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Protein Hydrolysates and Oil from Herring Rest Raw Material

Effect of Storage Conditions on Yield and Properties

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Preface

This thesis has been a part of the Master of Technology education in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU). The work was carried out as part of the project "Herring protein for human consumption"[1] and the thesis written with Professor Turid Rustad at the Department of Biotechnology at NTNU as a supervisor. The experiments were conducted partly at NTNU and partly at SINTEF Fisheries and Aquaculture.

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Thanks to my family for all help and support while growing up, making it possible for me to be accepted to and complete my education at NTNU. Lastly I want to thank all my fellow students for five wonderful years. They will never be forgotten.

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Abstract

In Norway there is an increasing trend of filleting herring (*Clupea harengus*) before export. This leads to generation of large amounts of rest raw material that often is discarded or only used as animal feed. Rest raw material of marine origin, such as herring, contain valuable biomolecules like proteins and lipids that can be extracted from the rest raw material and find various applications in the food, pharmaceutical and cosmetic industries. With a growing world population there is an increasing need for reducing waste and for increasing food production. Therefore an optimal use of already available resources is becoming more and more important.

The aim of this study was to investigate the effect of the raw material storage conditions on the yield and properties of proteins and lipids extracted from the rest raw material with enzymatic hydrolysis. The rest raw material from Norwegian Spring Spawning herring consisted of all parts of the fish except for the fillet, and contained 14,2 ± 0,9 % protein, 16,2 ± 0,2 % lipid and 3,5 ± 0,2 % ash. The raw material was stored 0-120 hours at either 4 °C or 10 °C before enzymatic hydrolysis was performed with focus on producing protein hydrolysates and crude oil with a high yield and a high quality, at the same time as minimising the lipid content of the protein hydrolysates and avoiding formation of an emulsion fraction.

Crude oil was extracted prior to enzymatic hydrolysis after heating the raw material to 50 °C. The quality of crude oil was high when analysed with respect to peroxide value (PV), anisidin value (AV) and amount of free fatty acids (FFA). All values were within recommended limits for crude oil intended for human consumption, irrespective of the storage conditions of the raw material. The level of FFA increased with storage time and was higher for storage at 10 °C compared to 4 °C. The correlation between oxidation status and storage conditions was less clear. The crude oil produced from fresh raw material had PV 11,9 ± 0,2 meq/kg, AV 1,4 ± 0,1, TOTOX 25,2 ± 0,4 and FFA 0,5 ± 0,08 %.

Protein hydrolysates were produced by enzymatic hydrolysis with the enzymes papain and bromelain for 60 min at 50 °C. The protein content (81-85 %, conversion factor 6,25) decreased slightly with increasing storage time of raw material, and was higher for storage at 4 °C compared to 10 °C. The protein recovery (33-39 %) remained relatively constant throughout the storage period and was at times higher for storage at 10 °C compared to 4 °C. No correlation was found between lipid content (1,5-6,8 %) and storage time, but storage at 10 °C resulted in a higher

lipid content than storage at 4 °C. In all cases the lipid content was higher than what was aimed for (<0,5 %). Degree of hydrolysis (19-26 %) increased with increasing storage time, while amount of acid soluble peptides (38-46 %) remained constant or decreased slightly. Both parameters were higher for storage at 10 °C compared to 4 °C. The molecular weight distribution was not affected by the storage conditions and the most pronounced peptide fractions were estimated to be of size ∼800 Da, ∼300 Da and ∼150 Da. The hydrolysates were found to contain bioactive peptides with strong inhibitory activity against angiotensin I converting enzyme *in vitro*, an enzyme which is responsible for elevated blood pressure *in vivo*. The inhibiting effect was independent of the storage conditions.

The yield of protein hydrolysate did not change with storage time of rest raw material. The yield of crude oil was first decreasing with increasing storage time, but after 48-72 hours the trend reversed and the yield returned to initial value. Storage at 10 °C resulted in higher yields compared to storage at 4 °C. The protein content, protein recovery and yield of the protein hydrolysates improved with hydrolysis time, the greatest change being observed between 0 and 30 min of hydrolysis. The hydrolysates after 0 min of hydrolysis were typically fluffy, porous and bright in colour, while the hydrolysates after 60 min of hydrolysis had a denser texture and a darker colour. The hydrolysates after 60 min of hydrolysis were in general too dark for incorporation into food products, especially the ones with a high lipid content.

This study shows that production of high quality protein hydrolysates and crude oil from herring rest raw material is possible. However, challenges still remain with e.g. respect to the high lipid content and dark colour of the hydrolysates. The high degree of hydrolysis might imply that the hydrolysates are better suited for use as a pharmaceutical ingredient in treating e.g. hypertension, rather than as a functional ingredient in the food industry. As per today, with the challenges existing for production of high quality herring hydrolysates, production of crude oil might be the best option for utilising the herring rest raw material.

Sammendrag

I Norge er det en økende trend i filetering av sild (*Clupea harengus*) innen eksport. Dette resulterer i dannelse av store mengder restråstoff som ofte kastes eller kun blir brukt til dyrefôr. Restråstoff fra sild og annet marint opphav inneholder verdifulle biomolekyler som proteiner og lipider. Disse biomolekylene kan ekstraheres fra restråstoffet og har potensiale for å brukes i næringsmiddel-, farmasøytisk- og kosmetisk industri. Med en voksende verdensbefolkning øker behovet for alternative matkilder og for å redusere mengden avfall, og dermed øker også viktigheten av å utnytte allerede tilgjengelige ressurser optimalt.

Målet med dette studiet var å undersøke effekten av restråstoffets lagringsforhold på utbyttet og egenskapene til proteiner og lipider ekstrahert fra restråstoffet ved hjelp av enzymatisk hydrolyse. Restråstoffet fra norsk vårgytende sild bestod av alle deler av fisken med unntak av fileten, og inneholdt 14,2 ± 0,9 % protein, 16,2 ± 0,2 % lipid og 3,5 ± 0,2 % aske. Restråstoffet ble lagret 0-120 timer ved enten 4 °C eller 10 °C før enzymatisk hydrolyse ble utført med fokus på å produsere proteinhydrolysater og råolje med høyt utbytte og av høy kvalitet, samtidig som lipidinnholdet i proteinhydrolysatene ble minimert og dannelse av en emulsjonsfraksjon unngått i den grad det var mulig.

Råoljen ble ekstrahert før enzymatisk hydrolyse ved å varme restråstoffet til 50 °C. Kvaliteten på oljen var høy ved analyse av peroksid verdi (PV), anisidin verdi (AV) og mengde frie fettsyrer (FFA). Alle resultater var innenfor anbefalte grenseverdier for råolje ment for humant konsum, uavhengig av lagringsforholdene til restråstoffet. Mengden FFA økte med lagringstid og var høyere når restråstoffet var lagret ved 10 °C sammenlignet med 4 °C. Sammenhengen mellom oksidasjonsstatus og lagringsforhold var mindre klar. Råoljen fra ferskt restråstoff hadde PV 11,9 ± 0,2 meq/kg, AV 1,4 ± 0,1, TOTOX 25,2 ± 0,4 og FFA 0,5 ± 0,08 %.

Proteinhydrolysatene ble produsert ved å utføre enzymatisk hydrolyse med enzymene papain og bromelain i 60 min ved 50 °C. Proteininnholdet (81-85 %, omregningsfaktor 6,25) sank noe med økende lagringstid av restråstoffet, og var høyere ved lagring på 4 °C sammenlignet med 10 °C. Proteingjennvinningen (33-39 %) holdt seg relativt konstant gjennom lagringsperioden og var til tider høyere ved lagring på 10 °C sammenlignet med 4 °C. Det ble ikke funnet noen sammenheng mellom lipidinnhold (1,5-6,8 %) og lagringstid, men lagring ved 10 °C resulterte i høyere lipidinnhold enn lagring ved 4 °C. I alle tilfeller var lipidinnholdet høyere enn ønsket (<0,5 %). Hydrolysegraden (19-26 %) økte med økende lagringstid,

mens mengden syreløselige peptider (38-46 %) forholdt seg konstant eller sank noe. Begge parametree var høyere ved lagring på 10 °C sammenlignet med 4 °C. Molekylvektsfordelingen var ikke påvirket av lagringsforholdene til restråstoffet og de mest utbredte peptidfraksjonene ble estimert til å være av størrelse ∼800 Da, ∼300 Da and ∼150 Da. Hydrolysatene inneholdt bioaktive peptider med sterk inhiberende effekt overfor angiotensin I konverterende enzym *in vitro*, et enzym som er ansvarlig for forhøyet blodtrykk *in vivo*. Den inhibirende effekten var uavhengig av lagringsforholdene til restråstoffet.

Utbyttet av proteinhydrolysat forholdt seg konstant over lagringsperioden av restråstoffet. Utbyttet av råolje sank først med økende lagringstid, men etter 48-72 timer reverserte trenden og utbyttet returnerte til startverdi. Lagring av restråstoff ved 10 °C resulterte i høyere utbytte sammenlignet med lagring ved 4 °C. Proteininnholdet, proteingjenvinningen og utbyttet økte med hydrolysetid, og den største endringen skjedde mellom 0 og 30 min hydrolyse. Hydrolysatene etter 0 min hydrolyse var typisk porøse og lyse i fargen, mens hydrolysatene etter 60 min hydrolyse hadde en tettere tekstur og var mørkere i fargen. Hydrolysatene etter 60 min hydrolyse var generelt for mørke for å kunne inkorporeres i mat, spesielt de med et høyt lipidinnhold.

Denne studien viser at produksjon av høykvalitets proteinhydrolysater og råolje er mulig. Flere utfordringer gjenstår imidlertid, deriblant en uønsket mørk farge og et høyt lipidinnhold i hydrolysatene. Den høye hydrolysegraden kan innebære at hydrolysatene er bedre egnet som farmasøytisk ingrediens i blant annet behandling av hypertensjon, enn som funksjonell ingrediens i næringsmiddelindustrien. Per dags dato, med de eksisterende utfordringene for produksjon av høykvalitets sildeproteinhydrolysater, kan det tyde på at produksjon av råolje er den beste måten å utnytte restråstoff fra sildeindustrien på.

Contents

Glossary

Abz anthranilic acid or 2-aminobenzoic acid

AC ash content

BSA bovine serum albumin

CHL cholesterol

CPH chicken protein hydrolysate

DG diglyceride

DH degree of hydrolysis

DHA docosahexaenoic acid

DM dry matter content

EC European Commission

EFSA European Food Safety Authority

EPA eicosapentaenoic acid

EU European Union

FFA free fatty acids

FPH fish protein hydrolysate

FPLC fast protein liquid chromatography

IC_{50} half maximal inhibitory concentration

LC lipid content

MG monoglyceride

n-3 omega-3

NCBI The National Center for Biotechnology Information

NSS Norwegian spring-spawning

NSSH Norwegian spring-spawning herring

NTNU Norwegian University of Science and Technology

PL phospholipid

PrC protein content

PUFAs polyunsaturated fatty acids

SEC size-exclusion chromatography

SHR spontaneously hypertensive rats

SINTEF Stiftelsen for industriell og teknisk forskning ved Norges tekniske høgskole

TCA trichloroacetic acid

TG triglyceride

1 Introduction

1.1 Background

While China is the world-leading country in export of fish and fish products, Norway is a good number two [1]. Norway exports seafood to about 140 countries all over the world, and in 2014 the value of Norwegian seafood export totalled NOK 69 billion [2]. The development in Norwegian seafood export throughout the last years is shown in Figure 1. Both the aquaculture and the fishery industry are clearly of big importance to the Norwegian export economy, only being exceeded by the petroleum industry.

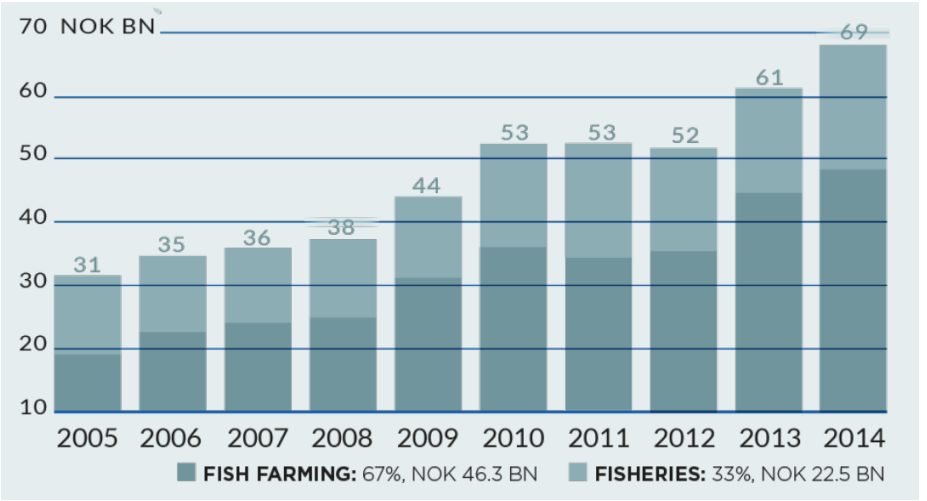

Figure 1: The development in Norwegian seafood export between 2005-2014, expressed in billion NOK[3].

In 2013, 3,07 million tons of fish and shellfish were produced from the fishery and aquaculture industry. Out of this, 0,87 million tons (28 %) were by-products[4]. By-products are often understood as all the rest raw material that is left after production of the main product, either it being edible or not. Some of the by-products are, when recycled and processed, suitable for human consumption. To distinguish this recyclable part from the waste material, the term rest raw material will be used in this context rather than the term by-product.

Today's value of the rest raw material lays between 2 and 2,5 billion NOK [5]. In 2013, 69 % of the rest raw material was utilised, adding up to around 600 000 tons. At the same time this means that 270 000 tons were discarded. One aim for future business could be to utilise more of the rest raw material that is produced. Another aim could be to look into how the already utilised part is utililised. Of the 69 % that was utilised, about 90 % went into production of animal feed. Only 10 % went into value-added products for human consumption[6]. On the other hand, these value-added products constitute about 50 % of the total economic value of the rest raw material. Today's total value can be multiplied several times if the rest raw material to a larger degree is utilised as human food and as functional ingredients in foods, nutraceuticals, cosmetics, special feed etc., rather than as animal feed. It is clear that there is a large and unexploited potential within the area of processing rest raw material.

This project's aim is to investigate the possibilites for making value-added products from herring rest raw material. Crude oil and protein hydrolysates have been made from a variety of fish species earlier, including salmon (*Salmo salar*), cod (*Gadus morhua*) and herring (*Clupea harengus*). The reason for choice of herring in this project is the huge amount of herring rest raw material that every year is landed from Norwegian vessels. There is an increasing trend of filleting the herring before export, and therefore also an increasing amount of herring rest raw material produced [7]. The increase is expected to continue due to installation of equipment with increased filleting capacity in Norwegian processing plants.

Figure 2 shows the value of Norwegian herring export over the last years. Note that the decrease in export from 2012 to 2013 is due to a lower quota for sustainable harvesting. The most important species for the Norwegian herring industry is the commonly called Norwegian spring-spawning herring, NSSH. This fish stock is sustainable[8] and rest raw material from NSS herring will be used for production of crude oil and protein hydrolysates in this project.

According to RUBIN's yearly commodity flow analysis [9], the pelagic industry exported 660 000 tons of herring and 170 000 tons of mackerel in 2007 [7]. This resulted in 212 000 tons of rest raw material, mainly originating from the filleting of herring; 6 % was dumped and 94 % utilised. In 2011, 231 000 tons of rest raw material from pelagic fish were produced; 3 % was dumped and 97 % utilised. The utilised part went for production of either fishmeal, fish oil or ensilage. These three products are all used in the feed industry, but the economic benefit of this processing is low. A higher financial contribution from

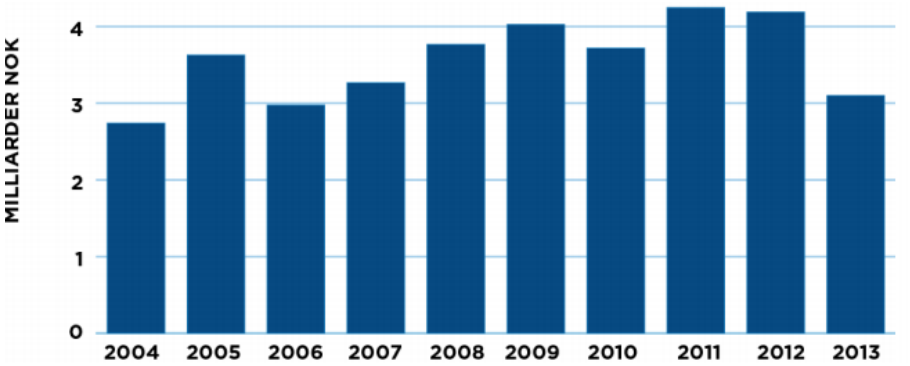

Figure 2: The trend in Norwegian herring export between 2004-2013, expressed in billion NOK [3]. Note that the decrease in export from 2012 to 2013 is due to a stricter quota for sustainable harvesting.

the rest raw material will be an important element for improved profitability in the pelagic industry.

1.2 Hydrolysis and fish protein hydrolysate

From rest raw material, when handled properly, both high quality fishmeal, fish oil and ensilage can be produced. Production of fish protein hydrolysates (FPH) is a newer way of utilising the proteins. Protein hydrolysates are proteins that have been broken down into smaller proteins and peptides of different sizes through a process called hydrolysis [10]. Compared to traditional fishmeal production, this production process results in a product with other properties, thus making hydrolysates an interesting value-added product. Figure 3 illustrates the process of protein degradation.

Hydrolysis is a process where a linkage between two molecules are broken and water is consumed. Figure 4 on page 5 shows how a water molecule cleaves the peptide bond in protein hydrolysis. The hydrolysis process can be influenced by several factors, including composition of raw material, enzyme type, water addition, inactivation of endogenous enzymes and process conditions like pH, temperature and time. These factors and more are further described in the sections below.

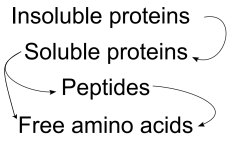

Figure 3: A simplified description of protein degradation. Insoluble proteins are solubilised and the solubilised proteins further degraded into smaller peptides of different sizes. The mixture is then often referred to as fish protein hydrolysate (FPH). The proteins and peptides might also liberate free amino acids. All steps are reversible, e.g. peptides might interact to form insoluble proteins.

Hydrolysis can be performed both chemically and enzymatically. The chemical hydrolysis uses acid or base, while the enzymatic hydrolysis is catalysed by enzymes. Chemical hydrolysis is commonly applied in the food industry due to being a simple and relatively inexpensive method. On the other hand, the process is often difficult to control and also uses extreme pH, pressure and temperature compared to enzymatic hydrolysis. This often leads to products with reduced nutritional qualities and inconsistent functional properties [11]. Functional properties are further described in Section 1.8. Other disadvantages are use of excessive amounts of NaCl after neutralization of the digest, destruction of the essential amino acid tryptophan and restricted usage of the hydrolysates as flavour enhancers, one of the reasons why proteins traditionally have been hydrolysed [10]. Acid hydrolysis is more popular than alkaline because alkali reagents often have an adverse effect on the nutritive value and possibly also functional properties of the hydrolysate[11]. Enzymatic hydrolysis has been predicted a brighter future due to higher specifity and use of milder process conditions, resulting in products with more consistent functionality and nutritive value [10, 11].

1.3 Enzymatic hydrolysis of proteins

Enzymes are biochemical catalysts which accelerate chemical reactions within the cell by lowering the activation energy. The highly specific active site of the enzyme forms a complex with the substrate, the substrate is converted to product and the product released so that the enzyme is regenerated. Without enzyme present the reaction could otherwise take very long time to complete, or not take place at all. Enzymes are to a large degree exploited in the food

industry, catalysing desired reactions and converting raw material into high-value products[10]. One example is the use of rennet, consisting of the enzyme rennin, for making cheese out of milk.

Proteolytic enzymes are economically the most important group of enzymes [10]. Proteolytic enzymes are also called proteases, proteinases and peptidases[12]. Depending on their specifity the proteolytic enzymes cleave the peptide bond between two amino acids, yielding peptides of varying sequences and lengths. The process is illustrated by hydrolysis of a dipeptide in Figure 4. Endopeptidases cleave peptide bonds within the peptide chain, generating large peptides, while exopeptidases cleave peptide bonds from the end of the chain, generating dipeptides and free amino acids [13]. The enzymes can also be classified according to their active seat, e.g. cysteine endopeptidases are endopeptidases that contain a highly reactive cystein residue in their active site[12].

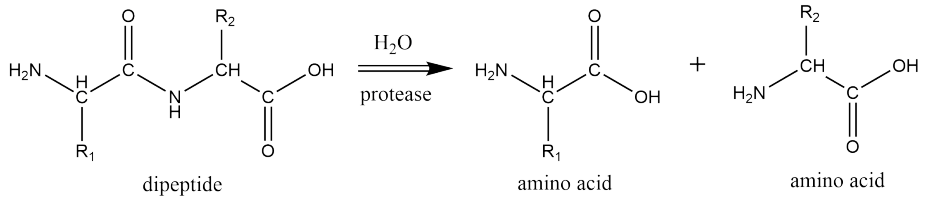

Figure 4: Proteases hydrolyse proteins and peptides into smaller peptides and free amino acids. Here a dipeptide is hydrolysed into two free amino acids. A water molecule is consumed in the process.

Enzymatic hydrolysis of proteins can be achieved by utilising endogenous enzymes already present in the raw material, or by addition of commercial enzymes. Endogenous enzymes can be more efficient in the hydrolysis process than commercial enzymes[14], solubilising more protein and increasing the hydrolysate yield. Raw material fractions such as liver and intestines are associated with high endogenous proteolytic activity[15], while fractions as e.g. backbones are not. The simultaneous use of endogenous and commercial enzymes can result in higher hydrolysate yields than if using commercial enzymes alone[16]. However the use of commercial enzymes alone are likely to offer better possibilities for controlling the properties of the product [17]. A controlled and reproducable process is of uttermost importance, and therefore, depending on the type of raw material, endogenous enzymes are sometimes inactivated prior to addition of commercial enzymes. Commercial and endogenous enzymes are

further described in Section 1.5 and Section 1.6, respectively.

Prior to addition of commercial enzymes, the minced rest raw material is mixed with water. This is to allow the enzymes easy access to the substrate by dissolving soluble components from the sediment phase, leading to increased protein recovery and hydrolysate yield[16, 18]. Šližytė[18] reported that addition of water influenced the yield more than what type of enzyme used did. On the negative side, the oil yield is likely to decrease and the emulsion yield to increase[16, 18]. Additionally the water added has to be removed upon drying or concentrating later. The cost of hydrolysis increases both with the inactivation of endogenous enzymes and the addition of water and commercial enzymes, and the necessity of these actions must be considered in relation to desired product quality.

1.4 Raw material for enzymatic hydrolysis

In this project, rest raw material from NSS herring harvested outside Trøndelag, Norway in October 2014 will be used for enzymatic hydrolysis. The composition of NSS herring varies throughout the season due to its natural cycle, maturity stage and geographical location[19, 20]. NSS herring has its wintering period between September and January and its spawning migration period between mid-January and April. In these two periods the herring does not feed, leading to changes in chemical composition[21]. Between April and September the herring feeds, and the herring harvested in October will therefore have a high lipid content.

The herring will be filleted and delivered to the locations of SINTEF Fisheries and Aquaculture by Grøntvedt Pelagic. The rest raw material will consist off all parts of the fish apart from the fillet, including viscera. Before enzymatic hydrolysis is performed, the raw material will be minced into a homogenous mass. A uniform mixture is important in order to produce a good substrate for the enzymes to degrade [6]. The enzymatic hydrolysis will be performed at 50 °C for 60 minutes. The pH value of the hydrolysis mixture will not be monitored, but will be determined by the pH value of rest raw material and water in a ratio of 1:1 (w/w). Keheller et al.[22] reported that the pH value of muscle tissue from three different pelagic fish was in the range 6,0-6,5. Similar values are therefore expected for the herring in this project. Because the raw material contains viscera it might be that the pH is slightly lower than it would be if only muscle tissue was present.

1.5 Commercial enzymes

A wide range of commercial enzymes have been used to produce protein hydrolysates and these include alcalase (subtilisin), bromelain, chymotrypsin, cathepsin, Corolase 7089, Corolase PP, Denazyme AP, ficain (ficin), Flavourzyme, pancreatin, papain, pepsin, plasmin, Protamex R, Proteinase K, Protex 6L, Neutrase, Seabzyme, thermolysin and trypsin in addition to several other proteases derived from plants, microorganisms and animal tissues[10, 11, 23, 24, 25, 26, 27][28, and references cited therein].

Choice of commercial enzymes

When choosing which commercial enzymes to work with there are several factors that needs to be considered. One of the most undesirable properties of the hydrolysates is their bitter flavour [29]. The formation of bitter components during hydrolysis is amongst other influenced by the type of enzyme used[24, 30] and will be further described in Section 1.7. Other factors include price, availability, efficiency and human acceptance. For increased yield, enzymes with a broad specifity should be used. Extensive hydrolysis by nonspecific hydrolases, such as papain, causes solubilisation of even poorly soluble proteins[29]. Such hydrolysates usually contain low-molecular-weight peptides of the order 2-4 amino acid residues[29]. On the contrary, if the aim is to obtain specific bioactive peptides, enzymes with a narrow specificity might be necessary to use to ensure that the peptides are not extensively broken down. Extensive hydrolysis may also lead to loss of functional properties[29]. Bioactive peptides and functional properties are further described in Section 1.8. When the requirements for the desired product are set, the process conditions during hydrolysis can be chosen based on these requirements at the same time as assuring that the enzymes will be active at these conditions.

In this project enzymatic hydrolysis will be performed with bromelain and papain extracts from pineapple and papaya, respectively. These are abundant and relatively cheap enzymes. The fact that they are extracted from plants could possibly mean that they will gain easier acceptance by customers than enzymes derived from animals or microbes. Information about the enzymes is given in Table 1. Use of papain alone has been reported to yield bitter hydrolysates [31], while the combination of papain and bromelain has shown promising results for herring hydrolysates [24].

Table 1: Enzymes that will be used for hydrolysis of herring rest raw material stating origin, type, activity, optima for temperature and pH, and source [32].

enzyme	**origin**	**type**	**activity [TU/mg][a]**	**temp [°C]**	**pH**	**source**
bromelain	*Ananas comosus*	cysteine proteinase	100	20-65	5-8	Enzybel
papain	*Carica papaya*	cysteine proteinase	100	40-80	5-9	Enzybel

[a] TU, tyrosine unit, defined as the amount of enzyme that releases the equivalent of 1 mg of tyrosine per minute from a specified casein substrate under the conditions of the assay[32].

Papain

The commercial papain enzyme mixture takes its name from the main enzyme papain, which cleaves peptide bonds near the amino acids glycine or leucine [25]. The refined micro granular papain from Enzybel consists of a mixture of the four proteases papain, caricain, chymopapain and glycyl endopeptidase. They are all cysteine endopeptidases and members of the peptidase family C1 [12]. This means they cleave peptide bonds within the peptide chain and contain a highly reactive cystein residue in their active site. The active site of papain consists of seven subsites (S1-S4 and S1'-S3'), each subsite able to accommodate one amino acid residue of a substrate [12]. As with most endopeptidases in family C1, all four enzymes accept hydrophobic residues in S2 and S3. Preference is for a bulky non-polar amino acid such as phenylalanine in S2 position. However, other residues are also accommodated in these subsites, and the rules are therefore not absolute. The S1 subsite of papain is not as selective as the S2 subsite, but there is some preference for arginine or lysine in S1 position [33] and valine is not accepted [34]. In general all four enzymes show a fairly broad specifity.

Bromelain

The commercial bromelain enzyme mixture takes its name from the main enzyme bromelain, either extracted from the stem or the fruit of the pineapple

plant. Fruit bromelain has higher proteolytic activity compared to stem bromelain [35]. The refined micro granular bromelain from Enzybel consists of a mixture of the four proteases fruit bromelain, stem bromelain, ananin and comosain. They are, like the papain mixture, all cysteine endopeptidases and members of the peptidase family C1[12]. All four enzymes show a fairly broad specifity with a preference for polar amino acids in the S1 and S1' position, hydrophobic amino acids in S2 position[35] and proline in S3 position [36].

1.6 Endogenous enzymes

Quantitative and qualitative knowledge of the endogenous enzymes in the rest raw material is of high importance for having control during processing and for achieving the right quality of final product [37]. The lysosome, a small circular organelle of the animal cell, contains numerous enzymes capable of degrading both carbohydrates, lipids, nucleic acids and proteins [38]. This degradation of the animal body is called autolysis[39].

Lipases and phospholipases hydrolyse lipids into diglycerides (DG), monoglycerides (MG), glycerol and free fatty acids (FFA) as illustrated in Figure 5. These molecules are susceptible to interaction with other fish constituents, leading to a lowering in the nutritional and sensory values [40, 41]. Presence of lipases and phospholipases in the rest raw material is thought to be the most important reason for formation of these compounds, but they can also origin from microbial activity. FFA do not contribute much to flavour, but both FFA, mono- and diglycerides can work as prooxidants. They do so by lowering the surface tension at the interface and thereby increase the oxygen diffusion rate into the animal body[42]. In addition to working as prooxidants, FFA are more susceptible to oxidation than esterified fatty acids like mono-, di- and triglycerides[43]. The level of FFA is therefore considered an important quality parameter. FFA, mono- and diglycerides also have emulsifying properties, a property that can be both desired and undesired, depending on where in the process they appear. For example during hydrolysis and subsequent separation of the protein, oil and sediment fraction, formation of an additional emulsion fraction is not desired.

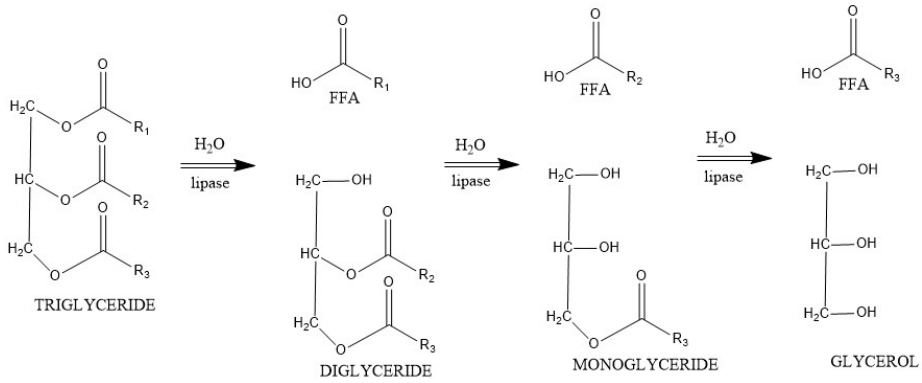

Figure 5: Lipases hydrolyse triglycerides (TG) into diglycerides (DG), monoglycerides (MG), glycerol and free fatty acids (FFA). Both FFA, MG and DG can work as prooxidants and their formation is therefore best limited.

Proteases degrade proteins into smaller proteins, peptides and free amino acids. This is illustrated in Figure 4 on page 5 where a dipeptide is hydrolysed into two free amino acids. Free amino acids play an important role as taste and flavour components [15]. The content of free amino acids in muscle tissue of aquatic species is normally higher than in muscle tissue of land animals, and wild fish tend to contain more free amino acids than cultured fish[15]. Endopeptidases like calpains and cathepsins break down large proteins, while exopeptidases, mainly dipeptidylpeptidases and aminopeptidases, cleave of small peptides and free amino acids from the end of proteins and peptides [13].

Endogenous enzymes are found in all tissues, but the distribution of enzymes and their activity show considerable variation [15]. The highest activities are found in gut fractions such as viscera and liver [15]. It is well documented that protein digestion in fish of different species occur both in the acidic region in the stomach and in the alkaline region of the intestines[44, 45, 46]. Stoknes[47] reported that the proteolytic activity in intestinal and liver fractions of herring dominated over fractions such as bones, skin and muscle. The maximum activities found in herring intestines were 30 times greater than what was found in herring muscle, and muscle tissue from cod showed considerably lower activity than muscle tissue from herring. Also Johansen[48] found that both proteolytic and lipolytic activity was substantially higher in herrring intestine versus herring muscle. The rest raw material in this project is from herring and contains viscera, and endogenous activity is therefore expected to be significant.

The reaction rates of the autolytic processes are highly temperature-dependent [38]. Chilling and freezing can reduce the reaction rate, but some endogenous enzymes are also active at low temperatures, especially in the presence of light or other catalysts[38]. Søvik[49] reported that lipases from cod were active even at cold temperatures and that rapid sorting was necessary in addition to low temperature storage in order to hold lipolytic activity down. The temperature dependence of endogenous enzymes is further described in the section below. In addition to tissue location and environmental temperature, the activity of endogenous enzymes also depends on pH, the life cycle of the fish and certain activators (mostly reducing compounds and metal ions) or inhibitors (antioxidants) [15]. In viscera, the relative concentration and activity of an enzyme will also be influenced by the feed[37].

Often the activity of endogenous enzymes is considered a negative aspect of the raw material, the activity being difficult to control and e.g. resulting in prooxidants that promotes oxidation. On the contrary, endogenous enzymes can also contribute in a positive direction with increased yield and e.g. hydrolysates containing specific bioactive peptides. Bioactive peptides are further described in Section 1.8.

Temperature dependence of endogenous enzymes

The influence of temperature on enzyme activity is described by the Arrhenius equation, a formula for the temperature dependence of reaction rates. Changing the temperature by 1 or 2 °C may introduce changes in reaction rates of 10-20 %[50]. Increased reaction rate with increased temperature can be explained with increased kinetic energy of the reactants and hence increased possibility for collisions to occur between them. The reaction rate profile of an enzyme as a function of temperature is shown in Figure 6. The first part of the reaction rate profile follows the Arrhenius equation. The reaction rate eventually enters a plateau where the enzyme begins to denature and lose activity. At even higher temperature the enzyme is fully denatured, and no activity remains. The point where this happens depends on the structure of the enzyme[51]. An enzyme's optimal temperature is a trade-off between the temperature dependence described by the Arrhenius equation (the higher temperature, the faster reaction) and the instability of the enzyme as it approaches its denaturation temperature[51].

A general rule of thumb originating in Arrhenius equation states that a 10 °C

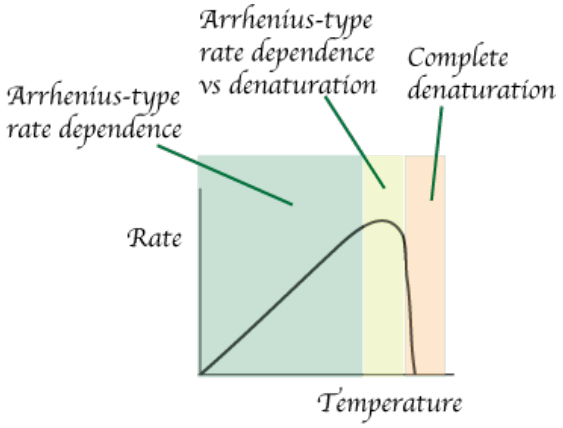

Figure 6: The reaction rate profile of an enzyme as a function of temperature [51].

increase in temperature doubles the enzyme reaction rate[51]. However, whether this is a good estimation or not depends on the activation energy of the enzyme involved in the reaction. Osvik[52] reported the activation energy of lipolytic and proteolytic enzymes in cod roe to be 20 kJ/mol and 40 kJ/mol, respectively. Using these values in combination with Arrhenius equation gives that a 10 °C increase in temperature would increase the reaction rate with approximately 35 % for lipolytic enzymes and 80 % for proteolytic enzymes.

Endogenous enzyme activity during storage of raw material

In this project the rest raw material will be stored at either 4 °C or 10 °C. Based on the temperature dependence of enzymatic reactions described by the Arrhenius equation and the activation energies found by Osvik[52] for enzymes in cod roe, it can be estimated that the raw material stored at 10 °C will have approximately 20 % higher lipolytic activity and 45 % higher proteolytic activity during storage, compared to the raw material stored at 4 °C.

Endogenous enzyme activity during hydrolysis

The endogenous enzyme activity is expected to be higher during hydrolysis than during storage due to the temperature dependence of enzymatic reactions

described by the Arrhenius equation (page 11). The pH value of raw material in this project is expected to be in the lower neutral pH range (Section 1.4 on page 6). Johansen[48, 53] examined endogenous enzyme activity between pH 5-7 in water extracts of herring roe (40-60 °C), intestine (40 °C) and muscle (40 °C) and reported of maximum proteolytic activity at pH 5 and maximum lipolytic activity at pH 7. Stoknes[47] detected maximum proteolytic activity at pH 3 - 4,5 (45-60 °C) for all herring and cod tissues investigated, i. e. muscle, liver, intestines (without liver), and head, skin and bones. Some activity also remained at pH = 8. Søvik[37] investigated endogenous enzyme activity of cod rest raw material from different fishing grounds (the Barents Sea, the Icelandic Sea and the coast outside Ireland) and found that proteolytic activity was strongly affected by harvest location, the maximum activity in cod viscera from the Icelandic Sea and the coast of Ireland being detected at pH 7 and pH 3 (<65 °C), respectively[37]. Søvik[49] also reported of high lipolytic activity at pH 5-7 (20-50 °C), the maximum activity being detected at pH 7. Summing up the results from previous studies[37, 47, 48, 49, 53], proteolytic activity seems to be most pronounced in the acidic region and lipolytic activity in the neutral/alkaline region. Both proteolytic and lipolytic activity was present at temperatures between 20 - 65 °C, indicating that endogenous enzymes in the herring raw material used in this project will be active during heating and hydrolysis at 50 °C. The activity will however depend on the pH value of the hydrolysis mixture. The commercial enzymes used in this project will also be active during hydrolysis due to a temperature optima between 20-65 °C for bromelain and 40-80 °C for papain (Table 1 on page 8). Both Stoknes[47] and Johansen[48] found that the gut fractions of herring showed higher proteolytic activity than other tissues. Since the rest raw material in this project contains viscera, a high endogenous activity is expected.

Endogenous enzyme activity in herring versus other species

The free amino acid content tend to be higher in muscle tissue of aquatic species than terrestrial animals[15] (page 10). This can possibly mean that proteases of marine origin are more active than proteases of terrestrial animals. Marine proteases tend at least to be more active at lower temperatures, at the same time as being less resistant to thermal denaturation[54].

There is limited research published with respect to endogenous enzymes in fish, and therefore little knowledge exists about the endogenous enzymes in herring

compared to other fish species. Several studies have reported increased proteolytic activity in fish muscle during sexual maturation[55, 56, 57, 58], making it difficult to compare the endogenous activity between species as they all have different maturation periods. Some differences between the activity of endogenous enzymes in herring and cod were briefly described in the previous section (page 10 and page 12). Furthermore Stoknes[47] reported that, in terms of enzyme units, cod muscle extracts showed lower levels of activity than those of herring. Stoknes[47] also found a difference in the heat stability of alkaline proteases. While herring muscle extract retained almost all its alkaline activity after 60 min preincubation at 60 °C, the activity of cod muscle extract was halved. The alkaline proteases from herring were also more activated by heat than those from cod. Proteolytic activity in herring muscle extract increased by 30 % after 10 min preincubation at 65 °C, with only a 10 % increase for cod muscle extract. These findings, suggesting that the endogenous enzymes are more active in herring than in cod, should be taken into account when comparing results from this project with studies on hydrolysates from other fish species. There has also been found differences in the proteolytic activity between North Sea herring and Baltic herring[59] during the ripening process of making salted herring products, indicating that differences are also found between the same species due to different harvest locations. Søvik[37] concluded with the same for cod from different fishing grounds, as described in the previous section (page 13).

1.7 Yield and quality of raw material and final product

High quality protein hydrolysates have a high protein content and a low content of ash, water and lipids. A high ash content lowers the relative amount of protein in the hydrolysates, water facilitates microbial growth and a pronounced lipid content results in a deteriorated product with less appealing taste, smell and colour due to lipid oxidation. It is essential to start with rest raw material of good quality in order to retain a high quality during processing. Selection, storage and correct handling of the raw material are therefore essential steps when processing the rest raw material [38]. The need for each of these steps depends on the demands of the end user, e.g. whether the product is to be used for animal feed or human consumption. When utilising the rest raw material for human consumption the nutritive value must be retained and the product must not have any undesired smell or taste. However to maximise the profit a high yield is also always important, and neither high yield or high quality can be neglected on an industrial scale.

Final quality and yield can be affected by many of the same factors and some of them will be briefly mentioned here. The level of contaminants building up in the food chain depends on the geographic region. The location of harvest therefore influences the final content of e.g. dioxins, PCBs and heavy metals in the rest raw material [38]. Geographical variations like water temperature, genetic variations like fish species and seasonal variations like spawning time, food availability and lipid content also affect the raw material composition and quality. After harvesting, storage conditions like time, temperature and atmosphere are important for handling the catch right and after filleting additional storage conditions might apply before the raw material is further processed. Sorting and selection of rest raw material after filleting can affect the final composition of raw material to be processed, e.g. including viscera will contribute to higher levels of endogenous enzymes and possibly also bitter components from bile. The mincing process is an important step, assuring a homogenous mixture and producing a good substrate for the enzymes to degrade[6]. After mincing the processing conditions during enzymatic hydrolysis can strongly influence the yield and quality of final product. These processing parameters include e.g. enzyme type, enzyme concentration, amount of water added prior to hydrolysis, temperature and pH during hydrolysis, hydrolysis time and time and temperature for inactivation of enzymes. A good centrifuge is essential for separation of the hydrolysis mixture into oil, protein and sludge. In this project the centrifuged mixture will be frozen down and while still sufficiently frozen, the fractions will be manually separated by use of spatula and knife. The separation conditions are expected to be more automatic and better performed in the industry. Finally, post-treatment of dried hydrolysates may influence final yield and quality[11], e.g. removal of bitter peptides in final product have the potential of reducing both the yield and the nutritional value[30].

Lipid oxidation

The oil fraction is mainly valuable because of its long-chain omega-3 (n-3) polyunsaturated fatty acids (PUFAs). Oil from plant sources are often rich in saturated, monounsaturated and polyunsaturated C_{18} fatty acids, but lack n-3 fatty acids such as 20:5n-3 (EPA) and 22:6n-3 (DHA) which are found in fish oil [60]. These fatty acids are very susceptible to oxidation, thus being a major cause of reduction in seafood quality. A variety of hydrocarbons, alcohols, furans, aldehydes, ketones and acid compounds are formed during their oxidation, thereby being responsible for off-flavours in oxidised oils [61]. Oxidised

lipids also react with proteins and amino acids with formation of objectionable off-flavours [62].

Lipid oxidation in fish muscle can be caused by endogenous enzymes[41] or by non-enzymatic processes such as autooxidation and photosensitised oxidation[63]. A simplified description of autooxidation is given in Figure 7. Autooxidation happens when free lipid radicals are spontaneously formed and react with atmospheric triplet oxygen to form oxidation products. Photooxidation happens in the presence of light and a photosensitiser such as myoglobin, converting triplet oxygen to singlet oxygen. Singlet oxygen then reacts directly with intact lipids, avoiding the need for formation of lipid radicals for oxidation to occur. Lipooxygenase and cyclooxygenase are examples of endogenous enzymes which cause lipid oxidation[64].

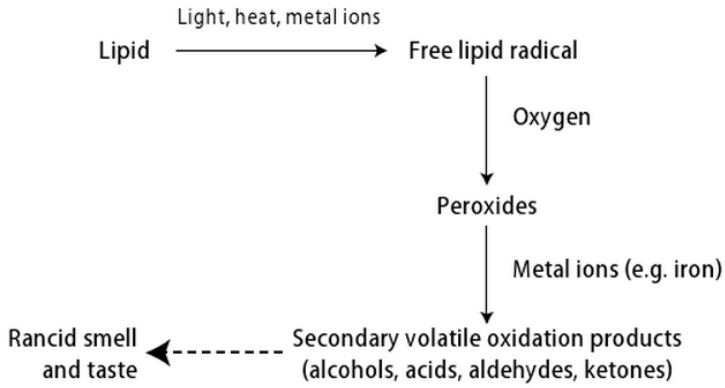

Figure 7: A simplified description of autooxidation [38]. Light, heat and metal ions can induce the spontaneous formation of lipid radicals. Lipid radicals are highly reactive in the precense of oxygen and react to form primary oxidation products called peroxides. The peroxides are unstable and break down to secondary volatile oxidations products, leading to a rancid smell and taste.

Lipid oxidation will not only occur in the rest raw material and in the extracted crude oil, but also in the protein hydrolysates if they contain significant amounts of fat. In addition to development of off-flavours, spoilage also comes in the form of textural, nutritional and colour changes that reduce the product quality and lead to decreased shelf life[41]. The nutritional value is lowered due to

interactions between proteins and oxidised lipids [65]. These interactions lead to lower digestibility because the covalent bonds formed are not hydrolysed by proteases under the digestion conditions in humans [65]. In additon the resulting imine bonds (protein-CH_2-N=CH-CH_2-lipid) in the lipid-protein interactions reduce the availability of essential amino acids like lysine, tyrosine, tryptophan and methionine [65]. Since the interactions lead to products that are less soluble and less easily moistened, they also cause deteriorated textural properties [65].

Lipid oxidation can induce darkening of the hydrolysates through the Maillard reaction[10, 62]. In the Maillard reaction brown pigments are formed from aldol condensation of carbonyls produced from lipid oxidation upon reaction with basic amino groups in proteins[66]. Hydrolysis of fresh raw material has been shown to give powders which are brighter in colour due to less pronounced oxidation[67]. Antioxidant-acting enzymes in fish can have a protective role against lipid oxidation[38]. Immediately after catch of herring the antioxidant level is generally high and most oxidative attacks are inhibited[68]. However, with time after death, changes like increased free iron content and membrane disintegration will take place, leading to increased lipid oxidation[68].

It has been suggested that the hydrolysates should contain no more than 0,5 % lipid for stability reasons[69]. A high lipid content in the raw material makes separation of the protein and oil fraction more difficult, thereby leading to a higher lipid content in the hydrolysate[18]. To remove excessive lipid requires additional treatments during production[10], and from an economical point of view, raw material from lean species is the preferred substrate. However if the challenge related to the high lipid content in hydrolysates from pelagic fish can be overcome, this abundant and underutilised rest raw material can possibly be a good source for production of high quality protein hydrolysates[10, 24].

Protein oxidation

Proteins may also be oxidised. Oxidation occurs at both the protein backbone and at the side chains, resulting in formation of further reactive species such as hydroperoxides and peroxides[38]. Because oxidative compounds such as free lipid radicals are produced during processing and storage of raw material[29], marine proteins are vulnerable to oxidative attack[70].

Protein oxidation can result in so major physical changes in protein structure that the nutritional value is lost[70]. The amino acids most susceptible to ox-

idation are methionine, cysteine, tryptophan, histidine and tyrosine[29]. As an example of protein oxidation, the process of methionine oxidation is shown in Figure 8. This chemical alteration makes methionine, which is an essential amino acid, biologically unavailable[29]. Oxidation of proteins can also lead to polymerisation, again lowering the biological value [29]. Lastly, oxidised proteins have the possibility of interacting with intact lipids and hence influence the oxidative stability of the lipids [38].

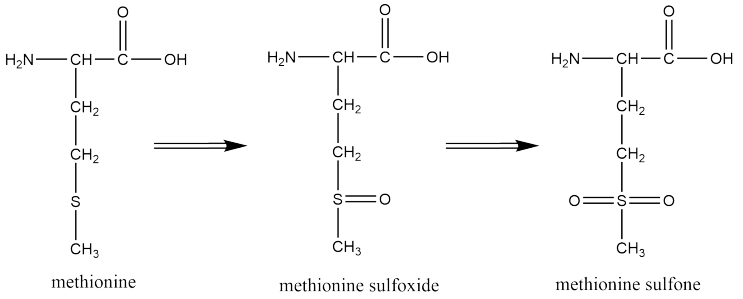

Figure 8: The process of methionine oxidation. Once oxidised to methionine sulfone the amino acid becomes biologically unavailable. Methionine is an essential amino acid.

Heat denaturation of proteins and enzymes

Moderate heat treatment improves the digestibility of proteins without developing toxic derivatives[29]. On the other side, both heat inactivation of endogenous enzymes prior to hydrolysis, temperature applied during hydrolysis and inactivation of enzymes after hydrolysis may have damaging effects on the raw material and final product[30]. Even though leading to quality loss, e.g. in the form of darkening of the hydrolysates[24], heat treatment after hydrolysis is essential to prevent the enzymes in degrading the hydrolysis mixture more than is desired for obtaining the right properties.

Heat inactivation of endogenous enzymes prior to hydrolysis can make the hydrolysis process easier to control (Section 1.3 on page 5). Heat inactivation of endogenous enzymes also has the benefit of reducing rancidity, textural changes, discolouration and formation of off-flavours during storage of rest raw material[29]. On the contrary, endogenous enzymes may also contribute posi-

tively to increase the yield of liberated oil and solubilised proteins. Endogenous enzymes were described in Section 1.6.

Extensive heat treatment during hydrolysis can result in insolubilisation of the proteins in the raw material and make them highly resistant to enzymatic breakdown due to hydrogen bonding, sulfhydryl reactions and hydrophobic- and ionic interactions [29]. Severe heat treatment can also inactivate the commercial enzymes, leading to reduced ability of these enzymes to hydrolyse the proteins[18]. Most food proteins are denatured when exposed to moderate heat treatments (60-90 °C, 1 h or less)[29]. In this project enzymatic hydrolysis will be performed at 50 °C for 1 hour.

Bitterness of hydrolysates

Bitterness is an important quality parameter. Many studies have looked at the formation of bitter components during hydrolysis, mainly bitter peptides. The formation of these bitter peptides depends on both the amino acid sequence of the proteins, as well as type of enzyme used and process conditions applied during hydrolysis[24, 30]. Several studies have shown that both small and hydrophobic peptides increase the risk of a bitter taste in the product[30]. There is ample evidence that bitterness is related to hydrophobicity, and that peptides with a mean residue hydrophobicity of >1,4 kcal/mol are bitter[71]. The bitterness can be reduced by inactivating endogenous enzymes[72] and by using a mixture of endo- and exopeptidases so that the bitter peptides will be further broken down into fragments with hydrophobicity <1,4 kcal/mol[29]. By choosing the right enzymes to work with and by monitoring the degree of hydrolysis during the process one can more easily control the formation of bitter peptides in the product, as well as obtain the right degree of protein degradation for optimal functional properties.

However, even though the major focus has been on formation of bitter components during the hydrolysis process, another possible source of bitter flavour is the raw material itself[30]. Dauksas et al.[30] reported that bitterness in the hydrolysates increased linearly with increasing lipid content. Bitterness was also clearly influenced by the presence of bitter components from bile when the raw material contained viscera. Removal of the gallbladder and keeping the lipid content in the hydrolysate low might therefore be just as important parameters to prevent bitterness in the final product as minimising the formation of bitter peptides during hydrolysis.

Colour of hydrolysates

In addition to bitterness and smell, colour is one of the most important sensory properties of the hydrolysate. Colour is the first sensory property evaluated by the consumer and is especially important if the hydrolysate is to be incorporated into food where it can influence the colour of the final product[73]. If the hydrolysate is mixed into a white fish pudding the hydrolysate should take on the same colour itself, while if taken as a pure protein powder the consumer might be more flexible with respect to darker tones. Regardless of the final application, the consumer prefers consistency in visual appearance.

The hydrolysate colour is difficult to control, and hydrolysates are often reported to have an undesirable yellow colour[25]. Colour intensity have been reported to be influenced by both type of raw material[74], enzyme used[66] and processing parameters applied [24]. Hydrolysates from rainbow trout, anchovy sprat and herring rest raw material have been reported to be darker and more yellowish in colour than hydrolysates from chicken or chicken rest raw material[25, 74, 75]. Raw material containing large amounts of entrails compared to bones results in darker hydrolysates and Sathivel et al.[76] reported that hydrolysates from herring gonads were darker in colour than hydrolysates from whole herring. The raw material to be used in this project will contain a normal ratio of bones and entrails.

Hydrolysates with a high lipid content might become darker due to the Maillard reaction[10, 62] (Section 1.7 on page 17). Hydrolysis of fresh raw material has been shown to give hydrolysates which are brighter in colour due to less pronounced lipid oxidation[67]. Furthermore, darkening of hydrolysates may also be the result of oxidation of melanin and myoglobin pigments. These pigments are susceptible to oxidation, and dark flesh fish such as herring contain high amounts of myoglobin[66, 75]. It is also known that browning reactions, including the Maillard reaction, are enhanced by thermal treatment [31]. Carvajal [24] reported that herring hydrolysates darkened during heat inactivation of enzymes at 90 °C. This supports the statement that processing temperature affects the colour of the product [24].

In summary there are many processing parameters that should be evaluated. Studies done by Slizyte[16] on enzymatic hydrolysis of cod showed that it is not possible to obtain all desirable quality indicators at the same time. These quality indicators include maximum oil and hydrolysate yield, minimum sediment yield and no emulsion formation together with a high protein recovery and a low

lipid content of the hydrolysates. Therefore, the aim and requirements for the final products should be prioritised and defined very clearly before the process is designed, taking into account the composition of raw material [16].

1.8 Applications of fish protein hydrolysates

The herring oil is of particular interest due to its valuable long-chain n-3 polyunsaturated fatty acids (Section 1.7 on page 15). Production of crude oil might be cause enough alone for processing herring rest raw material, but the total value increases significantly if high quality protein hydrolysates can be produced in addition to crude oil. The protein fraction is nutritionally rich and have interesting properties that can be exploited in the industry. Protein hydrolysates from NSS herring rest raw material, the same fish species that will be used in this project, were reported by Carvajal[24] to contain all the essential amino acids required for a balanced diet in adults.

Hydrolysis with enzymes is done in order to improve the functionality of the protein fraction in terms of nutritional, pharmaceutical, sensory, textural and physiochemical properties[11, 27]. Physiochemical properties include e.g. foaming, gelling, solubility and emulsifying properties. There is also an interest in hydrolysed proteins for e.g. reducing allergenicity[29, 77] and for the production of hydrolysates containing bioactive peptides[10]. Since the protein fraction has a wide range of properties it is important to control the extent of protein degradation in order to achieve the desired properties for that specific purpose [27].

A summary of currently known properties of protein hydrolysates is given in Table 2.

Table 2: The main functional, nutritional and bioactive properties of protein hydrolysates that have been reported[10, 24, 78, 79]

Functional properties	**Nutritional properties**	**Bioactive properties**
Foaming	High nutritional value	Antioxidative
Emulsifying	Easily digestible	Antihypertensive
Gel forming	Antigenic	Hypocholesterolemic
Heat tolerating (without precipitating)	Complete amino acid profile	Anticoagulant and antiplatelet
Cryoprotective		Immunomodulatory
Solubility (over a wide pH range)		Opioid agonistic (pain relieving)
Water holding capacity		Hormone-like peptides and growth factors
Oil absorbing (retain/hold oil)		Cytomodulatory and anticancer
Protect proteins from drying		Mineral binding (accelerated calcium absorption)
		Antithrombotic
		Antimicrobial
		Prebiotic
		Obesity modulatory

Bioactive peptides

In addition to having desired functional and nutritional properties, several bioactive properties have been identified in fish protein hydrolysates [24]. Bioactive peptides are defined as food-derived components that exert a physiological effect in the body in addition to having a nutritional value [80]. They are inactive within the original protein, but once released they function as regulatory compounds with hormone-like activity [80]. They can be liberated from the parent protein during both gastrointestinal digestion in the body and during food processing [81, 82]. Bioactive peptides usually contain 2-20 amino acid residues [80] and the activity depends on the chemical structure, i.e. the amino acid composition and chain length [83]. Some bioactive peptides have also been reported to be >20 residues [84].

It has been postulated that the bioactive peptides can produce local effects as early as in the gastrointestinal tract[85]. However it should be kept in mind that for reaching other target tissues than the gastrointestinal tract, the activity depends on transport of the peptides in their active form across intestinal walls and membranes[82]. Bioactivity of peptides is usually tested *in vitro*, and this is therefore not necessarily comparable to the bioactivity observed *in vivo*. Yet it has been suggested that because the bioactive peptides origin from already degraded proteins they have partial or total resistance to hydrolysis and may pass the digestive enzymes without being destructed[85]. Because of their low molecular size they can possibly also enter the blood system intact and from there exert systemic effects[85].

Naturally derived bioactive peptides often show a lower activity than synthetically prepared peptides, but compared to synthetic medication they have the advantages of being natural products with extra benefits, such as providing essential amino acids and acting as a nutritional supplement [23]. In addition, natural bioactive peptides seemingly show no side effects normally associated with synthetic medication [82, 86]. Food-derived bioactive peptides have been shown to display a wide range of physiological functions where some of them were mentioned in Table 2 (page 22). The antihypertensive effect is further described in the section below.

Hypertension and inhibitory peptides

Hypertension, better known as elevated blood pressure, is a significant health problem affecting over 25 % of the world's population in both industrialised and developing countries[23]. Prevention and treatment of hypertension is efficient in reducing the risk of cardiovascular disease and bioactive peptides have been shown to exhibit antihypertensive effect. While angiotensin I converting enzyme (ACE) is responsible for elevating the blood pressure, ACE-inhibitory peptides have the potential of reducing it.

The blood pressure is regulated by the renin-angiotensin hormone system as shown in Figure 9. When the blood flow rate through the kidneys is lowered, the enzyme renin is secreted by kidney cells[87]. Renin cleaves the precursor peptide called angiotensinogen into the smaller, but still inactive, angiotensin I (Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu). It is referred to Appendix N for translation of the three letter codes into the full names of the amino acids. ACE furthermore cleaves of the dipeptide His-Leu from angiotensin I, yielding the active peptide angiotensin II (Asp-Arg-Val-Try-Ile-His-Pro-Phe). This results in an increase in the blood pressure through vasoconstriction, i.e. through narrowing of the blood vessels. ACE also degrades the vasodilator bradykinin, a peptide that normally enlarges the blood vessels and causes the blood pressure to fall[87].

Several studies have reported ACE-inhibitory effect of hydrolysates derived from enzymatic treatment of numerous protein rich sources. These include milk, soy bean, wheat, egg, meat, human plasma and different sources of marine protein, e.g. seaweed, salmon pectoral fin, cuttlefish and catfish[23, 82]. The main sources of ACE-inhibitory peptides so far has been dairy products and fish[82]. The hydrolysates are considered a non-pharmalogical alternative for the prevention and control of hypertension due to their natural origin and lack of side effects[80]. Synthetic drugs are associated with adverse side effects such as cough, allergic reactions, taste disturbances and skin rashes [86]. Although these synthetic inhibitors are remarkably effective as antihypertensive drugs, an increased focus on identifying safer and natural inhibitors could be necessary for future prevention and treatment of hypertension[87].

The hypertensive effect has mostly been observed *in vitro*, but the hydrolysates may also show an antihypertensive effect *in vivo*[80]. *In vitro* inhibition of ACE and information obtained from simulated gastrointestinal systems are only indicators of the potential of a peptide to act as an antihypertensive agent *in*

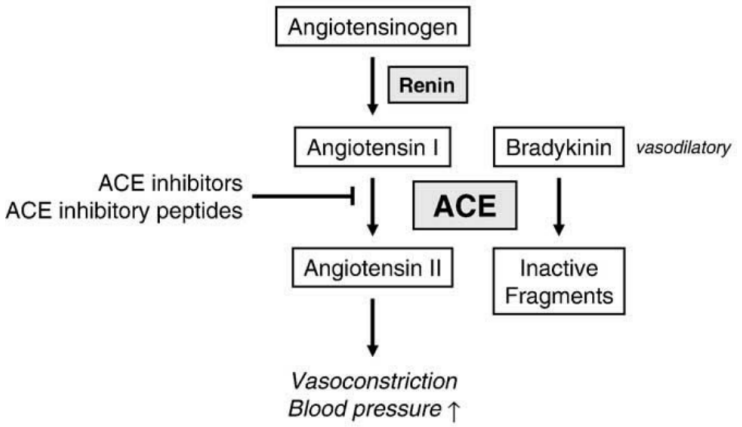

Figure 9: The renin–angiotensin system [82]. Renin converts the inactive precursor angiotensinogen to the inactive angiotensin I. ACE converts angiotensin I to angiotensin II, an active compound that causes elevated blood pressure through vasoconstriction. ACE also degrades the vasodilator bradykinin.

vivo. Due to the often unknown bioavailability of the peptides following oral administration, it is difficult and unreliable to predict the *in vivo* effect based on measured activity *in vitro*[82]. While a number of ACE-inhibitory peptides have shown antihypertensive effects in spontaneously hypertensive rats (SHR) after intravenous or oral administration, there is still lack of information from human clinical studies whether the peptides have an effect or not, and how large the dosage should be to exert an effect[82]. Numerous products that contain potentially antihypertensive peptides are already on the market.

Bioactive peptides in protein sequences of herring

Antihypertensive peptides from fish sources were first identified in sardine meat over twenty years ago, but since then there have been several reports of fish protein hydrolysates containing bioactive peptides with antihypertensive actions[88]. Pampanin et al.[88] found bioactive peptides in herring rest raw material mainly with cardiovascular, antioxidative and immunomodulatory activities. The majority of bioactive peptides were found in the soluble protein fraction and the dipeptide VK (Val-Lys) was the most present. Also Sathivel et al. [76] found

antioxidative activity in hydrolysates from herring. Table 3 gives an overview of peptides found in herring rest raw material for which antioxidative and cardiovascular properties have been reported in the literature, according to Pampanin et al.[88]. Identification was done by combining mass spectrometry with bioinformatics and database search.

Table 3: Peptides with antioxidative and cardiovascular properties found in herring rest raw material. Each letter corresponds to an amino acid for which the translation is given in Appendix N. Freely adapted from Pampanin et al.[88]

Antioxidative properties
AH, DLYA, EL, GPPGPPGPP, GPPGPPGPPG,
GPPGPPGPPGPPG, LQGM, MY, SLYA, QGAR, VW
Cardiovascular properties
AEL, AFL, AIYK, ALEP, ALPM, APL, AVF, FAL, FY, GPL, HHL,
HLP, IAE, IAP, IAPG, IHPF, IKP, IIAEK, IKW, IPP, IPY, IVVE,
IW, KFYG, LGP, LKP, LQGMP, LRP, NIPP, PLPLL, PPK, PSY
VAF, VIKP, VIY, VK, VPP, YN, YNKL, YP, YPK, YQEP, YQY

1.9 Background for analytical methods

1.9.1 Total volatile basic nitrogen (TVB-N)

Before proceeding with further processing of the rest raw material it is important to ensure that the quality is high enough for the desired purpose of the product. Total volatile basic nitrogen (TVB-N) is a common method for estimating the freshness of a fish product, and if exceeding a certain value, the product should be discarded and regarded as unfit for human consumption[89]. The chosen method in this project is the method recommended by the European Union (EU) and included in the Commission Regulation (EC) No 2074/2005 of 5 December 2005[90]. The volatile nitrogenous bases that are present are extracted from the sample using a solution of perchloric acid. After alkalinisation the extract undergoes steam distillation and the volatile base components are absorbed by an acid receiver. The TVB-N concentration is determined by titration of the absorbed bases [90].

1.9.2 Yield

Yield is an important parameter for the industry and dry matter analyses are used to determine the yield of the different fractions. Mass balances are used to control that the total dry matter content of rest raw material is approximately the same before and after hydrolysis. This is done by comparing the dry matter content of the rest raw material before hydrolysis with the sum of the dry matter content of all four fractions obtained after hydrolysis. Some loss is expected during the hydrolysis and separation process.

1.9.3 Oxidation status in crude oil

Oxidation status is used as a parameter for evaluating the quality of the crude herring oil. The oil is susceptible to lipid oxidation due to its long-chain n-3 polyunsaturated fatty acids. Several analytical methods for studying lipid oxidation in oil exist. These methods can be classified into four groups based on what they measure: absorption of oxygen, loss of initial substrate, formation of free radicals and formation of primary and secondary oxidation products[91]. In this project the formation of primary and secondary oxidation products will be analysed. Lipid oxidation leads to the formation of many different oxidation products other than hydroperoxides (PV) and non-volatile aldehydes (AV)[24], but traditionally estimates of PV and AV have been used to calculate the total oxidation status of the oil (TOTOX = 2PV + AV).

No standard quality criteria are set for crude oils. According to the European Food Safety Authority (EFSA) this is difficult to do because there exists no relationship between PV and AV in crude oil compared to refined oil[92]. However the application range of the oil is often determined by the crude oil's quality and several recommended values are published for both crude and refined oils. These values are summerised in Table 4.

Table 4: Quality guidelines for crude and refined fish oils.

Quality guidelines	**Crude oils[93]**	**Refined oils**
Free fatty acids [% oleic acid]	1-7, often 2-5	0,05-0,7[94]
Peroxide value [meq/kg]	3-20	max. 5[95]
Anisidin value [-]	4-60	max. 20[95]
TOTOX (2PV + AV)	10-60	max. 26[95]

1.9.4 Free fatty acids in crude oil

Free fatty acids (FFA) work as strong prooxidants in both bulk and emulsified oils[94]. FFA contain both a hydrophobic hydrocarbon tail and a hydrophilic carboxylic acid head; a combination that allows these molecules to concentrate at the water–oil and headspace-oil interface[96]. At the headspace-oil interface FFA increase the diffusion rate of oxygen into the bulk oil[97], and at the water-oil interface they attract prooxidative transition metals, thereby promoting oxidation[98]. Recommended FFA values for crude and refined fish oil are listed in Table 4.

1.9.5 Degree of hydrolysis

Degree of hydrolysis, %DH, is used to monitor the extent of protein degradation in the hydrolysates and can be defined as:

$$\%\mathrm{DH} = \frac{h}{h_{tot}} \cdot 100 \tag{1}$$

where
h = number of hydrolysed peptide bonds
h_{tot} = total number of peptide bonds present

There is no standard method for determining degree of hydrolysis, but a number of methods have been developed. In this project %DH will be determined with formol titration. An amino group, NH_2, that is not part of a peptide bond

is referred to as a free amino group. Formaldehyde is added to the protein hydrolysate to adjust the equilibrium between protonated and non-protonated free amino groups. An illustration of the equilibrium is shown in Figure 10. Formaldehyde will react with the amino groups, pushing the equilibrium to the right and liberating H^+ ions[99]. By titration with NaOH up to pH = 8,5 the concentration of free amino groups can be found, as the amount of amino groups is directly proportional to the amount of NaOH added. One should be aware of that also the amino group in the side chain of lysine and arginine gets taken into account when using formol titration to determine %DH. Additionally herring is known to contain a number of nitrogen extractives that do not originate from proteins[100], but that contribute to a constant background level in the measurements. The reason for titration up to pH = 8,5 is that the procedure earlier involved an indicator with a colour change around this pH value, but now a pH meter is used instead.

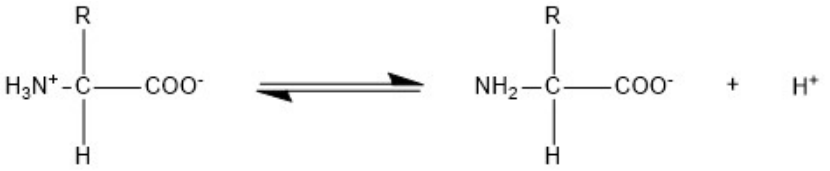

Figure 10: Dissociation of H^+ from the amino group in the amino acid.

1.9.6 Acid soluble peptides

This method, described by Hoyle & Merritt[31], can give an indication of how hydrolysed the hydrolysates are, but the method does not measure %DH directly as it does not determine the number of peptide bonds broken[101]. The amount of protein that is soluble in trichloroacetic acid (TCA) is determined. Most intact protein will be precipitated by TCA and afterwards separated by centrifugation. The supernatant is therefore assumed to consist of only small peptides and free amino acids, even though larger peptides might be present. The average length of TCA soluble peptides has been reported to be 3-4 amino acid residues with 10 % TCA[102] and 2-20 amino acid residues with 12 % TCA[103]. In addition to size, hydrophobicity is also important for the solubility of the peptides[104].

The protein content of the supernatant is determined by the Lowry method[105].

The Biuret reaction, which is important for the colour formation in the Lowry method, is considered to require peptides with >3 amino acid residues for detection[106]. This may lead to an underestimated result when dipeptides and free amino acids are present in the supernatant.

1.9.7 Molecular weight distribution

Fast protein liquid chromatography (FPLC) gel filtration is used to determine the molecular weight distribution of the peptides found in the hydrolysates. Chain length of peptides is of special interest in relation to organoleptic characteristics such as bitterness and colour, as well as functional characteristics such as emulsion capacity and solubility[25].

Size-exclusion chromatography (SEC) separates the peptide molecules according to their hydrodynamic volume. It is assumed that the affinity for the column is the same for all peptides and that the only parameter separating them is size and not charge. The smaller peptides are able to enter the pores of the column media and will spend longer time inside the column than the larger peptides who are excluded and not retained. This results in a continuous range of retention volumes, the larger peptides eluting first. An illustrative chromatogram is shown in Figure 11. Standards with known molecular weights are used to estimate the molecular weight of the peptide fractions detected. The column is adapted to separate peptides within a certain molecular weight range and molecules outside this given range cannot be properly detected unless another column is used.

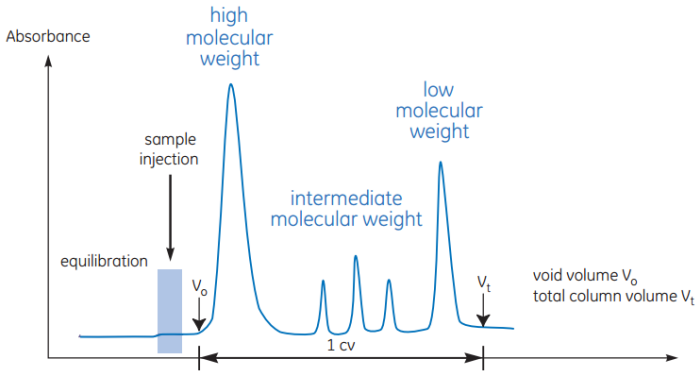

Figure 11: Illustrative chromatogram from gel filtration[107]. The detection of peptides is presented as a function of volume eluted. All molecules too large to enter the pores of the column are eluted together in the void volume, V_0, at the same speed as the buffer flow. cv = column volume.

1.9.8 ACE-inhibitory activity

Angiotensin I converting enzyme (ACE) is a peptidyl-dipeptidase A that removes C-terminal dipeptides from different oligopeptide substrates that have a free C terminus[108]. ACE has been shown to cleave other peptides in addition to angiotensin I and bradykinin from the renin-angiotensin hormone system (Section 1.8 on page 25). One example is the tripeptide o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO_2)-Pro) which will be used in the analysis. Hydrolysis of this substrate generates the fluorescent product o-aminobenzoylglycine (Abz-Gly) which can be easily quantified fluorimetrically using appropiate excitation and emission wavelengths. This will give an indication on how active ACE is when ACE-inhibitory peptides are present. The method, described by Sentendrau and Toldrá[108], avoids extraction with organic solvents, uses commercially available reagents and can process a high number of samples in a short amount of time when a microplate fluorometer is available[108].

1.10 Chauvenet's criterion

Chauvenet's criterion[109] will be used to eliminate values that are suspected to deviate significantly from a mean value of a set of data. The criterion states that if the expected number of measurements, at least as deviant as the suspect value is from the mean value, is less than one half (<0,5), then the suspected measurement should be rejected and a new mean value calculated. Once the criterion has been applied once, it should not be applied again for the same set of data.

For N measurements the mean ($\bar{x}$) and standard deviation (σ_x) is calculated. If one of the measurements (x_{sus}) differs from the mean so much that the value is suspected to be an outlier, then the number of standard deviations (t_{sus}) of which this value differs from the mean is calculated as shown in Equation 2.

$$t_{sus} = \frac{x_{sus} - \bar{x}}{\sigma_x} \tag{2}$$

The number of measurements (n) that is expected to differ from the mean by at least t_{sus} standard deviations is calculated by multiplying the probability for the measurement to be t_{sus} standard deviations away from the mean (prob[outside t_{sus}]) with the total number of measurements (N), as shown in Equation 3.

$$\mathrm{n} = \mathrm{N} \cdot \mathrm{prob[outside}\ t_{sus}] \tag{3}$$

If the expected number is less than one half (<0,5), then, according to Chavenet's criterion, the suspected value is rejected.

1.11 Aim of project

The main aim of this project is to investigate the effect of storage conditions and hydrolysis time on yield, chemical composition and quality of protein hydrolysates and crude oil obtained from enzymatic hydrolysis of rest raw material from NSS herring. The raw material will be divided into nine parts and stored under different storage conditions. Some raw material will be frozen for possible later studies, to see how the freezing and thawing process will affect quality and yield.

The reason for studying storage conditions of raw material is that a potential commercial production might take place in a centralised location where transport might be both long and not very frequent. The rest raw material will be stored at either 4 °C or 10 °C for up to 120 hours. Sampling will be done after 0 h (day 0), 24 h (day 1), 48 h (day 2), 72 h (day 3) and 120 h (day 5) of storage. Storage at 4 ° C simulates the upper limit in the cooling chain, and 10 °C a worst-case-scenario when the temperature has not been correctly controlled, or if due to choice of transport, cooling is not available at all. 120 hours of storage at 10 °C does not represent storage conditions that are likely to happen or to be aimed for. These conditions are more meant to represent an extreme limit when evaluating the consequences of inappropiate storage.

This study, seen as a whole together with my project carried out during fall 2014 and submitted in December 2014, can be divided into five parts.

1) Part one of the project consists of performing enzymatic hydrolysis on rest raw material from herring, separate the fractions and produce crude oil and fish protein hydrolysate (FPH) with the potential of being fit for human consumption.

2) Part two of the project aims at investigating how the chosen storage conditions influence the chemical composition (protein, lipid and ash content) and quality (TVB-N content) of the rest raw material.

3) Part three of this project aims at investigating how the storage conditions influence the final yield of the four fractions obtained after enzymatic hydrolysis. Mass balances are included in this part, and the yield will also be investigated as a function of hydrolysis time.

4) Part four of this project aims at investigating how the storage conditions influence the quality of the oil fraction. The quality of oil will be assessed by determination of oxidation status and by analysing the free fatty acid content.

5) Part five of this project aims at investigating how the storage conditions influence the chemical composition and properties of the fish protein hydrolysate. This will be assessed by determination of protein and lipid content, degree of hydrolysis, acid soluble peptides and molecular weight distribution. The chemical properties will also be investigated as a function of hydrolysis time. The project aims at producing fish protein hydrolysates with bioactive properties and it will therefore be investigated if there are detectable levels of ACE-inhibitory peptides, and if there is a correlation between ACE-inhibitory effect, storage

conditions of raw material and hydrolysis time. Also visual inspection of the hydrolysates in terms of colour and texture will be examined.

2 Material and methods

In this project enzymatic hydrolysis was performed on rest raw material from Norwegian spring-spawning herring, NSSH, delivered by Grøntvedt Pelagic the 23rd of October 2014. The fish was caught outside Trøndelag, Norway and can have been stored up to three or four days before being filleted by Grøntvedt Pelagic. The rest raw material was delivered to the locations of SINTEF Fisheries and Aquaculture only a few hours after filleting. Apart from the fillet, the rest raw material consisted of all parts of the fish, including viscera.

Figure 12 shows the handling of rest raw material prior to hydrolysis. Immediately after receiving the raw material, after 0 hours of storage, two parallels of hydrolysis were performed. The rest of the incoming rest raw material was divided in two parts and either stored at 4 °C or 10 °C. After 24 h, 48 h, 72 h and 120 h of storage four hydrolyses were performed, two for each storage temperature.

2.1 Enzymatic hydrolysis

Figure 13 shows the stepwise procedure of enzymatic hydrolysis and production of hydrolysates. The rest raw material (approx. 2,5 kg) was minced in a Hobart mincer (10 mm holes) to produce a homogenous substrate. A portion of the minced mass was frozen down for later analyses of composition and quality, the rest was prepared for two parallels of hydrolysis (2 x 1000 g). The enzyme solution used for hydrolysis was prepared by dissolving bromelain (0,37 g) and papain (0,37 g) in a minimal amount of water. The final concentration of enzymes in the hydrolysis mixture was 0,1 % w/w, bromelain and papain contributing 0,05 % w/w each.

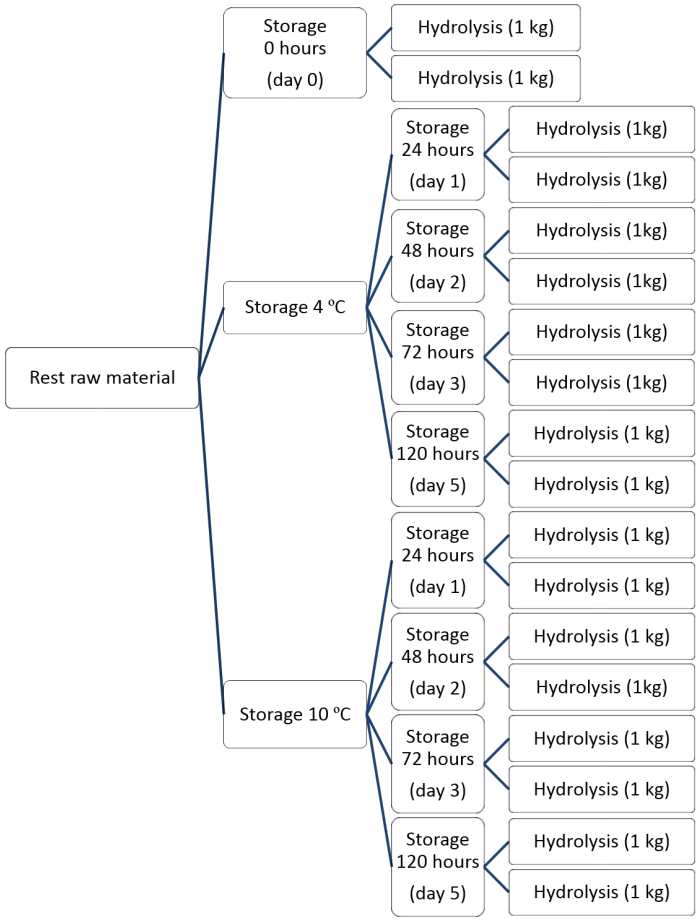

Figure 12: Flow diagram illustrating the handling of rest raw material prior to hydrolysis.

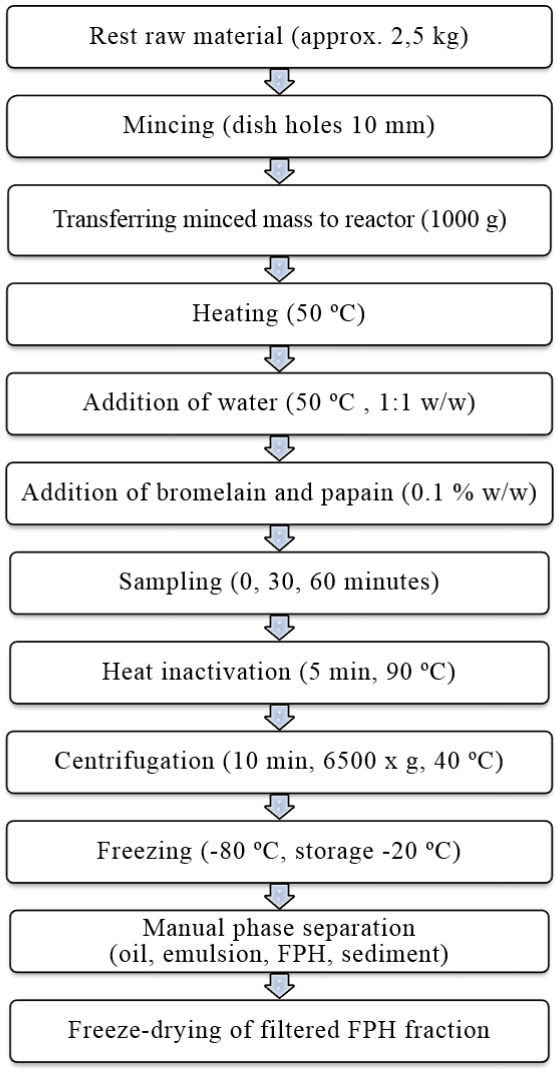

Figure 13: Flow diagram illustrating the steps in production of hydrolysates after storage of raw material at either 4 °C or 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

The minced mass (1000 g) was weighed out and added to a reactor immersed in a waterbath holding 50 °C. The content was stirred at approximately 100 rpm. Water (1000 ml, 50 °C) was added to the reactor, and the final temperature was controlled to be 50 °C before proceeding. A zero sample (500 g) was taken out of the reactor before addition of the prepared enzyme solution. Hydrolysis was assumed to start immediately after addition. Samples were taken out after 30 min (500 g) and 60 min (the rest). All samples were heated and the enzymes inactivated for 5 min at 90 °C using a microwave oven.

Figure 14 shows the experimental set-up for performing enzymatic hydrolysis at the laboratory at SINTEF Fisheries and Aquaculture.

Figure 14: Experimental set-up for performing enzymatic hydrolysis. The 3 L closed batch reactor is placed in a water bath and the content is stirred using an electrical stirrer. The enzyme solution is added through the open tube in the lid and the lid is taken of for transferring rest raw material and water in and out of the reactor.

The inactivated samples were transferred and distributed into 50 ml bottles for centrifugation (6500 x g, 10 min, 40 °C) and subsequent rapid freezing

at -80 °C. The samples were later moved to another freezer for storage at -20 °C. While still sufficiently frozen, manual separation of the samples were conducted with a metal spatula and a knife. The four fractions obtained after separation are shown in Figure 15. The protein (FPH) fraction was thawed and filtered, while the lipid fraction was thawed and centrifuged in order to enhance separation. The weight of leftover water in the oil fraction was added to the weight of the FPH fraction for mass balance purposes. Dry matter content analyses (Section 2.2.1) were performed on the FPH, emulsion and sediment fractions. The oil was assumed to be 100 % dry. The filtered FPH fractions were freeze-dried and stored in exicators at room temperature until further analysed. Figure 16 shows some of the filtered FPH fractions, ready for freeze-drying. The three other fractions were frozen and stored at -20 °C in case of later studies.

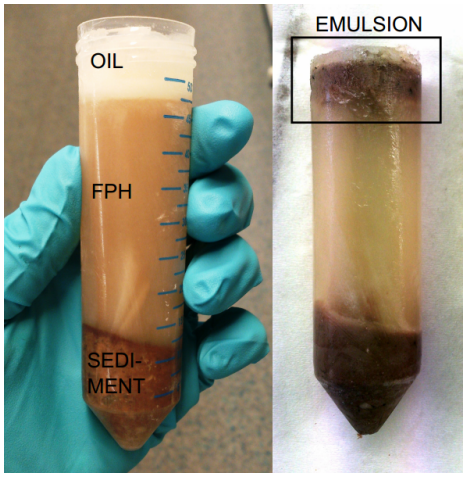

Figure 15: The picture to the left shows a sample without an emulsion fraction. The picture to the right shows a sample with a large emulsion fraction, consisting of two layers, after the oil has been removed.

The hydrolysis process described above was done as part of my project thesis during fall 2014. The centrifuged bottles with hydrolysed raw material from reactor 1 were separated as part of this project. Dry matter analyses of the obtained fractions and some analyses of the freeze-dried hydrolysates were also performed during fall 2014. Centrifuged bottles from reactor 2 were separated during spring 2015 and followed by dry matter analyses, quality assessment of

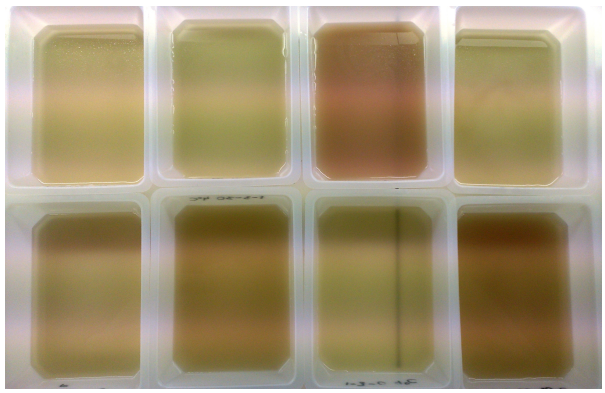

Figure 16: Filtered FPH fractions, ready for freeze-drying. The samples came in a variety of colours.

crude oil and additional analyses of the freeze-dried hydrolysates, this time of hydrolysates from both reactors. It is referred to Appendix A for an overview over what was done when and by who.

2.2 Methods of analysis

2.2.1 Composition of raw material and hydrolysate

The lipid content, protein content and dry matter content was determined for the raw material and the hydrolysates. For the raw material the ash content was also determined.

Protein content

The nitrogen content was determined by using a CHN-S/N elemental analyser 1106 (Costech Instruments ECS CHNSO Analysator). Measurements were conducted in either four or eight parallels. The samples (0,8-2 mg) were weighed out (Mettler UM 3) and packaged in tin cups. The samples were combusted in a stream of oxygen at 900 °C, using helium as a carrier gas. A thermal conductivity detector detected the oxides of nitrogen, after reduction to nitrogen on a hot copper catalyst. Crude protein was estimated by multiplying the nitrogen

content by a conversion factor of 6,25[110]. The instrumental analyses after packaging in tin cups were carried out by Marte Schei.

Protein recovery

Protein recovery was calculated as given in Equation 4.

$$\begin{aligned}
&\text{Protein recovery [\%]} \\
&= \frac{\text{protein in FPH from 100 g raw material}}{\text{protein in 100 g raw material}} \cdot 100\ \% \\
&= \frac{\text{yield of FPH from 100 g raw material} \cdot \text{protein content in FPH}}{\text{protein in 100 g raw material}} \cdot 100\ \% \\
&= \frac{\frac{\text{DW of FPH}}{\text{100 g raw material}} \cdot \frac{\text{PrC in FPH [\%]}}{\text{\%DM in FPH}}}{\text{protein in 100 g raw material}} \cdot 100\ \% \qquad (4)
\end{aligned}$$

where
DW of FPH / 100 g raw material = dry weight fraction of FPH (yield)
PrC in FPH [%] = protein content of FPH
%DM in FPH = dry matter content of FPH

Lipid content

The total lipid content was determined by using two different versions of the Bligh and Dyer method [111], the macro method and the micro method. The macro method was used for analysing the wet rest raw material, the micro method for analysing the freeze - dried hydrolysates. The analyses were carried out by Merete Selnes.

In the macro method the raw material was analysed in duplicates. The samples (10 g) were accurately weighed out in centrifugation bottles and homogenised (Ultra Turrax, 10 000 rpm) for 2 minutes with water (16 ml), chloroform (20 ml) and methanol (40 ml). The homogenisation was performed under a fume hood while keeping the bottles on ice. Chloroform (20 ml) was added to the samples,

and the mixtures were homogenised for 40 seconds. Water (20 ml) was then added and the mixtures homogenised for 40 seconds more. The samples were centrifuged for 15 minutes (Sigma 3K-2, 4100 x g, 4 °C), separating them into three fractions; an aqueous methanol phase, a lipid containing chloroform phase and a bottom fraction. For determination of total lipid content, a part of the chloroform phase (2 ml) was transferred to a pre-weighed test tube using a glass pipette. The chloroform was removed by evaporation at 60 °C under a stream of N_2 for 20 minutes. The samples were left to cool in a desiccator for 1 hour at room temperature before the lipid content was determined by weighing.

In the micro method the hydrolysates were also analysed in duplicates. Samples (10-50 mg) were accurately weighed out in centrifugation tubes and homogenised (Ultra Turrax, 12 000 rpm) for 1 minute with distilled water (0,8 ml), methanol (2 ml) and chloroform (1 ml). The homogenisation was performed under a fume hood while keeping the test tubes on ice. Chloroform (1 ml) was added to the samples, and the mixtures were homogenised for 20 seconds. Water (20 ml) was then added to the samples and the mixtures homogenised for 20 seconds more. The samples were centrifuged for 1 min (Hettich Zentrifugen, Universal 16A, 3500 x g, 4 °C), separating them into three fractions; an aqueous methanol phase, a lipid containing chloroform phase and a bottom fraction. A part of the chloroform phase (0,5 ml) was transferred to a pre-weighed and annealed glass tube. To determine the total lipid content the remaining chloroform was evaporated at room temperature within the fume hood for 14 hours before the samples were weighed.

The total lipid content was calculated using Equation 5.

$$\text{TLC } [\%] = \frac{\text{vaporised fat } [\text{g}] \cdot \text{added chloroform } [\text{ml}]}{\text{vaporised chloroform } [\text{ml}] \cdot \text{weighed sample } [\text{g}]} \cdot 100\ \% \qquad (5)$$

where TLC = total lipid content.

Dry matter content

The dry matter content of raw material, emulsion, wet FPH, sediment and freeze-dried FPH was determined gravimetrically by drying. The dry matter content of the oil fraction was assumed to be 100 %. Samples (1-2 g) were weighed out in pre-weighed glass crucibles and placed in an incubator (Termacks A/S, TS8000) for 24 hours at 105 °C. Samples were then cooled in a

desiccator for 1 hour before the crucibles were weighed again. Measurements were conducted in duplicates for the raw material, emulsion fraction and FPH fraction, and in triplicates for the sediment fraction, the latter due to a larger extent of inhomogeneity.

The dry matter content was calculated using Equation 6.

$$\text{DM } [\%] = \frac{\text{DW of fraction}}{\text{W of fraction}} \cdot 100\ \% \tag{6}$$

where DM = dry matter content, W = wet weight and DW = dry weight.

Ash content

Ash content was determined for the raw material according to the method described by AOAC Official Methods of Analysis[112]. Samples (1-2 g) were weighed out in pre-weighed ceramic crucibles and placed in the incubator for 24 hours at 105 °C. The crucibles were cooled in a desiccator for 1 hour, weighed, charred at 550 °C for 24 hours, cooled in a desiccator for 1 hour and weighed once again.

The ash content was calculated using Equation 7.

$$\text{AC } [\%] = \frac{\text{AW of fraction}}{\text{W of fraction}} \cdot 100\ \% \tag{7}$$

where AC = ash content, W = wet weight and AW = weight after charring.

2.2.2 Total volatile basic nitrogen (TVB-N)

The different procedures for determination of total volatile basic nitrogen were studied. The chosen method is included in the Commission Regulation (EC) No 2074/2005 of 5 December 2005[90], being the recommended procedure for regulation of certain fishery products [89].

The set-up of the apparatus for steam distillation is shown in Figure 17. The sample (10 ± 0,1 g) was mixed with perchloric acid (90 ml, 6 g / 100 ml) and

homogenised with a blender (Ultra Turrax, 2 min) before being filtrated using glass wool. The extract (50 ml) was transferred to a round-bottomed flask (marked C in Figure 17) together with a few drops of phenolphtalein. NaOH (6,5 ml, 20 g / 100 ml) was added right before steam distillation began. To start the distillation a tube (B) connected to the boling water (A) was inserted into the round-bottomed flask (C). To manage 100 ml of destillate in 15 min it was necessary to substitute the boiling water flask (A) with a flask with a much larger ground area than the one seen in Figure 17. Even with this set-up it was not possible to produce enough distillate in 10 min as the manual suggested[90]. It was also necessary to cover the tubes and flasks (B, C and D) with insulation material to prevent the vaporised water from condensing too early. The distillation outflow tube (D) was submerged in a receiver (E) filled with 100 ml boric acid solution (3 g / 100 ml) and a few drops of indicator solution (2 g methyl-red and 1 g methylene-blue in 1000 ml of 95 % ethanol). The distillation was ended after 15 min and the amount of volatile bases collected in the receiver (E) was determined by titration with standard hydrochloric acid solution (0,05 M). A solution consisting of perchloric acid (50 ml) served as a blank.

The TVB-N value was calculated according to Equation 8.

$$\text{TVB - N [mg / 100 g sample]} = \frac{(V_1 - V_0) \times 14 \times 2 \times 0,05 \times 100}{m} \qquad (8)$$

where
V_1 = volume of 0,05 M hydrochloric acid solution in sample [ml]
V_0 = volume of 0,05 M hydrochloric acid solution in blank [ml]
m = weight of sample [g]

2.2.3 Yield

Yield, expressed as dry weight of fraction per 100 g raw material, were calculated using data from the dry matter content analyses and the mass of fraction obtained after hydrolysis and separation. It should be noted that the (wet) weight of fraction was divided by the total weight of all four fractions (<500 g, due to some loss during separation), and not the original mass taken out of the reactor after end of hydrolysis (500 g). The dry weight was then calculated

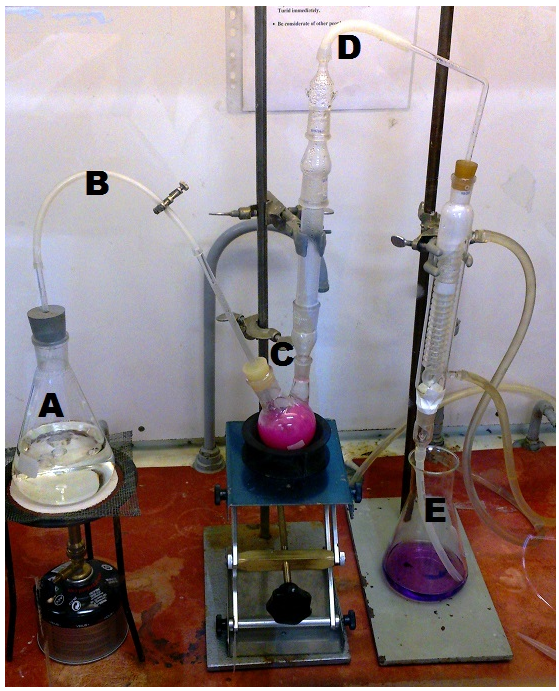

Figure 17: Early set-up during the trials. The set-up was changed many times before it was possible to run the experiment. The final set-up has not been photographed, but consisted of a larger flask of boiling water (A) and insulated material to cover the tubes (B and D) and the round-bottomed flask (C).

using Equation 9.

$$\frac{\text{DW of fraction}}{\text{100 g raw material}} = \%\text{DM} \cdot \frac{\text{W of fraction}}{\text{100 g raw material}} \tag{9}$$

where
W = (wet) weight
DW = dry weight
%DM = dry matter content

2.2.4 Mass balances

Mass balances were performed using the calculated dry weight, DW / 100 g raw material. The dry weight of all four fractions together should be more or less equal to the dry weight of the rest raw material. The dry weights were therefore balanced as shown in Equation 10. Some loss must be expected during the process of distribution of samples in centrifugation bottles and when separating the fractions.

$$\frac{\text{DW of raw material}}{\text{100 g raw material}} = \frac{\text{DW of oil}}{\text{100 g raw material}} + \frac{\text{DW of em}}{\text{100 g raw material}} + \frac{\text{DW of FPH}}{\text{100 g raw material}} + \frac{\text{DW of sed}}{\text{100 g raw material}} \tag{10}$$

where DW = dry weight, em = emulsion and sed = sediment.

2.2.5 Oxidation status in crude oil

The oxidation status of the oil fraction was determined by measuring peroxide values (PV) and anisidin values (AV). In order to avoid further oxidation after heating to 50 °C, all samples were kept at -80 °C until analysed. Prior to analysis the oil was thawed at room temperature for about 30 min and centrifuged (6500 x g, 10 min, 40 °C) to remove impurities. A few samples were kept overnight in the fridge before being analysed.

PV value

The amount of lipid hydroperoxides was determined by the iodometric titration method described in AOCS Official method Cd 8b-90[113] using glacial acetic acid/iso-octane (3:2 v/v) as lipid solvent. The titration was performed using a TitraLab980 automatic titrator coupled with a single platinum electrode (M21Pt) and a reference electrode (REF 921) (all equipment from Radiometer Analytical ASA, Copenhagen, Denmark) according to the titration protocol[114]. The oil samples (2 g) were mixed with lipid solvent (25 ml) before a saturated solution of potassium iodide (0,25 ml) was added and the

content stirred on a magnet stirrer (250 rpm, 1 min). Distilled water (15 ml) was then added and the PV was determined by titration with 0,01 M standardised $Na_2S_2O_3$. Potentiometric determination of the titration end point was employed. The analyses were performed in triplicates and the results expressed in meq peroxides/kg oil.

AV value

The AV was determined according to AOCS Official Method Cd 18-90[115]. Oil (0,1 g) was dissolved in iso-octane (6 ml). The absorbance (A1) of samples (2,5 ml) was measured in glass cuvettes at 350 nm against a pure iso-octane blank. p-anisidine reagent (0,5 ml) was added to the cuvettes and the cuvettes were carefully mixed. The samples were placed in the dark for 10 minutes before a new spectrophotometric measurement (A2) was made. The analyses were performed in duplicates and each duplicate was measured twice, giving four cuvettes and one blank for each run.

AV was calculated according to Equation 11.

$$\text{AV [-]} = 6 \cdot \frac{1,2[(A_{S2} - A_{B2}) - (A_{S1} - A_{B1})]}{m} \tag{11}$$

where
6 = volume of iso-octane used to dissolve the sample [ml]
1,2 = correction factor for dilution of sample solution with 0,5 ml of anisidine reagent dissolved in acetic acid
AS1 and AS2 = first and second spectrophotometric measurement of samples
AB1 and AB2 = first and second spectrophotometric measurement of blanks
m = weight of sample [g]

2.2.6 Free fatty acids in crude oil

The amount of free fatty acids (%FFA) in the oil fraction was analysed according to the procedure proposed by Bernardez et al.[116]. Cyclohexane was substituted with iso-octane as the lipid solvent. Oil (0,1-0,2 g) was dissolved in iso-octane (5 ml) and mixed with a solution of 5 % cupric acetate-pyridine aqueous reagent (0,5 ml). The mixture was vortexed (30 s) and centrifuged (2,000 x g, 5 min, 20 °C). The upper layer was measured by spectrophotometry

at 715 nm against iso-octane in glass cuvettes. A standard curve prepared with oleic acid (0-20 μmol) was used to calculate %FFA. The samples were analysed in triplicates and the results were expressed as weight percentage of FFA on an oleic acid basis, i.e. % of oleic acid.

2.2.7 Degree of hydrolysis

Degree of hydrolysis, %DH, was used to monitor the extent of protein degradation in the hydrolysates. The amount of free amino groups was determined using formol titration [117]. This, together with the nitrogen content of the hydrolysates obtained as described in Section 2.2.1 on page 40, was used as a base for calculating degree of hydrolysis. A solution was prepared by dissolving hydrolysate (0,25 g) in distilled water (50 g). 0,1 M NaOH was added until pH 7,0 was reached. The solution was left to stand for 5 minutes after addition of formaldehyde (10 ml). Lastly, titration with 0,1 M NaOH was performed until a pH of 8,5 was reached.

The amount of free amino groups in the hydrolysate was calculated according to Equation 12.

$$\% \text{ free amino groups} = \frac{A \times B \times 14,007 \times 100\%}{C \times 1000} \tag{12}$$

where
A = NaOH used [ml]
B = concentration of NaOH [M]
C = weight of sample [g]
14,007 = molar mass of nitrogen [g/mol]

Degree of hydrolysis could then be calculated according to Equation 13.

$$\% \text{ DH} = \frac{D \times 100}{E} \tag{13}$$

where
D= % free amino groups
E = % nitrogen

2.2.8 Acid soluble peptides

The amount of acid soluble peptides was determined after the method described by Rohm et al.[118], except for that 5 % TCA was used for precipitation instead of 12 % TCA. The hydrolysates (0,1 g) were dissolved in doubly distilled water (10 ml). TCA 10 % (2 ml) was added to a small part of the solution (2 ml) to a final concentration of 5 % TCA. The solutions were mixed well on a whirlimixer before left in ambient temperature for at least 30 min. The samples were filtered by use of filter paper (Whatman®qualitative filter paper, grade 1) placed in funnels. The filtered solutions, containing acid soluble peptides, were diluted with 5 % TCA in a ratio of 1:20 before the Lowry method[105] was applied to determine the amount of acid soluble peptides. The analyses were done in triplicates.

A stock solution (1000 μg / ml) of bovine serume albumin (BSA) was utilised as a standard when running the Lowry method. The stock solution was diluted to concentrations of 12,5, 25, 50, 100, 150, 200 and 300 μg / ml. The alkaline copper reagent was prepared by adding 1 ml of 1 % $CuSO_4$ and 1 ml of 2 % potassium sodium tartrate to 100 ml of 2 % Na_2CO_2 in 0,1 M NaOH. The Folin-Ciocalteu reagent was prepared by adding one part Folin-Ciocalteu to two parts of doubly distilled water.

The standard solutions and hydrolysate extracts (0,5 ml) were transferred to test tubes. Alkaline copper reagent was added (2,5 ml) and the tubes instantly mixed vigorously on a whirlimixer. After 10 min Folin-Ciocalteu reagent (0,25 ml) was added and the tubes again mixed before they were left for 30 min in room temperature. The absorbance at 750 nm was read in a spectrophotometer (Ultrospec 2000, UV/Visible spectrophotometer, Pharmacia Biotech). A blank sample served as a reference. A standard curve was plotted and used to determine the percentage of acid soluble peptides with respect to original amount of dry hydrolysate (0,1 g).

2.2.9 Molecular weight distribution

The molecular weight distribution of peptide fractions in hydrolysate was investigated using size-exclusion chromatography (SEC) on a fast protein liquid chromaography (FPLC) system. The column (Superdex TM Peptide 10/300 gL) can separate peptides in a molecular weight range of 100-7000 Da. Samples were prepared by dissolving hydrolysate (25 mg) in sodium acetate buffer (0,05 M,

pH 5,0, 1 ml). The samples were mixed vigorously with a whirlimixer and then centrifuged (Eppendorf, Centrifuge 54115 R, 10 min, 9500 x g, 20 °C). The resulting supernatant (0,1 ml) was injected into the septum and onto the column with a syringe. Detection was done at 280 nm with a flow rate of 0,5 ml/min. The column had a total volume (V_T) of 24 ml and a void volume (V_0) of 8 ml. Three standards were prepared in a similar manner to establish a relationship between retention volume (V_R) and molecular weight (M_W). The relationship is given in Equation 14. The standards and their corresponding molecular weights are given in Table 5.

$$log(M_W) = a \cdot K_{\text{av}} + b \tag{14}$$

where a is the slope and b the intercept with the y - axis from the linear regression of the standard curve. K_{av} is called the available coefficient and is related to V_R by the formula given in Equation 15.

$$K_{\text{av}} = \frac{V_R - V_0}{V_T - V_0} \tag{15}$$

where V_0 = void volume, V_R = retention volume and V_T = total volume.

Table 5: Standards used to establish a relationship between retention volume, V_R, and molecular weight, M_W.

Standard	**M_W [Da]**
Aprotinin	6511
Vitamin B12	1355
Val-Glu-Ser-Ser-Lys	549

2.2.10 ACE-inhibitory activity

The hydrolysates (20 mg) were dissolved in distilled water (1 ml) and centrifuged (Eppendorf, Centrifuge 54115 R, 10 min, 20 °C, 9300 x g). The supernatants (50 μl) were transferred to the wells of a microtiter plate. Distilled water was added to an empty well to serve as a blank. A solution of ACE (50 μl) was added to the

samples and the microtiter plate was shaken automatically (10 s) and incubated (10 min, 37 °C) in the fluorometer (Tecan Infinite M200 Pro). After incubation the reaction was started by addition of substrate solution (Abz-Gly-Phe(NO_2)-Pro, 200 μl). The microtiter plate was shaken (10 s) and sampling was done every 30 s for a period of 30 min (λ(excitation) = 366 nm, λ(emission) = 420 nm). The analyses were performed in duplicates.

2.2.11 Bioactive peptides in protein sequences of herring

The database of National Center for Biotechnology Information (NCBI)[119] was used to search for sequences of the muscle proteins myosin and actin from herring (*Clupea harengus*) and zebrafish (*Danio rerio*). Myosin and actin were chosen due to being the most extensively studied proteins that are present in the raw material in significant amounts. Even though the raw material in this project did not consist of the fillet, there will always be some muscle tissue attached to the backbones and trimmings.

ClustalW2[120], a multiple sequence alignment program, was used to check for homogeneity in the sequenced parts of the myosin heavy chain of herring and zebrafish.

2.3 Uncertainty analyses

Microsoft Excel was employed for data processing and statistical analysis. Standard deviation was used to calculate the uncertainty in the results.

3 Results and discussion

Table 6 lists five randomly selected samples and an explanation to their sample ID. The first number in the sample ID indicates the temperature the raw material was stored at, the second number which reactor was used for hydrolysis, the third number for how many days (hours) the raw material was stored and the fourth number for how many minutes the raw material was hydrolysed. The raw material from day 0 was not stored, and therefore no storage temperature is available.

Table 6: Five randomly selected samples and an explanation to their sample ID.

Sample ID	**Temperature** [°C]	**Reactor** [-]	**Storage time** [days (hours)]	**Hydrolysis time** [min]
1-0-0	-	1	0 (0)	0
1-0-30	-	1	0 (0)	30
4-1-1-60	4	1	1 (24)	60
10-1-1-60	10	1	1 (24)	60
10-2-1-60	10	2	1 (24)	60

The enzymatic hydrolysis was performed for both reactors during fall 2014. After hydrolysis the mixtures were distributed into centrifugation bottles, centrifuged and the bottles frozen down. The bottles from reactor 1 were separated during fall 2014 and the bottles from reactor 2 during spring 2015.

3.1 Chemical composition of raw material

The protein, lipid and ash content was determined for the rest raw material. These results were earlier presented as part of my project thesis that was carried out during fall 2014. The results are now presented again as part of my master thesis because they are essential for a complete perspective on the studies.

The protein content of the raw material is compared with the amount of protein that is retrieved in the protein hydrolysates. The protein content gives an indication of how much protein can ideally end up in the FPH fraction, if all

proteins were soluble and no emulsion fraction occurred. The lipid content of the raw material is compared with the amount of lipids that is retrieved in the oil fraction after hydrolysis. The aim is to maximise the lipid content of the oil fraction while minimising the lipid content in the three other fractions. The ash content is a measure of inorganic matter, indicating how much bones, scales and other parts of the fish with a high mineral content that is present in the rest raw material. Ideally the sediment fraction would only consist of bones and some parts of the raw material that has not been thoroughly solubilised. A complete separation and solubilisation is hard to achieve and therefore the sediment fraction also contains lipids and insoluble proteins. Some lipids and proteins are also retrieved in the emulsion fraction and the aim is to minimise both the sediment and the emulsion fraction.

The chemical composition of fresh raw material (0 hours of storage) is given in Table 7, while Figure 18 shows the changes in chemical composition throughout the storage period. As expected, neither the values for lipid nor ash content changed significantly. The protein content was only measured for the fresh raw material.

Table 7: The protein, lipid and ash content of fresh rest raw material from NSS herring (0 hours of storage).

	Protein [%]	**Lipid** [%]	**Ash** [%]
raw material, 0 h	14,19 ± 0,85	16,20 ± 0,20	3,46 ± 0,19

Previous pilot scale studies done by Carvajal[24] in 2010-2012 found the chemical composition of rest raw material from NSS herring to be in the range 13-16 % protein, 13,5-24 % lipids and 2,8-4,0 % ash. 98,5-99,5 % of the lipids were triglycerides (TG), and only traces of phospholipids (PL) and cholesterol (CHL) were found. More specifically, herring harvested late in October 2012 consisted of around 13 % protein and 22 % lipid. The NSS herring that was used in this project was harvested 23rd of October 2014. While having a similar protein content (14 %), the lipid content (16 %) was significantly lower than what was reported by Carvajal, remembering that the composition of NSS herring is influnced by both seasonal and annual variations (Section 1.4 on page 6). Both the analyses in this project and the studies done by Carvajal[24] were conducted at the laboratories of SINTEF Fisheries and Aquaculture, using more or less the same procedures, equipment and personnel.

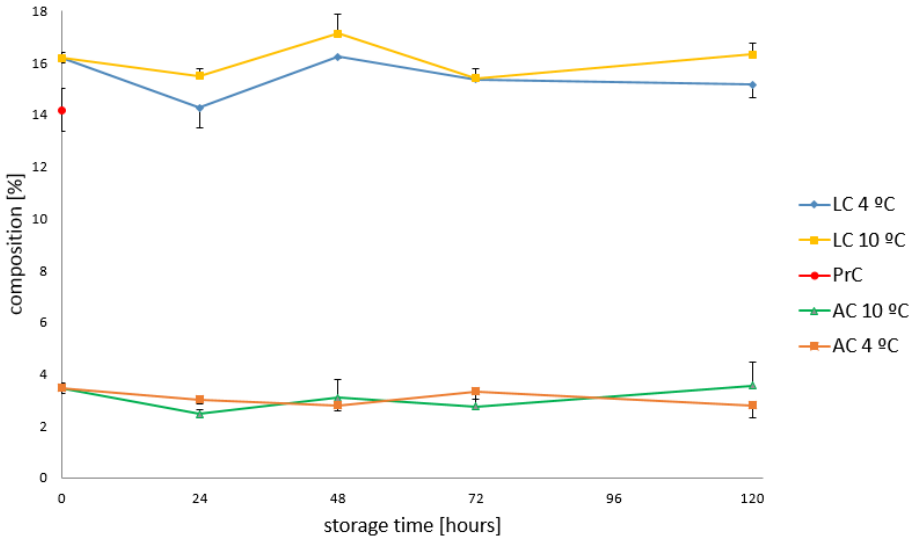

Figure 18: Protein, lipid and ash content in rest raw material from NSS herring as a function of storage time. The protein content was only measured for fresh raw material (0 hours of storage). PrC = protein content, LC = lipid content, AC = ash content.

3.2 Total volatile basic nitrogen (TVB-N)

The chosen method for determination of total volatile basic nitrogen (TVB-N) in the raw material is the recommended procedure for regulation of certain fishery products by the European Union (EU)[89]. The equipment was set up and the procedure tried out several times with small changes for every trial. The method was optimised until it was possible to produce 100 ml of distillate in 15 min. It was not possible to produce 100 ml of distillate in 10 min as the manual[90] suggested. The obtained results were comparable to earlier reported values (25-35 mg nitrogen/100 g of flesh [89]), but the set-up must be improved before the results are reliable. It was therefore decided not to go further with the analyses. It would probably have been easier to perform the experiment, producing enough distillate in 10 min and obtaining more reliable results, if an automatic distillation apparatus had been available.

3.3 Yield

With respect to yield, the desire is to maximise the yield of FPH and oil, while minimising the yield of sediment and emulsion. Yield is expressed as dry weight of fraction per 100 g raw material and all results are given as average values of reactor 1 and 2. It is referred to Appendix B and Appendix C for separate diagrams of reactor 1 and 2. Note that in the yield calculations of sample ID 10-1-2-60, one measured value of the FPH fraction was omitted. The dry matter content found for this single measurement was much larger than its parallel and all other FPH fractions. This high value is thought to originate in solid impurities in the filtrated FPH solution rather than a remarkably high dry matter content. Unfortunately this was not discovered until after the FPH fraction was freeze-dried, and no replica was therefore available.

Yield as a function of hydrolysis time

In Figure 19 the yield of the four fractions is shown as a function of hydrolysis time for fresh raw material (0 hours of storage). As hydrolysis proceeds more material is solubilised. This causes the sediment fraction to decrease and a simultaneous increase in the three other fractions are therefore observed. The changes are most pronounced between 0 and 30 min of hydrolysis.

The yield of FPH fractions after different storage conditions of the raw material is shown in Figure 20 as a function of hydrolysis time. The standard deviation is not shown in the diagrams due to limited space between the series. With dry weight values between 3,44-6,33 g/100 g raw material, the standard deviation did never exceed ± 0,52, the average standard deviation being ± 0,08. It is referred to Appendix B for separate diagram of reactor 1 and 2.

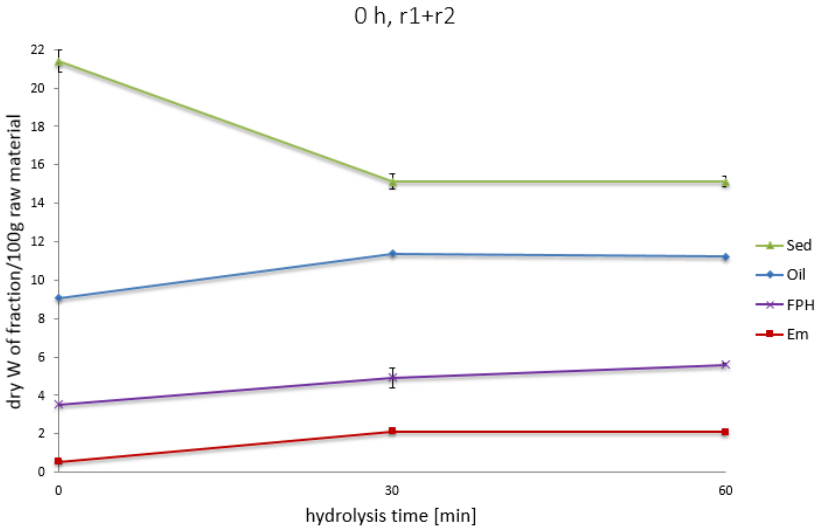

Figure 19: Yield of the four fractions, in dry weight per 100 g raw material, as a function of hydrolysis time. Sampling was done after 0 hours of storage of raw material. The oil is assumed to have a dry matter content of 100 % and no standard deviation is possible to calculate for this fraction. em = emulsion and sed = sediment.

As seen from Figure 20, the yield of FPH increases with increasing hydrolysis time, and it increases most between 0 and 30 min of hydrolysis. There is one exception to the increase in yield as hydrolysis proceeds. The FPH yield for raw material stored at 10 °C for 120 hours, i.e. the most extreme storage conditions used in this project, decreases between 30 and 60 min of hydrolysis. The decrease in yield is significant, but small, going from 5,52 ± 0,01 to 5,38 ± 0,03 g/100 g raw material. However the decrease is only observed for reactor 1, the yield of FPH from reactor 2 remaining constant. A likely explanation for the observed decrease for reactor 1 might be experimental errors due to the fact that the hydrolyis mixture is divided into centrifugation bottles, centrifuged and the frozen content manually separated by hand. This manual separation will always lead to some loss. The decrease could possibly also be due to the so-called plastein reaction, an effect observed with increasing degree of hydrolysis that causes precipitation of insoluble protein complexes to occur[121]. The normal scenario is that the proteolytic enzymes, both endogenous and commercial, aid

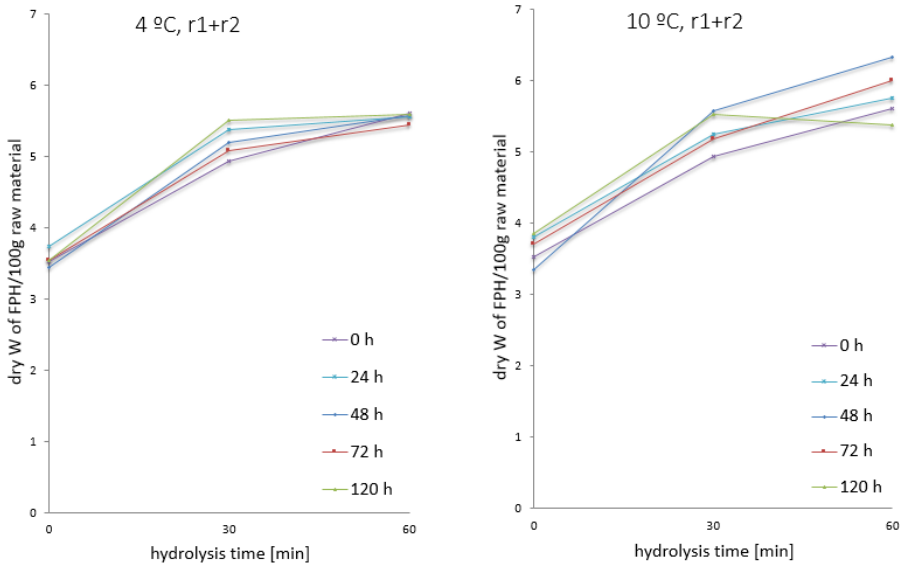

Figure 20: Yield of FPH fraction, in dry weight per 100 g raw material, as a function of hydrolysis time for raw material stored at 4 °C (left) and 10 °C (right). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

in hydrolysis and solubilisation of the proteins. However in the plastein reaction these enzymes catalyse the reverse reaction, i.e. formation of higher molecular weight polypeptides from small and soluble peptides. These polypeptides may to a higher extent be insoluble, leading to a loss of protein in the soluble FPH fraction that instead is retrieved in the sediment fraction.

The yield of oil fractions after different storage conditions of the raw material is shown in Figure 21 as a function of hydrolysis time. It is referred to Appendix B for separate diagrams of reactor 1 and 2. The yield of oil increases with increasing hydrolysis time, as was also the case for FPH. However there is a wider range of values, and the yield change between 30 and 60 min of hydrolysis is also less consistent.

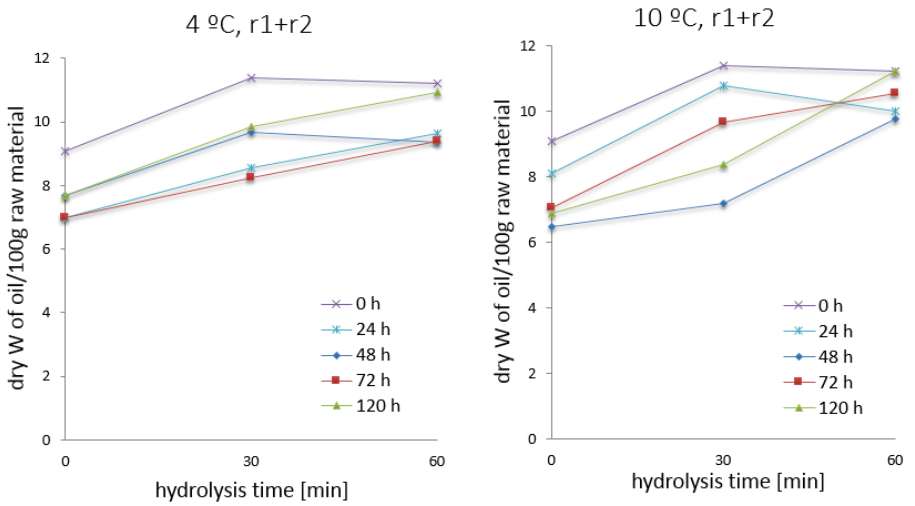

Figure 21: Yield of oil fraction, in dry weight per 100 g raw material, as a function of hydrolysis time for raw material stored at 4 °C (left) and 10 °C (right). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. The oil is assumed to have a dry matter content of 100 % and no standard deviation is possible to calculate for this fraction.

Yield as a function of storage time

The yield of each fraction after 60 min of hydrolysis is presented in Figure 22 as a function of storage time of the raw material. It is referred to Appendix C for separate diagrams of reactor 1 and 2. It should be noted that it was difficult to separate the emulsion fraction from the other fractions. Both the amount and visual appearance of emulsion varied from sample to sample, and no specific correlation was found between emulsion formation and storage conditions of raw material. Some emulsions contained mostly liquid, others mostly sediment. Some emulsions consisted of two layers, liquid on top and sediment below, while some samples hardly contained any emulsion at all. In Figure 15 (page 39) a picture of an emulsion fraction consisting of two layers is shown. All these factors resulted in large variations in emulsion yield, and experimental errors must therefore be expected. The composition of the emulsion fraction was not analysed in this project.

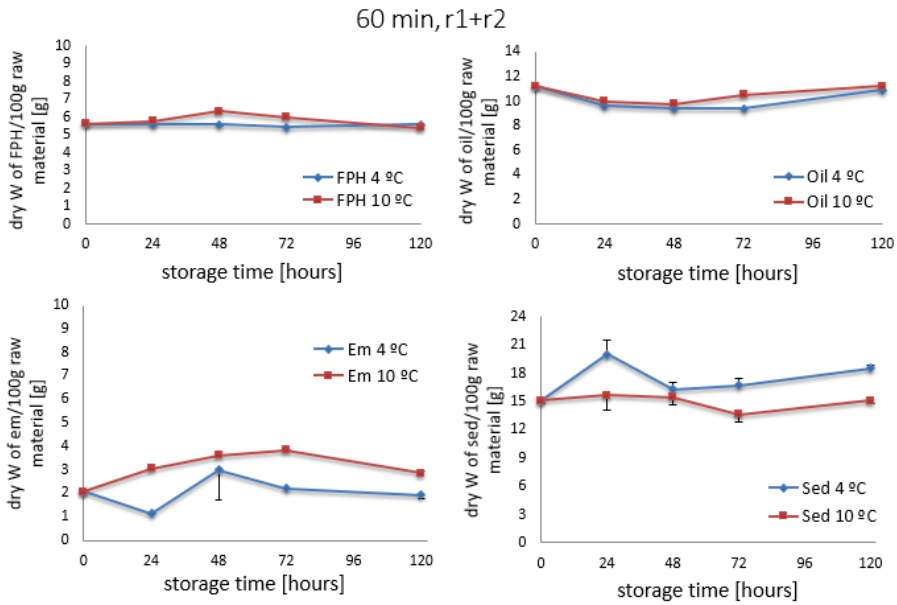

Figure 22: Yield of all four fractions, in dry weight per 100 g raw material, as a function of storage time for raw material stored at 4 °C and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. The oil is assumed to have a dry matter content of 100 % and no standard deviation is possible to calculate for this fraction. em = emulsion and sed = sediment.

Figure 22, upper left corner, shows that the yield of FPH is more or less constant throughout the storage period. The yield is slightly larger for the raw material stored at 10 °C compared to 4 °C, which is thought to origin in the temperature dependence of the endogenous enzymes (Section 1.6 on page 11). The proteases and lipases present in the rest raw material have their optima at different temperatures and pH values, and the pH should ideally have been measured in this project during both storage and hydrolysis. Keheller et al.[22] reported that the pH value of muscle tissue from three different pelagic fish was in the range 6,0-6,5 (Section 1.4 on page 6). The only pH values known in this project are the ones of freeze-dried hydrolysates that have been redissolved in water and prepared for formol titration. These pH values were in the range 6,3-7,1. Both proteolytic and lipolytic activity was detected in previous studies

of herring rest raw material at these pH values (Section 1.6 on page 12). This combined with the temperature dependence of enzymatic reactions described by the Arrhenius equation (Section 1.6 on page 11) can explain the difference in yield when the raw material has been stored at different temperatures.

Figure 22, upper right corner, shows that the yield of oil is first decreasing with storage time before returning to an approximate start value at the end of the storage period. This correlates well with the opposite development in yield of emulsion fraction, as seen in lower left corner in Figure 22. The yield of emulsion is first increasing with storage time, but later it decreases and returns close to start value. Many factors may influence the development of an emulsion fraction. One major factor is that partly degraded proteins have better emulsifying properties than intact proteins [67]. Increased storage time might result in degradation of proteins by endogenous proteases so that the FPH fraction gets increased emulsifying properties and can bind more lipids in the emulsion fraction. It is possible that the amount of protein needed to form an emulsion is so small that a decrease in yield of FPH fraction is not observed. The emulsifying properties of degraded proteins will increase with increasing degradation until a certain critical point is reached. Then the process reverses because of extensive degradation, resulting in loss of emulsifying properties[67]. The endogenous enzymes might, already prior to addition of commercial enzymes, have degraded the proteins so much after 48-72 hours of storage that the emulsifying properties to some degree are lost. This could then explain why the emulsion yield decreases and the oil yield increases at the end of the storage period. Another major factor that might lead to the development of an emulsion fraction is that extensive degradation of fat cell membranes upon storage can cause phospholipids and triglycerides to be released. Endogenous lipases can further degrade the liberated triglyserides into di- and monoglyserides and free fatty acids. These compounds have emulsifying properties (Section 1.6 on page 9) and therefore the change in emulsion fraction could also be caused by a change in the distribution of phospholipids, di- and monoglycerides and free fatty acids[67].

Figure 22, lower right corner, shows that the yield of sediment is changing with storage time. Storage at 10 °C results in more solubilised raw material due to the endogenous enzymes being more active at a higher storage temperature (Section 1.6 on page 12). The increase of sediment yield at an early stage of the storage period might possibly correlate to the simultaneous decrease in oil, being caused by formation of insoluble complexes between lipids and proteins[16]. This should however then be accompanied with a decrease in the yield of FPH, but

this is not observed. To strengthen or discard this theory, analyses of the lipid content in the sediment fraction should be conducted. The increase in sediment yield at the end of the storage periode could origin in the plastein reaction (page 57), but this phenomenon should perhaps also be followed by a decrease in yield of FPH which is not observed.

To sum up the yields of all four fractions were influenced by storage temperature of raw material. Storage at 10 °C resulted in higher yields of oil, FPH and emulsion compared to storage at 4 °C. A simultaneous lower yield of sediment was observed. The oil, emulsion and sediment fractions were more influenced by storage time than the FPH fraction, but in general the storage temperature seemed to have a stronger impact on yield compared to the storage time. To the best of my knowledge there has been little research conducted on how freshness of rest raw material influences the yield of oil and FPH fractions.

3.4 Mass balances

The mass balances were done on the basis of yield expressed as dry weight of fraction per 100 g raw material. The dry weight of raw material should equal the sum of dry weight of the four fractions obtained after hydrolysis. The mass balances are shown in Table 8 and seem to correlate fairly well, since dry weight into the reactor approximately equals the dry weight out. Some loss of weight must be accounted for due to evaporation during hydrolysis, distribution into centrifugation bottles and separation of the fractions. On the contrary, it can be observed that the sum of dry weight of the four fractions often exceeds the dry weight of raw material; an observation that is not possible in practical terms. The raw material contains on average 33 % dry matter. The sum of dry weight of the four fractions should therefore theoretically add up 33 g / 100 g raw material, and not more.

A higher sum is partly a consequence of the calculations being based on the weight of hydrolysate left after separation (<500 g due to some loss) and not the original amount of sample taken out of the reactor (500 g), thus leading to somewhat larger dry weight fractions. Also, the mass balances are based on the calculated %DM and therefore the systematic errors in these experiments should be taken into account as well. For example, as seen from Table 8, the dry weight of fractions obtained for reactor 1 after storage of raw material at 4 °C for 72 hours (0 min of hydrolysis) is estimated to be 43 g / 100 g raw material. This large value origins an uneven distribution of bones in the dry

Table 8: Mass balances for raw material and the sum of all four fractions obtained after 0, 30 and 60 min of hydrolysis. rm = raw material, W = weight and DW = dry weight.

		Reactor 1			Reactor 2		
	Raw material	0 min	30 min	60 min	0 min	30 min	60 min
	[DW of rm / 100 g rm]	[sum DW of all four fractions / 100 g rm]			[sum DW of all four fractions / 100 g rm]		
0 h	33,4	32,7	32,4	34,0	36,3	34,8	34,1
24 h, 4 ºC	33,2	30,6	31,9	36,5	31,8	32,9	36,2
24 h, 10 ºC	31,6	34,1	31,7	34,1	35,4	34,1	34,9
48 h, 4 ºC	35,0	33,9	30,2	34,1	34,7	32,2	34,3
48 h, 10 ºC	33,2	35,4	27,9	33,6	36,5	32,5	36,7
72 h, 4 ºC	32,5	43,0	33,1	33,5	35,2	35,8	33,9
72 h, 10 ºC	31,5	33,5	32,3	34,1	34,4	36,2	33,9
120 h, 4 ºC	32,8	34,2	31,3	38,3	32,7	33,5	35,7
120 h, 10 ºC	33,2	31,7	33,0	33,8	33,5	32,1	35,4

matter analyses of the sediment fraction, resulting in an unlikely high %DM. The suspect value for the mentioned sample was not rejected by the Cauvernet's criterion, and the value was therefore included in the calculations, even though leading to an overestimated result.

3.5 Oxidation status in crude oil

The oxidation status of crude oil extracted from herring rest raw material is expressed as peroxide value (PV), anisidin value (AV) and total oxidation status (TOTOX = 2PV + AV). Crude oil was extracted from raw material after heating to 50 °C (0 min of hydrolysis), i.e. prior to enzymatic hydrolysis. The oxidation status of crude oil extracted from fresh raw material (0 hours of storage) is given in Table 9. It should be noted that the PV is unexpectedly high to be crude oil from fresh raw material. This can have many reasons, one of them being access to oxygen during heating to 50 °C because of the open tubes in the lid of the reactor. However all values in Table 9 are within the recommended limits

(Table 4 on page 28) for crude oil intended for human consumtion. The Global Organization for EPA and DHA omega-3s (GOED) claims that the maximum TOTOX-value for refined fish oil is 26[95]. The TOTOX-value of crude oil in this project is below this limit prior to refining, indicating oil of good quality.

Table 9: The PV, AV and TOTOX value of crude oil extracted from fresh raw material (0 hours of storage) after heating to 50 °C (0 min of hydrolysis).

	PV [meq/kg]	**AV [-]**	**TOTOX value [-]**
crude oil, 0 h, 0 min	11,9 ± 0,2	1,4 ± 0,1	25,2 ± 0,4

Significant differences in PV of crude oil was found between reactor 1 and 2. This could be the result of the different stirring mechanism observed for the two reactors, one of them possibly incorporating more oxygen into the mixture, leading to more severe oxidation. The PV of crude oil is presented in Figure 23 as a function of storage time. The result is not presented as average values between the two reactors to illustrate that even though reactor 2 shows consistently higher values, both reactors follow the same trend throughout the storage period. The PV and AV of crude oil is presented together in Figure 24 as a function of storage time of raw material. The values are averages of reactor 1 and 2. It is referred to Appendix D for separate AV diagrams of reactor 1 and 2.

The development in PV and AV follows the expected trend when the raw material has been stored at 4 °C. Peroxides are formed at an early stage of the storage period so that the PV rises. The peroxides are unstable and break down to secondary oxidation products after a short while, resulting in an increased AV. The same trend cannot be seen when the raw material has been stored at 10 °C, but that the PV varies in an unsystematic manner is not uncommon. It should be emphasised that some formation of peroxides can already have happened prior to hydrolysis upon storage of raw material, as well as during heating to 50 °C in the reactor. Most importantly, all values reported for the crude oil in this project are within the limits for crude oils intended for human consumption, irrespective of the storage conditions of the raw material. No standards exist, but recommended values are PV between 3-20 meq/kg lipid and AV between 4-60 [122]. The PV in this project varied between 11-16 ± 0,7 meq/kg and the AV between 1,1-2,8 ± 0,2.

Aidos et al.[123] reported that crude herring oil produced by thermal treatment

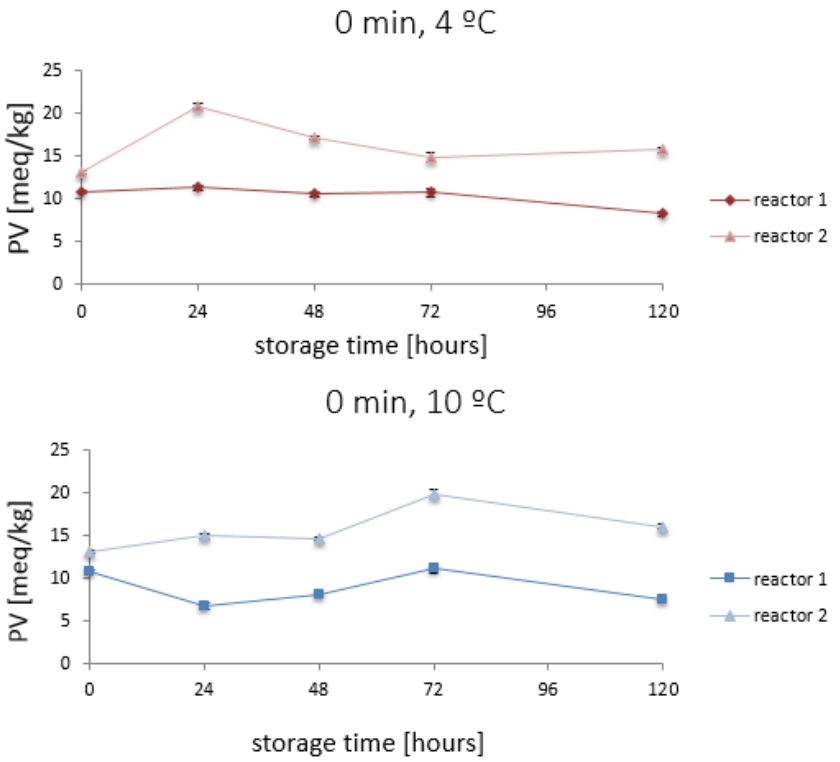

Figure 23: The PV as a function of storage time for crude oil extracted from raw material stored at either 4 °C (top) or 10 °C (bottom) and heated to 50 °C (0 min of hydrolysis). Sampling was done after 0 h, 24 h, 49 h, 72 h and 120 h of storage.

of fresh rest raw material (including viscera) at 95 °C had PV 0,7 ± 0,2 meq/kg lipid and AV 0,4 ± 0,06. These values are lower than the ones obtained in this project (Table 9 on page 64), but should be seen in correlation with that heating to 50 °C in this project involved both stirring and the presence of oxygen. It is possible to combine thermal treatment with enzymatic hydrolysis[124]. Then the raw material is first heated and the oil extracted (without stirring) before enzymes are added and enzymatic hydrolysis performed (with stirring).

Carvajal[24] reported that crude NSS herring oil produced by enzymatic hydrolysis of fresh raw material (including viscera) had PV 3,1 ± 0,3 meq/kg and

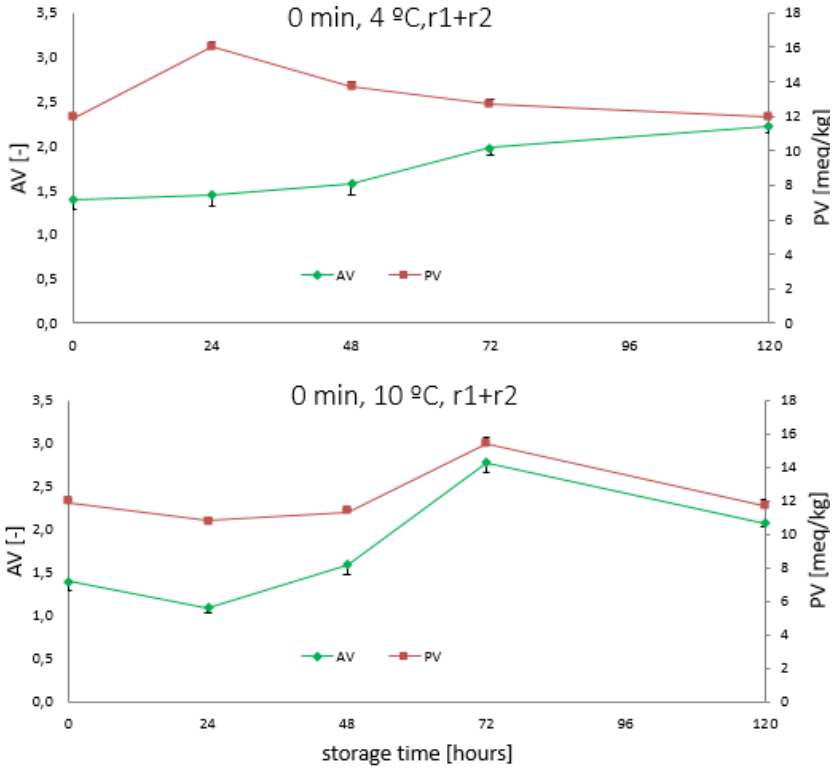

Figure 24: The PV (red) and AV (green) as a function of storage time for crude oil extracted from raw material stored at either 4 °C (top) or 10 °C (bottom) and heated to 50 °C (0 min of hydrolysis). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

AV 1,5 ± 0,05. Lower PV was achieved by adding antioxidants to the raw material prior to heating and hydrolysis[24]. Also these values are lower than the ones obtained in this project, but it should be noted that Carvajal performed the hydrolysis in a pilot plant on-site and not in a laboratory off-site. The idea behind this was that the filleting factory itself would produce crude oil, and not export the rest raw material to a second location for further processing. The use of a pilot plant on-site is expected to result in lower oxidation values because of fresher raw material and better control of the process, in addition to having

a closed system with less access to oxygen. Additionally the oil from the pilot plant was flushed with nitrogen before being frozen down for later analyses of oxidation status. The production of oil in this project was not performed under anoxic conditions and the presence of oxygen in the system during heating might have increased the PV. It is also possible that flushing the reactor with nitrogen first and adding nitrogen continuously to the reactor during heating, would have lead to lower PV as well[124]. Lastly, the chemical composition of herring varies from year to year and between different locations (Section 1.4 on page 6), and this might also influence the oxidation status of crude oil. Carvajal[24] found that the AV increased from 0,7 to 19 after thermal treatment when the raw material had been stored for 72 hours at 10 °C. This increase in AV shows that improper storage of raw material affects the quality of crude oil.

3.6 Free fatty acids in crude oil

Amount of free fatty acids (%FFA) is an important quality parameter for crude oil. The level of FFA in crude oil extracted from raw material heated to 50 °C (0 min of hydrolysis), is presented as a function of storage time of raw material in Figure 25. The values are averages of reactor 1 and 2. It is referred to Appendix E for separate diagrams of reactor 1 and 2.

Figure 25 shows that the %FFA increases with increasing storage time of raw material. Longer storage time leads to an increase in %FFA due to lipid degradation by endogenous lipases[24, 123, 125], the increase being larger when the raw material has been stored at 10 °C compared to 4 °C due to the temperature dependence of these endogenous lipases (Section 1.6 on page 12). 72 hours of storage at 10 °C lead to an increase in %FFA from 0,5 to 2,7 %, while Carvajal[24] reported that the same storage conditions of herring rest raw material (including viscera) resulted in an increase from 0,3 to 3,4 %.

Overall the level of FFA seemed to be more affected by the storage temperature than the parameters relevant for the protein hydrolysates, e.g. protein content and protein recovery (Section 3.7), degree of hydrolysis (Section 3.8), acid soluble peptides (Section 3.9), molecular weight distribution (Section 3.10) and ACE-inhibitory activity (Section 3.11). This supports the findings of Stoknes[47], Søvik[37, 49] and Johansen[48, 53], all concluding with that lipolytic activity is highest in the neutral/alkaline pH range and proteolytic activity highest in the acidic pH range (Section 1.6 on page 12). With an assumed pH value of around

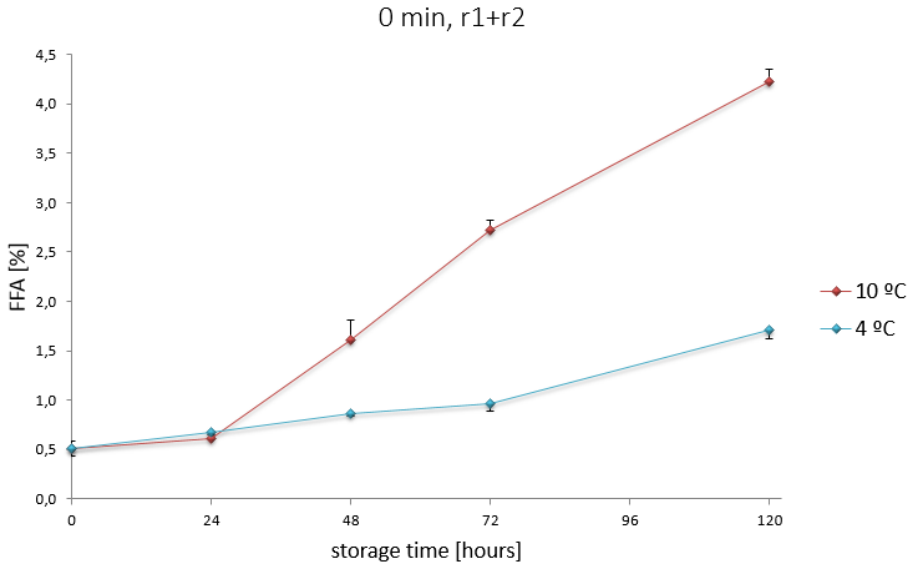

Figure 25: The amount of free fatty acids (%FFA) as a function of storage time for crude oil extracted from raw material stored at either 4 °C or 10 °C and heated to 50 °C (0 min of hydrolysis). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

6-7 in the herring rest raw material used in this project, lipolytic activity is expected to be more significant than proteolytic activity during storage.

The %FFA of crude oil from fresh raw material (0 hours of storage) in this project was found to be 0,51 ± 0,08 %. Aidos et al.[123] reported that the %FFA of crude herring oil produced by thermal treatment (95 °C) from fresh rest raw material (including viscera) was 0,60 ± 0,01 %. Carvajal[24] found that enzymatic hydrolysis of herring rest raw material (including viscera) resulted in higher levels of FFA in crude oil compared to thermal treatment. The %FFA was 0,23 ± 0,03 % after enzymatic hydrolysis with papain and bromelain and 0,15-0,20 ± 0,02 % after thermal treatment, depending on which temperature was used for oil extraction. This difference is probably due to the enzymatic hydrolysis involving stirring, presence of oxygen and happening over a longer time interval, even though using a lower temperature than thermal treatment. Another reason for the lower levels of FFA by thermal treatment could be that

the endogenous enzymes are still active during enzymatic hydrolysis at 50 °C, but inactived by thermal treatment at 95 °C. Søvik [49] reported that heating for 10 min at temperatures above 60 °C was required for inactivation of lipases in rest raw material from cod. Differences in FFA levels can also be caused by quality and freshness of rest raw material. The important point is however that all values obtained in this project are below the recommended limits (5-7 %[93]) for crude oil intended for human consumption, irrespective of the storage conditions of the raw material. Together with acceptable PV and AV, the crude oil shows promising results as being fit for human consumption.

To the best of my knowledge few studies have looked at how freshness of raw material influences the quality of oil. One interesting study was done by Wu and Bechtel[126] on the quality of crude oil extracted by thermal treatment (95 °C, 15 min) from salmon heads and viscera stored at 15 °C for 4 days. The concluding remark was that the oil remained a good source of long chain n-3 fatty acids and had a higher quality than many would anticipate. More specifically it was found that after storage of raw material the antioxidant level in crude oil was still 25 % of initial value, %FFA still below 7 % and no change in the levels of n-3 fatty acids observed. These findings together with the results from this project support the concept of rest raw material from remote locations being transported to a centralised facility for further processing. However, the storage conditions must be considered within certain limits for palatability. As an example, both the raw material stored for 120 hours at 10 °C and the crude oil extracted from this raw material had a very unpleasent smell that would not be appreciated by consumers.

More research has been done on how the quality of oil is affected by storage conditions of the oil itself, not the raw material. Studies done by Aidos et al.[123] looked at the stability of crude herring oil produced from fresh raw material. It was reported that the oil was still of good quality after prolonged storage (<50 °C, <155days), but that the storage temperature had a strong effect on the stability of herring oil in terms of changes in PV and AV. The amount of FFA stayed approximately constant throughout the storage period. The more or less constant FFA value was suggested by Aidos. et al.[123] to be due to inactivation of residual lipases and phospholipases during thermal treatment so that no or little hydrolysis occurred. Presence of lipases and phospholipases is thought to be the major factor for formation of FFA, with microbial activity being regarded as less important (Section 1.6 on page 9). However, as long as the crude oil does not contain water, lipases and phospholipases will not be present, or at least not active.

3.7 Chemical composition of hydrolysate

The protein, lipid and dry matter content was determined for the freeze-dried hydrolysates. Some of the results were earlier presented as part of my project thesis during fall 2014. These results are essential for a complete perspective on the studies and are therefore presented again as part of my master thesis. Furthermore, the results are now shown as average values of reactor 1 and 2.

Dry matter content of hydrolysates

The dry matter content of six freeze-dried hydrolysates is presented in Figure 26. From these results the average dry matter content of each reactor was estimated as shown in Table 10. Hydrolysates from reactor 1 and 2 were dried during fall 2014 and spring 2015, respectively, and the difference in time might explain why the dry matter content differs slightly between the two reactors. It should be kept in mind that the freeze-drying process is not standardised. The frozen FPH fractions (stored at -80 °C) are dried until they look dry rather than being based on achieving a specific dry matter content. The hydrolysates were stored in exicators at room temperature until analysed.

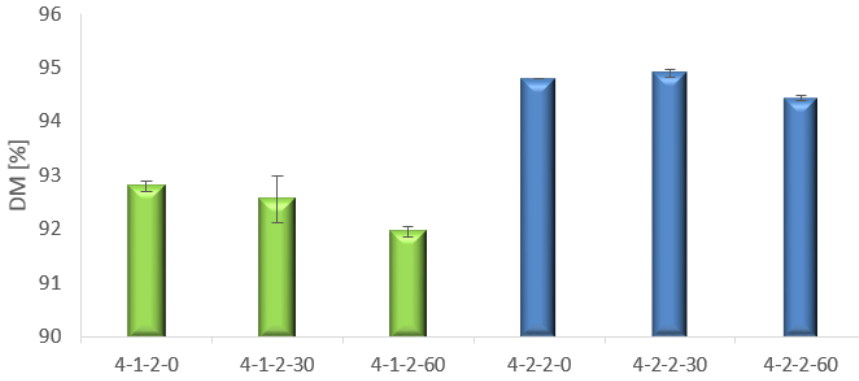

Figure 26: The dry matter content, %DM, of freeze-dried hydrolysates from reactor 1 (green) and reactor 2 (blue). The raw material was stored for 48 hours at 4 °C and sampling was done after 0, 30 and 60 min of hydrolysis.

Table 10: The average dry matter content, %DM, of freeze-dried hydrolysates from reactor 1 and 2.

	DM [%]
Reactor 1	92,4 ± 0,15
Reactor 2	94,7 ± 0,03

Protein content and protein recovery of hydrolysates

Determination of protein content by analysis of total nitrogen (N) multiplied by a specific factor is a common procedure in fish analyses. Traditionally the conversion factor has been 6,25, however this might not be suitable for all protein sources as they vary in amino acid composition[127]. Studies have shown that for rest raw material from fish, a conversion factor of 5,82 might be more correct[128, 129]. Also in fish products there can be a significant nitrogen contribution from other sources than amino acids structures, including e.g. nucleic acids, nucleotides, urea and trimethylamine N-oxide (TMAO), and therefore a conversion factor of 4,94 has also been suggested[130]. The traditional conversion factor 6,25 will be used in all further calculations, unless otherwise is stated.

Hydrolysates from reactor 1 were analysed for protein content during fall 2014, while hydrolysates from reactor 2 were analysed during spring 2015. The protein content of hydrolysates from reactor 2 was unexpectedly high. To see if %DM of the hydrolysates could explain the differences in protein content, data from Figure 26 was used to calculate the protein content of 100 % dry hydrolysates. Using sample ID 4-1-2-60 and 4-2-2-60 as examples (Table 11), the difference in protein content is reduced from 7,61 % to 5,70 % when %DM is accounted for, but the difference is still significant. Selected hydrolysates from both reactors were therefore analysed for protein content a second time to double check the first results. The protein content of hydrolysates from reactor 1 turned out to be the same, but for reactor 2 the values differed significantly. Both new and old values are presented in Figure 27 as a function of storage time of raw material. The average ratio between new and old values was found to be 1,13, and this factor was used to downscale the protein content of hydrolysates from reactor 2 that were not analysed twice (including the hydrolysates after 0 and 30 min of hydrolysis not presented in Figure 27).

Table 11: The dry matter content and protein content of two selected hydrolysates, given both as protein content in hydrolysates as measured and as protein content in 100 % dry hydrolysates. DM = dry matter content and PrC = protein content.

Sample	DM in FPH [%]	PrC in FPH [%]	PrC in 100 % dry FPH [%]
4-1-2-60	91,95 ± 0,10	81,78 ± 0,58	88,9 ± 0,6
4-2-2-60	94,43 ± 0,04	89,39 ± 0,26	94,7 ± 0,3
Difference	2,48	7,61	5,70

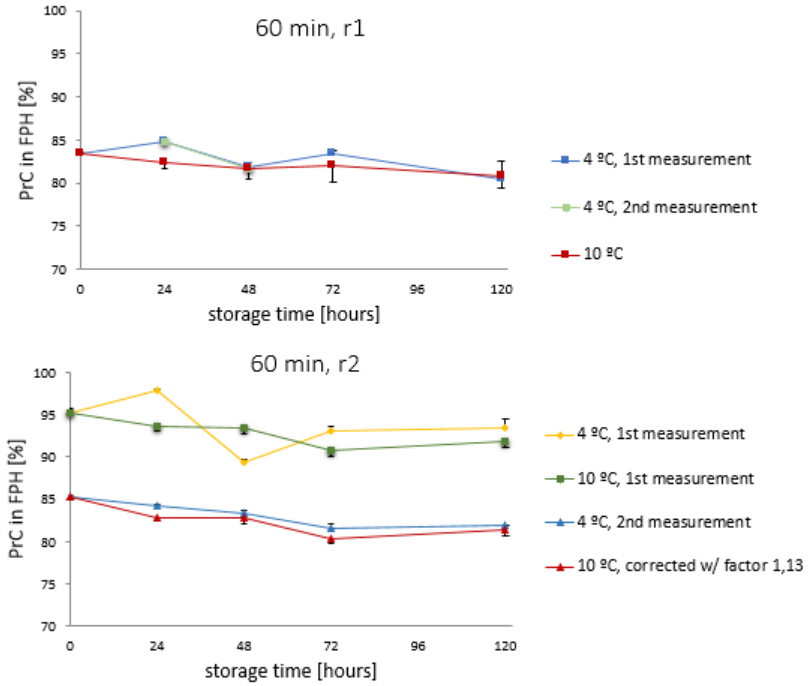

Figure 27: Protein content of hydrolysates from reactor 1 (top) and reactor 2 (bottom) after 60 min of hydrolysis as a function of storage time of raw material. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. PrC = protein content.

In Figure 28 the protein content is presented with two different conversion factors for illustrative purposes. Furthermore the values are averages of reactor 1 and 2 after the protein content of some hydrolysates has been corrected with a factor 1,13. It is referred to Appendix F for separate diagrams of reactor 1 and 2.

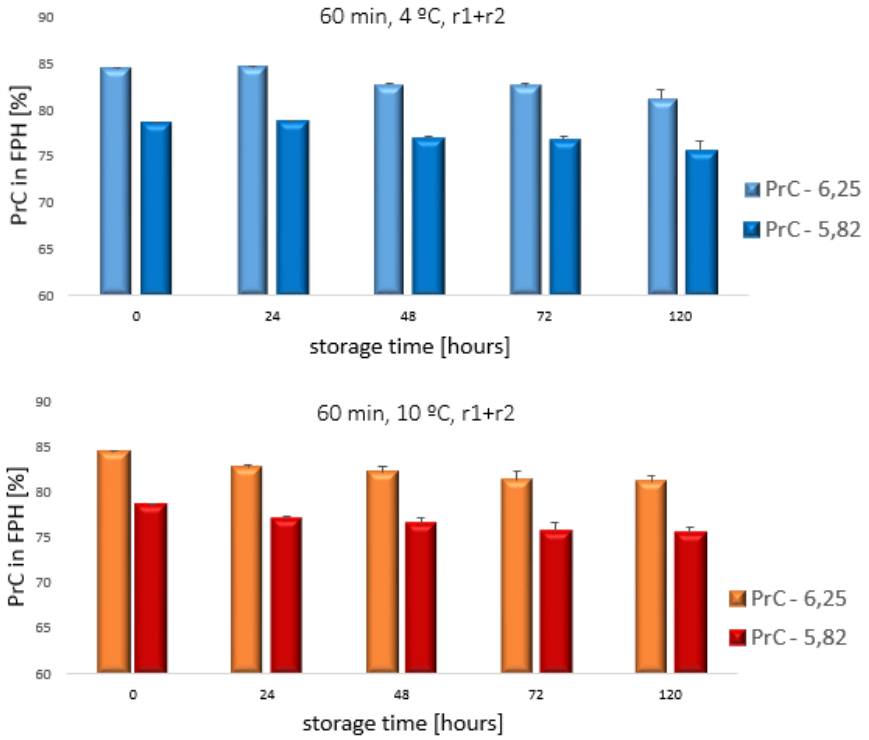

Figure 28: Protein content of hydrolysates after 60 min of hydrolysis as a function of storage time of raw material. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage at either 4 °C (top) and 10 °C (bottom). The protein content is based on two different conversion factors, the traditional value 6,25, and a suggested value for fish, 5,82. PrC = protein content.

The protein content and protein recovery is given as a function of hydrolysis time in Figure 29. The values are presented as averages of reactor 1 and 2. The standard deviation is not shown in Figure 29 due to limited space between

the series in the diagrams. With a protein content in the range 68,45-84,52 % the standard deviation did never exceed ± 1,07, the average standard deviation being ± 0,45. It is referred to Appendix F for separate diagrams of reactor 1 and 2. Figure 29 shows the importance of performing hydrolysis for at least 30 min to increase the protein content and protein recovery of the hydrolysates. It can also be seen that with respect to these two parameters 60 min of hydrolysis might be unnecessary long, as the protein content and protein recovery either stagnates or decreases slightly between 30 and 60 min. This decision will depend on the desired properties of the proteins since properties are related to degree of hydrolysis[27], and degree of hydrolysis is related to hydrolysis time. This is further discussed in Section 3.8 on page 84.

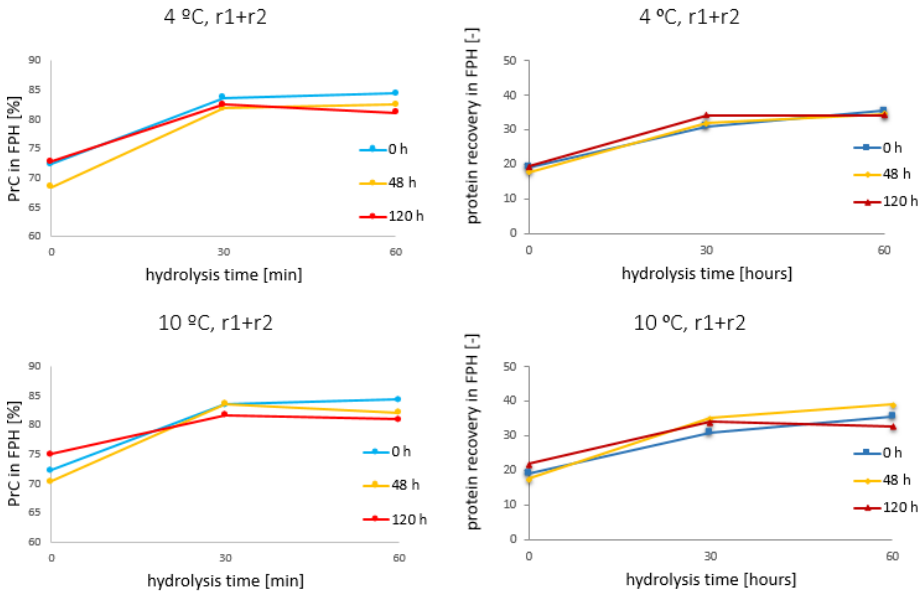

Figure 29: Protein content (left) and protein recovery (right) of hydrolysates as a function of hydrolysis time for storage of raw material at 4 °C (top) and 10 °C (bottom). Sampling was done after 0 h, 48 h and 120 of storage. PrC = protein content.

The protein content and protein recovery of hydrolysates as a function of storage time of raw material is presented in Figure 30. The values are presented as averages of reactor 1 and 2. It is referred to Appendix F for separate diagrams

of reactor 1 and 2. The protein content is declining with increasing storage time, and is higher when the raw material has been stored at 4 °C compared to 10 °C. The lower protein content when the raw material has been stored at a higher temperature and over a longer period of time could be the result of more severe degradation by endogenous enzymes, leading to incorporation of more impurities. Impurities may include bones and skins that have been thoroughly solubilised. Also incorporation of lipids due to better emulsifying capacity will lead to a lowering in the product quality with respect to purity (and stability) of the product. The protein recovery remains constant or decreases slightly as storage time increases, and is at times higher when the raw material has been stored at 10 °C compared to 4 °C. That the protein recovery is higher when the raw material has been stored at 10 °C, without the protein content increasing equivalently, possibly do imply that more impurities are incorporated into the product when the raw material is stored at 10 °C.

The protein content of hydrolysates after 60 min of hydrolysis was in the range 81-85 % and 75-79 % with a conversion factor of 6,25 and 5,82, respectively. Previous studies by Carvajal[24] on enzymatic hydrolysis of rest raw material from NSS herring reported the protein content of hydrolysates to be 79,0 ± 0,5 % with a conversion factor 5,82. The same enzymes were used, namely papain and bromelain. It should be mentioned that for sample ID 2-0-60, 4-1-3-60, 4-1-5-60, 10-1-1-60 and 10-1-3-60, one measurement (out of either four or eight in total) was eliminated from the average value by the method of Chauvenet's criterion (Section 1.10 on page 32). The deviating measurements of sample ID 4-2-2-0, 10-2-2-0 and 10-1-5-60 could not be regarded as outliers and a somewhat larger standard deviation is therefore associated with the protein content of these hydrolysates.

Lipid content of hydrolysates

The lipid content was determined for all hydrolysates from reactor 1 and the result is presented as a function of hydrolysis time in Figure 31. Only the lipid content after 0 and 60 min of hydrolysis is included for a simplified view. It is referred to Appendix G for diagrams where data for 30 min of hydrolysis is included. The standard deviation is not shown in Figure 31 due to limited space between the series in the diagrams. With a lipid content in the range 1,19-8,13 % the standard deviation did never exceed ± 0,45, the average standard deviation being ± 0,11.

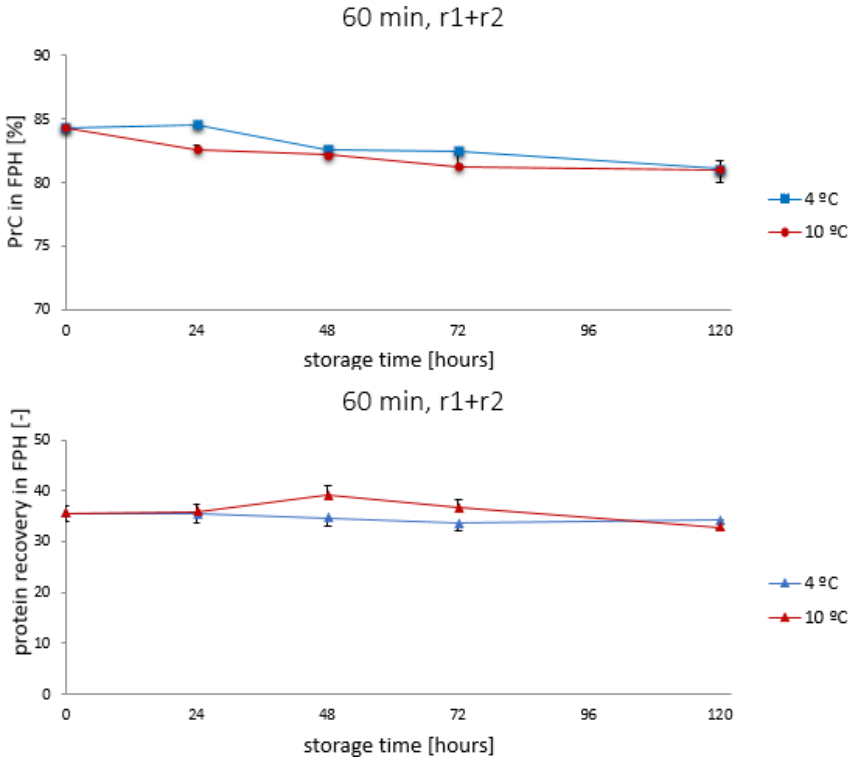

Figure 30: Protein content (top) and protein recovery (bottom) of hydrolysates after 60 min of hydrolysis as a function of storage time of raw material. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage at either 4 °C or 10 °C. PrC = protein content.

There is, with the samples 48 h - 4 °C, 48 h - 10 °C and 120 h - 10 °C as exceptions, a decrease in the oil content from 0 to 60 min of hydrolysis. The significant increase in lipid content for the three exceptions is unexpected and hard to explain. Referring to Figure 33 (page 80) and Figure 34 (page 82) for reactor 1, it could be postulated that there is a relationship between the simultaneous increase in lipid content and degree of hydrolysis after 48 hours of storage of raw material. It is referred to Section 3.8 on page 80 for further discussion. A storage time of 1-3 days for the raw material could be the normal situation during commercial production. If big changes in lipid content of hydrolysates

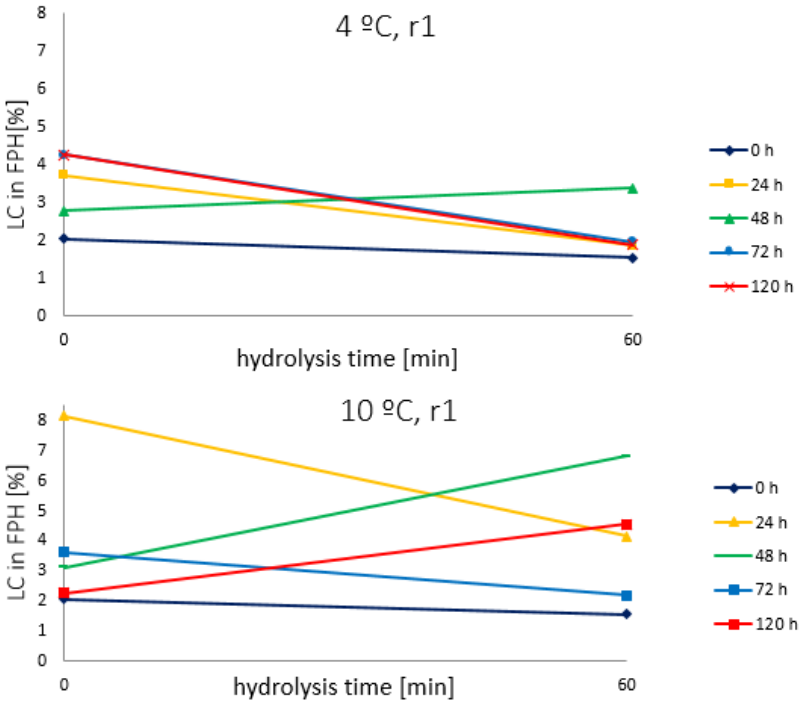

Figure 31: Lipid content of hydrolysates from reactor 1 as a function of hydrolysis time for raw material stored at 4 °C (top) and 10 °C (bottom). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. Only the lipid content after 0 and 60 min of hydrolysis is included for a simplified view. LC = lipid content.

happen in this period, it will be important to look further into this phenomenon. The lipid content of hydrolysates from reactor 2 should be analysed before any conclusion is drawn.

The lipid content of hydrolysates after 60 min of hydrolysis is presented as a function of storage time of raw material in Figure 32. The lipid content varies inconsistently throughout the storage period and no correlation was found between lipid content and storage time of raw material. The lipid content is higher when the raw material has been stored at 10 °C compared to 4 °C. A higher storage temperature can possibly lead to more extensive degradation of fat cells, releasing emulsifiers like diglycerides, monoglycerides and free fatty

acids (Section 3.3 on page 61). Also a more extensive protein degradation can lead to better emulsifying properties by the proteins themselves, thereby having the capacity of binding more oil and increasing the lipid content in the hydrolysates (Section 3.3 on page 61).

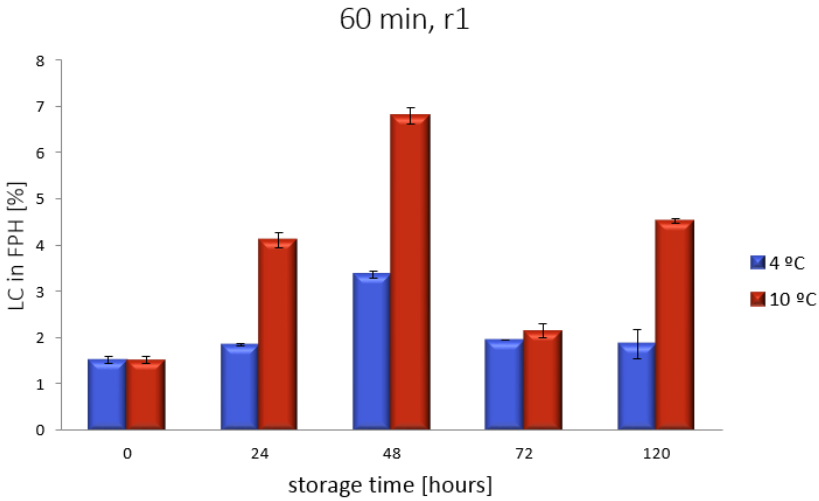

Figure 32: Lipid content of hydrolysates from reactor 1 after 60 min hydrolysis for raw material stored at 4 °C and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. LC = lipid content.

The lipid content of hydrolysates was found to be in the range 1,5-6,8 % after 60 min of hydrolysis. These values are unacceptable high, as it has been suggested that the lipid content in protein hydrolysates should be no more than 0,5 % in order to prevent alterations in the lipids upon storage[69]. A good centrifuge is essential for good separation, and in several centrifugation bottles solid impurities was found in the FPH fraction after centrifugation. Most of the solid impurities were removed during filtration, but some liquid oil remained. In Figure 16 (page 40) it is shown how shiny the filtrated solutions are due to a high lipid content. A better and larger centrifuge should be used in the industry for minimising this problem. Carvajal[24] reported the lipid content of herring hydrolysates to be 1,7 ± 0,6 % [24], i.e. slightly higher than what was found for the hydrolysate from fresh raw material in this project (1,5 ± 0,1 %), but significantly lower than what was found when the raw material was stored at 10 °C

(2,2-6,8 %). It should be noted that Carvajal[24] produced the hydrolysates in a pilot plant, using a more automatic process and a better centrifuge. Because the fat content of NSS herring varies significantly through the season and from year to year, it is also essential to take the total lipid content of the rest raw material into account when comparing values. The herring raw material in this project contained 14-17 % lipids, while the herring raw material used by Carvajal[24] had a lower lipid content (12,3 ± 0,3 %).

Studies on chicken protein hydrolysates (CPH) done by Møen Tveit[25] in the same laboratories of SINTEF Fisheries and Aquaculture as in this project, reported a lipid content of 0,8-1,6 % in the hydrolysates, the value depending on hydrolysis time and type of enzymes used. The treatment with papain and bromelain gave the lowest lipid content. The lipid content (17,5 ± 2 %) of the chicken rest raw material was slightly higher than the lipid content of rest raw material in this project. However it is possible that the lipid is bound differently in chicken and herring, making it easier to achieve a good separation with chicken as the rest raw material. The main lipid depots in herring are found under the skin and in white muscle, with smaller depots found in head, dark muscle, backbone, abdominal epithelium and around entrails[131]. The collagen fibres that stabilise the tissue where the lipids are stored have a significant influence on the tissue's capacity of withstanding mechanical stress, and factors that weakens the collagen fibres may lead to enhanced release of lipid[131]. Muscle cells are surrounded by a more complex network of collagen and are therefore more resistant to mechanical stress than adipose tissue[131]. Most of the lipid in herring is stored in adipose tissue, but a minor part is also stored in muscle cells. To the best of my knowledge, little information exists about the lipid depots in chicken. It is commonly known that the lipid mainly is stored under the skin of chicken, but this should be looked further into to ensure that the values of lipid content in chicken and herring hydrolysates can be directly compared with each other.

3.8 Degree of hydrolysis

Degree of hydrolysis, %DH, in the hydrolysates is presented in Figure 33[2] as a function of hydrolysis time. Values from reactor 1 and 2 have this time not been

[2]Sample ID 10-1-5-0 and 10-2-5-0 were analysed twice due to precipitation on the pH electrode when formaldehyde was added (page 81). The old results are plotted in the diagram, while the new results are given here: 22,1 ± 0,7 % (10-1-5-0) and 24,7 ± 0,1 % (10-2-5-0).

presented as an average because %DH differs notably between the two reactors. The standard deviation is not shown in Figure 33 due to limited space between the series in the diagrams. With %DH in the range 16-35 %, the standard deviation did never exceed ± 0,7, the average standard deviation being ± 0,02. It is referred to Appendix H for diagrams of average values of reactor 1 and 2.

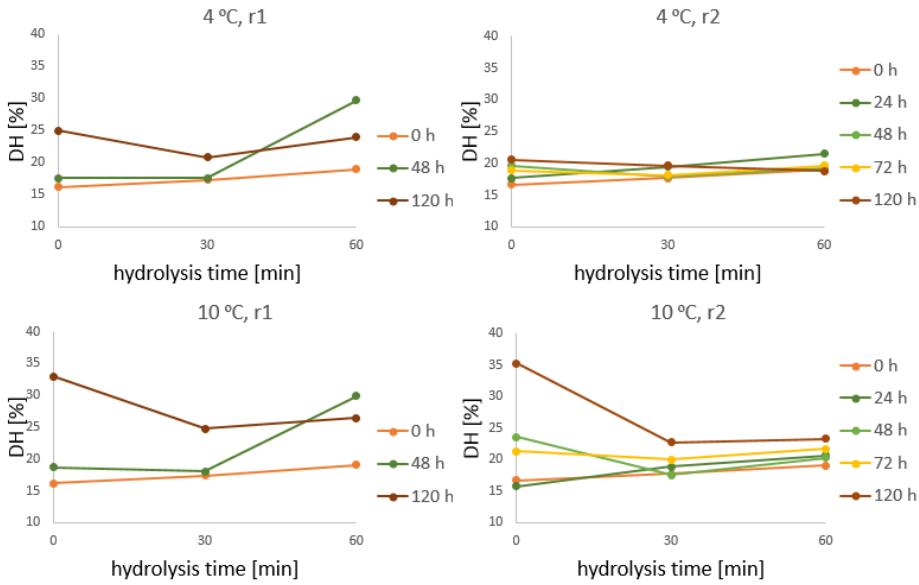

Figure 33: Degree of hydrolysis as a function of hydrolysis time for hydrolysates from reactor 1 (left) and reactor 2 (right). Sampling was done after 0 h, 24 h (only reactor 2), 48 h, 72 h (only reactor 2) and 120 h of storage of raw material at either 4 °C (top) or 10 °C (bottom).

When the raw material has been stored for 48 hours at both 4 °C and 10 °C there is an unexpectedly large increase in %DH in hydrolysates from reactor 1 between 30 and 60 min of hydrolysis. The reason for this is unknown, but there could be a correlation with an increased lipid content in these hydrolysates. This was briefly mentioned in Section 3.7 on page 76 and shown in Figure 31 and Figure 32. The same large increase in %DH is not observed in hydrolysates from reactor 2, and since the lipid content in these hydrolysates was not analysed it is difficult to draw any conclusion regarding this phenomenon.

The degree of hydrolysis will normally increase as hydrolysis proceeds. This

follows from the definition of %DH (Section 1.9.5 on page 28). However as seen in Figure 33, %DH decreases as hydrolysis proceeds for several of the hydrolysates, e.g. for all hydrolysates when the raw material has been stored for 120 hours. This could possibly be explained by the fact that the raw material after 120 hours of storage is so degraded by endogenous enzymes that when commercial enzymes are added, the plastein reaction occurs (Section 3.3 on page 57). For sample ID 10-1-5-0 and 10-2-5-0, the %DH is very high already after 0 min of hydrolysis. However these values may not be reliable due to precipitation on the pH electrode when formaldehyde was added. This may have resulted in incorrect readings of the pH value and therefore also wrong titration volumes and unrealistic large %DH. When the two hydrolysates were analysed again by Vesela Chalova, %DH was lower even though precipitation still occurred. The reason for precipitation occurring is not known, but can possibly explain why the %DH is decreasing with increasing hydrolysis time. Also when raw material has been stored at 4 °C for 120 hours the %DH is remarkably high already after 0 min of hydrolysis, at least for reactor 1. In this case no visible precipitation was formed.

However the most likely explanation to why the %DH is almost constant and/or declining as hydrolysis proceeds for nearly all hydrolysates, may be due to the following. The commercial enzymes papain and bromelain are endopeptidases[12], while the endogenous enzymes are thought to be a mixture of endo- and exopeptidases. The endopeptidases cut proteins within the chain and thereby aid in the solubilisation of proteins that would otherwise end up in the sediment fraction. The exopeptidases cut from the end and will therefore mostly just degrade already solubilised proteins (Section 1.6 on page 10). To calculate %DH the amount of free amino groups is divided by the total number of peptide bonds present in the hydrolysate. The amount of free amino groups is independent of whether the peptide bonds are cut with endo- or exopeptidases. The total number of peptide bonds present is estimated out from total nitrogen content, and since the commercial enzymes solubilise more proteins, the hydrolysates will have a higher nitrogen content when hydrolysis has been performed. Thus both the numerator and denominator of the %DH expression increase, resulting in that the overall expression decreases or remains approximately constant. Figure 29 (page 74) shows how the protein content is increasing with hydrolysis time, leading to a decrease in %DH even though proteins indeed are degraded into smaller fragments as hydrolysis proceeds. Nitrogen extractives present in significant amounts in herring might also influence %DH and make the interpretation more complex (Section 1.9.5 on page 29).

Degree of hydrolysis after 0 and 60 min of hydrolysis as a function of storage time of raw material is presented in Figure 34 (reactor 1) and Figure 35 (reactor 2). 0 min of hydrolysis is included to show the contribution of endogenous enzymes to the degradation of proteins during storage and subsequent heating to 50 °C. Standard deviations are included, but since they are of magnitude ± 0,1-0,5 % they are not visible. It is referred to Appendix H for diagrams of average values of reactor 1 and 2.

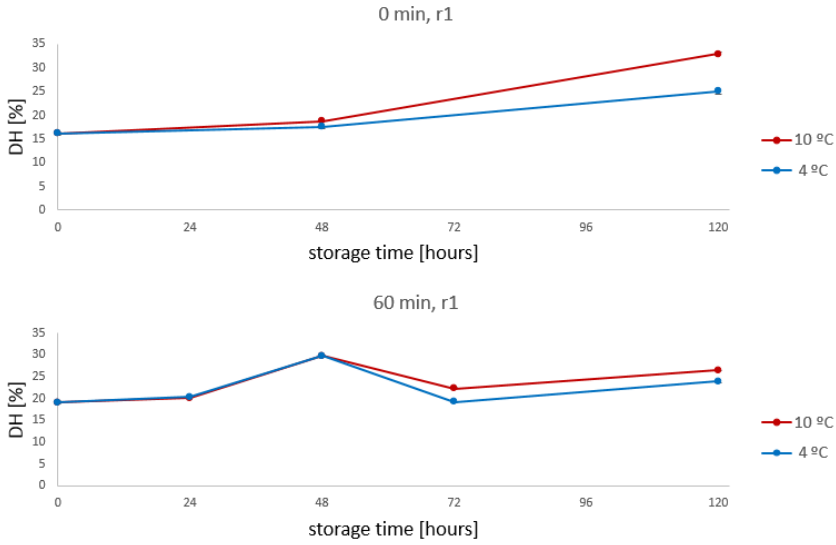

Figure 34: Degree of hydrolysis as a function of storage time of raw material for hydrolysates from reactor 1 after 0 min (top) and 60 min (bottom) of hydrolysis. Sampling was done after 0 h, 24 h (only 60 min), 48 h, 72 h (only 60 min) and 120 h of storage at either 4 °C or 10 °C.

The degree of hydrolysis is expected to increase with increasing storage time due to the endogenous enzymes degrading the raw material prior to addition of commercial enzymes. This increase is shown in Figure 34 and Figure 35 and is present after both 0 and 60 min of hydrolysis. The endogenous enzymes are more active when the raw material is stored at 10 °C (Section 1.6 on page 12), thus leading to more protein degradation and a higher %DH compared to storage at 4 °C. The difference in %DH between the two storage temperatures increases with storage time, but addition of commercial enzymes and performing hydrolysis for 60 min reduces the gap. For raw material stored for 120 hours

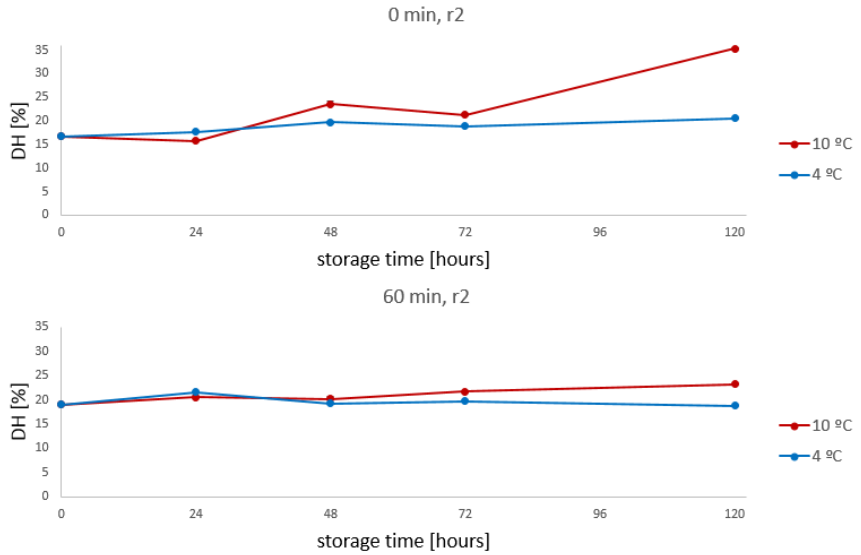

Figure 35: Degree of hydrolysis as a function of storage time of raw material for hydrolysates from reactor 2 after 0 min (top) and 60 min (bottom) of hydrolysis. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage at either 4 °C or 10 °C.

the difference is reduced from 8,0 % to 2,4 % (reactor 1) and from 14,7 % to 4,4 % (reactor 2) going from 0 to 60 min of hydrolysis. As for Figure 33, also Figure 34 illustrates the remarkably high degree of hydrolysis observed for both storage temperatures after 48 hours of storage for reactor 1.

In this project, %DH of hydrolysates from herring was in the range 19-26 % (reactor 1) and 19-23 % (reactor 2) after 60 min of hydrolysis. Previous studies on herring hydrolysates done by Møen Tveit[25] reported %DH ranging between 19-22 % after 60 min of hydrolysis. 22 % was achieved when the herring raw material contained viscera, and 19 % when viscera was removed. The same enzymes were used as in this project, and %DH was determined with formol titration. Sathivel et al.[76] reported lower %DH of herring hydrolysates, but here alcalase (0,5 % w/w) was used and %DH was determined by the method described by Hoyle and Meritt[31], i.e. precipitation with 10 % TCA. When other methods than formol titration are used, the results are difficult to compare as the different methods all give different outcomes. A standardised method is

not available, but my fellow student Kristine Grønstad is at present studying the different methods to determine degree of hydrolysis as part of her master thesis.

The desired degree of hydrolysis depends on the desired properties of the hydrolysates. A small %DH is necessary for obtaining good emulsifying properties. It has been suggested that optimum emulsifying properties are achieved when %DH is relatively low (<10% [29, 132]). On the other hand hydrolysates with high %DH, while not being suited for being a functional ingredient in food, might be well suited as speciality food in human nutrition due to reduced allergenicity and often higher bioactivity[29, 70]. Several studies have found that the most effective ACE-inhibitory peptides, lowering the blood pressure in spontaneously hypertensive rats (SHR), are the peptides of smaller size and higher %DH [133, 134, 135]. Almost regardless of the application area the formation of bitter peptides must be taken into account. A %DH value between 4-40 % has been reported to have a high risk of bitterness [11]. This problem can be minimised by controlling the process conditions (Section 1.7 on page 15 and 19).

3.9 Acid soluble peptides

The amount of acid soluble peptides was determined for the hydrolysates after 60 min of hydrolysis. The hydrolysates from fresh raw material (0 hours of storage) were also analysed after 0 and 30 min of hydrolysis. The amount of acid soluble peptides is presented as a function of hydrolysis time and storage time of raw material in Figure 36 and Figure 37[3], respectively. Reactor 1 was analysed by Fataneh Melstad as part of her project thesis during fall 2014 and reactor 2 by me during spring 2015. The average values of reactor 1 and 2 have not been presented together due to the results from reactor 1 being significantly larger than the results from reactor 2. This was thought to be caused by the hydrolysates from reactor 1 being diluted with water and not trichloroacetic acid (TCA). Additional analyses with both water and TCA as dilution media were done by Maiken Lindgjerdet, confirming that the dilution media has an effect on the outcome of the Lowry method[105]. The Biuret reaction in this method is influenced by acid present because it involves a reaction between copper ions and peptides bonds in alkaline solution. Dilution should preferably

[3]The absorbance measurement of sample ID 10-2-2-60 was unstable and this sample was therefore analysed a second time by Maiken Lindgjerdet. The new value is included in Figure 37.

be standardised for easier comparison with data from the literature.

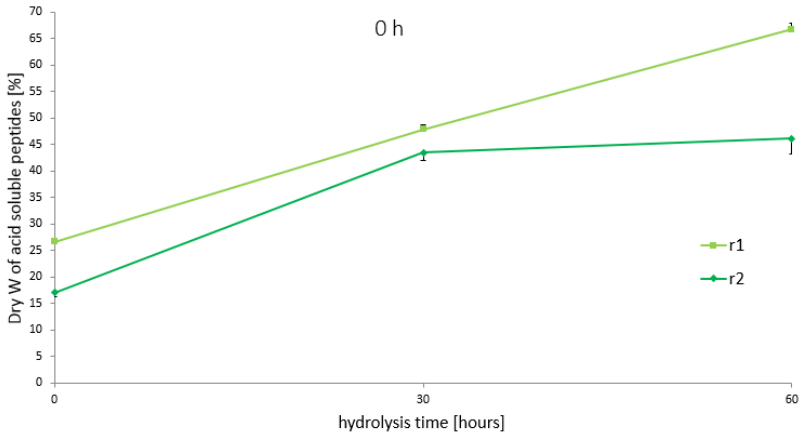

Figure 36: Amount of acid soluble peptides as a function of hydrolysis time for fresh raw material (0 hours of storage). Sample solutions from reactor 1 and 2 were diluted with water and TCA, respectively, and therefore larger values were obtained for reactor 1.

According to Hultmann[15] the amount of acid soluble peptides can be used as a method for detecting proteolytic activity of exopeptidases[15]. Endopeptidases do not release small enough peptides to be acid soluble, suggesting that the observed increase in acid soluble peptides in Figure 36 as hydrolysis proceeds is caused by endogenous exopeptidase activity rather than the activity of endogenous or commercial endopeptidases. However the endopeptidases probably aid in this increase as they serve the exopeptidases with more substrate when they break down larger proteins. These results supports the assumption of the endogenous enzymes being active during heating and hydrolysis at 50 °C (Section 1.6 on page 12).

Hultmann[15] studied how the amount of acid soluble peptides was changing in salmon and cod fillets stored on ice for 4 and 10 days. The amount of acid soluble peptides was notably higher in the fillets stored for 10 days. A temperature abuse during the storage period of 4,25 hours at 20 °C did not result in significant differences compared to a control group that was kept on ice all the time. It was postulated that the small, but not significant, increase was due to the activation of endogenous enzymes by storage at a higher temperature.

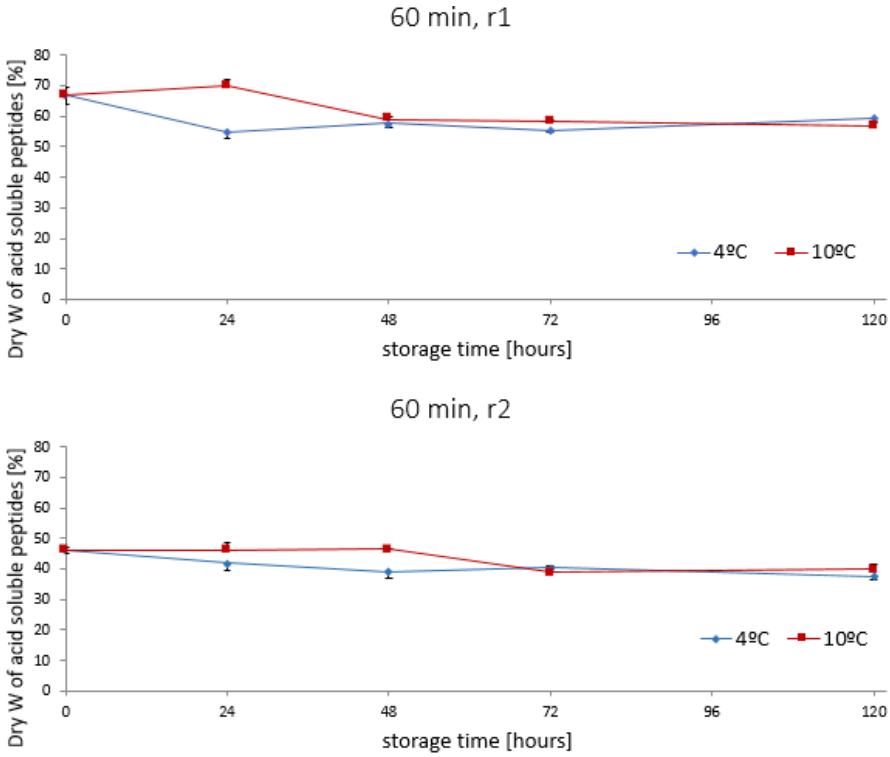

Figure 37: Amount of acid soluble peptides as a function of storage time for raw material stored at either 4 °C or 10 °C. Sampling was done after 0h, 24 h, 48 h, 72 h and 120 h of storage. Sample solutions from reactor 1 and 2 were diluted with water and TCA, respectively, and therefore larger values were obtained for reactor 1.

Figure 37 shows that the amount of acid soluble peptides is decreasing slightly with increasing storage time, even though an increasing or constant value is expected. The observed decrease might origin in the Biuret reaction not detecting peptides smaller than three amino amino acid residues (Section 1.9.6 on page 29). The hydrolysates from raw material stored at 10 °C contain slightly higher amounts of acid soluble peptides compared to storage at 4 °C, but the difference is small and not present throughout the whole storage period. It could be postulated that after >48 hours of storage there is no longer enough substrate easily accessible for the endogenous enzymes to degrade before commercial en-

zymes have aided in the degradation process, and therefore the difference in acid soluble peptides between the two storage temperatures decreases as storage time increases.

3.10 Molecular weight distribution

The molecular weight distribution is important for both organoleptic and functional properties of the hydrolysates (Section 1.7 on page 19 and Section 1.9.7 on page 30). As will be discussed in Section 3.13 on page 95, the molecular weight distribution can also influence the colour of the product through the Maillard reaction. The results from the molecular weight distribution analyses are presented in Figure 38, Figure 39, Figure 40 and Figure 41. A standard curve was established and the size of detected peptide fractions was estimated. The estimated molecular weights are presented in Table 12. The standards used were listed in Table 5 (page 50). It is referred to Appendix J for the standard curve used to calculate the molecular weights and for additional chromatograms. The three peaks furthest to the right in the chromatograms, having the largest retention volumes (29 ml, 30 ml and 35 ml), represents peptide fractions of sizes $\sim$25 Da, $\sim$15 Da and $\sim$5 Da. These values are very small compared to the molecular weight of the standards used and are therefore not reliable. Furthermore 110 Da is the average molecular weight of an amino acid and 75 Da the molecular weight of the smallest amino acid, glycine[136], suggesting that even the smallest retention volume (26 ml) included in Table 12 does not describe the size of an amino acid structure. The column can separate peptide fractions in the range 100-7000 Da, but all structures that are absorbed at 280 nm can contribute to the retention volume peaks, not just proteins, peptides and amino acids. In the results presented below, attention should therefore only be paid

to the retention volume peaks in the left half of the chromatograms (< 25 ml).

Table 12: Estimated molecular weight of peptide fractions in the hydrolysates.

Retention volume peaks [ml]	**Estimated molecular weight [Da]**
8 (void volume)	> 24 000
18	800
22	300
24	150
26[a]	60

[a] This retention peak is not likely to represent an amino acid structure as the estimated molecular weight is smaller than the molecular weight of glycine (75 Da).

In Figure 38 the molecular weight distribution in hydrolysates from fresh raw material (0 hours of storage) is given as a function of hydrolysis time for reactor 1 and 2, showing that the distribution is similar in the two reactors. The most pronounced peaks resemble peptide fractions of size ∼800 Da (18 ml), ∼300 Da (22 ml), ∼150 Da (24 ml) and ∼60 Da (26 ml). Some of the proteins and peptides that in the beginning are eluted with the void volume are broken down as hydrolysis proceeds, now being detected by the column and giving rise to peptides in the molecular weight range of 800-24 000 Da. This is illustrated by the increase in absorbance between retention volume 8-17,5 ml as hydrolysis proceeds.

The composition of muscle protein is largely dominated by the proteins myosin and actin. Myosin has an approximate molecular weight of 500 kDa and actin an approximate molecular weight of 42 kDa[137]. Because myosin and actin are salt soluble, they will not be soluble in water unless they are broken down by enzymes first. Additionally proteins and peptide fractions of size >24 000 Da are too large to enter the pores of the column. Therefore myosin and actin will first be eluted with the void volume when they are sufficiently broken down and solubilised by endogenous and commercial proteases.

Figure 39 shows that the molecular weight distribution does not change significantly with storage time when the raw material has been stored at 4 °C, meaning the same peptide fractions are detected throughout the storage pe-

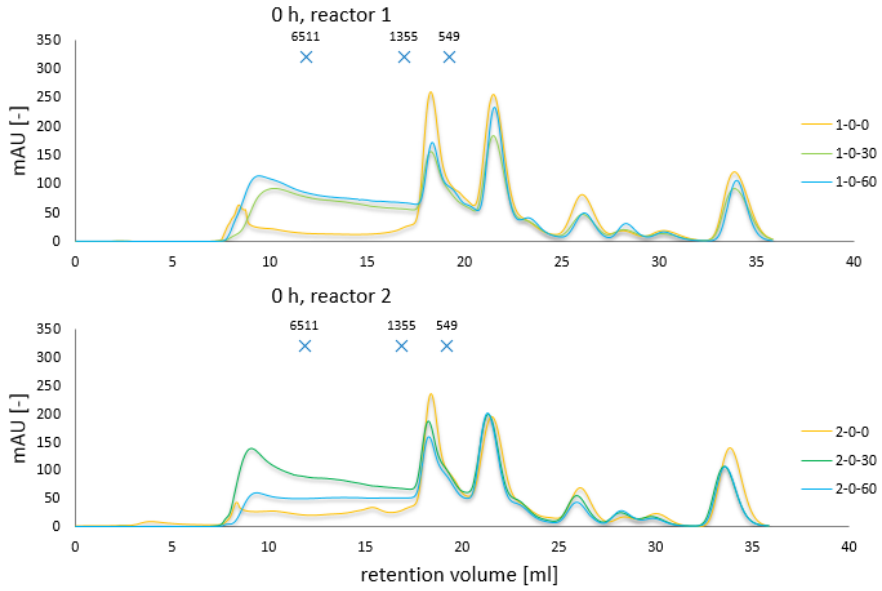

Figure 38: Molecular weight distribution of peptides in hydrolysates from reactor 1 (top) and reactor 2 (bottom) after 0 min (yellow), 30 min (green) and 60 min (blue) of hydrolysis. Sampling was done after 0 hours of storage of raw material. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

riod. However the molecular weight distribution changes with hydrolysis time, as already illustrated for hydrolysates from fresh raw material in Figure 38. The content of proteins which are degraded into peptide fractions of size ∼800-24 000 Da increases when going from 0 to 60 min of hydrolysis. This can clearly be seen when comparing the absorbance values in the top and bottom diagram of Figure 39 with each other. The peak at a retention volume of 18 ml is also decreasing and the peak at 22 ml increasing, indicating that peptide fractions of ∼800 Da are broken down and peptide fractions of ∼300 Da are formed. The same trend regarding storage and hydrolysis time is observed in Figure 40, representing the molecular weight distribution in hydrolysates from raw material stored at 10 °C.

Figure 41 shows that the molecular weight distribution does not change significantly with storage temperature. The same peaks are dominant in hydrolysates

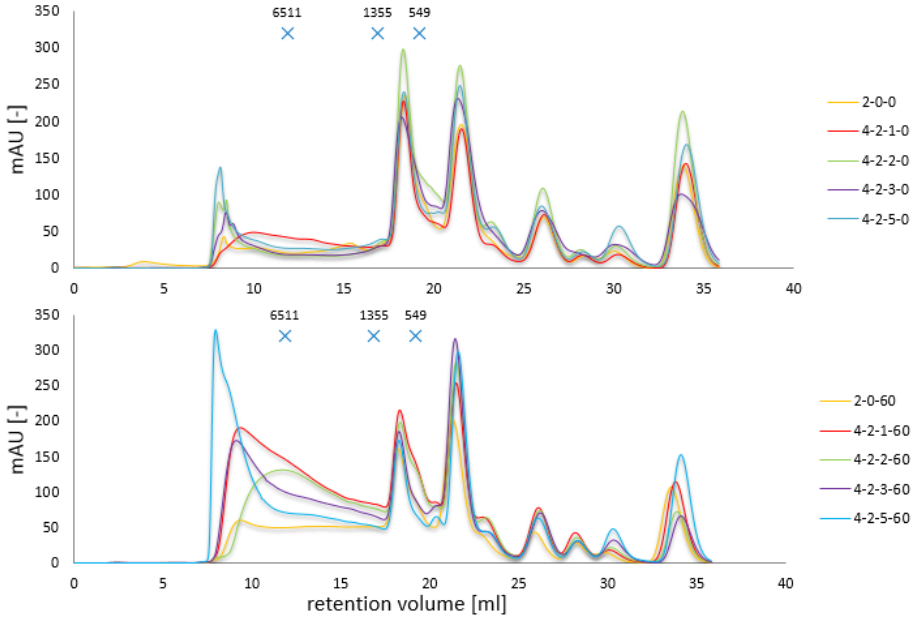

Figure 39: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (top) and 60 min (bottom) of hydrolysis for raw material stored at 4 °C. Sampling was done after 0 h (yellow), 24 h (red), 48 h (green), 72 h (purple) and 120 h (blue) of storage. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

from raw material stored at both 4 °C and 10 °C. Seemingly the molecular weight distribution is affected neither by storage time nor temperature.

3.11 ACE-inhibitory activity

The ACE-inhibitory activity was measured for 13 selected hydrolysates from reactor 2 and is given as % inhibition / mg FPH when the hydrolysate is present in the mixture, compared to if no hydrolysate (i.e. no inhibitor) is present at all. It should be emphasized that the ACE-inhibitory activity has been evaluated for the hydrolysate as a whole. The specific bioactive peptides have not been separated and purified prior to analysis. The hydrolysates contain

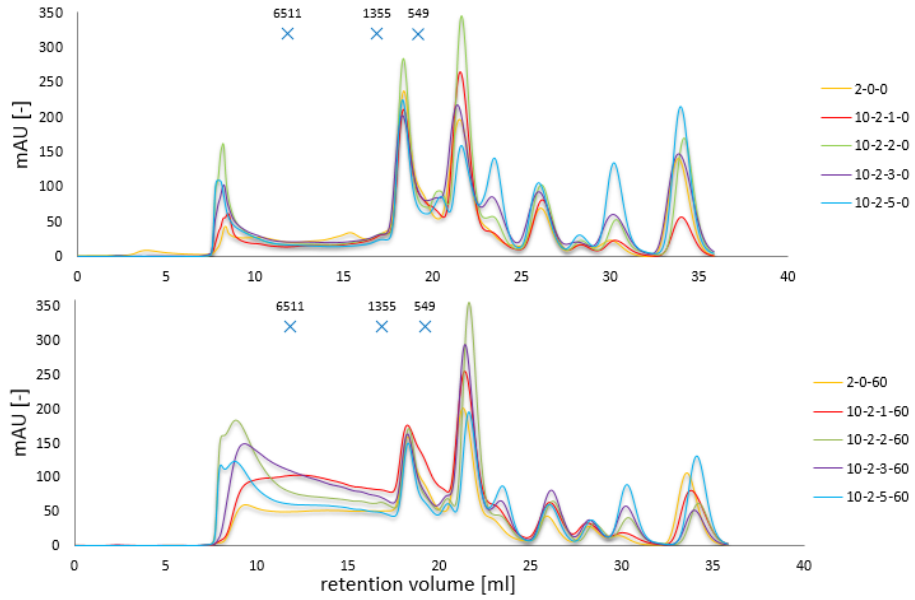

Figure 40: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (top) and 60 min (bottom) of hydrolysis for raw material stored at 10 °C. Sampling was done after 0 h (yellow), 24 h (red), 48 h (green), 72 h (purple) and 120 h (blue) of storage. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

68-85 % protein in addition to other water soluble components resulting from the enzymatic hydrolysis, and it is their collective ability to inhibit ACE that has been evaluated.

The ACE-inhibitory activity found for the selected hydrolysates is presented in Figure 42 and should only be used to compare the different hydrolysates from this project with each other. Normally an IC_{50}value is reported, i.e. the concentration which inhibits the enzyme by 50 %. In this project it was focused on investigating the effect of storage conditions and hydrolysis time rather than which concentration is needed to observe an effect. Therefore the ACE-inhibitory activity was only analysed for one single concentration and no IC_{50} value is obtainable. Figure 42 shows that the ACE-inhibitory effect was not affected by storage conditions of the raw material. However there is a clear

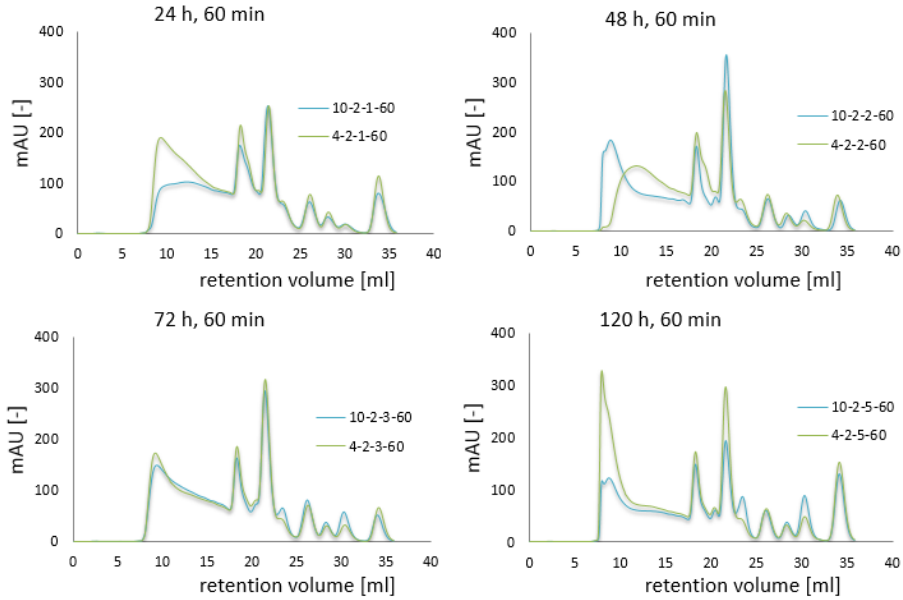

Figure 41: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 60 min of hydrolysis for raw material stored at 4 °C (green) and 10 °C (blue). Sampling was done after 24 h (upper left), 48 h (upper right), 72 h (lower left) and 120 h (lower right) of storage.

difference in the ability to inhibit ACE with respect to hydrolysis time, especially between 0 and 30 min of hydrolysis. The difference is less pronounced between 30 and 60 min of hydrolysis, but it is expected that a larger difference would be observed if the concentration of prepared hydrolysate solution is reduced from 20 mg/ml to e.g. 10 mg/ml. The lowest inhibitory effect (27,4 %[4]) was observed for the hydrolysate after 0 hours of storage of raw material and after 0 min of hydrolysis (sample ID 2-0-0). This is probably because the endogenous enzymes have had the least amount of time to degrade the proteins and no commercial enzymes have yet been added. The hydrolysates after 30 min of hydrolysis showed an inhibitory effect of 88-93 % and the hydrolysates after

[4]A value of 27,4 % inhibition / mg FPH means that 1 mg of the hydrolysate inhibited 27,4 % of the ACE activity when 0,375 μg of ACE and 0,09 mmol of substrate were present in a total volume of 300 μl.

60 min of hydrolysis an inhibitory effect of 90-93 %. These high values indicate that the hydrolysates are almost able to fully inhibit the ACE activity *in vitro*. However, this does not necessarily mean that the same effect is observed *in vivo* (Section 1.8 on page 24). It is referred to Appendix K for diagrams of ACE-inhibitory activity as a function of storage and hydrolysis time.

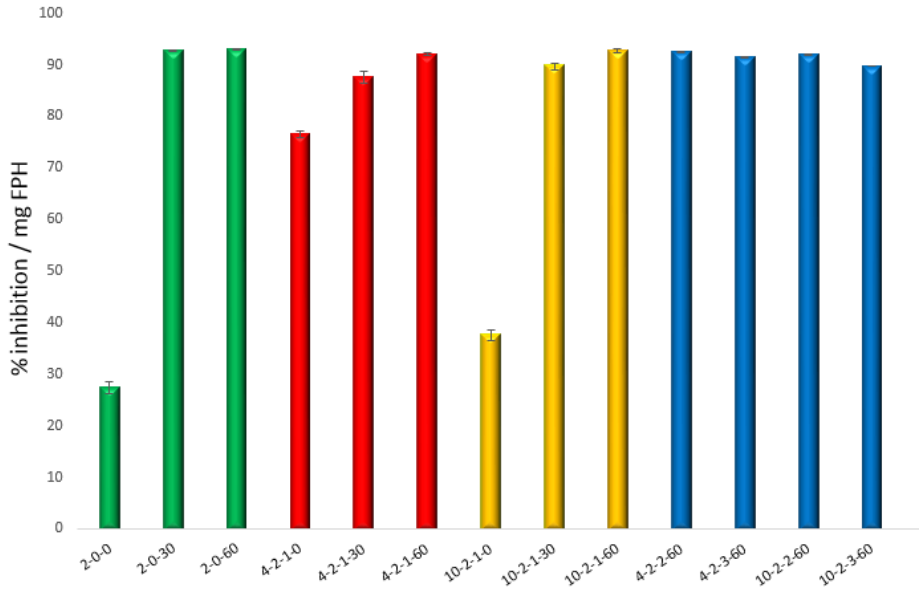

Figure 42: ACE-inhibitory activity given as % inhibition/mg FPH when the hydrolysate is present in the mixture, compared to if no hydrolysate (i.e. no inhibitor) is present at all. All 13 hydrolysates are from reactor 2.

Few studies have looked at ACE-inhibitory peptides from herring rest raw material. He et al.[138] tested the ACE-inhibitory activity of 12 different marine proteins and found that hydrolysates from herring skin, codfish skin, oyster and scallop had an IC_{50} >10 mg/ml, while hydrolysates from shark meat, shrimp and mackerel bone had an IC_{50} <1 mg/ml. No specific value for herring skin was available. The method used for analysis was the method described by Cushman and Cheung[139]. There are many studies on marine organisms other than herring, but reported values cannot be compared to the results obtained in this project since no IC_{50} value was measured.

3.12 Bioactive peptides in protein sequences of herring

The database of National Center for Biotechnology Information (NCBI)[119] was used to search for sequences of the muscle proteins myosin and actin from herring (*Clupea harengus*). The three relatively short sequences found are given in Appendix L. In Table 13 the peptides that was reported by Pampanin et al.[88] to have antioxidative or cardiovascular properties (Table 3 on page 26) and that appeared in these sequences, are listed. It is possible that some of these peptides contribute to the ACE-inhibitory effect found and discussed in Section 3.11. It should be noted that the myosin and actin proteins are large molecules and that more bioactive peptides probably would have been detected if larger parts of the proteins were sequenced.

The myosin protein of zebrafish (*Danio rerio*) is more extensively sequenced and ClustalW2[120] was used to check for homogeneity in the sequenced parts of the myosin heavy chain of zebrafish and herring. This yielded a high score (89-90 %), meaning the amino acids are similarly aligned. Since the sequenced parts of herring protein is so limited it might be possible to use sequenced parts of zebrafish protein to screen for bioactivity, at least to estimate what bioactive peptides can possibly result from degradation of herring protein. The sequenced part of the myosin heavy chain of zebrafish is given in Appendix M.

Table 13: Peptides found in the myosin and actin sequences of herring and reported to have antioxidative and cardiovascular properties[88]. Each letter corresponds to an amino acid for which the translation is given in Appendix N. Coloured peptides means they appear in the sequences more than once, the number in paranthesis how many times they appear.

Antioxidative properties
AH, DLYA, EL (4), MY (2), SLYA, VW
Cardiovascular properties
APL, AVF, FY, IAE, VK (6), IW, YN (3), YP (2)

3.13 Colour and texture of hydrolysate

The colour of the hydrolysates was investigated by visual expection. Colour measurements with a colorimeter can be performed for a more objective result, but this was not done as part of this project. Several factors can influence the colour of the hydrolysates (Section 3.13 on page 95). Figure 43 and Figure 44 show how the colour and texture of freeze-dried hydrolysates changes with hydrolysis time. The hydrolysates after 0 min of hydrolysis are typically fluffy, porous and bright in colour, while the hydrolysates after 60 min of hydrolysis have a denser texture and a darker, browner and more yellowish colour. Carvajal[24] reported that the the hydrolysates from herring darkened with hydrolysis time. This darkening can possibly be explained by the formation of more peptides and therefore a greater possibility for formation of brown products in the Maillard reaction[24]. Also previous studies by Kim and Lee[140] indicate that the %DH and peptide chain length greatly influences the Maillard reaction and the colour of hydrolysates, because increased %DH signifies presence of more free amino groups that can react with the carbonyl group of reducing sugars.

Figure 43: The colour and texture of hydrolysates after 0 (left), 30 (middle) and 60 (right) min of hydrolysis. The raw material was stored for 48 hours.

Figure 45 shows how the colour of hydrolysates changes with storage conditions of the raw material. It should be noted that the hydrolysates from reactor 1 were freeze-dried during fall 2014 and therefore have been stored in exicators at room temperature for a longer time than the hydrolysates from reactor 2. Also note that the hydrolysates furthest to the left in the picture (0 hours of storage) by a mistake have swapped places, the darkest sample belonging to reactor 1.

Figure 44: The colour of hydrolysates from reactor 2 after 0, 30 and 60 min of hydrolysis. The raw material was stored for 120 hours at either 10 °C (top) or 4 °C (bottom).

Figure 45: The colour of hydrolysates after 60 min of hydrolysis. From left to right: 0 h, 24 h, 48 h, 72 h and 120 h of storage of raw material. From top to bottom: reactor 1 - 10 °C, reactor 1 - 4 °C, reactor 2 - 4 °C and reactor 2 - 10 °C. Note that the hydrolysates furthest to the left in the picture (0 hours of storage) by a mistake have swapped places, the darkest sample belonging to reactor 1.

The hydrolysates from reactor 1 show a higher colour inconsistency than reactor 2, possibly due to the variable lipid content found for these hydrolysates. If the colour of the darker hydrolysates from reactor 1, namely 24 h - 10 °C, 48 h - 4 °C, 48 h - 10 °C and 120 h - 10 °C, is seen in relation to their lipid content, one can possibly draw the conclusion that these hydrolysates are darker in colour due to a higher lipid content and a more pronounced lipid oxidation. Lipid oxidation can induce darkening of the hydrolysates through the Maillard reaction[10, 62] (Section 1.7 on page 17). The lipid content in hydrolysates from reactor 1 was

presented in Figure 32 (page 78). No picture was taken of the hydrolysates from reactor 1 during fall 2014, but they might have been brighter in colour then. The hydrolysates from reactor 2 show a higher colour consistency, but if a new picture is taken in a few months, the colour might have changed in the hydrolysates with a high lipid content.

The herring hydrolysates in this project were expected to be darker than both chicken hydrolysates and herring hydrolysates without entrails (Section 1.7 on page 20). However the hydrolysates after 30 and 60 min were in general too dark to be incorporated into food, especially the hydrolysates with a high lipid content. The hydrolysates after 0 min of hydrolysis were brighter in colour, but the results presented in this project show that a minimum of 30 min of hydrolysis is necessary in order to improve other important properties of the hydrolysates, especially protein content and protein recovery.

3.14 Sum up of results

The herring rest raw material contained 14,2 ± 0,9 % protein, 16,2 ± 0,2 % lipid and 3,5 ± 0,2 % ash. Crude oil and protein hydrolysates were produced from the raw material by heating to 50 °C and performing enzymatic hydrolysis for 60 min. The hydrolysates after 0 min of hydrolysis were typically fluffy, porous and bright in colour, while the hydrolysates after 30 and 60 min of hydrolysis had a heavier and denser texture and a darker colour. The hydrolysates after 30 and 60 min of hydrolysis were in general too dark for incorporation into food products, especially the ones with a high lipid content.

The average dry yield of oil and FPH after 60 min of hydrolysis was 10,2 g/100 g raw material and 5,7 g/100 g raw material, respectively. Between 0 and 60 min of hydrolysis the yield increased for oil and FPH, the increase being most pronounced between 0 and 30 min. The oil yield was first decreasing with increasing storage time of raw material, but after 48-72 hours the trend reversed and the yield returned to initial start value. The FPH yield was not affected by storage time. Storage of raw material at 10 °C resulted in higher yields compared to 4 °C.

The PV, AV, TOTOX and FFA value of the oil were all within recommended limits for crude oil intended for human consumption, independent of the storage conditions of the raw material. However the FFA value increased with storage time and was higher for storage at 10 °C compared to 4 °C. The correlation

between storage conditions and oxidation status was less clear. The crude oil produced from fresh raw material had PV 11,9 ± 0,2 meq/kg, AV 1,4 ± 0,1, TOTOX 25,2 ± 0,4 and FFA 0,5 ± 0,1 %.

The protein hydrolysates had a dry matter content of 92 % (reactor 1) and 95 % (reactor 2). The protein content after 60 min of hydrolysis was in the range 81-85 % (conversion factor 6,25) and the protein recovery in the range 33-39 %. Protein content and protein recovery increased with hydrolysis time, and the increase was most pronounced between 0 and 30 min. The protein content decreased slightly with increasing storage time of raw material, and was higher for storage at 4 °C compared to 10 °C. The protein recovery remained relatively constant throughout the storage period and was at times higher for storage at 10 °C compared to 4 °C. The lower protein content at 10 °C compared to 4 °C signifies a less pure product, i.e. that other components like lipids and dissolved inorganic matter are present to a higher degree. The simultaneous higher protein recovery at 10 °C does therefore not necessarily imply that more protein from the raw material is retrieved in the FPH fraction, it could also be that more of the mentioned impurities are incorporated into the product. The lipid content of the hydrolysates was high, being in the range 1,5-6,8 % after 60 min of hydrolysis. In most cases the lipid content decreased with hydrolysis time, but for some hydrolysates the lipid content increased. No correlation was found between lipid content and storage time of raw material, but storage at 10 °C resulted in a higher lipid content compared to 4 °C. In all cases the lipid content was higher than what was aimed for (<0,5 %).

After 60 min of hydrolysis %DH was in the range 19-26 %. A decline or constant level of %DH was found with increasing hydrolysis time. %DH increased with storage time of raw material and was higher for storage at 10 °C compared to 4 °C. Amount of acid soluble peptides was in the range 38-46 % and 55-70 % after dilution with 5 % TCA and water, respectively. Amount of acid soluble peptides increased with hydrolysis time. The level remained constant or decreased slightly with increasing storage time of raw material, and was at times higher for storage at 10 °C compared to 4 °C. The molecular weight distribution was not affected by the storage conditions of the raw material. Proteins and peptides that in the beginning were larger than 24 000 Da were broken down and subsequently detected as hydrolysis proceeded. The most pronounced peptide fractions were estimated to be of size ∼800 Da, ∼300 Da and ∼150 Da. The hydrolysates were found to contain bioactive peptides with strong inhibitory activity against angiotensin I converting enzyme *in vitro*. The inhibiting effect increased between 0 and 30 min of hydrolysis, but between 30

and 60 min no difference was found. The inhibiting effect was independent of the storage conditions of raw material.

All in all, the storage conditions of raw material did not seem to have a detrimental effect on the properties of crude oil and protein hydrolysates. However, it should be emphasised that both the crude oil and protein hydrolysates after >72 hours of storage of raw material had a very unpleasent smell, especially if stored at 10 °C. Sensory analyses of smell and taste are just as important factors as the parameters evaluated in this study before it can be concluded under what conditions the raw material should and can be stored.

4 Conclusion

This study has shown that it is possible to utilise herring rest raw material for production of crude oil and protein hydrolysates (FPH). The herring rest raw material consisted of 14,2 ± 0,9 % protein, 16,2 ± 0,2 % lipid and 3,5 ± 0,2 % ash. Enzymatic hydrolysis gave good hydrolysis yields for oil and FPH after 60 min of hydrolysis. Storage of raw material at 10 °C resulted in higher yields compared to storage at 4 °C. The yield of oil was at its lowest after 48-72 hours of storage, while the FPH yield did not change with storage time. The protein hydrolysates after 0 min of hydrolysis were typically fluffy, porous and bright in colour, while the hydrolysates after 60 min of hydrolysis had a denser texture and a darker colour. Other properties like protein content and protein recovery were improved with hydrolysis time, the change being greatest between 0 and 30 min of hydrolysis.

The quality of crude oil was high and may find applications in both the food, feed and cosmetic industry. For all storage conditions of the raw material the quality parameters were within recommended limits for crude oil intended for human consumption. The crude oil produced from fresh raw material had PV 11,9 ± 0,2 meq/kg, AV 1,4 ± 0,1, TOTOX 25,2 ± 0,4 and FFA value 0,5 ± 0,1 %.

The protein hydrolysates after 60 min of hydrolysis had a similar protein content (81-85 %) and protein recovery (33-39 %), with the higher values originating in fresher raw material. The lipid content (1,5-6,8 %) varied inconsistently throughout the storage period of raw material and was higher than what was aimed for (<0,5 %). Storage of raw material at 10 °C resulted in higher protein recovery, but lower protein and higher lipid content compared to 4 °C. The hydrolysates after 30 and 60 min of hydrolysis showed good ACE inhibiting effect, irrespective of the storage conditions of the raw material. The molecular weight distribution (∼800 Da, ∼300 Da and ∼150 Da), high %DH (19-26 %) and high level of acid soluble peptides (38-46 %) support the fact that the hydrolysates contain small peptides with bioactive properties.

The findings from this study may indicate that the hydrolysates are well suited for applications in human nutrition and in the pharmaceutical industry, as these applications often require peptide fractions of smaller sizes. For incorporation into food as a functional ingredient, a lower %DH is needed. Additionally the dark colour is undesired if the hydrolysates are to be incorporated into fish pudding and other light coloured food products. In general, due to the undesirable colour and high lipid content of protein hydrolysates, it might be that per to-

day production of crude oil is the best option for utilisating the herring rest raw material.

5 Future outlook

This study has shown that enzymatic hydrolysis is a promising method for increasing the value of herring rest raw material by production of high quality crude oil and protein hydrolysates. Thermal treatment can be used prior to enzymatic hydrolysis to extract the crude oil under milder conditions. Factors like enzyme type and hydrolysis time can influence both sensory, functional, nutritional and bioactive properties of the protein hydrolysates, and the enzymatic hydrolysis should therefore be designed according to the desired application area.

Before the results from this research can be used for designing an industrial process for making human grade oil and protein hydrolysates, there are still some challenges that should be looked further into. The process of enzymatic hydrolysis should be optimised in a way that minimises formation of emulsion. In relation to this the effect of e.g. vacuum, defoamers and stirring velocity should be investigated closer. Since a good part of the raw material is retrieved in the sediment fraction, it should be investigated what possible applications exist for the sediment. Utilising as much as the raw material as possible and achieving a high yield is of uttermost importance for the industry.

For the production of human grade oil, a refining process for converting the crude oil into refined oil must be developed. For the production of protein hydrolysates attention should be paid to the quality challenges identified in this project, i.e. too dark colour and too high lipid content in the product. Sensory challenges with respect to smell and bitter taste should also be investigated. The ACE inhibiting effect *in vitro* of the hydrolysates shows promising results for use in the pharmaceutical industry, but more attention should be paid to the necessary dosage needed to observe this effect *in vivo*. Furthermore there is a need for characterization of proteolytic activities during spawning and/or life cycle of different fish species. This may generate knowledge about when the activities of different endogenous enzymes increase, thereby making the enzymatic hydrolysis process more predictable.

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A Overview over what was done when and by who

An overview of what was done when and by who in this project is shown in Table 14.

Table 14: An overview of what was done when and by who in this project. F14 = fall 2014 and S15 = spring 2015.

What	Reactor	When	By who
Enzymatic hydrolysis	1	F14	Erle Saltvedt, Fataneh Meldstad and
	2	F14	people from SINTEF
Separation of frozen phases	1	F14	Erle Saltvedt, Fataneh Meldstad
	2	S15	Erle Saltvedt, Maiken Lindgjerdet
Dry matter of fractions	1	F14	Erle Saltvedt
	2	S15	Erle Saltvedt, Maiken Lindgjerdet
Total volatile basic nitrogen	1	N/A	N/A
	2	S15	Erle Saltvedt
Protein content in RM	N/A	F14	Marte Schei
	N/A	N/A	N/A
Protein content in FPH	1	F14	Erle Saltvedt
	2	S15	Erle Saltvedt
Lipid content in FPH	1	F14	Merete Selnes
	2	N/A	N/A
Molecular weight distribution	1	S15	Erle Saltvedt
	2	S15	Erle Saltvedt
Degree of hydrolysis	1	F14	Erle Saltvedt
	2	S15	Maiken Lindgjerdet
Acid soluble peptides	1	F14	Fataneh Meldstad
	2	S15	Erle Saltvedt
PV and AV	1	S15	Erle Saltvedt
	2	S15	Erle Saltvedt
Free fatty acids	1	S15	Erle Saltvedt
	2	S15	Erle Saltvedt
ACE-inhibition	1	N/A	N/A
	2	S15	Erle Saltvedt
Database search and sequence	N/A	N/A	N/A
alignment	N/A	S15	Erle Saltvedt

B Yield as a function of hydrolysis time

Yield is expressed as dry weight of fraction per 100 g raw material and in Figure 46, Figure 47, Figure 48 and Figure 49 the yield of either the FPH or oil fraction is given as a function of hydrolysis time for each reactor separately. The standard deviation is not shown for the FPH fraction due to limited space between the series in the diagrams. No standard deviation is available for the oil fraction because it was assumed to have a dry matter content of 100 %.

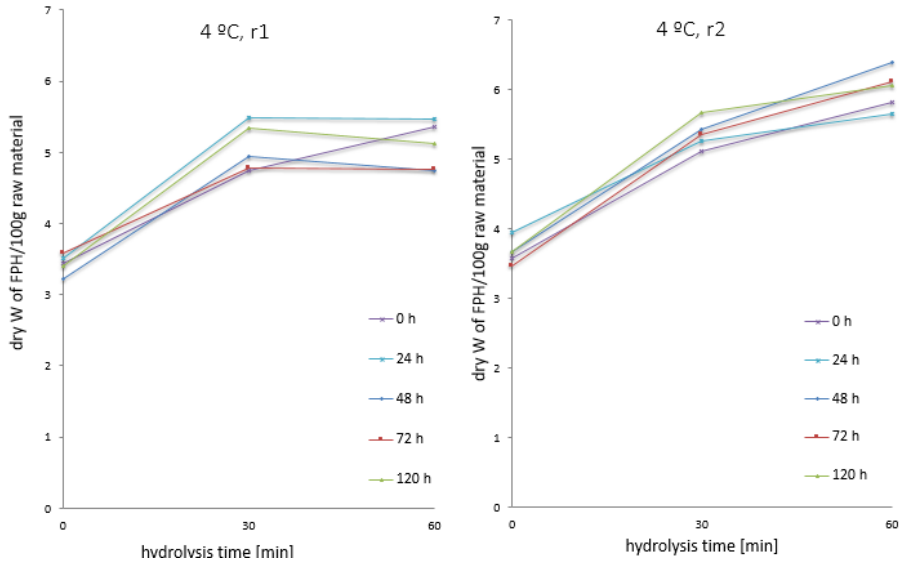

Figure 46: Yield of FPH fraction, in dry weight per 100 g raw material, for reactor 1 (left) and reactor 2 (right) as a function of hydrolysis time for raw material stored at 4 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

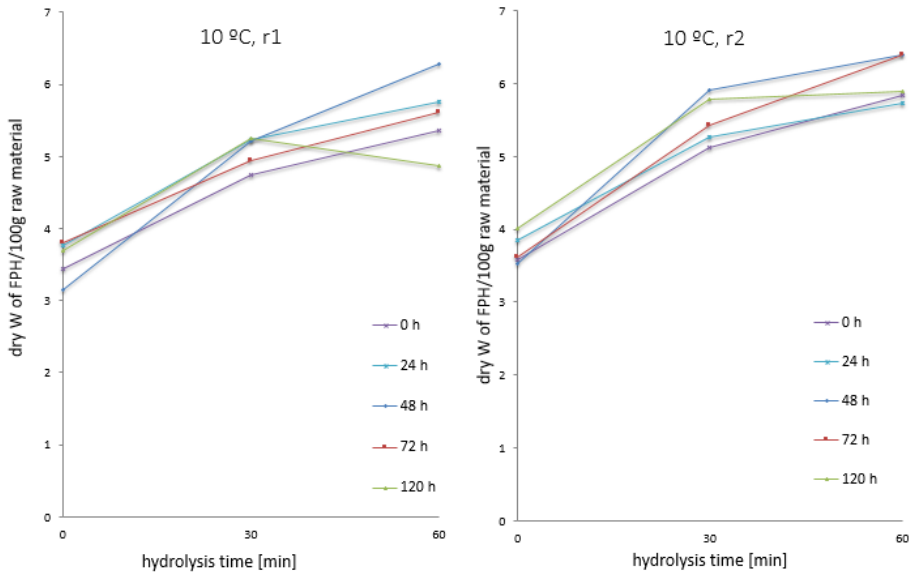

Figure 47: Yield of FPH fraction, in dry weight per 100 g raw material, for reactor 1 (left) and reactor 2 (right) as a function of hydrolysis time for raw material stored at 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

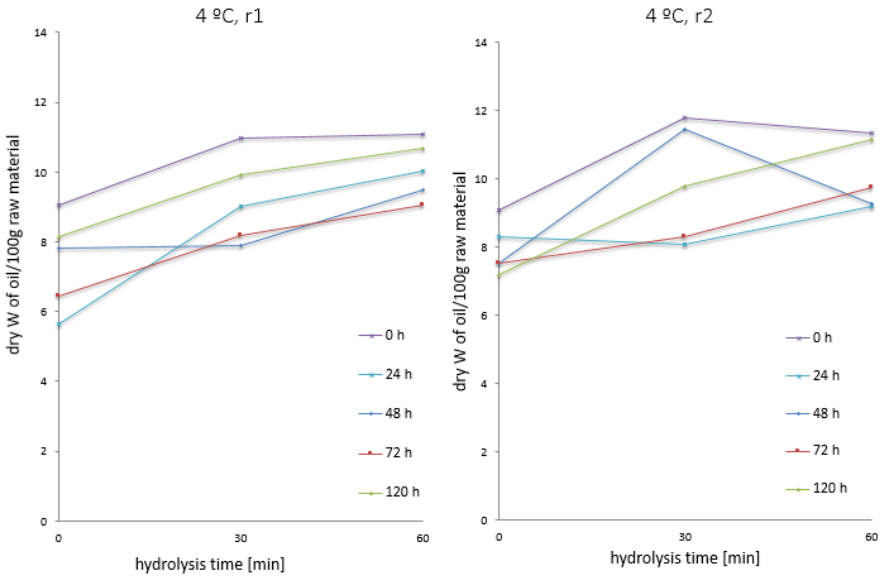

Figure 48: Yield of oil fraction, in dry weight per 100 g raw material, for reactor 1 (left) and reactor 2 (right) as a function of hydrolysis time for raw material stored at 4 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

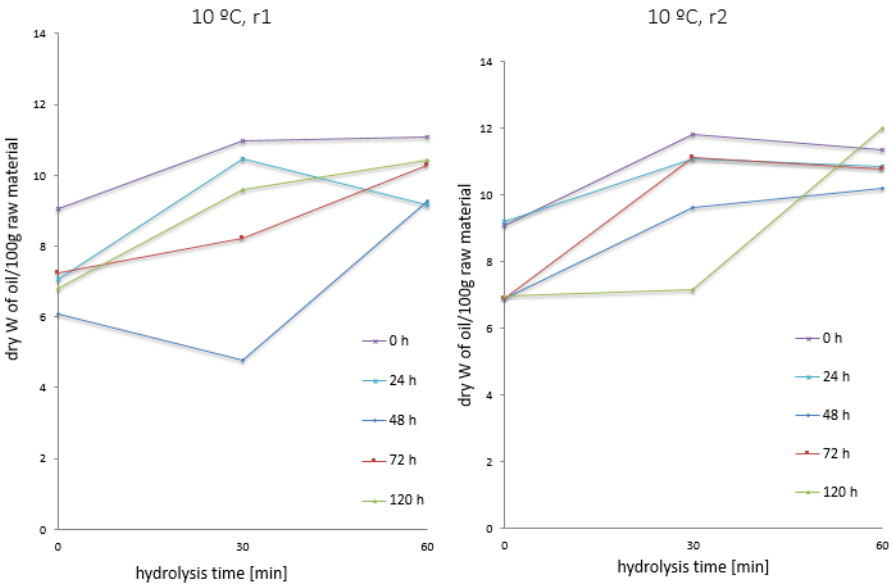

Figure 49: Yield of oil fraction, in dry weight per 100 g raw material, for reactor 1 (left) and reactor 2 (right) as a function of hydrolysis time for raw material stored at 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

C Yield as a function of storage time

Yield is expressed as dry weight of fraction per 100 g raw material and in Figure 50 and Figure 51 the yield of the FPH and oil fraction is given as a function of storage after 60 min of hydrolysis time for each reactor separately. The standard deviation for the FPH fraction is included, but too small to be visible. No standard deviation is available for the oil fraction because it was assumed to have a dry matter content of 100 %.

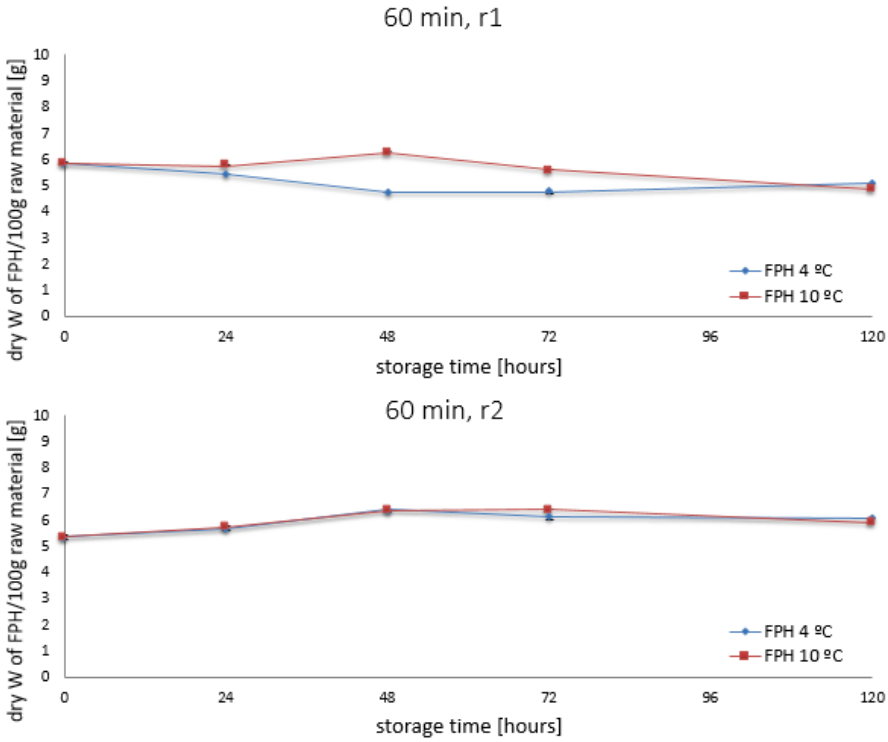

Figure 50: Yield of FPH fraction, in dry weight per 100 g raw material, after 60 min of hydrolysis for reactor 1 (top) and reactor 2 (bottom) as a function of storage time for raw material stored at 4 °C and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

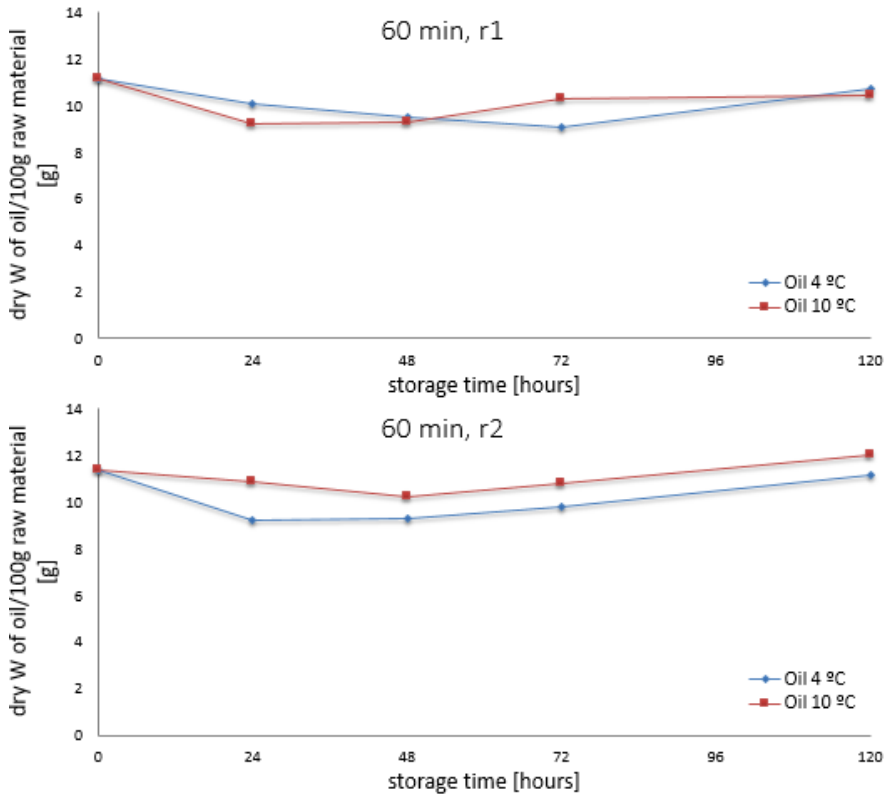

Figure 51: Yield of oil fraction, in dry weight per 100 g raw material, after 60 min of hydrolysis for reactor 1 (top) and reactor 2 (bottom) as a function of storage time for raw material stored at 4 °C and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

D Oxidation status in crude oil

In Figure 52 the anisidin value (AV) of crude oil is shown as a function of storage time for each reactor separately.

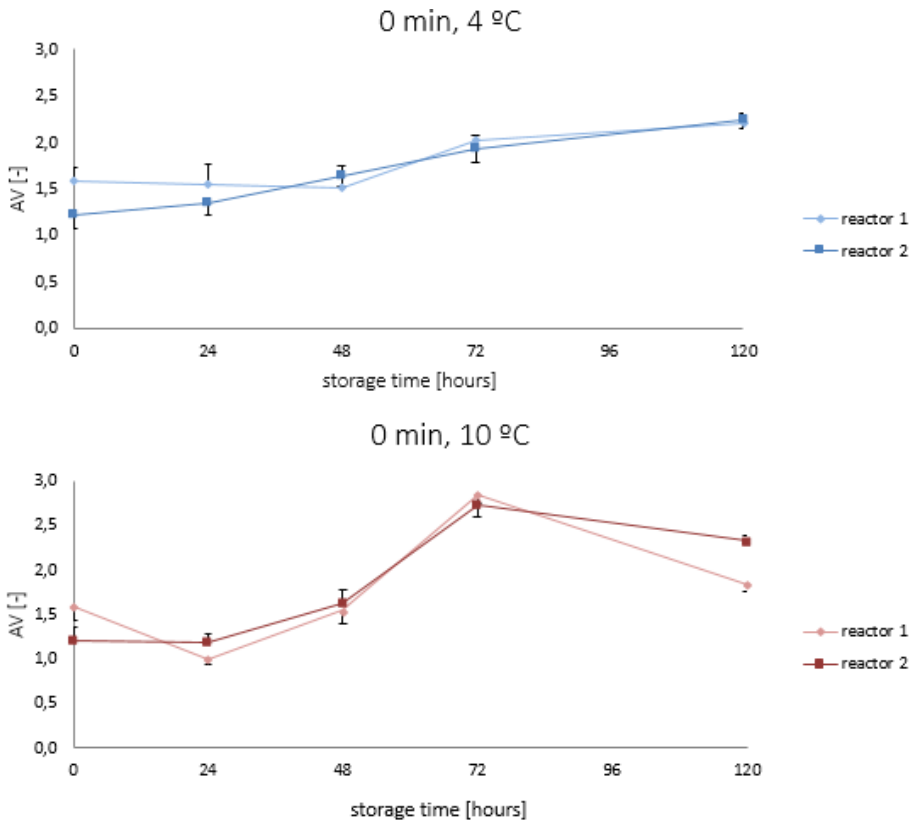

Figure 52: The AV as a function of storage time for crude oil from raw material stored at either 4 °C (top) or 10 °C (bottom) and after heating to 50 °C (0 min of hydrolysis). Sampling was done after 0 h, 24 h, 49 h, 72 h and 120 h of storage.

E Free fatty acids in crude oil

The standard curve used for determination of amount of free fatty acids (FFA) is presented in Figure 53. The amount of FFA as a function of storage time is given in Figure 54 for each reactor separately.

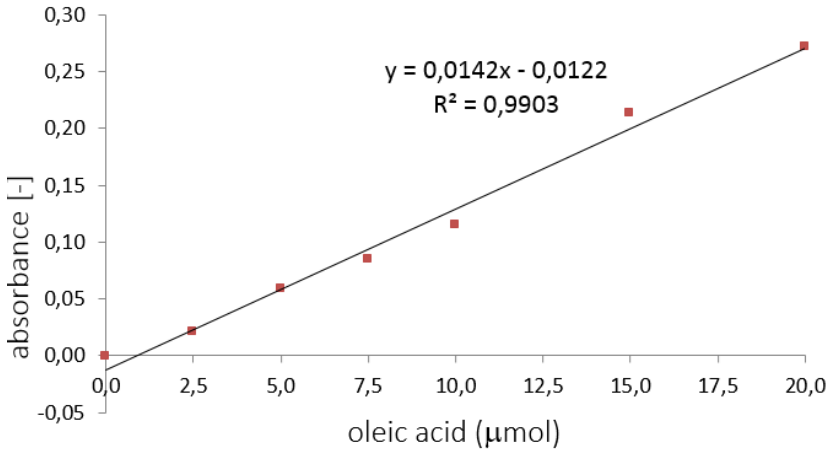

Figure 53: Standard curve for determination of amount of FFA in crude oil. Measured absorbance as a function of concentration of oleic acid.

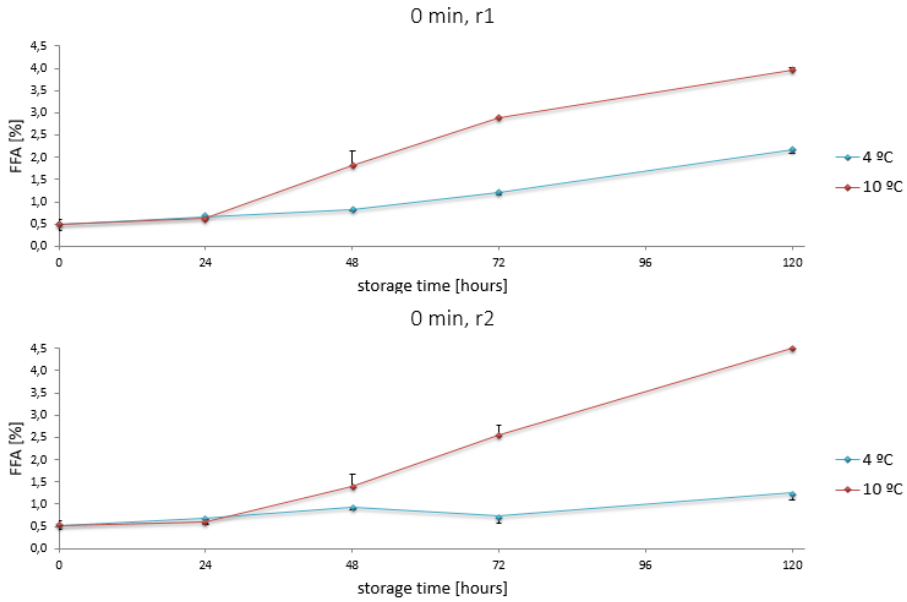

Figure 54: The amount of free fatty acids (%FFA) as a function of storage time for crude oil from reactor 1 (top) and reactor 2 (bottom). The raw material was stored at either 4 °C or 10 °C and heated to 50 °C (0 min of hydrolysis). Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage.

F Protein content and protein recovery of hydrolysate

Figure 55 shows the protein content and protein recovery after 60 min of hydrolysis of hydrolysates from reactor 1 and 2 separately, as a function of storage time. Figure 56 shows the protein content and Figure 57 the protein recovery of hydrolysates as a function of hydrolysis time. The standard deviation is not shown in Figure 56 and Figure 57 due to limited space between the series in the diagrams.

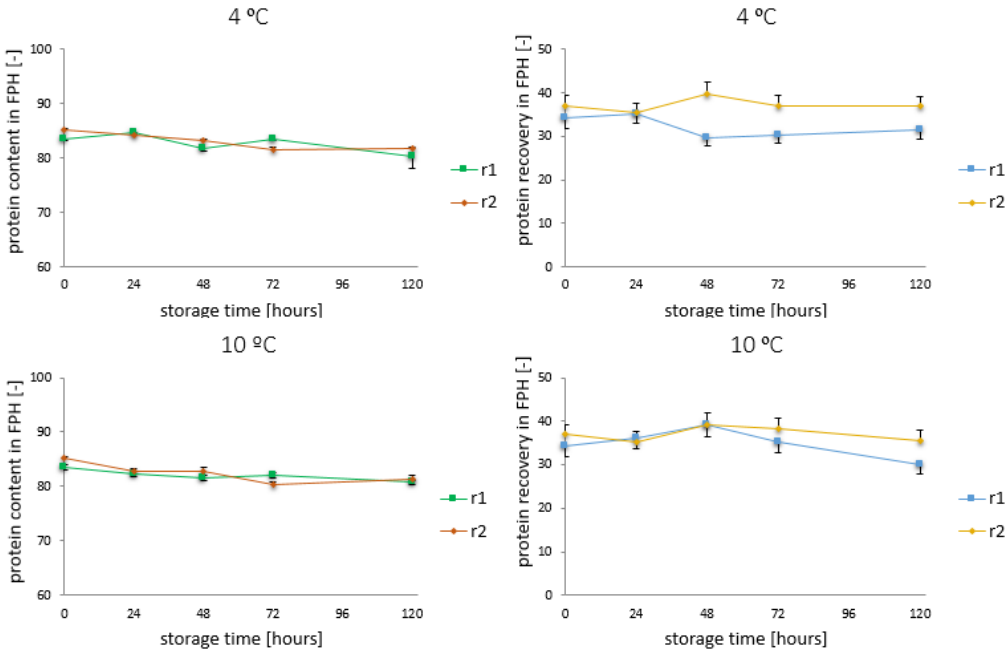

Figure 55: Protein content (left) and protein recovery (right) of hydrolysates after 60 min of hydrolysis as a function of storage time for raw material. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage at either 4 °C (top) or 10 °C (bottom). PrC = protein content.

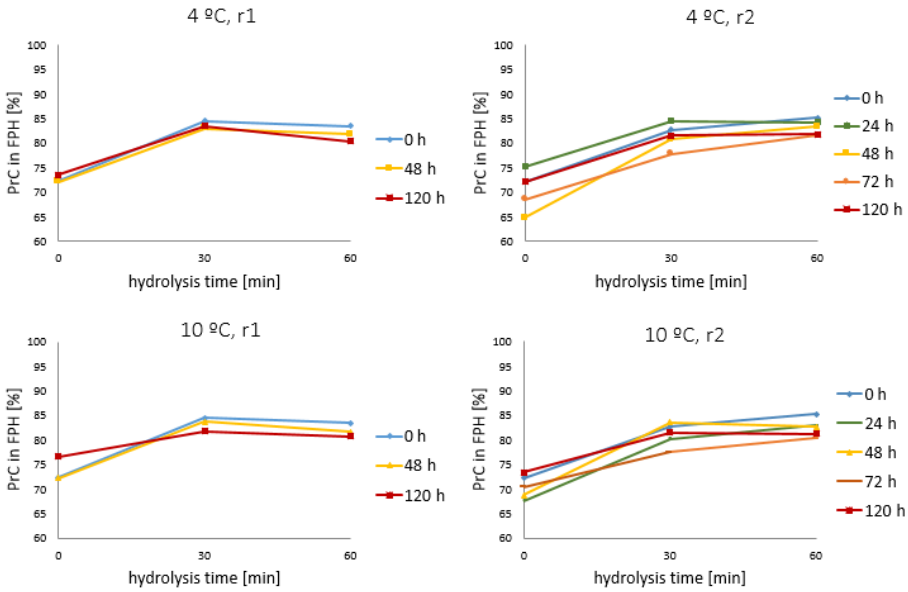

Figure 56: Protein content of hydrolysates as a function of hydrolysis time for hydrolysates from reactor 1 (left) and reactor 2 (right) when raw material was stored at 4 °C (top) and 10 °C (bottom). Sampling was done after 0 h, 24 h (only reactor 2), 48 h, 72 h (only reactor 2) and 120 h of storage. PrC = protein content.

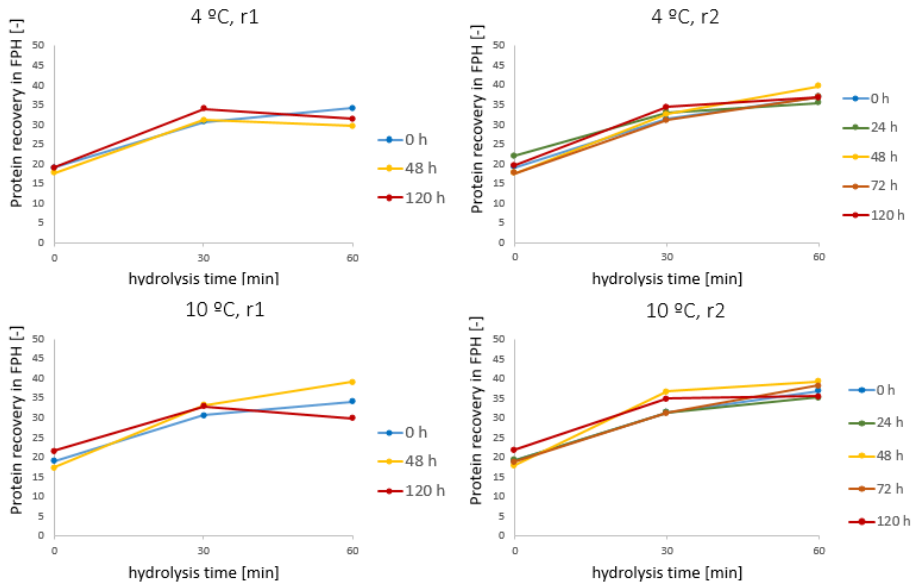

Figure 57: Protein recovery of hydrolysates as a function of hydrolysis time for hydrolysates from reactor 1 (left) and reactor 2 (right) when raw material was stored at 4 °C (top) and 10 °C (bottom). Sampling was done after 0 h, 24 h (only reactor 2), 48 h, 72 h (only reactor 2) and 120 h of storage.

G Lipid content of hydrolysate

The lipid content of hydrolysates as a function of hydrolysis time, with sampling at 30 min included, is shown in Figure 58. The standard deviation is not shown due to limited space between the series in the diagrams.

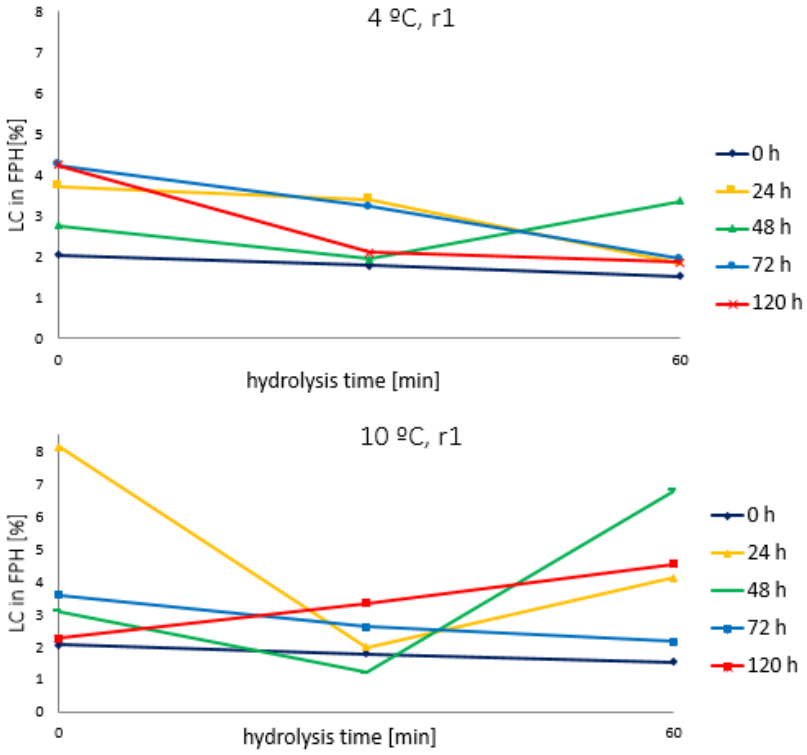

Figure 58: Lipid content of hydrolysates from reactor 1 as a function of hydrolysis time for raw material stored at 4 °C and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h and 120 h of storage. LC = lipid content.

H Degree of hydrolysis

The degree of hydrolysis in hydrolysates is shown in Figure 59 as a function of hydrolysis time and in Figure 60 as a function of storage time. The values are averages of reactor 1 and 2. The standard deviation is not shown in Figure 59 due to limited space between the series in the diagram.

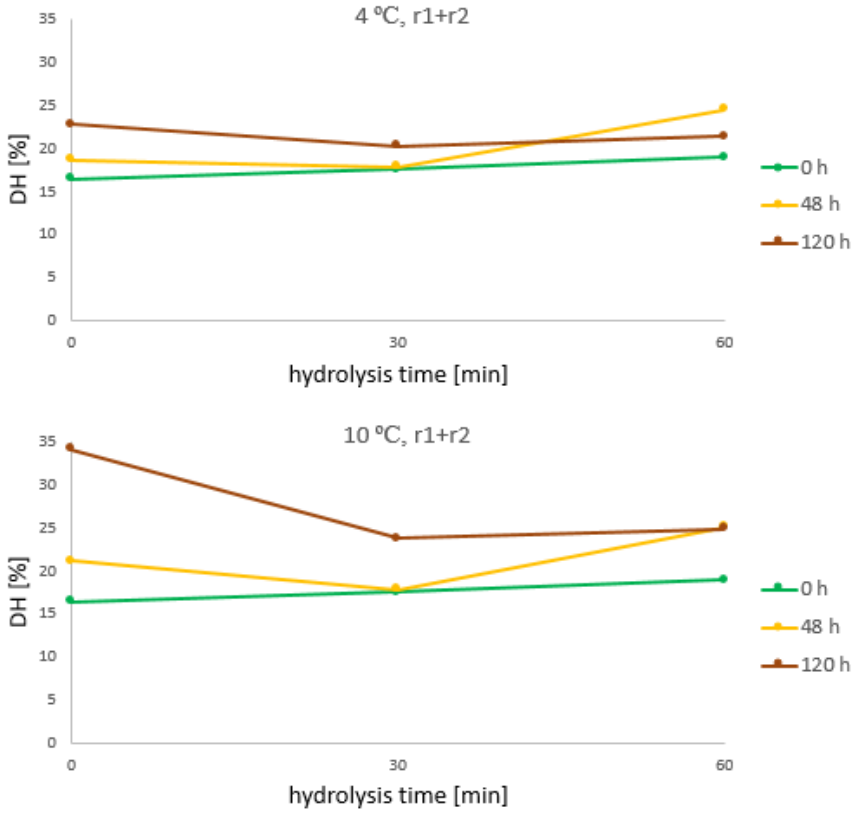

Figure 59: The degree of hydrolysis as a function of hydrolysis time for raw material stored at either 4 °C (top) or 10 °C (bottom). Sampling was done after 0 h, 48 h and 120 h of storage.

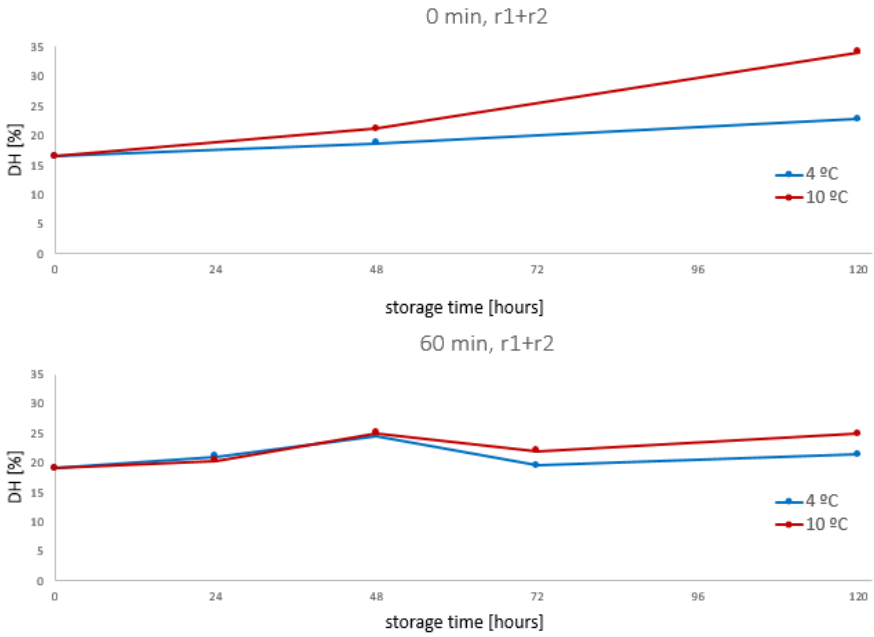

Figure 60: The degree of hydrolysis as a function of storage time of raw material after 0 min (top) and 60 min (bottom) of hydrolysis. Sampling was done after 0 h, 24 h (only 60 min), 48 h, 72 h (only 60 min) and 120 h of storage at either 4 °C or 10 °C.

I Acid soluble peptides

Standard curves used for determination of amount of acid soluble peptides are presented in Figure 61, Figure 62 and Figure 63.

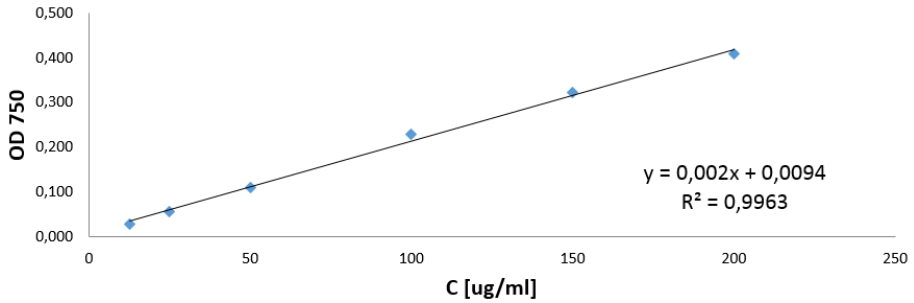

Figure 61: Standard curve 1 for determination of amount of acid soluble peptides in hydrolysates. Measured absorbance as a function of concentration of bovine serume albumin (BSA).

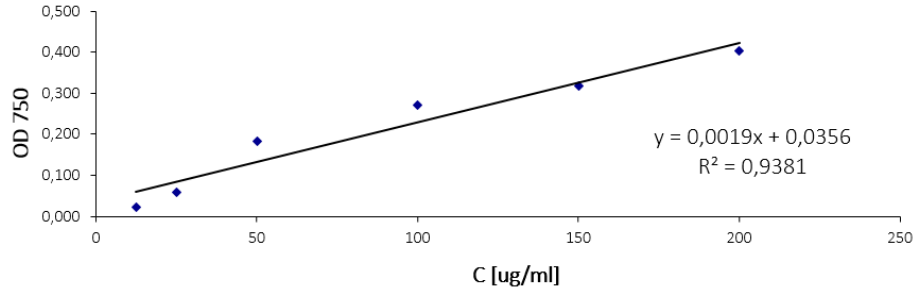

Figure 62: Standard curve 2 for determination of amount of acid soluble peptides in hydrolysates. Measured absorbance as a function of concentration of bovine serume albumin (BSA).

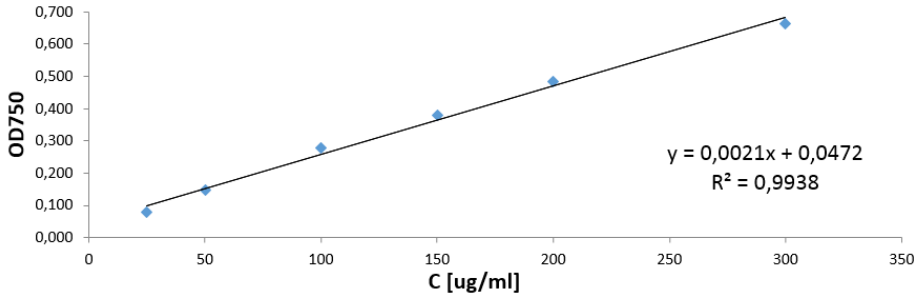

Figure 63: Standard curve 3 for determination of amount of acid soluble peptides in hydrolysates. Measured absorbance as a function of concentration of bovine serume albumin (BSA).

An overview over which standard curve was used for which samples and who analysed them, is given in Table 15.

Table 15: An overview over which standard curve was used for which samples and who analysed them.

Standard curve	**Who**	**Samples**
1	Erle Saltvedt	Reactor 2[a]
2	Fataneh Meldstad	Reactor 1
3	Maiken Lindgjerdet	10-2-2-60

[a] Except for sample ID 10-2-2-60 analysed by Maiken Lindgjerdet

J Molecular weight distribution

The standards used for estimating molecular weight fractions in the hydrolysates are listed in Table 16 together with their known molecular weight, obtained retention volume, V_R, and calculated available coefficient, K_{av}. Values for V_R were found by analysing the standards with fast protein liquid chromatography (FPLC) gel filtration and K_{av} was calculated by using Equation 16.

Table 16: Standards used to establish a relationship between molecular weight, M_W, retention volume, V_R, and available coefficient, K_{av}.

Standard	**M_W [Da]**	**V_R [ml]**	**K_{av}**
Aprotinin	6511	11,84	0,24
Vitamin B12	1355	16,87	0,55
Val-Glu-Ser-Ser-Lys	549	19,21	0,70

$$K_{av} = \frac{V_R - V_0}{V_T - V_0} \tag{16}$$

where V_0 = void volume = 8 ml, V_R = retention volume and V_T = total volume = 24 ml.

The three standards with known molecular weight were used to establish a relationship between molecular weight and retention time for peptide fractions of unknown weight. The relationship found is given in Equation 17 and the standard curve used is presented in Figure 64. K_{av} is calculated from the retention volume found by analysing the samples with FPLC.

$$log(M_W) = -2,3196 \cdot K_{av} + 4,3862 \tag{17}$$

In Table 17 the most pronounced retention volume peaks from different chromatograms are lised together with estimated molecular weight for the peptide fractions.

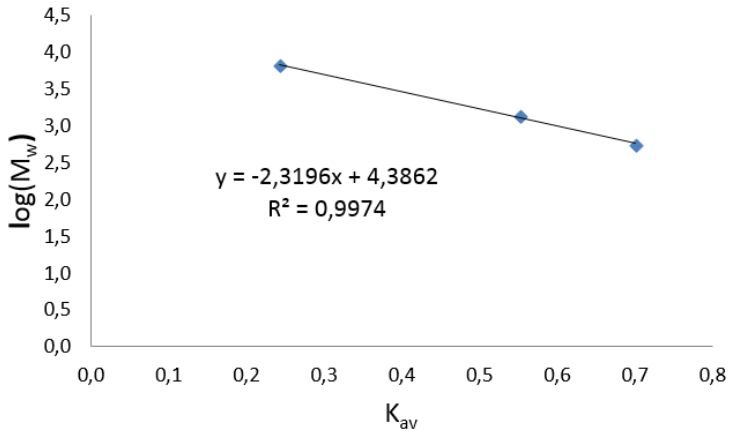

Figure 64: Standard curve for estimating molecular weight of peptide fractions in the hydrolysates. The logarithm of molecular weight, $\log(M_w)$, as a function of calculated available coefficient, K_{av}.

Table 17: Retention volume peaks from different chromatograms listed together with estimated molecular weight for the peptide fractions.

Retention volume peak [ml]	**K_{av} [-]**	**$\log(M_w)$ [-]**	**Estimated Mw [-]**
8	0,00	4,39	24333
18	0,64	2,89	782
22	0,84	2,43	269
24	0,97	2,14	138
26	1,14	1,75	56
29	1,28	1,41	26
30	1,39	1,15	14
34	1,63	0,62	4

The molecular weight distribution of peptide fractions in hydrolysates from reactor 2 from raw material stored at either 4 °C or 10 °C is presented in Figure 65, Figure 66, Figure 67 and Figure 68.

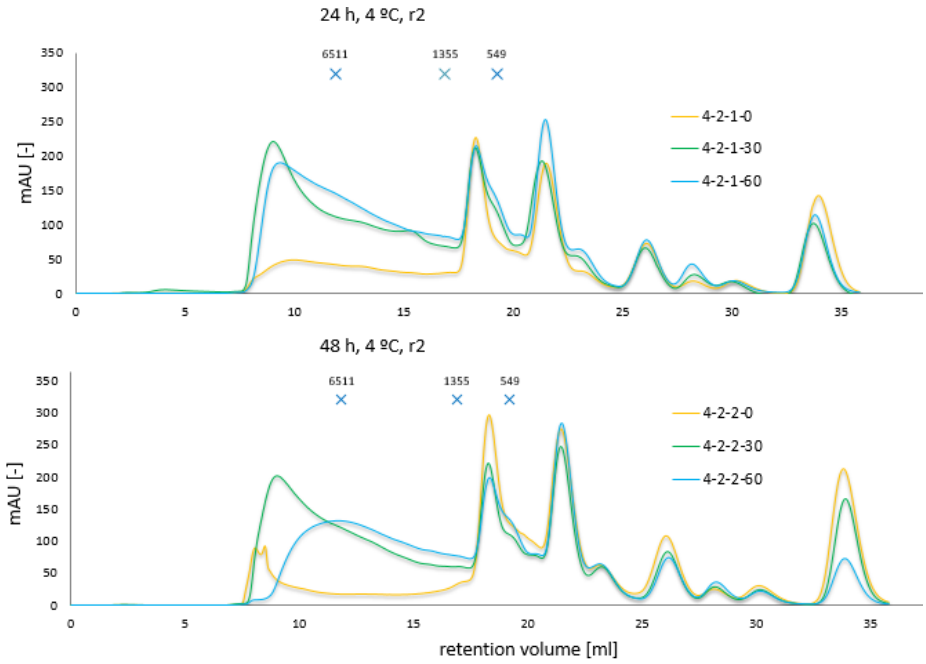

Figure 65: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (yellow), 30 min (green) and 60 min (blue) of hydrolysis for raw material stored at 4 °C. Sampling was done after 24 h (top) and 48 h (bottom) of storage. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

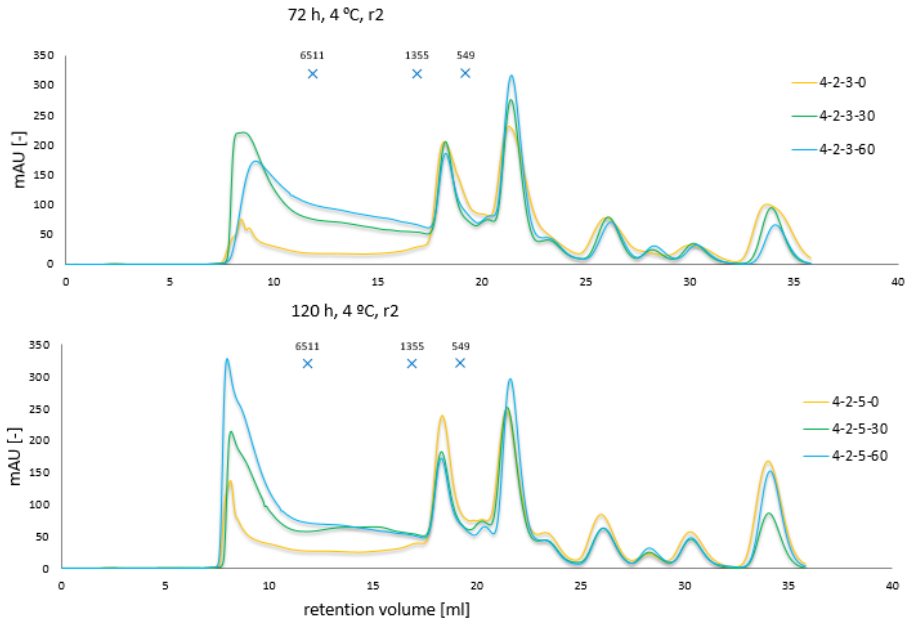

Figure 66: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (yellow), 30 min (green) and 60 min (blue) of hydrolysis for raw material stored at 4 °C. Sampling was done after 72 h (top) and 120 h (bottom) of storage. Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

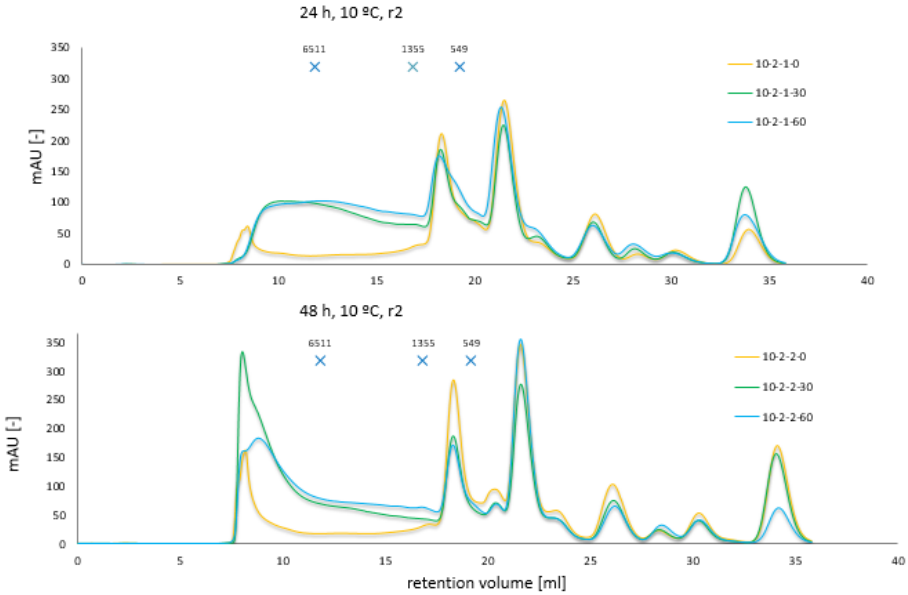

Figure 67: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (yellow), 30 min (green) and 60 min (blue) of hydrolysis for raw material stored at 10 °C. Sampling was done after 24 h (top) and 48 h (bottom) of storage. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

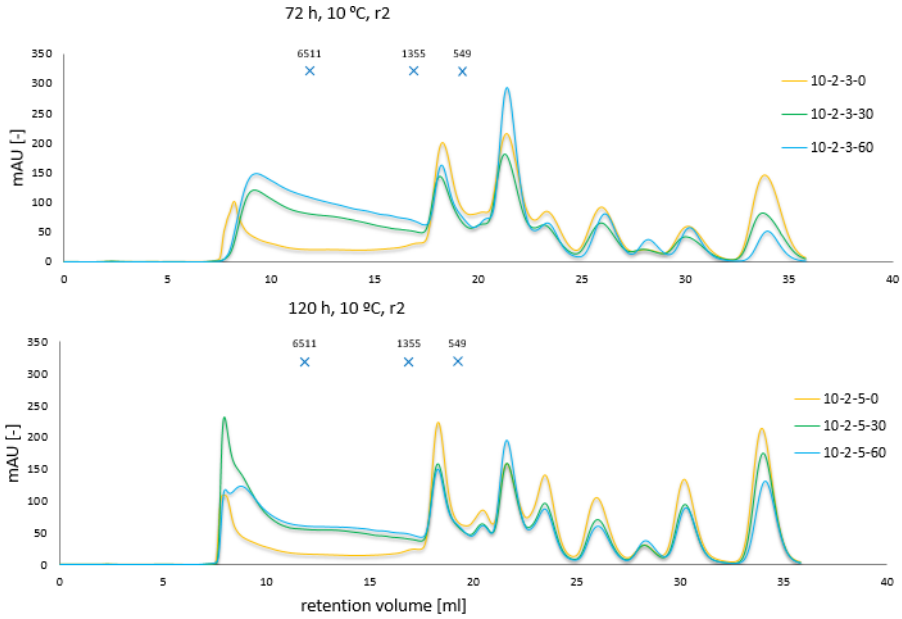

Figure 68: Molecular weight distribution of peptides in hydrolysates from reactor 2 after 0 min (yellow), 30 min (green) and 60 min (blue) of hydrolysis for raw material stored at 10 °C. Sampling was done after 72 h (top) and 120 h (bottom) of storage. Standards: Aprotinin (6511 Da), Vitamin B12 (1355 Da) and Val-Glu-Ser-Ser-Lys (549 Da).

K ACE-inhibitory activity

The ACE inhibitory activity of hydrolysates from reactor 2 is given as a function of hydrolysis time and storage time in Figure 69 and Figure 70, respectively.

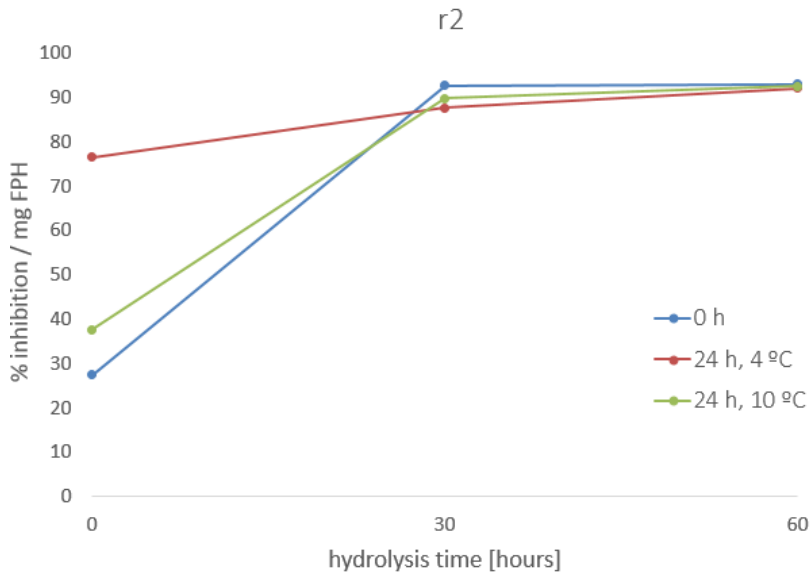

Figure 69: ACE-inhibitory activity as a function of hydrolysis time, given as % inhibition/mg FPH when the hydrolysate is present in the mixture, compared to if no hydrolysate (i.e. no inhibitor) is present at all. Sampling was done after 0 h and 24 h of storage of raw material at either 4 °C or 10 °C.

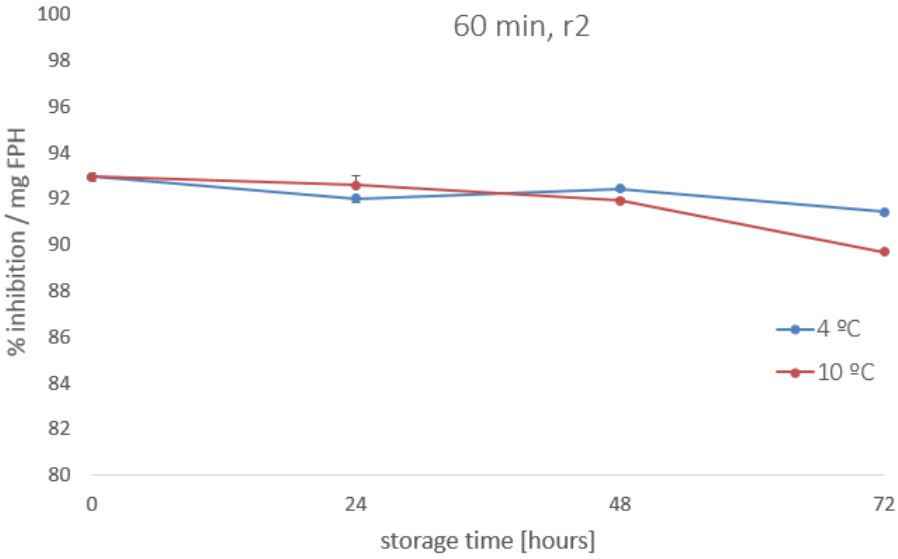

Figure 70: ACE-inhibitory activity as a function of storage time of raw material, given as % inhibition/mg FPH when the hydrolysate is present in the mixture, compared to if no hydrolysate (i.e. no inhibitor) is present at all. Sampling was done after 0 h, 24 h, 48 h and 72 h of storage at either 4 °C or 10 °C.

L Actin and myosin sequences of herring

The database of National Center for Biotechnology Information (NCBI)[119] was used to search for sequences of the muscle proteins myosin and actin from herring (*Clupea harengus*). The three sequences found are given below. Each letter corresponds to an amino acid for which the translation is given in Appendix N.

1) >gi|257792927|gb|ACV67302.1| alpha actin [Clupea harengus][5]
MCDDDETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMV
GMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNE
LRVAPEEHPTLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSL
YASGRTTGIVLDSGDGVSHNVPIYEGYALPHAIMRLDLAGRDLTDYLM
KILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEKSY
ELPDGQVITIGNERFRCPETLFQPSFIGMESAGIHETAYNSIMKCDIDIR
KDLYANNVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSV
WIGGSILASLSTFQQMWITKQEYDEAGPSIVHRKCF

2) >gi|14030595|gb|AAK52972.1|AF367621_1 skeletal muscle myosin heavy chain light meromyosin, partial [Clupea harengus][6]
EQDTSSHLERMKKNLEVTVKDLQHRLDEAENLAMKGGKKQLQKLES
RVRELEGEVEGEQRRGVDAVKGVRKYERRVKXLTYQTEEDKKNVTRL
QDLVDKLQLKVKAYKRQAEEAEEQANTHLSKCRKVQHELEEAEERAD
IAESQVNKLRAKGRDGGKGKEAAE

3) >gi|13274533|gb|AAK17967.1|AF329901_1 myosin heavy chain, partial [Clupea harengus][7]
LQRIKQKLEKEKSEYKMEIDDLSSNMEAVAKAKGNLEKMCRTLEDQLS
EIKAKSDENSRQLNDMNAQRARLQTENGEFSRQLEEKEALVSQLTRG
KQAFTQQIEDLKRHVEEEVKAKNALAHAVQSARHDCD

[5] `http://www.ncbi.nlm.nih.gov/protein/257792927?report=fasta` Accessed: 26.05.2015

[6] `http://www.ncbi.nlm.nih.gov/protein/14030595?report=fasta` Accessed: 26.05.2015

[7] `http://www.ncbi.nlm.nih.gov/protein/13274533?report=fasta` Accessed: 26.05.2015

M Myosin heavy chain sequence of zebrafish

The database of National Center for Biotechnology Information (NCBI)[119] was used to search for sequenced parts of the muscle protein myosin of zebrafish (*Danio rerio*). The sequence (1935 aa) is given below. Each letter corresponds to an amino acid for which the translation is given in Appendix N. Figure 72 and Figure 71 shows the output for sequence alignment of myosin heavy chain from zebrafish and herring.

```
Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: gi|66472732|ref|NP_001018321.1|          1935 aa
Sequence 2: gi|14030595|gb|AAK52972.1|AF367621_1      164 aa
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 89.02
Guide tree file created:   [clustalw2-I20150128-142011-0531-78487051-oy.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2      Score:3312
Alignment Score 844
```

Figure 71: Output for sequence alignment of myosin heavy chain from zebrafish and herring (sequence 2 in Appendix L).

```
Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: gi|66472732|ref|NP_001018321.1|          1935 aa
Sequence 2: gi|13274533|gb|AAK17967.1|AF329901_1      132 aa
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 90.15
Guide tree file created:   [clustalw2-I20150128-135909-0335-89339576-oy.dnd]

There are 1 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2      Score:2718
Alignment Score 691
```

Figure 72: Output for sequence alignment of myosin heavy chain from zebrafish and herring (sequence 3 in Appendix L).

>gi|66472732|ref|NP_001018321.1| myosin heavy chain 4 [Danio rerio][8]

MSTDAEMAVYGKAAIYLRKPEKERIETQNKPFDAKSACYVVDDKELY
VKGTIKSKDGGKVTVITLDTKEERVVKEDDVHPMNPPKYDKIEDMA
MMTHLNEPSVLYNLKERYAAWMIYTYSGLFCATVNPYKWLPVYDAEV
VAAYRGKKRMEAPPHIFSVSDNAYQFMLTDRENQSVLITGESGAGKTV
NTKRVIQYFATVAVQGGDKKKEQAPGKMQGSLEDQIIAANPLLEAYGN
AKTVRNDNSSRFGKFIRIHFGTTGKLASADIETYLLEKSRVTFQLPDERG
YHIFYQMMTNHKPELIEMTLITTNPYDFPMCSQGQITVASIDDKEELMAT
DSAIDILGFTGEEKMGIYKFTGAVLHHGNMKFKQKQREEQAEPDGTEEA
DKISYLLGLNSAELLKALCYPRVKVGNEFVTKGQTVPQVYNSVSALSKSIYE
KMFLWMVIRINQMLDTKQQRNFFIGVLDIAGFEIFDFNSMEQLCINFTNEK
LQQFFNHHMFVLEQEEYKKEGIVWEFIDFGMDLAACIELIEKPMGIFSILEE
ECMFPKATDVSFKNKLYDQHLGKCNAFQKPRPQKGKAEAHFSLVHYAG
TVDYNVNGWLDKNKDPLNESVVQLYQKSSVKLLATLYPPVVEETGGGKKGG
KKKGGSMQTVSSQFRENLGKLMTNLRSTHPHFVRCLIPNESKTPGLMENFLV
IHQLRCNGVLEGIRICRKGFPSRILYGDFKQRYKVLNASVIPEGQFIDNKKAS
EKLLGSIDVNHDEYRFGHTKVFFKAGLLGTLEEMRDEKLATLVTMTQALCRAY
LMRREFVKMMERRESIYTIQYNIRSFMNVKHWPWMKVYYKIKPLLKSAETE
KELATMKEDFVKCKEDLVKAEAKKKELEEKMVALLQEKNDLQLAVASEAENL
SDAEERCEGLIKSKIQLEAKLKETTERLEDEEEINAELTAKKRKLEDECSELKK
DIDDLELTLAKVEKEKHATENKVKNLTEEMASQDESIAKLTKEKKALQEAHQQ
TLDDLQAEEDKVNTLTKSKTKLEQQVDDLEGSLEQEKKLRMDLERAKRKLEG
DLKLAQESIMDLENDKQQSEEKIKKKDFETAQLLSKIEDEQSLGAQLQKKIKEL
QARIEELEEEIEAERAARAKVEKQRADLSRELEEISERLEEAGGATAAQIEMN
KKREAEFQKLRRDLEESTLQHEATAAALRKKQADSVAELGEQIDNLQRVKQK
LEKEKSEYKMEIDDLSSNMEAVAKAKANLEKMCRTVEDQLSEIKSKNDENLRQ
INDLSAQRARLQTENGEFGRQLEEKEALVSQLTRGKQAFTQQIEELKRQIEEE
VKAKNALAHAVQSARHDCDLLREQFEEEQEAKAELQRGMSKANSEVAQWRT
KYETDAIQRTEELEESKKKLAQRLQEAEEQIEAVNSKCASLEKTKQRLQGEVED
LMIDVERANALAANLDKKQRNFDKVLAEWKQKYEEGQAELEGAQKEARSLSTE
LFKMKNSYEETLDQLETLKRENKNLQQEISDLTEQIGETGKSIHELEKAKKTVETE
KAEIQTALEEAEGTLEHEESKILRVQLELNQVKGEIDRKLAEKDEEIEQIKRNSQR
VTEAMQSTLDSEVRSRNDALRIKKKMEGDLNEMEIQLSHANRQAAEAQKQLR
NVQAQLKDAQLHLDDAVRGQEDMKEQVAMVERRNTLMQSEIEELRAALEQT
ERGRKVAEQELVDASERVGLLHSQNTSLLNTKKKLESDLVQIQGEVEDTVQEAR
NAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNLEITVKDLQHRLDEAEN
LAMKGGKKQLQKLESRVRELESEVEAEQRRGADAVKGVRKYERRVKELTYQTE
EDKKNINRLQDLVDKLQLKVKAYKRQSEEAEEQANSHLSKLRKVQHELEEAEER
ADISESQVNKLRAKSRDAGKAKEE

[8] `http://www.ncbi.nlm.nih.gov/protein/66472732?report=fasta`
Accessed: 26.05.2015

N One and three letter codes of amino acids

One and three letter codes for the amino acids are given in Table 18[9]

Table 18: One and three letter codes for selected amino acids.

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamine or glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	trp	W
tyrosine	tyr	Y
valine	val	V

[9]Table accessed from the website of Food and Agriculture Organization of the United Nations (FAO) 21.05.2015, `http://www.fao.org/docrep/004/y2775e/y2775e0e.htm`