Protamex™ was the chosen enzyme used to hydrolyse cod heads for 30 and 60 minutes, and two separate phases of hydrolysate and sediment were obtained.

The composition and characteristics of the resulting fish protein hydrolysates (and sediments to some extent) were analyzed to evaluate the differences due to raw material status, thawing methods and hydrolysis time; the results are shown and discussed in this chapter.

4.1 Sensory characteristics of hydrolysates

Figure 12 shows the appearance and color of frozen hydrolysate samples for day 1, day 20 (thawed in water), and day 21 (thawed in air) hydrolysed for zero, 30 and 60 minutes.

The zero minute hydrolysate fraction produced from raw material that was frozen for 20 and 21 days was not in liquid state and had a fibrous or fleshy-like texture (Figure 12), which can be an indication of possible interactions between different components during storage and low degradation rate during thawing.

The 30 minutes hydrolysed samples for the minced cod heads thawed in both air and water were not fractioned properly (Figure 12, second raw, second column), which made it difficult to separate the hydrolysate from the sediment phase. It can be suggested that the 60 minutes hydrolysis be used for these conditions of freezing and thawing, and if it is necessary to use only 30 minutes hydrolysis, the centrifugation step should be modified to separate the fractions more adequately.

The minced head zero minute sample was much darker than the other zero samples, which can be due to hemoglobin content of the sample released by the mincing stage.

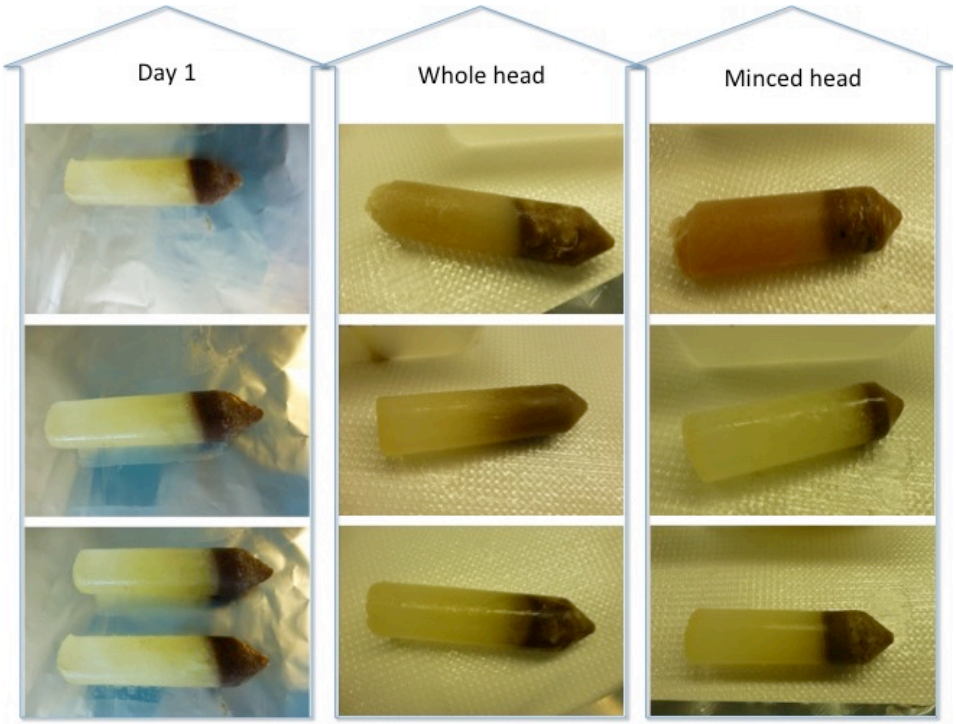

Figure 12. A comparison between the appearances of the frozen centrifuged cod head hydrolysates on the first day (the left column), the whole head thawed in water (the middle column) and minced head thawed in water (the right column); Protein hydrolysate in the aqueous phase (left part), and the sediments at the bottom (right), for zero, 30 and 60 minutes from top.

4.2 Raw material Composition

The composition of the raw material prior to each hydrolysis is shown in table 3, in order to demonstrate the changes in raw material during freezing storage and the influence of the two thawing methods used.

Table 3. Gross composition (g/100 g) of raw material used for enzymatic hydrolysis of cod head for fresh raw material and after freezing – thawing

Composition	R-H	R-WH-W	R-MH-W	R-WH-A	R-MH-A
Dry matter (%)	23.36	21.41	21.15	21.76	22.04
Ash (%)	7.17	5.97	6.88	6.86	7.27
PC (%)	13.52	13.57	11.93	12.45	12.36
LC (%)	3.91	3.74	3.19	3.40	3.74

R-H: fresh raw material (day one); R-WH-W: frozen whole head (20 days frozen storage) thawed in running water (6-10ºC for 3 hours); R-MH-W: frozen minced head (20 days frozen storage) thawed in running water (6-10ºC for 20 minutes) and stored in 4ºC for 2.5 hours prior to hydrolysis; R-WH-A: frozen whole head (21 days frozen storage) thawed in air (4ºC for 20 hours); R-MH-A: frozen minced head (21 days frozen storage) thawed in air (4ºC for 20 hours)

4.2.1 Total dry matter content

The dry matter (DM) content is lower for the frozen/thawed samples than the fresh raw material, which indicates some biochemical changes during freezing and thawing (Table 3). As mentioned before, the texture of the 0 min hydrolysate was different for the frozen raw material compared to the 0 min hydrolysate of fresh raw material. The minced head thawed in air showed the lowest decrease in DM among frozen/thawed samples, which can be due to its vacuum packaging that prevented more interaction with the environment. While the other vacuum packed minced-head that was thawed in water appeared to have the maximum reduction of dry matter and gain of moisture. There might be some penetration of water in the sample through the packaging while it was immersed in water. The whole head thawed in water had the second lowest DM, perhaps because it adsorbed some water while thawing in water for 3 hours.

4.2.2 Total ash content

The ash content was 7.17% for the raw material, and 5.97% to 7.27% for the other samples (Table 3). The lowest ash content was observed in the whole head thawed in water. The whole heads were left immersed in running water for 3 hours, and therefore lost a considerable amount of loose particles and soft tissues that might be water-soluble and washed out. In addition, the removal of those external items that might be included in cod head by water can be an explanation of the low concentration of ash in the product.

The other raw material samples did not show any large difference in ash content compared to the fresh raw material. The ash results in this project are higher than what was observed in a project by Slizyte, et al. (2005), which was maximum 4.4 g/100 g for the cod viscera sample and 3.6 g/ 100 g for the backbone cod sample. The reason can be the high bone content of the cod heads used as the raw material.

4.2.3 Total protein content

The protein content for the fresh raw material was 13.52% of dry weight and for the stored samples ranged between 11.93 and 13.57% of dry weight (Table 3). The highest belonged to the whole head thawed in water and the lowest for the minced

head thawed in water. The differences that were observed between the whole head thawed in water and the rest can be attributed to either removing of soft tissues and some water-soluble components (such as neck), or to the exposure of the more hidden components in the cod head that promotes removal of water-soluble protein content.

The raw material thawed in air had more or less the same protein content for both whole and minced head. The protein content was determined using CN method, which showed a large fluctuation (standard deviation) in the results for the six-fold analysis of the same sample. Perhaps using more homogenous sample would give a better estimation of protein content.

4.2.4 Total lipid content

The lipid content ranged between 3.19 and 3.74% dry weight for the frozen stored samples (freeze dried), while the day one raw material contained 3.91% lipid (Table 3). Lower lipid content in frozen stored raw material can be due to degradation of lipid content into other components during storage and thawing. The minced head thawed in water and whole head thawed in air showed the lowest and second lowest lipid content, respectively. The lipid content in the raw material was measured after freeze-drying, which is one more processing step that might influence the composition of the raw material.

4.3 Composition of fractions

The compositional differences between the FPH samples that were produced from fresh cod head and the raw material with different freezing/thawing methods provides information on the influence of storage time, thawing method used, and hydrolysis process on the characteristics of the product, including the yield of FPH.

The dry matter and ash content were determined for the wet hydrolysate, and moisture, lipids, proteins, and free amino acid content were the compositional elements that were measured for freeze-dried FPH in this study.

4.3.1 Dry matter content

In Figure 13, the dry weight of FPH fractions per 100 g of each sample is compared for 0, 30 and 60 minute hydrolysis time. The FPH samples follow more or

less a similar pattern with an almost linear increase in the dry weight after 30 min and more change that is rapid after 60 min hydrolysis, indicating the importance of time, at which the raw material is hydrolysed. The frozen whole head that was thawed in water showed the lowest dry weight for FPH for 0 and 30 minutes samples, but after 60 minutes, the concentration is comparable to the other samples.

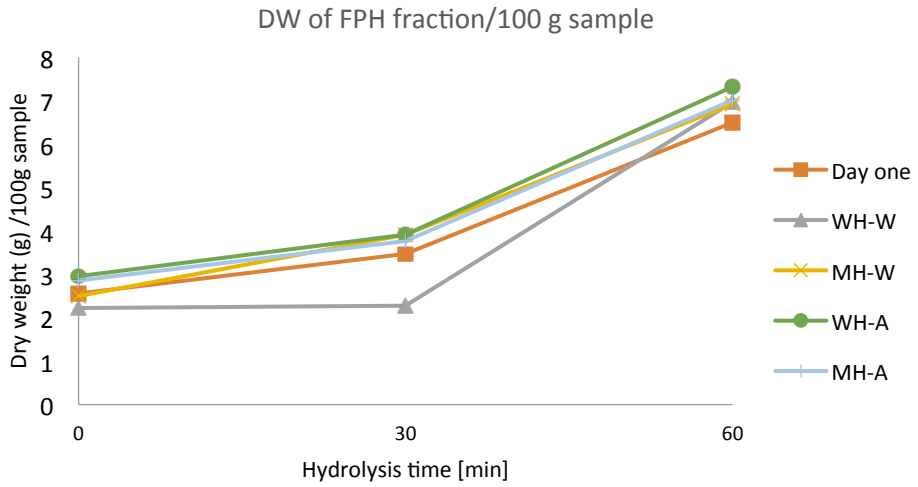

Figure 13. The dry weight of FPH fractions per 100 g of each sample after 0, 30 and 60 minute hydrolysis; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

The sediment fraction is a heterogeneous mixture that should be thoroughly mixed prior to analysis to avoid uneven distribution of bones. Unfortunately, the size of the samples taken for the analysis was not sufficient to provide an acceptable representative of the whole fraction, and therefore, the dry weight of the sediment fraction for this project is not reliable (results not shown). A suggestion to have a proper estimation of the composition of the sediment fraction would be to take a much larger sample (e.g. 10-20 g instead of 1-2 g) after vigorous stirring and blending of the entire sediment sample.

4.3.2 Yield of dry matter in fractions

The yield of dry matter in FPH and sediment fractions per 100 g wet weight of raw material for day one is compared to the dry matter yield produced from frozen and thawed raw material (Table 4).

The yield of dry matter in hydrolysate fraction after 60 minutes hydrolysis is rather similar in all samples (between 10.6 and 12.3 g/100 g raw material), but there is

a significant difference between 0 min and 30 min hydrolysis samples. In a study by Slizyte, et al. (2009), frozen cod backbone was used to produce FPH, and the yield of dried FPH after 60 minutes enzymatic hydrolysis was estimated to approximately 5.6 g per 100 g wet weight of raw material.

The lowest yield after 30 minutes hydrolysis is observed in the whole head thawed in water. As it is mentioned in Chapter 4.1 (Figure 12), the fractions were not separated properly in centrifugation and therefore, a large amount of hydrolysate ended up in the sediment fraction.

Table 4. Yield of dry matter in hydrolysate and sediment fraction per 100 g wet weight of raw material after 0, 30 and 60 min hydrolysis; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

	FPH fraction					Sediment fraction				
	Day 1	WH-W	MH-W	WH-A	MH-A	Day 1	WH-W	MH-W	WH-A	MH-A
0-min	9.2	6.7	6.3	9.6	8.3	14.1	14.7	14.8	12.2	13.7
30-min	14.2	4.0	14.9	15.2	9.2	9.2	17.4	6.3	6.6	12.8
60-min	11.0	12.3	10.6	10.8	11.3	12.3	9.1	10.5	10.9	10.7

The highest yield of dry matter in sediment fraction is seen in the day one sample and the lowest in the whole head thawed in water, where the latter showed the highest yield in the FPH fraction (Table 4).

4.3.3 Mass balance

The mass balance for the dry weight (%) of raw material and the sum of the two fractions (hydrolysate and sediment) after 0, 30 and 60 minutes hydrolysis is shown in Table 5.

The dry matter (DM) content of the day zero (the day of delivery) cod head was 23.4 per 100 g of raw material (results not shown) and the first column in Table 5 shows the dry matter content of the raw material used for hydrolysis with and without freezing and thawing.

The sum of the DM for the two fractions obtained after hydrolysis is supposed to be more or less comparable to the DM of the corresponding raw material. However, in some cases, there is slightly less or more DM than the raw material, which might

be because of: either the loss of some sample during processing (e.g. distribution into centrifugation tubes, fractionation, evaporation, etc.), or difficulties in sampling (particularly for sediment fraction, Chapter 4.3.1).

Table 5. Mass balance for dry weight of raw material and sum of all fractions after 0, 30 and 60 min hydrolysis per 100 g of samples; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

Sample	Raw material	0 min	30 min	60 min
Day 1	20.29	21.42	21.4	24.79
WH-W	21.41	23.94	22.6	20.66
MH-W	21.15	25.2	19.72	23.33
WH-A	21.76	23.9	19.76	24.88
MH-A	22.04	25.28	20.72	22.76

4.3.4 Moisture content

The water content in the freeze-dried FPH samples is shown in Figure 14. The amount of water in the samples was in the range between 2.25 and 3.79 percent of dry FPH, where the lowest belonged to the day one raw material and the highest was for the minced head thawed in water. The other dry samples showed more or less similar water content.

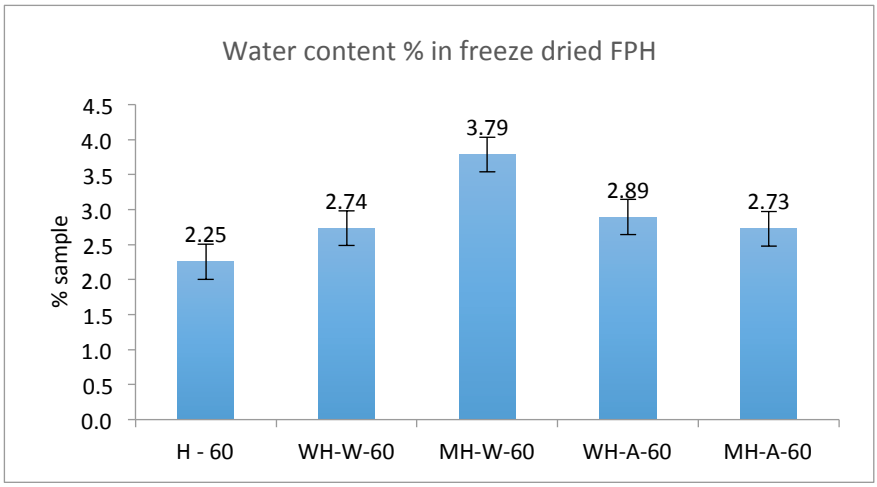

Figure 14. Water content (%) in freeze-dried FPH samples after 60 min hydrolysis time with Protamex™; Error bars represent standard deviation, n=4; H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

Low moisture content in the FPH powders is desirable, due to its positive influence on storage stability, however it might encourage lipid oxidation in samples with higher concentration of lipid (Arason, et al., 2009).

In spite of using the same freeze drying procedure for all samples; there is variation between water content of the dried samples, which can be attributed to the freeze-drying process. Differences in the composition of the FPH sample could influence its melting point and hygroscopicity (i.e. the ability of FPH to attract and hold water molecules from the surrounding environment).

The principle of freeze-drying is based on freezing the sample and then reducing the atmospheric pressure to allow the frozen water in the material to sublimate directly from the solid phase, below the melting point of the solvent to the gas state. Therefore, to ensure that sublimation (rather than melting) will occur, the sample should be cooled down below its triple point, which is the lowest temperature at which the three phases (gas, liquid and solid) can coexist in thermodynamic equilibrium. If a sample melts during primary drying step (due to its low melting point), it will lead to splattering of the sample, which results in sticky crumbs with low solubility instead of powdered sample.

In addition, the batch-to-batch variation (e.g. variation in atmospheric pressure and temperature) between the different freeze drying rounds is the other possibility for the differences in water content of the dried samples. Since all the samples with different hydrolysis times and degrees were freeze dried simultaneously and under similar freezing and preparation conditions, some of the samples with lower melting point were melted when vacuum condition was applied. I would suggest to freeze-dry similar samples (e.g. all 30 minute hydrolysates) together, so we could adjust the conditions of the operation according to the characteristics of the sample.

4.3.5 Ash content

Regarding the ash content of the dry FPH samples, day one sample showed the highest ash content, whereas the whole head thawed in water contained the lowest concentration of ash and the other three samples were in between (Figure 15). The sediment fraction of hydrolysed whole heads thawed in water also contained the

lowest percentage of ash, which can be attributed to low ash content in the corresponding raw material (Table 3).

Although as mentioned before, the sediment sample might not be a good representative of the whole fraction, and it should be considered when comparing the results.

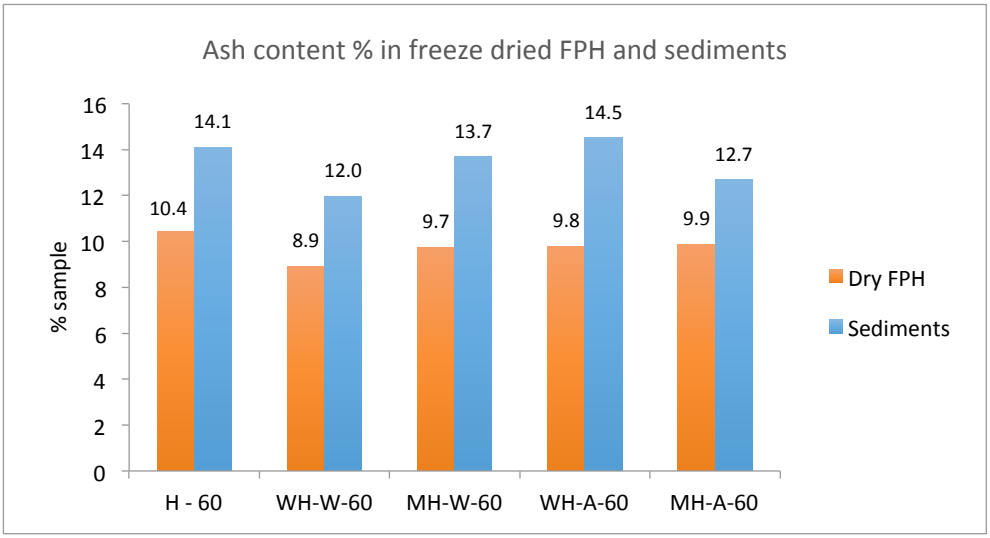

Figure 15. Ash content (%) in freeze-dried FPH samples and sediment fraction for the samples with 60 min hydrolysis by ProtamexTM; H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

The ash content of FPH and sediment fractions represents the mineral content, including the mineral part of the bones and inorganic content of the raw material that ends up in the fraction. But the external objects might also take part in the raw material, for instance the gill in the raw material containing head might include small amount of stones, sand, shells, etc., which can add up to the total ash in the hydrolysis fractions. However, these types of particles are more concentrated in internal organs (e.g. viscera). In this project, the raw material used for hydrolysis was solely cod head, which consists mainly of bones.

The importance of high ash content is due to its effect on the percentage of total weight of the FPH by increasing the amount of soluble components and altering the yield of hydrolysate fraction, which depends on the composition of ash (Slizyte, 2004). For instance, potassium and sodium are readily water-soluble ions and can end up in the FPH fraction (Liaset & Espe, 2008).

In addition, the amount of inorganic compounds can influence the composition of oil fraction, for example, the concentration of FAME (fatty acid methyl esters) in oil fraction can be strongly influenced by changes in ash and water content (Daukas, et al., 2004). However, the lipid content of cod head was not adequate for separation of oil fraction, and in this project, the composition of lipid content in neither of the fractions was analyzed.

4.3.6 Lipid content

The concentration of lipid in cod heads was not high enough to form a separate oil fraction after hydrolysis process, and therefore, the lipid content was distributed between the sediment and hydrolysate fractions. According to Slizyte (2004), in order to obtain the oil fraction, when cod viscera and backbone mixture is used as raw material, a minimum concentration of 6 g lipid per 100 g of wet weight is required. The cod heads used for this project had a lipid concentration of between 3.19 and 3.91% for the different raw materials (Table 3), which is not enough to separate an oil fraction.

The lipid content in the raw material was slightly different between samples, but the difference became more noticeable in the fractions obtained after hydrolysis, meaning that the distribution of lipid between the fractions was not uniform in all samples. The majority of lipid was extracted in the sediment fraction with lipid content of between 4.57 and 5.09% (Figure 16). The maximum lipid content was observed in the hydrolysate processed from the whole head thawed in air with 0.71% of dry weight, while the lipid content of the other FPH samples ranged between 0.37 and 0.50%. Despite the highest concentration of lipid in day one raw material (3.91%, Table 3), the lipid content was the lowest for this FPH sample (0.37%, Figure 16).

Regarding the freeze-dried sediment fractions, the lowest level of lipid was seen in the sediment of minced head thawed in water sample associated with the corresponding FPH sample containing the second highest lipid content among freeze dried hydrolysates. While day one sample contained the lowest lipid content in FPH and quite low proportion of lipid in its sediment fraction (second lowest lipid content among sediment fractions).

Since the presence of lipid can promote lipid oxidation and formation of undesirable products and decrease stability of fish protein hydrolysate during storage, FAO suggested a limit of 0.5% lipid concentration in dry FPH (FAO, 1971). The results showed that the concentration of lipid is within the limit, which ensures the oxidative stability for all FPH powder samples except for the whole head thawed in air.

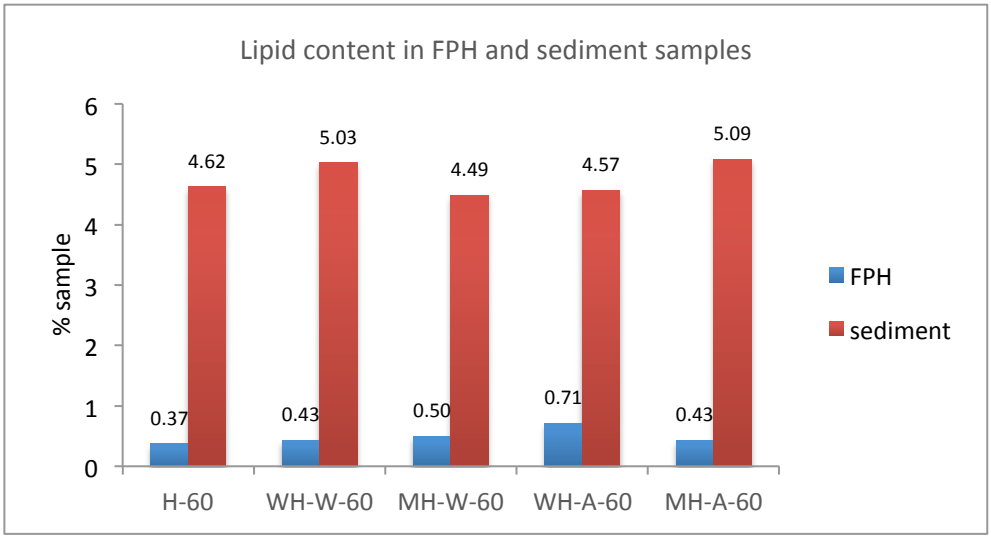

Figure 16. Lipid content (% of dry weight) in the freeze-dried FPH and sediment samples after 60 min hydrolysis time with Protamex™; R. Raw material, H: Head day 1, WH: frozen Whole Head, MH: frozen Minced Head, W: Water thawed, A: Air thawed.

4.3.7 Protein recovery in FPH powder

Figure 17-a shows the protein concentration in the freeze-dried FPH samples after 60 minutes hydrolysis, and Figure 17-b shows the amount of protein recovery in FPH samples. The highest protein content belongs to the FPH produced from fresh cod head (88.4%), and the lowest is seen in the whole head thawed in air (67.7%). However, the variation between the FPH produced from frozen samples regarding their protein content is rather small (67.7-71.6%).

The difference between the samples regarding protein concentration is more pronounced, when the protein recovery for FPH samples is compared (Figure 17-b). The protein recovery is also somewhat lower in all FPH samples obtained from the frozen stored raw material compared to day-one hydrolysate (73.6-81.2 % vs. 85.0%). In addition, the lowest amount of protein recovered in FPH is seen in whole head

thawed in water; while the lowest total protein content is seen in whole head thawed in air.

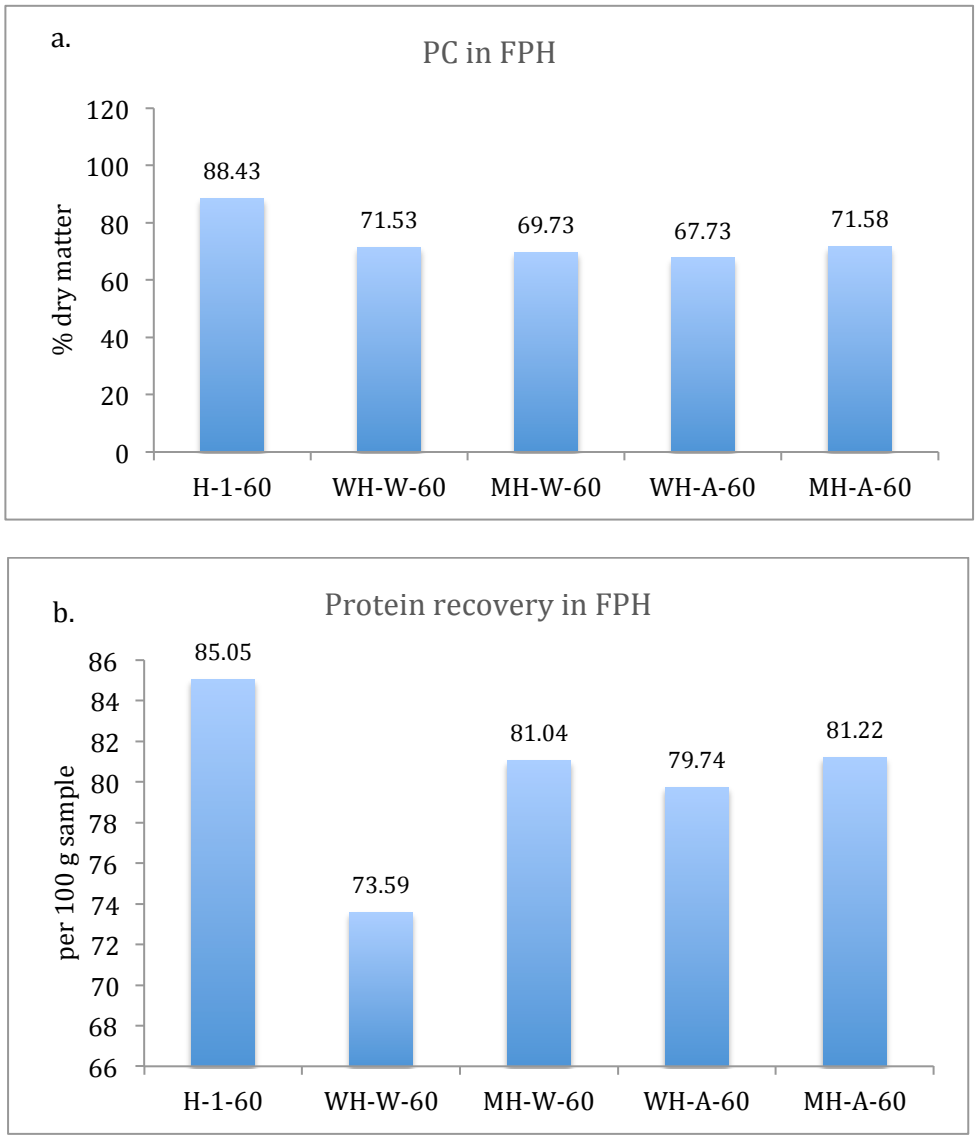

Figure 17. Protein content (% dry matter) in: a. Freeze-dried FPH samples after 60 min hydrolysis time with Protamex™; b. Protein recovery in Freeze-dried FPH samples; R. Raw material, H: Head day 1, WH: frozen Whole Head, MH: frozen Minced Head, W: Water thawed, A: Air thawed

The whole heads thawed in water had the same protein concentration as the day one raw material (Chapter 4.2, Table 3); however, after hydrolysis, the protein recovery is much lower than day one FPH and all other samples. It can be due to the interactions during frozen storage and particularly thawing, including protein-protein interactions and protein-lipid complexes that can reduce solubilization of protein content, and cause aggregation and precipitation of a part of nitrogen content in the sediment fraction. Although the whole head lost some particles and soluble

components in water during thawing, it cannot be used as a reason for this change. Since the raw material sample that was taken after thawing and mincing contained high amount of protein. Therefore, all the changes that cause low recovery of protein in FPH fraction is due to interactions that occurred during hydrolysis and further steps.

In a report by Slizyte (2004), protein concentration of 78 to 83% was obtained for FPH produced from cod backbone and liver with 6-12% lipid content. It was observed in a couple of projects on hydrolysis of cod byproducts that the protein concentration in FPH fraction is dependent on the amount of lipids in the raw material (Slizyte, et al., 2005) and vice versa (Slizyte, 2004). Because the lipid-protein complex can reduce the solubility of protein and decrease the concentration of protein in the hydrolysate phase (aqueous phase), while protein can entrap the oil and prevent separation of oil in the fractionation step. However, since the concentration of lipid in cod head was not sufficient to obtain a lipid fraction, the influence of these two substances on each other is more difficult to determine.

4.4 Degree of hydrolysis (DH)

The degree of hydrolysis (DH) is defined as "*the number of peptide bonds broken as a proportion of the total number of peptide bonds present*" (Rutherfurd, 2010). In other words, the degree of hydrolysis is a reflection of the number of broken peptide bonds, and consequently the average size of the peptides in the product.

In this study, the degree of hydrolysis was calculated for the FPH powders obtained from cod head, and showed a range between 13.63 and 20.60% (Figure 18). The two highest DH value is seen in the whole head thawed in water and minced head thawed in air, respectively. Both raw materials were thawed under conditions that provided opportunity for the endogenous enzymes to be more active prior to hydrolysis compared to the other raw materials.

Since all samples were subjected to the same hydrolysis conditions, it can be suggested that autolysis of raw material played a role in the number of broken peptide bonds and cause the differences among fresh and frozen/thawed samples. In addition, due to freezing, there would be some damages to the cell membranes, which leads to better contact between substrates and endogenous enzymes. Furthermore, the reaction

rate may be increased in the partly frozen tissue, because of the higher concentration of substrates in the liquid phase, because of melted water. In either case, increased enzyme activity leads to formation of small size peptides and increased DH.

In a study by Sližytė, et al. (2005), the degree of hydrolysis for cod byproducts including viscera with and without initial inactivation of endogenous enzymes was measured and the results showed a rather large difference due to autolysis, namely, 19.8 to 23.6% for FPH obtained from initially heated samples and between 31.1 and 34.9% for initially unheated samples.

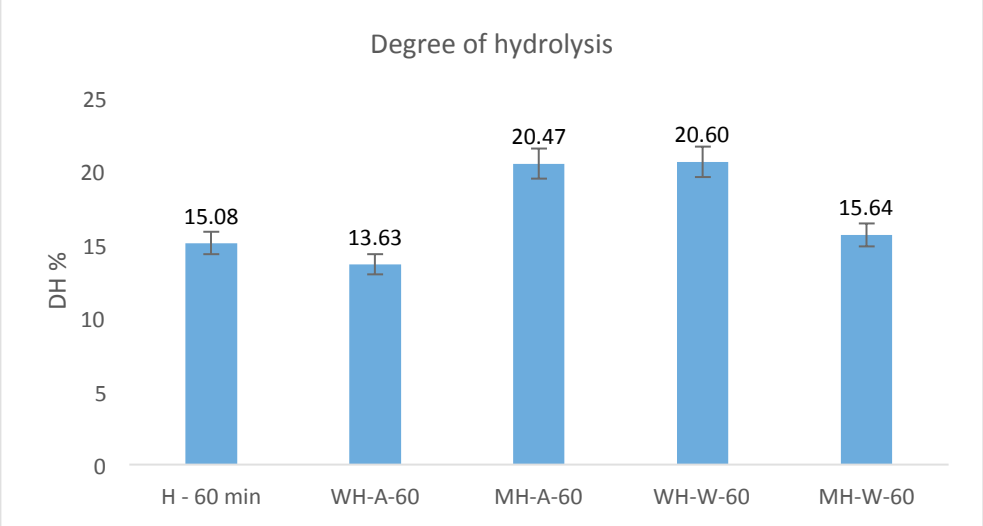

Figure 18. Degree of hydrolysis for FPH samples after 60 min hydrolysis time with Protamex™; Error bars represent standard deviation, n=2; H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

Regarding the thawing of whole heads in water, they were soaked in 10ºC water for about 3 hours, giving enough time to the peptidases to function at a comparatively higher temperature. On the other hand, the minced heads were vacuum packed in a thin layer that provided an increased contact surface area for the defrosting medium (water or air), which shortened the time needed for thawing compared to the whole head (Chapter 3.1), causing lower autolysis rate.

The differences between the degree of hydrolysis for FPH produced from minced head thawed in water and air illustrates the influence of thawing method on the raw material and consequently the hydrolysate. Moreover, the different DH for the whole head and minced head thawed in either water or air demonstrates the influence of mincing before freezing and storage.

4.5 Determination of hydrolysis: TCA-solubility vs. total soluble peptides

The extent of hydrolysis can be determined by measuring the TCA-soluble nitrogen (SN-TCA), which is the amount of soluble nitrogen in the presence of a precipitating agent (e.g. TCA), instead of measuring the number of peptide bonds broken. The method is based on the assumption that only amino acids and small peptides can be dissolved in TCA (10% trichloroacetic acid), while big-size proteins are precipitated. However, it is likely that a proportion of the intact proteins remain solubilized and participates in the result (Rutherfurd, 2010).

Figure 19 shows the concentration of total soluble peptides versus acid soluble peptides in the FPH samples that were hydrolysed for zero, 30 and 60 minutes with Protamex™.

It appears that the zero-minute FPH contains significantly lower concentration of both soluble peptides and acid soluble peptides, compared to the other two samples in each group (i.e. 30 and 60 min), which can be translated into high percentage of insoluble large size proteins with higher hydrophobicity and low solubility. However, during hydrolysis, the insoluble proteins are degraded into soluble and acid soluble peptides detectable by this method.

The general pattern is more or less similar in most samples, indicating an increase in the percentage of both total soluble peptides and SN-TCA after 30 minutes and for some samples further increase after 60 minutes hydrolysis. There is a slight difference between acid soluble peptides in the 30 and 60 minutes hydrolysed FPH, suggesting that adequate degree of hydrolysis might be obtained after 30 minutes. In addition, the acid soluble peptide content is approximately equal to the percentage of total soluble peptides in most samples after 30 and 60 min hydrolysis, which indicates that a large portion (almost 100%) of the total soluble peptide content of the hydrolysates are small enough to be acid soluble.

The highest total soluble and acid soluble peptides for all three hydrolysis times belonged to day one FPH sample. The cod heads received at day zero were removed from the body and stored at 4ºC for about 24 hours until day 1 and the first hydrolysis process. Although the cod heads were not frozen or stored for a long time, there was sufficient time for endogenous enzymes to work on the protein content of the heads

and increase the soluble peptide content. However, it seems that the storage time was not long enough neither for excess degradation into volatile nitrogen compounds, nor for aggregation and precipitation or complex formation of protein content that would appear as a decrease in the soluble peptide content.

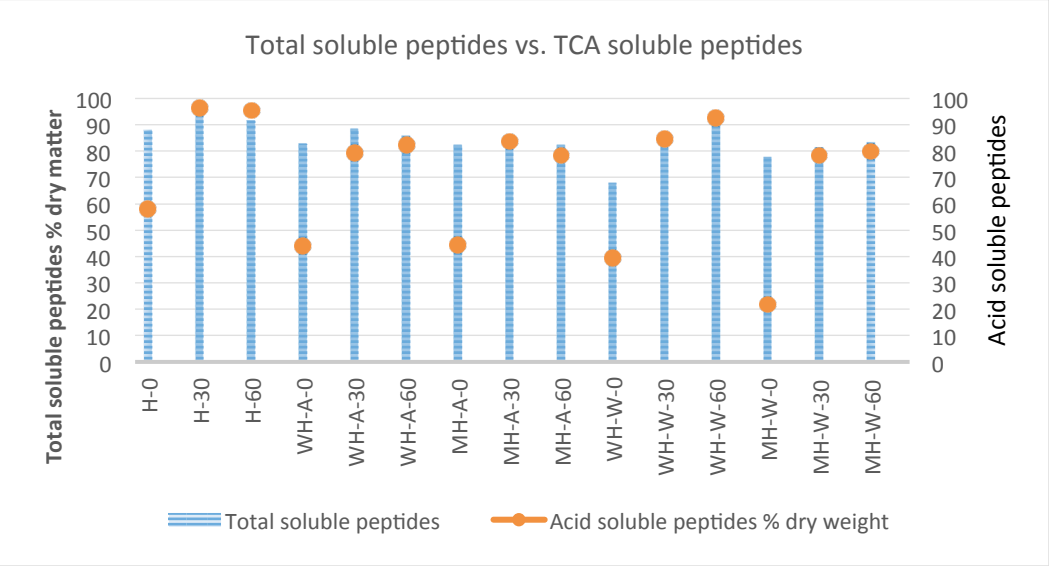

Figure 19. Indirect evaluation of hydrolysis for total water soluble peptide versus TCA-soluble peptides in dry FPH samples using Lowry method (Lowry, 1951) after 0, 30 and 60 min hydrolysis time with Protamex™; H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

The amount of acid soluble peptides in the 0 min samples is the lowest at the minced head raw material thawed in water. If the concentration of acid soluble peptides in 0 min hydrolysates is an indication of endogenous enzyme activity during freezing and particularly thawing, it can be suggested that the enzymatic activity is lowest in these conditions. Since all zero-minute samples produced from frozen and thawed cod head showed lower total soluble and acid soluble peptides than the fresh raw material, it can be proposed that freezing and thawing have negative effect on the concentration of small size peptides in the raw material. The protein content is measured to be lower in the thawed samples than in fresh cod head (Chapter 4.3.7), therefore, it can be suggested that the protein content is either degraded further down into untraceable compound for this method (e.g. TVN), or denatured and aggregated during freezing and thawing and was separated in the sediment.

The day one FPH samples and the ones produced from minced head raw material thawed in air contained slightly lower acid soluble peptides after 60 minutes compared to 30 minutes (Figure 19). It could be due to further degradation of the acid

soluble proteins into TVN that cannot be detected in this analysis method, or aggregation and precipitation of the peptides in the presence of TCA.

The nitrogen content in the fish protein hydrolysate can be divided into 4 major groups based on their solubility, namely: insoluble proteins, water-soluble proteins, peptides and amino acids (i.e. acid soluble peptides), and total volatile nitrogen (TVN) compounds. A great portion of the raw material belongs to the insoluble proteins, which can be partly transformed into soluble protein components either by endogenous enzymes during storage and/or also by exogenous enzymes during the hydrolysis process (Yin, et al., 2008). Although a very small amount of insoluble proteins is expected in FPH, due to difficulties in separation of sediment and hydrolysate phases, it is possible to have an increased amount of this kind of nitrogen compounds in some FPH samples (Chapter 4.1, Figure 12).

The solubility of the small molecular size peptides is due to the presence of more polar residues that can form hydrogen bonds with water and contribute in the protein solubility (Kristinsson & Rasco, 2000). The major interactions that can influence solubility of the proteins are hydrophobic interactions that decrease solubility by promoting protein-protein interactions, and ionic interactions, which improve protein-water interactions and therefore, increase solubility of FPH (Kristinsson & Rasco, 2000). Ionizable carboxyl groups of amino acids are exposed by hydrolysis, which leads to an increase in the hydrophobicity of the fish protein hydrolysates, and consequently, decrease the solubility. Simultaneously, the hydrolysis process increases the net charge by generating two end-carbonyl and -amino groups (NH_3^+ and COO^-) and decreases the molecular size of the polypeptide chain (Kristinsson, 2010).

The electrostatic repulsion between ionic residues on the surface of protein molecules and repulsion between hydration shells around ionic groups are the causes for increased protein solubility. TCA changes the pH value in the solution, which consequently can influence the solubility of small size peptides by influencing the charge on the weakly acidic and basic side chain groups and increase solubility of proteins as pH moves away from isoelectric point (pI) (Linder, et al., 1995).

Denaturation of protein is another factor that can influence protein size and solubility, and it can be induced during heat inactivation of enzymes in the end of hydrolysis process. According to Kristinsson (2010), fish protein muscles from cold-water species have more tendency to denaturation (lower heat stability) compared to the tropical water species. Protein denaturation increases resistance to enzymatic breakdown, and therefore, decreases the number of small soluble peptides in hydrolysates (Slizyte, et al., 2005).

On the other hand, excessive hydrolysis will cleave the peptide bonds more intensely resulting in a decrease in peptide size and degradation into single amino acids and volatile compounds. TCA-soluble peptide method cannot detect the proteins that are decreased into TVN, and it is suggested that determination of TVN also be considered to see how much of the protein has been degraded to volatile nitrogen compounds.

4.6 Degree of hydrolysis: Formol titration vs. TCA-solubility method

Two different methods were used in this project to determine the extent of hydrolysis, namely formol titration and TCA-solubility measured by the Lowry method (Lowry, et al., 1951). In Figure 20, the relationship between the results obtained using these two methods is shown.

The graph shows that there is no correlation between degree of hydrolysis and TCA-soluble peptides. Therefore, if the results of both methods are considered reliable, then the number of acid soluble peptides does not increase by the increased degree of hydrolysis, except for one sample that was hydrolysed from whole head thawed in water. However, the two methods measure different parameters that make them incomparable (Rutherfurd, 2010). To be specific, the formol titration method measures the number of NH_2 groups and compares it to an estimate of the total number of peptide bonds. This estimate is based on the protein content. While the TCA-method detects the solubility of the nitrogen content in the sample, and there is a possibility that large size (unhydrolysed) proteins also end up in the supernatant and appear in the result (Rutherfurd, 2010). Therefore, the extent of hydrolysis can be overestimated when SN-TCA is measured, compared to formaldehyde titration.

In a study by In, et al. (2002), three different methods including the two used in this project were used to determine the DH for hydrolysis of hemoglobin using a serial digestion with an endopeptidase (Esperase) followed by exopeptidase (Flavourzyme) at three different concentrations. They found out that there is very little correlation between the methods used; for example, the values for SN-TCA method were two or four times higher than those obtained using formol titration method, depending on the enzyme used for hydrolysis.

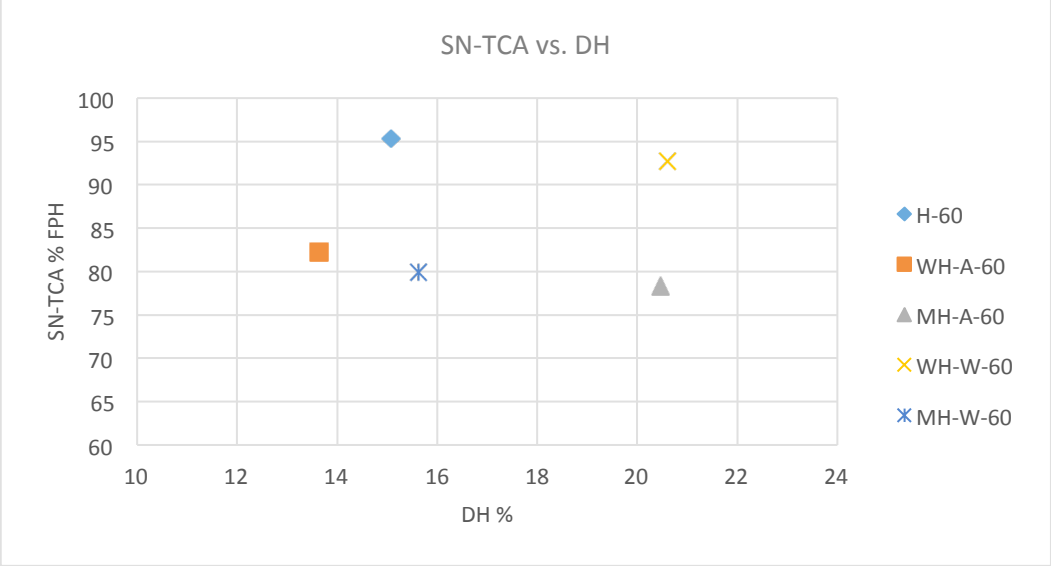

Figure 20. Relationship between degree of hydrolysis (DH %) and TCA-soluble nitrogen content in FPH samples hydrolysed by Protamex^{TM} for 60 minutes, H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

It appears that there are a number of variables (other than the type of enzyme used), which can influence the result of each DH method, such as pH, extent of hydrolysis and the origin of protein (Rutherfurd, 2010).

4.7 Amount of free amino acids

Figure 21 shows that the amount of total free amino acids (FAA) in FPH samples increases by time of hydrolysis, meaning that there is a higher concentration of free amino acids in the 60 minutes hydrolysed sample compared to 30 minutes (and 0-min) sample for the same raw material. The highest FAA content in 60-min FPH belonged to the whole head thawed in air (54.3 mg/g dry FPH) and the second was the minced head thawed in water (53.6 mg/g dry FPH). While in 30 min samples, the minced head thawed in water and then minced head thawed in air had 38.9 and 38.5 mg/g, respectively, contained the two highest concentration of total FAA. The zero

min samples showed a large diversity in the total FAA content as well, ranging from 12.5 mg/g dry FPH produced from minced head thawed in air to 16.5 mg/g in whole head thawed in water.

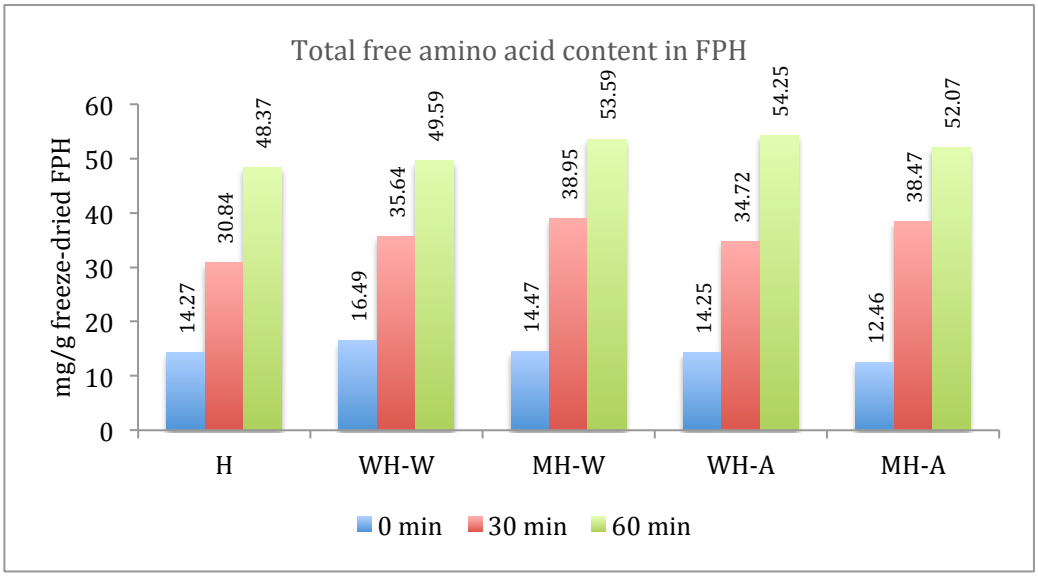

Figure 21. Total amino acid content for hydrolysate samples after 0, 30 and 60 minutes hydrolysis; H: Head day 1; WH: frozen Whole Head; MH: frozen Minced Head; W: Water thawed; A: Air thawed

In a study by Slizyte, et al. (2005), the amount of free amino acids was calculated for FPH produced from different combinations of cod rest raw materials (e.g. viscera, backbone, etc.) to a range between 15-97 mg/g dried powder. In another article, the amount of free amino acids was measured 66 mg/g for FPH produced from a mixture of cod lever and backbone (Slizyte, et al., 2008).

The dominating free amino acid differed for 0-min and hydrolysed samples for 30 and 60-min (Appendix II). In 0-min samples, the three top dominating amino acids were alanine, glycine/arginine, and serine respectively, while in 30 and 60 minute hydrolysed FPH, leucine was the most dominating amino acid followed by glycine/arginine and then alanine.

4.8 Suggestions to improve the process and analyses

Some modifications and adjustments are required that can improve the process setting and the accuracy of the analyses results.

The centrifugation speed and time used for fractionation of hydrolysed samples was not adequate, particularly for frozen whole head raw material and 30 minutes

hydrolysis time. Improved fractionation can enhance the yield of fish protein hydrolysate.

The sediment fraction used for analysis was not mixed uniformly before sampling and therefore, the sample was not an appropriate representative of the whole batch. Particularly, since the sediment sample contains different sizes of bones. In addition, in some samples due to insufficient fractionation, either there was a higher liquid content in the sediment or some sediment was lost in the hydrolysate fraction. Other than more proper fractionation, there are two solutions for this problem, either blend the sample more thoroughly, or take a larger sample to get a better representative of the total sample.

The whole heads have a smaller surface area to volume compared to the minced head samples (thickness), and therefore took longer time in both air and water to defrost. Furthermore, the whole heads immersed in running water for 3 hours lost a large amount of particles that differed them from the other samples. The amount of lost particles was not determined. Furthermore, the quality of water was not determined in this project.

The suggestions to improve the process conditions and obtain more accurate and reproducible results can be summarized as follow:

- The water used for thawing:
    - Using a water bath with controlled temperature
    - Indirect contact of the raw material with water
    - Quality control of water (if there is a direct contact between the sample and thawing water)
- Optimization of the hydrolysis process by:
    - Using more suitable storage conditions between removing of head and processing
    - Adjusting the time of hydrolysis
    - Better time-speed centrifugation combination to separate the phases

- Using larger blocks of minced cod heads to estimate the changes according to what is done in industrial practice

- Improving the analyses:

  - Better sediments sampling

  - Additional analysis according to the usage of the end products (e.g. functional and bioactive properties)

5 Conclusion

The main objective of this study was to determine the possibility of utilizing frozen marine cod head for further processing into high-quality and high-yield fish protein hydrolysates. The project included determination of the effect of mincing versus whole head, frozen storage and thawing methods on the composition and yield of FPH.

The results show that freezing and thawing of cod head lead to small changes in the composition of raw material and consequently the resulting fish protein hydrolysate. The lipid content in freeze dried FPH ranged from 0.37-0.71% dry-weight for all samples, where the lowest belonged to the day one and the highest for the whole head thawed in air. The protein content in freeze dried FPH was 88.4% for day one and in the other samples, the concentration was more or less similar and slightly lower than the day one sample (67.7-71.6%). While protein recovery was 85.0% dry weight for FPH produced from fresh cod head compared to 73.6 to 81.2% dry weight for frozen samples.

The degree of hydrolysis showed variations among FPHs produced from different raw materials and showed higher values for the whole head thawed in water and minced head thawed in air and rather low for the day one sample. There was no correlation between DH determined using formol titration and TCA-soluble peptides. For example, despite the low DH value, there was a very high concentration of TCA-soluble peptides in day one sample.

Regarding the yield of the dry matter in 60 min hydrolysed FPH factions; there is a slight difference between the samples (10.6-12.3%). The yield obtained in this project is higher than similar experiments, which makes it profitable to produce FPH from frozen stored cod heads.

Economically, it is more beneficial to store and thaw the minced head using an appropriate thickness of packaging, because of the smaller space that is required for the storage of the heads on board. In addition, if the right thickness and size is used for packaging, the time that is needed to thaw them will be much shorter. Due to

small differences between the compositions of minced and whole head, it is suggestible to store the samples in minced state.

Some optimizations are required for the hydrolysis process and analyses, including using more appropriate fractionation, adjusting the time of thawing according to the raw material to avoid excess changes during thawing and blending of sediments prior to sampling for analysis.

Everything considered, in order to determine the suitability of the used processing methods, and whether or not cod head can be used as raw material to produce high quality FPH, there should be a clear definition of “quality”. For instance, it should be determined in which section and for what purpose the end product is going to be consumed. If we are looking for the functional properties of the FPH, there are different characteristics that are required than when it is used for pharmaceutical purposes. Therefore, different analysis methods should be used to determine the changes in those particular required characteristics.

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APPENDIX I: Pre-experiment for freezing/thawing of whole and minced cod head and minced cod viscera

Applied and ran by Per Egil Gullsvåg at SINTEF Energy AS

Two live codfish was bought directly from the fishing boat ST-74-T, which was docked in Ravnkloa, approximately 6 kg (2 and 4 kg).

The fish were gutted and headed in the lab. Furthermore, products from both fish were minced and placed in zip-bags and the cod heads were minced and placed in a separate bag. The bigger cod head of the two was kept intact, because it was easier to place two pieces of I-button in the head to register the freezing and thawing progress. Similarly was made with minced product, to record the changes in temperature in the core and on the surface.

Furthermore, the products were frozen in an impingement-freezer at - 37 ° C for about 25 min. Then they were placed in a freezer at - 20° C to the next day to become equalized.

For the thawing, the products were placed in running cold water, at approximately +8°C temperature.

Table 6. Weight and size of the samples used for the pre-experiment

	Minced viscera	Minced heads	Whole head
Weight (g)	940	385	860
L × B × H (cm)	22 × 16 × 3.5	16 × 10 × 4	17 × 9 × 10

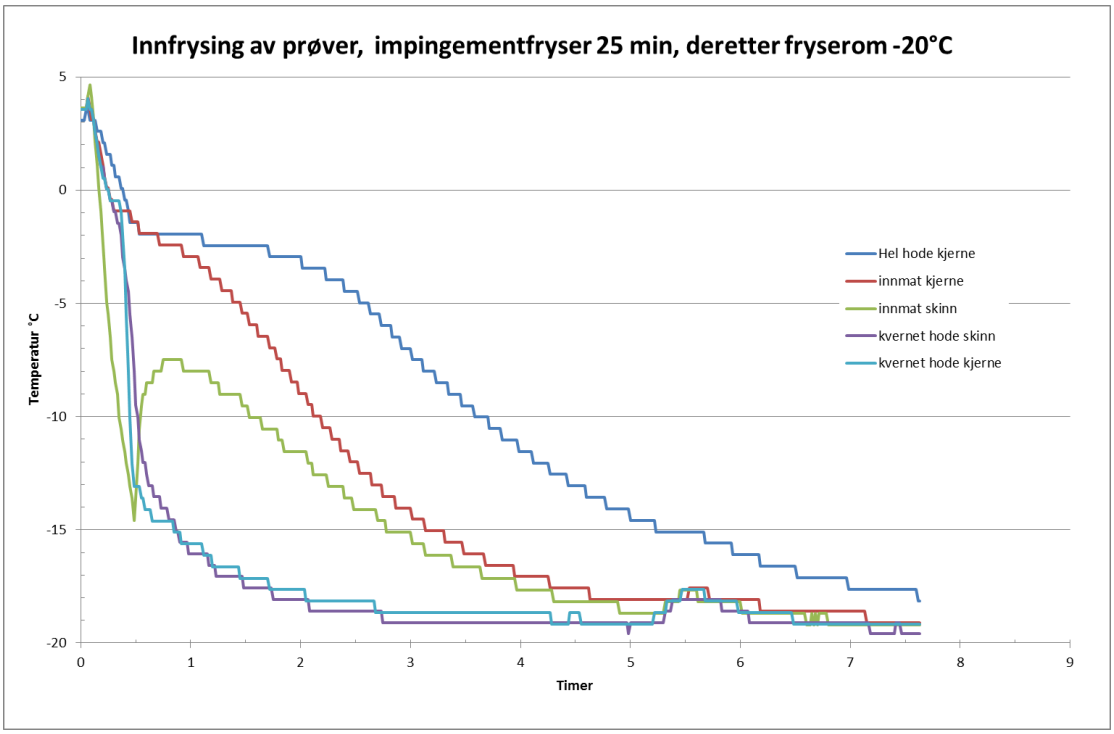

Figure 22. The graph shows the freezing progress by time, including the first 25 minutes in the impingement-freezer and later in the freezer-room. The log for the surface of the whole head was wrong and therefore it is not included here.

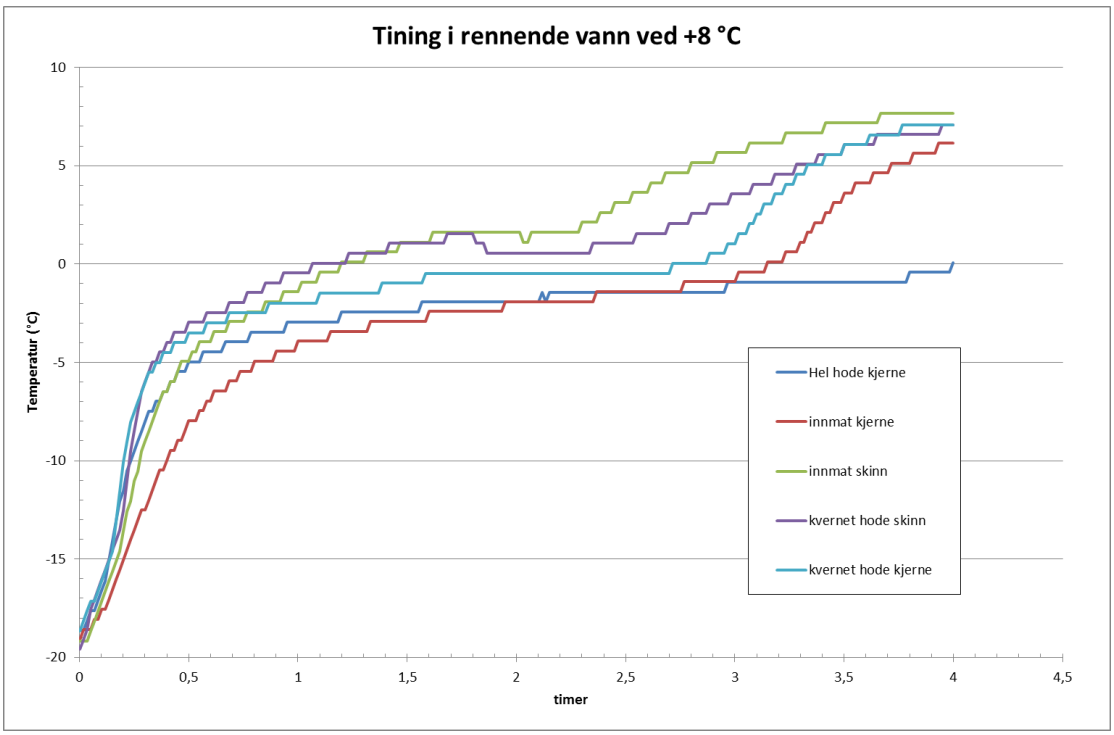

Figure 23. The thawing curves, demonstrating the changes in temperature on the core and surface of the samples by time. The core temperature in the minced viscera sample reached 0ºC after 3 hours.

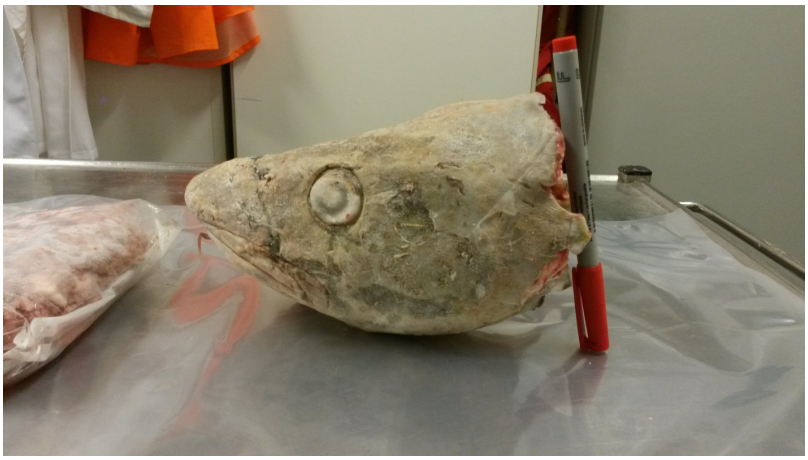

Figure 24. Whole and minced cod head and viscera samples used for pre-experiment

APPENDIX II: Dominating amino acids in FPH

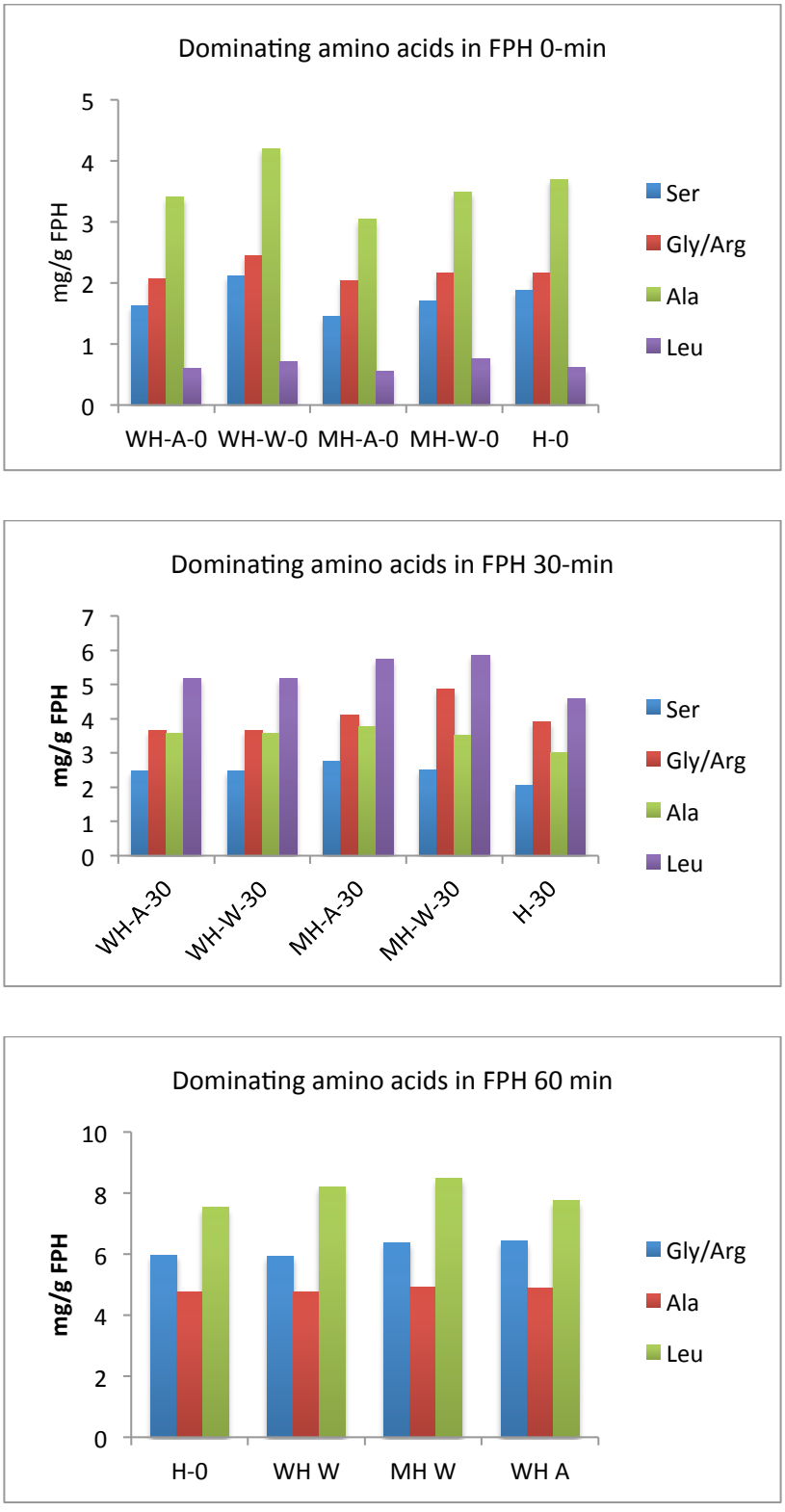

Figure 25. The dominating amino acid in freeze-dried FPH samples obtained by 0, 30 and 60 minutes hydrolysis determined by HPLC method; performed by Maiken Jeanette Lindgjerdet Grøndtveit