NTNU
Norwegian University of
Science and Technology

The Utilization of Mackerel (*Scomber scombrus*) Rest Raw Material Towards the Production of Oil and Protein

Effect of Storage Conditions on Yield, Quality, and Composition

Yazan Al-Janabi

Biotechnology (5 year)
Submission date: May 2016
Supervisor: Turid Rustad, IBT

Norwegian University of Science and Technology
Department of Biotechnology

NTNU – Trondheim
Norwegian University of
Science and Technology

The Utilization of Mackerel (*Scomber scombrus*) Rest Raw Material Towards the Production of Oil and Protein

Effect of Storage Conditions on Yield, Quality, and Composition

Yazan Al-janabi

MASTER THESIS

Department of Biotechnology
Norwegian University of Science and Technology

Biotechnology, a five-year integrated Master's degree

Filed: May 2016

Supervisor: Turid Rustad, NTNU Department of Biotechnology
Co-supervisor: Ana Karina Carvajal, Research manager at Process technology department, SINTEF Fisheries and Aquaculture AS

Preface

This study is a master's thesis as part of the five year study program in Biotechnology at the Norwegian University of Science and Technology, NTNU. The research was carried out through the SINTEF Fisheries and Aquaculture AS laboratories in cooperation with the biotechnology department at NTNU. It was conducted during the Fall-2015/Spring-2016 semesters (August-May) and counts for 60 credit points.

I would like to thank Ana Karina Carvajal and Turid Rustad for the invaluable guidance and much appreciated help over the course of this thesis. I would also like to thank engineer Merethe Selnes, engineer Marthe Schei, and engineer Vera Kristinova for their impeccable ability to impart practical knowledge, and keep a good atmosphere in the laboratory. Last but not least, i would like to thank research scientist Jannicke Fugledal Remme for running the molecular weight distribution analysis, leading to observations that were quite interesting.

I am as well grateful for the support and true selflessness that my parents have shown throughout my graduate and undergraduate years. Lastly i would especially like to thank my roommates for providing fun, laughter, and support.

Trondheim, 29.05.2016

Y.A.

Abstract

Norway has a fishing and aquaculture industry which does not utilize the North Atlantic mackerel (*Scomber scombrus*) resource to the fullest of its potential. This means that large amounts of rest raw material produced after filleting is either discarded, or utilized for feed production in the aquaculture industry, and is in general underused. The rest raw material has great potential in the production of high quality biomolecules for human consumption, and is therefore a major incentive for research. Mackerel rest raw material contains high amounts of beneficial and healthy omega-3 fatty acids, as well as being rich in protein. These valuable biomolecules can have various applications within the food, pharmaceutical, and cosmetic industries.

An increasing trend in global population leads to an increasing need to produce more food. Therefore, the repurposing of already available resources to a more optimal production of high quality, high yield valuable products, is needed. Optimization of resource usage is paramount to global welfare.

The aim of this master thesis was to study the effect of rest raw material storage conditions on the yield, quality, and functional properties of the lipid and protein molecules extracted by thermal treatment and endogenous hydrolysis. The rest raw material from North Atlantic mackerel consisted of all parts of the fish, except the fillet. The rest raw material contained 17.9 ± 0.4 % lipids, 12.7 ± 0.6 % protein, and 3.2 ± 0.01 % ash. The raw material was stored at either 4 or 10 °C for 0-120 hours, before being thermally treated for 15 minutes at 95 °C. The focus of the thermal treatment was towards the production of high quality, high yield products, i.e. crude marine oil and stickwater (protein). Quality parameters were also measured at an industrial standard.

Characterization of the chemical composition of the different rest raw material fractions, obtained from mackerel, was done on four different body parts; head, bellyflap, viscera, and backbone & tail. Lipid content was highest in bellyflap, 35 ± 0.8 %, lowest in viscera 7-8 ± 0.3 %, and between 15-20 ± 0.5 % in head and backbone & tail. Protein content ranged between 12-17 %, lowest in bellyflap, and highest in backbone & tail. While ash content was highest in head and backbone & tail 4.1-4.2 ± 0.1 %, and lowest in viscera 1.8 ± 0.01 %.

The storage experiment conducted, was done with a mixture of all rest raw material fractions. Crude oil extracted by means of thermal treatment (95 °C, 15 min) was done post endoge-

nous hydrolysis. The quality was high with respect to low peroxide value (PV), *para*-anisidine value (AV), and free fatty acid (FFA) content. The values obtained were well within the recommended limits for crude oil intended for human consumption, as long as the raw material was stored for 24 hours or less. Storage conditions play a major role in the quality of the crude oil, with respect to the total oxidation value (TOTOX). FFA levels increased with increases in storage time and were considerably higher in the raw material stored at 10 °C, as opposed to 4 °C. A correlation between storage conditions and oxidation status was found for several quality parameters. The crude oils oxidative stability was also analyzed, where increases in storage time and temperature lead to decreases in oxidative stability. Crude oil extracted from fresh raw material (0 hours of storage) had % FFA 0.5 ± 0.02, PV 5.3 ± 0.4 mEq/kg lipid, AV 1.0 ± 0.2, TOTOX 11.6 ± 0.3, and OSI 7.4 ± 0.1 hours.

The yield of crude oil increased slightly in the first 24 h, and was consistently a little higher for raw material stored at 10 °C, as opposed to 4 °C. Stickwater yield increased slightly in the first 48 hours, then decreased marginally between 48-120 h. The temperature of storage did not seem to consistently influence the stickwater yield. However, it should be noted that if the temperature did play a role, the higher temperature did have marginally increased stickwater yield.

Sludge yield decreases in the first 24 h, which is explained by increases in both oil and stickwater yields simultaneously.

Stickwater was simultaneously extracted by thermal treatment, post endogenous hydrolysis. The protein content in the stickwater ranged between 78.3 ± 0.6 % and 83.4 ± 0.2 % (using a total % (N) to protein conversion factor of 6.25). The protein content of the stickwater was higher for raw material stored at 10 °C as opposed to 4 °C. A correlation was found between the protein content and the storage time, protein content increased in the first 24 h - 48 h, then plateaued or decreased slightly after 48 h. The decrease may be correlated to the degree of hydrolysis and the incorporation of lipids into the stickwater fraction due to increased emulsifying ability.

The molecular weight distribution (MWD) of the proteins, peptides, and amino acids present in the stickwater fraction was analyzed. The MWD suggested that increased storage time leads to more endogenous hydrolysis, resulting in the degradation of large molecules to smaller ones. Cleavage happens due to the presence of exo- and endo-proteases. The increased

temperature of storage (10 °C) influenced the MWD by increasing the rate at which cleavage, by definition, hydrolysis proceeded. Higher temperature resulted in more rapid losses of concentration by large molecules, ultimately resulting in the rapid gain of concentration by the mid-range and small molecules.

Overall, yield and quality of crude oil and protein, were as expected, highly influenced by the raw materials storage conditions. However, the study has still shown that the production of high quality, high yield crude oil and protein from mackerel rest raw material is possible, and potentially very valuable. Challenges still remain in identifying the processes that mediate the high lipid content within the stickwater fraction, as well as dealing with the organoleptic properties (smell, taste, color) of the crude oil and protein products. The choice of process and storage condition is highly influenced by what the final product is intended for e.g. food industry, pharmaceuticals, or cosmetics. For the time being, with the challenges that exist for the production and processing of high quality mackerel rest raw material, production of crude oil might be the most feasible option.

Sammendrag

Fiskeri- og havburksnæringen i Norge utnytter ikke restråstoffet fra nord atlantisk makrell (*Scomber scombrus*) til sitt fulle potensiale. Dette betyr at store mengder restråstoff produsert etter filletering er enten kastet, eller brukt i produksjonen av fôr i akvakultur industrien. Restråstoffet er generelt underutnyttet. Restråstoffet har høy potensiale til produksjon av høy kvalitets biomolekyler til humant konsum. Derfor er det stor pågang i marin forskning. Makrell restråstoff inneholder høye mengder omega-3 fettsyrer som er helsegunstige, og er i tillegg rik på protein. Disse verdifulle biomolekylene kan ha varierte bruksområder innen næringsmiddel-, farmasyøtisk og kosmetisk industri.

En økning i verdensbefolkningen har ført til økt behov for alternative matkilder og for å optimalisere bruk av allerede tilgjengelige ressurser.

Målet med denne masteroppgaven var å studere effekten som lagringsforhold hadde på olje- og protein utbyttet og olje- og protein kvaliteten. Produktene ble ekstrahert ved hjelp av termisk behandling og endogen hydrolyse. Restråstoffet fra nord atlantisk makrell besto av hele fisken utenom filleten. Restråstoffet inneholdte 17.9 ± 0.4 % lipider, 12.7 ± 0.6 % protein og 3.2 ± 0.01 % aske. Råmaterialet ble lagret ved enten 4 eller 10 °C i 0-120 timer før påbegynt termisk behandling (15 min, 95 °C). Hovedfokuset var å produsere høykvalitets produkter med høy utbytte. Dette gjaldt produksjon av råolje (marine lipider) og limvann (protein). Kvaliteten ble også undersøkt i henhold til industrielle standarder.

Karakteriseringen av de forskjellige restråstoff fraksjonene ble gjort på fire forskjellige kroppsdeler: hode, buklist, innvoller, og ryggbein & hale. Lipid mengde var høyest i buklist fraksjonen og utgjorde 35.0 ± 0.8 %, laveste verdi var i innvollene 7-8 ± 0.3 %, og mellom 15-20 ± 0.5 % i hode og ryggbein & hale fraksjonene. Protein mengde varierte mellom 12-17 %, hvor laveste mengde var i buklisten og høyeste i ryggbein & hale fraksjonen. Aske innholdet var høyest i hode og ryggbein & hale fraksjonene 4.1-4.1 ± 0.1 %, og lavest i innvollene 1.8 ± 0.01 %.

Lagringsforsøket ble gjort på en blanding av all restråstoff (inkludert innvoller). Råolje ble ekstrahert vha. termisk behandling ved å varme restråstoffet ved 95 °C i 15 minutt. Kvaliteten var høy ved analyse av peroksid verdi (PV), anisidin verdi (AV) og mengde frie fettsyrer (FFA). Alle verdiene var innenfor de anbefalte grenseverdiene for råolje ment for humant konsum, sålenge

restråstoffet ble lagret i 24 timer eller mindre. Lagringsforholdene påvirket kvaliteten av råolje med hensyn til den totale oksidasjonsverdien (TOTOX). Mengden FFA økte med lagringstid og var høyere for restråstoff lagret ved 10 °C i motsetning til 4 °C. Det fantes flere sammenhenger mellom lagringsforhold og oksidasjonsstatus når det gjelder flere av kvalitetsparametrene. Råoljets oksidativ stabilitet ble også studert, hvor økt lagringstid og lagringstemperatur bidro til å minke den oksidative stibiliteten. Råolje ekstrahert fra fersk råmateriale (0 timer lagring) hadde % FFA 0.5 ± 0.02, PV 5.3 ± 0.4 mEq/kg lipid, AV 1.0 ± 0.2, TOTOX 11.6 ± 0.3, og OSI 7.4 ± 0.1 hours.

Utbytte for råolje økte i de første 24 timene med lagring og var høyere for råstoff lagret ved 10 °C sammenlignet med 4 °C. Limvannsutbyttet økte i de første 48 timene, og deretter minket marginalt mellom 48-120 timer. Lagringstemperaturen hadde ikke noe signifikant effekt på limvannsutbyttet.

Utbyttet til graksen minket i de første 24 timene, noe som er forklart av den samtidige økningen i utbytte hos både råolje- og limvanns fraksjonene.

Limvannsfraksjonen ble også ekstrahert vha. termisk behandling. Mengden protein i limvanns fraksjonen lå mellom 78.3 ± 0.6 % og 83.4 ± 0.2 % (ved bruk av forholdstallet 6.25 for beregning av protein fra total % (N)). Protein mengden i limvannet var høyere for restråstoff lagret ved 10 °C sammenlignet med 4 °C. Et forhold mellom protein mengde og lagringstid ble observert, hvor protein mengden økte i de første 24-48 timene, for så å platå eller minke etter 48 timer. Reduksjonen kan være korrelert til hydrolysegrad, der økt hydrolysegrad kan gi økt emulsifikasjonsegenskaper, noe som fører til økt inkorporering av lipider i limvanns fraksjonen.

Molekylvektsfordelingen (MWD) av proteinene, peptidene og aminosyrene som var tilstede i limvannet ble studert. MWD viste at økt lagringstid førte til mer endogen hydrolyse, noe som resulterte i økt degradering av store molekyler til små molekyler. Kløyving skjedde som følge av tilstedeværelsen av ekso- og endo-proteaser. Økt lagringstemperatur (10 °C) påvirket også molekylvektsfordelingen ved å øke mengden kløyving som fant sted, per definisjon; lengre hydrolyse. Høyere lagringstemperatur førte også til raskere nedbryting av de store molekylene, noe som førte til rask økning i mengden mellom-store og små molekyler.

Det konkluderes med at utbytte og kvaliteten av råolje og protein ble signifikant påvirket av restråstoffets lagringsforhold. Studien viser også at produksjon av høy kvalitets råolje og protein

fra makrell restråstoff er mulig, og potensielt veldig verdifull. Det finnes fortsatt flere utfordringer, deriblant å identifisere prosessen som fører til økt lipidinnhold i limvannet, og se på lukt, smak, og farge av både råolje og protein som ble ekstrahert. Valg av prosess og lagringsforhold er høyst påvirket av hva endeproduktet skal brukes til; næringsmiddel-, farmasøytisk, eller kosmetisk industri. Per dags dato, med de eksisterende utfordringene for produksjon av høylvalitets råolje og protein fra makrell resråstoff, så viser det seg at produksjonen av råolje er det beste alternativet.

Acronyms

0* Fresh raw material (0 hours of storage)

24_4C Raw material stored at 4 °C for 24 h

24_10C Raw material stored at 10 °C for 24 h

48_4C Raw material stored at 4 °C for 48 h

48_10C Raw material stored at 10 °C for 48 h

72_4C Raw material stored at 4 °C for 72 h

72_10C Raw material stored at 10 °C for 72 h

120_4C Raw material stored at 4 °C for 120 h

120_10C Raw material stored at 10 °C for 120 h

Abs Absorbance

AOCS American Oil Chemists Society

ALA α-Linolenic Acid (18:3n-3)

AV *para*-Anisidine Value

BB+T Backbone & tail

CHD Coronary Heart Disease

C.N. Total Carbon and Nitrogen

CVD Cardiovascular disease

DH Degree of Hydrolysis

DHA Docosahexaenoic Acid (22:6n-3)

DM Dry matter

DW Dry weight

EDTA Ethylenediaminetetraacetic Acid

EPA Eicosapentaenoic Acid (20:5n-3)

FAO Food and Agriculture Organization of the United Nations

FFA Free Fatty Acid

FPH Fish protein hydrolysate

FRS Free Radical Scavenger(s)

HPLC-MS High Performance Liquid Chromatography - Mass Spectrometry

ISO International Organization for Standardization

LA Linoleic Acid (18:2n-6)

LC Lipid content

LC-PUFA Long Chain - Polyunsaturated Fatty Acid

MO Molecular Orbital

MUFA Monounsaturated Fatty Acid

MWD Molecular Weight Distribution

O Oil

OSI Oil Stability Index

PC Protein content

PL Phospholipid

PUFA Polyunsaturated Fatty Acid

PV Peroxide Value

rm Raw material

rrm Rest raw material

SFA Saturated fatty acid

SL Sludge

sn Stereospecific numbering

SW Stickwater

TAG Triacylglycerole

TCD Thermal Conductivity Detector

TFA Trifluoroacetic Acid

TLC-FID Thin Layer Chromatography - Flame Ionization Detector

TOTOX Total oxidation value, (2× PV + AV)

WW Wet weight

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Background

In 2014 Norway produced 1.23 (aquaculture) and 2.20 (fisheries) million tonnes of fish and crustaceans [FAO, 2015]. According to the SINTEF yearly report in 2014 [Richardsen et al., 2015] on Norway's global raw livestock basis (aquaculture + capture), was around 3.43 million tonnes, which gave rise to a significant amount of rest raw material (885 thousand tonnes), around 26 % of the total mass. Approximately 70 % of the rest raw material (626 thousand tonnes) are utilized and further used mainly in the production of fishmeal and pet foods, as well as other components for human consumption in the form of marine foodstuffs and nutritional additives and/or supplements. The rest is considered waste. Marine rest raw material and by-products are common phrases used to explain the part of the fish or crustacean that is left after filleting. Rest raw materials naturally contain valuable and nutritional components, such as healthy lipids and proteins, further discussed in chapter 1.2.

Norwegian utilization of rest raw material is substantially high, but especially in the pelagic species such as herrings, mackerels, and capelins, the utilization of the "rest raw material" is close to a hundred percent [Richardsen et al., 2015].

In Norway, a major part of the rest raw material is utilized, however much is unknown about the potential that the pelagics sector may have. This is a result of the fact that the majority of mackerels are sold as round fish and no further processing is done in Norway. This ensures that no rest raw material is used, which in retrospect has potentially lead to a decreased incentive in

the utilization of mackerel rest raw material. The demand for a more process oriented industry is on the rise, and to improve knowledge of this valuable resource, this masters thesis will try to answer some questions about the composition of the North Atlantic Mackerel (*Scomber scombrus*), as well as give an overview of the chemical components present during rest raw material storage and how they affect the yield, quality, and shelf life of the products produced from the rest raw material (e.g. oil, proteins, minerals).

1.1.1 Scope and objectives

This master thesis characterizes the rest raw material of one of the most popular pelagic fish species, the North Atlantic mackerel (*Scomber scombrus*). The overall scope of this study is to provide insight into the composition and degradation of five different fractions of rest raw material. This includes analyzing the rest raw materials composition based on fish body part, and observe how differing temperatures and amounts of time influence the rest raw material during storage, as well as how they influence the products produced from said rest raw material. The results will provide valuable information regarding the possible value creation and possibilities for utilization of mackerel rest raw material.

1.1.2 Research questions

The central research questions in this thesis focus on both chemical composition and process parameters regarding the utilization of mackerel rest raw material. These questions have been studied previously in accordance with different fish species, country of origin, and type of extraction. However the rising industry of the pelagics within Norway gives rise to questions about the composition and processes, which need to be answered to increase value creation. Listed below are the research questions which this thesis attempts to answer:

- What is the composition and amount of rest raw material obtained from North Atlantic Mackerel (*Scomber scombrus*)?
- Which changes take place in the rest raw material during storage at different temperatures and as a function of storage time?

- How do storage conditions affect the outcome of the final products' (oil/protein production) yield, quality, and properties?

1.1.3 Thesis structure

A modified IMRaD format has been implemented throughout this thesis. Each chapter and their respective purposes has been explained in the list below. References and appendices are attached to the end of the report. A complete list of all acronyms used in this thesis are found at the beginning of the document.

- Introduction - Provides an introduction to the problem, gives context, and explains the objective.
- Methods and materials - Lists the methods and analyses used to generate, calculate, and analyze the data presented in the results chapter.
- Results and discussion - Presents the data gathered from the lab, and discusses the results and their implications, including their relationship in comparison to previous work.
- Conclusion - Answers the research questions.
- Future work - Discusses future prospects.

1.2 By-products and marine rest raw materials

The term *by-products* can be misinterpreted into being something negative, as it normally implies producing an *unwanted* product as a result of the production of the main product. That is why the best term(s) to use is either *marine rest raw materials* or co-products. Marine rest raw materials are everything left behind after the filleting of seafood. This includes the head, skin, viscera, backbone, fins, and any cutoffs. Rest raw materials are mainly defined by the fact that they require further processing before they can be sold [Rustad, 2006].

The definition of marine rest raw materials:

Rest raw materials are defined to be the non-primary products during the utilization of a resource. Primary resources in this case are fish and crustaceans that are farmed and/or harvested as a part of the Norwegian quota in Norwegian waters and/or is landed in Norway [Richardsen et al., 2015].

The definition may also depend on the species of marine organism. Some examples of delicacies such as; Cod tongue - A fried delicacy in northern Norway, Belly flaps, and Roe/Milt, are by the normal definition rest raw materials, however they are high value delicacies which are sold without further processing, and are not considered to be by-products.

The rest raw materials from Atlantic mackerel are head, backbone and tail, belly flaps, and viscera. Viscera includes heart, gills, stomach, liver, gallbladder, intestines, and roe/milt. Atlantic mackerel lies within the classification of being a pelagic species that is mainly harvested and not farmed in Norway. The pelagic sector includes herring (*Claupea harengus L.*), Atlantic mackerel (Scomber scombrus), and capelin (*Mallotus villosus*). Herring stands for the major portion of the rest raw materials produced, because as mentioned in chapter 1.1, mackerel is mainly sold as whole round fish [Richardsen et al., 2015]. In 2014 the Norwegian fleet generated 1.25 million tonnes of pelagics [Richardsen et al., 2015]. The 1.25 million tonne basis produced 162 thousand tonnes of rest raw materials, 13 % [Richardsen et al., 2015]. The rest raw materials are comprised of;

- Head - 42 000 tonnes 26 %.
- Backbone & tail - 59 000 tonnes 36 %.
- Bellyflap - 20 000 tonnes 12 %.
- Viscera - 35 000 tonnes 22 %.
- Skin - 6 000 tonnes 4 % - [Richardsen et al., 2015].

Dead fish, fish that are caught dead or have died during the harvesting process, and draft fish, fish that are of too low a quality to be processed directly into foodstuffs are considered as fractions that are practically used as rest raw materials (production of fishmeal and silage, however not allowed in the production of human food), but are in fact not measured/marked as being within the rest raw materials fraction. Rest raw materials from the pelagic fish species are mainly comprised of backbones/tails and heads [Richardsen et al., 2015]. The list above gives a

great incentive to utilize and exploit rest raw materials to the fullest extent. Rest raw materials are often divided into sub-groups:

- By-products that are only usable as fish/animal feed (e.g. dead fish, draft fish).
- By-products that are directly used as consumer products (e.g. liver, milt, roe, cod tongues).
- By-products which require extra processing in order to subsequently be sold for human consumption [Richardsen et al., 2015].

Rest raw materials are important in value creation and recycling. By-products that do not go directly to market in the form of foodstuffs still contain valuable and nutritional lipids and proteins [RUBIN, 2000]. Pelagic fish have high fat percentages and their by-products have a high potential for use as extracts for human consumption. Marine lipids are rich in polyunsaturated fatty acids(PUFAs). These include the two most important eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) [Aursand et al., 2011]. Marine lipids have also been proven to help reduce the incidence of CVD (Cardiovascular Disease), in particular the PUFAs EPA and DHA [Kris-Etherton et al., 2002]. The ratio of omega-3 to omega-6 PUFAs plays an important role in early brain development as well as neurotransmission, vital processes that are essential for normal brain function [Haag, 2003].Marine lipids are discussed in more detail in chapter 1.5.

There exists many different ways in which rest raw materials may be processed and refined. The production of fishmeal, silage, oils, and hydrolysates are the current processes. These methods extract the oil and protein within the rest raw materials for further utilization as nutritional products [Rustad et al., 2011].

The freshness of rest raw materials is an important factor to consider when utilizing rest raw material for human consumption. As mentioned above, rest raw material is divided into fractions based on what they are comprised of. These can further be divided into two groups: relatively stable by-products and easily degradable (unstable) by-products [Fennema et al., 2007]. The latter is comprised of by-products that contain large amounts of highly active endogenic enzymes (lipases and proteases found typically in blood and intestines), while backbones, heads, and skin contain less concentrations of endogenic enzymes, and are therefore more stable. The existence of high endogenic enzyme concentrations ultimately leads to the degradation of lipids into free fatty acids (FFA). This results in a higher rate of oxidation within the lipid fraction, and

leads to a lower oil quality [Rustad et al., 2011]. However the protein fraction benefits from the endogenic enzymes. Proteins get degraded into smaller peptides, which have higher nutritional value, and results in good functional properties. When using unstable by-products such as the viscera, freshness becomes extremely important. Viscera has very short shelf-life if not preserved or frozen [Rustad et al., 2011, RUBIN, 2000]. It is therefore paramount that the processing of viscera within the pelagic species happens directly after harvest. By utilizing more of the fish, and producing high quality extracts and foodstuffs, leading to products that have greater value.

1.3 Heat treatment

The separation of oil from fish requires the destruction of the cell membranes around the adipocytes (fat cells). This is achieved by heat treatment, as well as by the use of enzymes or low pH environments. Heat treatment is however the most widely used method for the extraction of oil, however other methods that are less practical and/or less viable do exist, including freezing or breaking adipocytes to liberate oils. Micro-scale extractions (laboratory-grade) include the usage of solvents, while macro-scale (industrial-grade) employs the use of mechanical/physical methods and prohibits the use of solvents due to the utilization of large quantities, and the issue of having solvent residues leftover in the products.

Traditional large scale extraction of marine oils involves heating the feedstock to high temperatures, pressing, and centrifugal separation. The negative sides to this method is the fact that many marine lipids can be degraded, leading to a higher possibility of oxidation within the fatty acids. This leads the industry towards finding other methods in which to extract marine oils without causing a higher risk of lipid oxidation to occur. The extraction of marine oils by enzymatic hydrolysis in comparison to the traditional method, gives higher yield and quality of oil [Aursand et al., 2011].

During hydrolysis, lipases will be active and lipolysis will occur in the rest raw material. This leads to a negative impact on the final product due to the susceptive nature of polyunsaturated fatty acids (PUFAs) to degrade and oxidize, resulting in the production of rancidity compounds. This is further discussed under chapter 1.7.

1.4 Hydrolysis

Hydrolysis is the process of cleavage of a molecules by the utilization of water. Hydrolysis of a protein happens when a water molecule attacks the peptide bond between the residues within the protein, figure 1.1. Hydrolysis of a lipid on the other hand cleaves the ester bond(s) between the triacylglycerole molecule and the individual fatty acids. Hydrolysis can either be performed chemically or enzymatically.

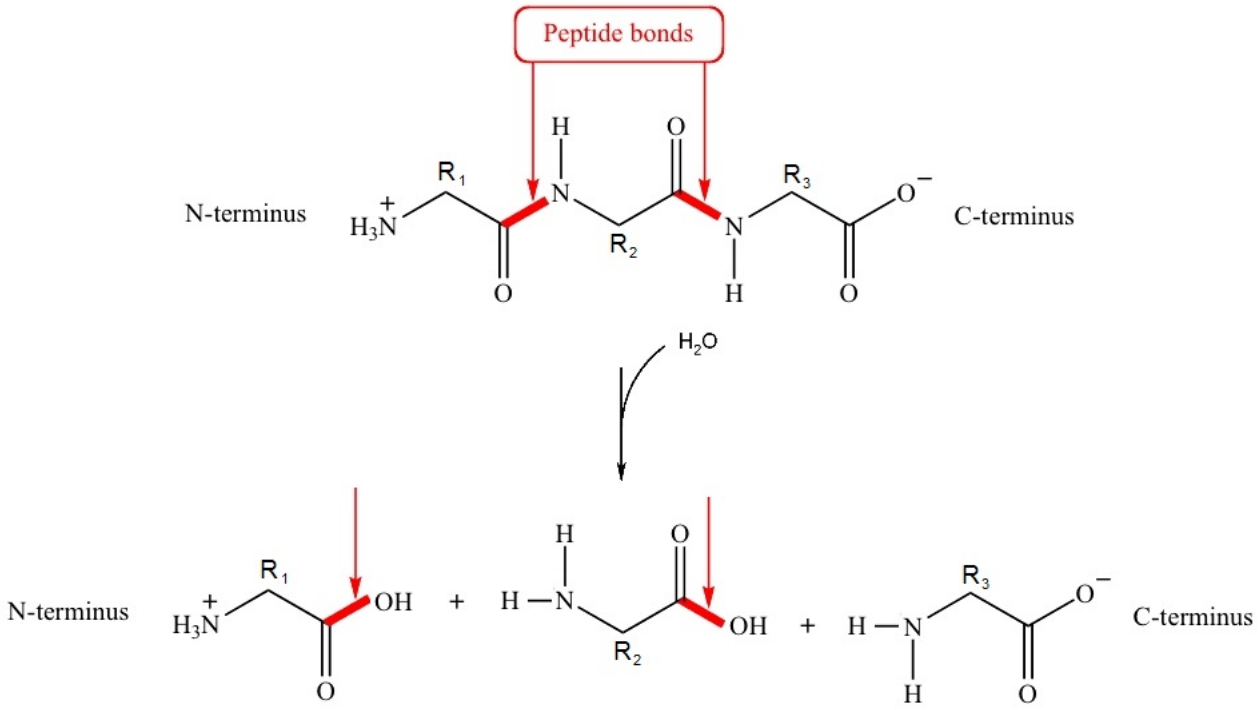

Figure 1.1: *Hydrolysis of a peptide bond* [Hardinger, 2015].

1.4.1 Chemical hydrolysis

Chemical hydrolysis is based on using acids or bases for the cleavage of the peptide bond between amino acid residues within proteins. This is economically favorable due to its simplicity and low-cost. However the negative sides of chemical hydrolysis is that the products are treated at high temperature, and low/high pH, which results in low quality products, low nutritional value, and loss of functional properties. Acidic hydrolysis results in the degradation of the essential amino acid Tryptophan. Chemical hydrolysis is also difficult to control and maintain at

constant rates. This results in the production of products that are varying in both composition and functional properties [Kristinsson and Rasco, 2000].

Chemical racemization

In conventional acid/base hydrolysis of marine rest raw materials the duration and temperature of the reaction play very important roles in influencing the end-product. Csapo et al. stated that the duration affects the degree of racemization of the hydrolyzate. Changing amino acids from their respective natural L- conformation to the D- conformation. This also affects the recovery yield. In comparison, when using acid/base protein hydrolysis, racemization is 1.2-1.6 times higher compared to the hydrolysis carried out at elevated temperatures of 160-180 °C (thermal treatment). This is due to the fact that proteins are hydrolyzed rapidly into free amino acids and racemization of free amino acids is always slower than that of amino acids bound to polypeptides at high temperatures. Proteins are hydrolyzed at a much slower rate during conventional acid hydrolysis, and the amino acids bound to polypeptide bonds are exposed to heat for a longer time, which results in racemization [Csapó et al., 2008].

1.4.2 Enzymatic hydrolysis

In the enzymatic version of protein/peptide hydrolysis, the enzymes are catalysts which lower the free energy of activation of the cleavage reaction, resulting in the amplified cleavage of the peptide bonds. Proteolytic enzymes cleave proteins and are called proteases, peptidases, and proteinases. Depending on their respective specificities and recognition of substrate, they produce different peptides when a protein is cleaved [Kristinsson and Rasco, 2000].

Enzymatic hydrolysis is achieved by the utilization of endogenous enzymes already present in the raw material, or by the addition of commercial enzymes. Endogenous enzymes are associated with digestional organs, such as the liver and intestines, which are contained within the viscera of most marine species. Hydrolysis is more efficient when using endogenous rather than commercial enzymes, however the use of commercial enzymes provides great control of the properties produced, and is therefore of utmost importance when producing high quality protein extracts. To provide high control and reproducibility when utilizing endogenous en-

zymes, it is important to have qualitative and quantitative knowledge of the enzymes in question [Fennema et al., 2007].

Enzymes are highly dependent and susceptible to temperature, as described by the Arrhenius equation, a formula for the temperature dependence of reaction rates. Change in temperature is highly correlated to a change in reaction rates and enzyme kinetics. Increase in temperature results in an increase in reaction rates due to the increase in kinetic energy of the reactants in the system. At a constant increase in temperature, enzyme kinetics and reaction rates increase, resulting in higher enzymatic activity, however the reaction will eventually reach a plateau where the enzyme becomes denatured and loses activity [Fennema et al., 2007].

Hydrolysis cleaves proteins into smaller peptides and separates oils. The fractions obtained after the hydrolysis of fish by-products are as follows;

1. Oil phase - top layer, containing mostly hydrophobic compounds.
2. Emulsion - a phase system containing hydrophobic- and hydrophilic compounds, as well as an emulgator that is amphiphilic.
3. Water phase - contains mostly hydrophilic compounds.
4. Sediment - Solid particles and sludge, bottom layer.

The oil phase contains a variety of different non-polar hydrophobic lipids. Emulsions are a mixture of oil and water that are emulsified by an emulgator that is amphiphilic. The water phase contains polar hydrophilic proteins and other water-soluble components. The sediment phase is mainly bone, insoluble proteins, and other components that are non-soluble.

Enzymatic racemization

The main advantage of enzymatic hydrolysis as opposed to chemical hydrolysis of proteins is that the process is much more gentle, and allows the production of fragile amino acid residues, such as glutamine, aspargine, and other residues that are normally destroyed by acid/base interaction. Enzymatic hydrolysis also does not result in any racemization during digestion, due to the stereospecific bonding of enzymes [Kim and Wijesekara, 2010].

1.5 Marine lipids

Solubility is the main physical attribute that defines lipids, they are soluble in non-polar, hydrophobic compounds. Lipids in general are hydrophobic (non-polar), and do not possess any common structural features. Naturally occurring fats, such as marine lipids, are inherently esters formed by propane-1,2,3-triol (glycerol) and fatty acids [Freemantle, 1995]. Lipids are normally divided into two distinct categories [Gunstone, 1996];

1. Neutral lipids
    - Triacylglyceroles (TAG) - figure 1.2
    - Wax - esters of a fatty acid and an alcohol.
2. Polar lipids
    - Phospholipids - figure 1.3
    - Sphingolipids

TRIACYLGLYCEROLES "NATURAL"

The esterification of three fatty acids to a glycerol molecule at three distinctly different positions results in the synthesis of a triacylglycerole (TAG). The fatty acids that are connected do not need to be identical, but they can be. Both lean and fatty fish store their lipids in the form of triacylglyceroles in adipose and muscle tissue. In fish, phospholipids are found mainly in the cell membrane providing fluidity and structural support [Phleger et al., 2002].

ETHYL ESTERS "SYNTHETIC"

Ethyl esters are a type of fatty acids that are cleaved from their respective glycerol molecule by the aid of hydrolysis. These are thought of as pre-digested and are easily absorbed through the intestinal wall [Phleger et al., 2002].

PHOSPHOLIPIDS

Mainly found in cell membranes, phopholipids are important in terms of structure and fluidity in membrane matrices. Phospholipids are defined by the characteristic of being amphiphilic (both hydrophilic and hydrophobic), due to their structure. A phospholipid molecule consists of a "head" group, which is hydrophilic, and a tail consisting of fatty acid long chains, thus ensuring the physical property of amphiphilia. Most "head" groups pertaining to phospholipids

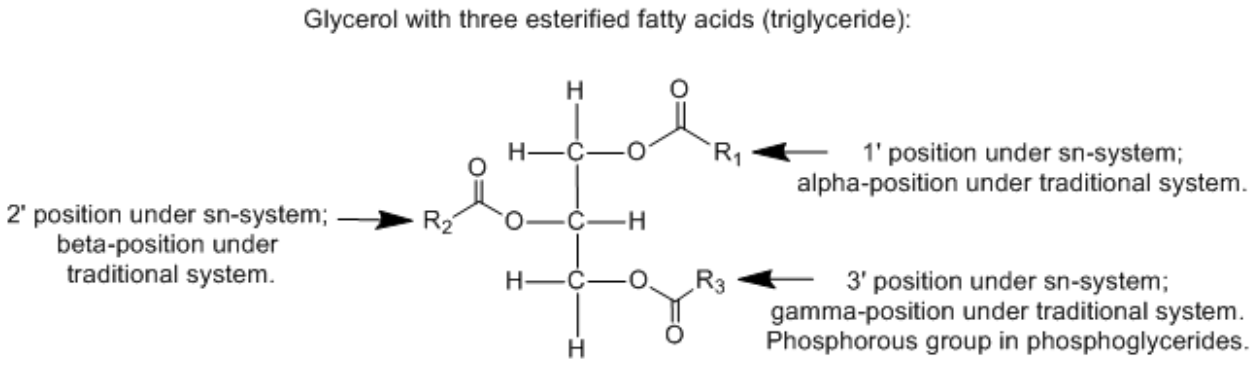

Figure 1.2: *Triacylglycerole: stereospecific numbering ("sn" system), as well as traditional glycerol numbering (alpha, beta, gamma system). Drawn using ChemDraw 15.0 software* [IUPAC, 1978].

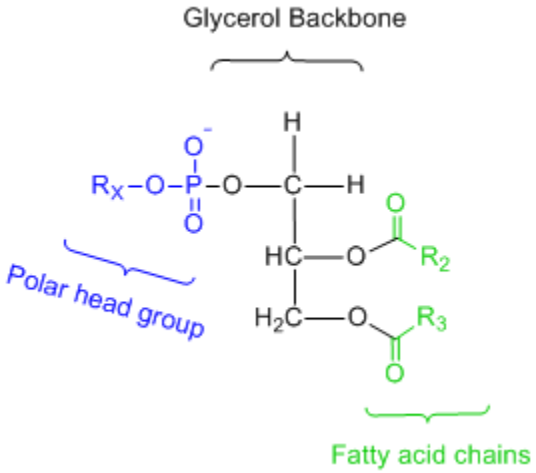

Figure 1.3: *A general illustration of a phospholipid comprised of the glycerol backbone (black), the fatty acid chains (green), and the polar head group (blue), which contains a phosphate group (polar). The illustration is adapted from Lehninger et al., and drawn using ChemDraw 15.0 software* [Lehninger et al., 2013].

contain one or more phosphate group(s), and belong to the phosphoglycerols family of compounds [Aursand et al., 2011].

Fatty acids as triacylglyceroles, ethyl esters, and phospholipids are relatively stable, meaning that they do oxidize, but not as easily as their counterpart, namely - free fatty acids (FFA). Free fatty acids can be considered as volatile (less than 6 carbon atoms) and are easily oxidized

[Aursand et al., 2011].

1.5.1 Fatty acids

Fatty acids are carboxylic acids containing a linear hydrocarbon chain (-CH_2-) that may vary in size from 2-22 carbon atoms. They have a carboxylic end (-$COOH$) and a methyl end (-CH_3) (also called terminal end). Fatty acids can be categorized into three different groups depending on their organic structure [Aursand et al., 2011].

1. **Saturated (SFA):** These fatty acids have the maximum amount of hydrogen atoms covalently bound. The general chemical formula for saturated fatty acids is; $CH_3-(CH_2)_n-COOH$. When n is low the acid is known as a short chain acid, and vice versa, when n is high the acid is known as a long chain acid [Freemantle, 1995].

2. **Monounsaturated (MUFA):** These fatty acids contain ONLY one double bond. They may also be distinguished as short or long chained acids [Freemantle, 1995]. The general formula is the same as above, just minus 2 hydrogen atoms, resulting in the formation of a double bond between two neighboring carbon atoms [Aursand et al., 2011].

3. **Polyunsaturated (PUFA):** Fatty acids that contain at least two or more double bonds within their carbon chain [Freemantle, 1995].

For most naturally occurring fatty acids within food, the number of carbon atoms that comprises the chain is an even number ranging between 12-18. The unsaturated double bonds of both MUFA and PUFA are in the *cis*-configuration, and when in proximity of several other double bonds within a fatty acid, they are methylene interrupted. Methylene interruption is the fact that the double bonds are not neighboring each other, but rather have a methylene unit/bridge (-CH_2-) between each double bond [Aursand et al., 2011].

Nomenclature

Fatty acids have different naming methods in which they are described structurally with numbers. Three naming systems that are used widely; the example fatty acid is eicospentaenoic acid (EPA), see table 1.1.

The three systems define a fatty acid as; the number of carbon atoms in the linear hydrocarbon chain, followed by a colon symbol (:), then the total number of double bonds. These are then (usually) followed up by the position of the first double bond from the terminal end (for the ω nomenclature), or the position of the double bond from the carboxylic end (for the Δ nomenclature) [Aursand et al., 2011]. However, of the three methods of nomenclature, the n-x system is the most popular when writing scientific documents, table 1.1.

Table 1.1: Fatty acid nomenclature [Rigaudy and Klesney, 1979].

System	Explanation	Example (EPA)
Trivial nomenclature	Non-systematic historical names.	Timnodonic acid
Systematic nomenclature	IUPAC name, derived from the standard IUPAC for the nomenclature of organic chemistry.	(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-eicosapentaenoic acid
Δ^X - (delta-*x*) nomenclature	The Δ^X indicates a double bond within the fatty acid structure, where the X indicates the location of the double bond based on the x'th carbon-carbon bond counting from the carboxylic end.	*cis, cis, cis, cis, cis*-Δ^5, Δ^8, Δ^{11}, Δ^{14}, Δ^{17}-eicosapentaenoic acid or; *cis*-Delta(5, 8, 11, 14, 17)-eicosapentaenoic acid
n-x nomenclature	n-x (also called ω-x) is the most widely used type of nomenclature for the definition of fatty acids. The x designates the first double bond from the terminal (methyl) end of the fatty acid. Keeping in mind that most long chain fatty acids are methyl interrupted.	n-3 or ω-3, normally accompanied by lipid numbers as mentioned under.
Lipid numbers	These numbers are represented as C:D, where C is the number of carbon atoms in the fatty acid (chain length), and D represents the number of double bonds present. If more than one double bond is present, then they are presumed to be methylene interrupted. These numbers are usually accompanied by n-x, ω-x, or the Δ-x designation.	20:5 (n-3), 20:5 (ω-3), or 20:5 (Δ-5)

Beneficial health effects related to marine lipid consumption and the essential fatty acids

In 1971 Bang et al. observed and reported findings connected to a lower level of cholesterol, β-lipoproteins, triacylglyceroles, and total plasma lipids in the isolated Inuit population of west Greenland. This sparked interest, as the population was known to have a PUFA rich diet with origins from marine resources. The control group was of Danish origin [Bang et al., 1971]. Observations lead to the well known fact that LC-PUFAs had preventative characteristics and protected against high levels of plasma cholesterol, which is greatly associated with coronary heart disease (CHD) [Baybutt et al., 2002, Abeywardena and Head, 2001]. The metabolism of triacylglyceroles and β-lipoproteins is closely connected to carbohydrate turnover and therefore also to Diabetes mellitus [Rutledge et al., 2010]. Their observations in this population therefore gave an explanation of the low frequency of occurrence, for CHD and Diabetes mellitus. Interest in dietary PUFAs arose and investigations have since shown that consumption of marine LC-PUFAs lowers susceptibility to CHD and Diabetes mellitus [Abeywardena and Head, 2001, Baybutt et al., 2002].

The two main classifications of marine PUFAs are; n-3 (ω-3) and n-6 (ω-6). The n-3 and n-6 PUFAs are obtained from their parent fatty acids, α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) respectively [Gurr, 1999, Frankel, 2014]. These parent fatty acids are essential, meaning that humans are not able to biochemically synthesize them, and therefore must be replenished through dietary sources [Frankel, 2014]. Structurally, all n-3 and n-6 fatty acids have their first unsaturated bond "double bond" located at the third carbon and sixth carbon respectively; counting from the terminal methyl end [Rigaudy and Klesney, 1979]. The metabolism of the long chain n-3 α-linolenic acid through multiple desaturations and elongations, results in the production of n-3 fatty acids, amongst them are two very important compounds, namely eicosapentaenoic acid (EPA, 20:5n-3) and docosahexanoic acid (22:6n-3). However this process is not effective in humans, the turnover and conversion ratios of the reaction are low [Anderson and Ma, 2009]. It is therefore usually recommended that EPA and DHA are supplemented regularly through the diet [NDH, 2015].

1.6 Lipid degradation

Lipolysis of fish lipids occurs *post mortem*, this entails the hydrolysis of fatty acids from TAG and PL molecules. This process results in several compounds, however, the most important being FFA. The highest activity of lipolysis is found in places with high concentrations of lipases (endogenous enzyme), such as liver and viscera of fish. Therefore rest raw materials containing viscera have generally higher amounts of FFA upon storage.

1.7 Lipid oxidation and rancidity

Lipid oxidation is a general term for the complex sequence of chemical reactions that ensue upon the interaction of lipids and oxygen. These result in the degradation of oil, and are responsible for the production of volatile substances, giving off an aroma that we associate with oil rancidity (oxidative rancidity). The oxidation reactions occur between the fatty acid molecules and oxygen, on triacylglyceroles, phospholipids, free fatty acids, and fatty acid esters. The oxidation reactions described further in this chapter. The oxidative stability of oil is dependent on;

- Oil type
- Fatty acid composition
- Conditions of storage:
    - Temperature
    - Light
    - Oxygen availability and type
- Emulsions present
- The amount of pro-oxidants and antioxidants present [Wilailuk et al., 2007].

The degree of oxidation is directly correlated to the amount of unsaturation (number of double bonds) present on the fatty acid. The more saturated the lipids are, the less likely they are to be oxidized, and vice versa, an increase in the degree of unsaturation will result in an increase

in the susceptibility of oxidation. Marine lipids contain high amounts of n-3 LC-PUFAs, which makes them highly susceptible to oxidation [McClements and Decker, 2000].

1.7.1 Mechanisms of oxidation; primary oxidation products

A direct approach of atmospheric oxygen to any fatty acid cannot occur naturally. Interaction is prohibited due to oxygen's molecular orbital (MO) build and that it is thermodynamically unfavorable because of spin conservation, figure 1.4. However, if any of the reaction substrates are in the activated form (higher energy), then the reaction is allowed and proceeds to produce off-flavor compounds making the oil less acceptable, or unacceptable for industrial use and/or consumers [Choe and Min, 2006]. The initiation step of oxidation in an oil can be classified as follows:

- Autoxidation; the spontaneous interaction between fatty acid radicals and atmospheric oxygen, via the activation of a fatty acid into a fatty acid radical [Choe and Min, 2006].

- Light induced oxidation (photosensitized oxidation); exposure to light activates photosensitizers, resulting in the activation of oxygen (singlet oxygen), which can directly interact with fatty acids [Choe and Min, 2006].

- Enzymatic oxidation; catalyzation of marine lipid oxidation by enzymes present in fish (endogenous) or enzymes added (exogenous) [Aursand et al., 2011].

Both autoxidation and photo-oxidation are responsible for the oxidation of edible oils during transport, storage, or processing, and these are dependent on the type of oxygen species present. There are two different types of oxygen species that can react with edible oils; atmospheric triplet oxygen ($^3O_2$) and activated singlet oxygen ($^1O_2$). Triplet oxygen can only react with fatty acids that are radicalized (activated form), and causes autoxidation, which is a free radical chain reaction. Singlet oxygen on the other hand is a product of the activation of triplet oxygen by photosensitizers, and can oxidize fatty acids due to its "active" and short-lived nature [Choe and Min, 2006]. Both mechanisms are further discussed in detail below.

Autoxidation and the free radical chain reaction

Triplet oxygen's ($^3O_2$) chemical properties towards lipid radicals is explained by it's molecular orbital diagram, figure 1.4. The ground state of $^3O_2$ has two unpaired electrons in the 2pπ antibonding orbitals, which results in a permanent magnetic moment (para-magnetic molecule). $^3O_2$ has three hybridized energy states and is therefore called triplet oxygen. The two unpaired electrons make $^3O_2$ a radical. Because of the electrons spin-moment, they react with radicalized food compounds (fatty acids, etc.) and retain their spin, e.g. spin conservation. If the food compound is not a radical, then spin conservation will hinder the thermodynamically unfavorable reaction to occur [Choe and Min, 2006].

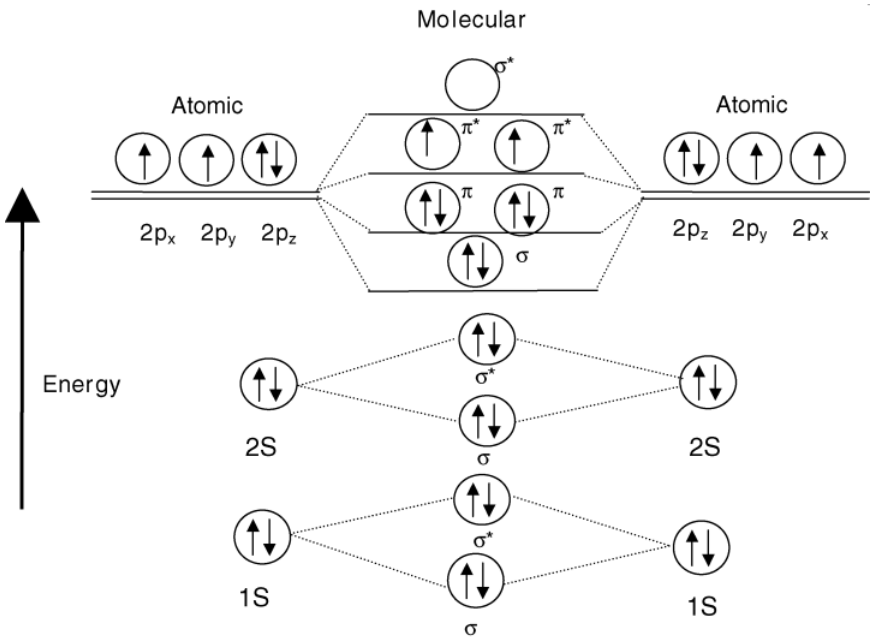

Figure 1.4: *Molecular orbital of triplet oxygen, ($^3O_2$). Adopted from Shriver et al.* [Shriver, 2014].

The free radical chain reaction is a sequence of steps that define the autoxidation of edible oils. Initiation, propagation, and termination.

Initiation:

$$RH \quad \rightarrow \quad R^* + H^* \tag{1.1}$$

Propagation:

$$R^* + {}^3O_2 \quad \rightarrow \quad ROO^* \tag{1.2}$$

$$ROO^* + RH \quad \rightarrow \quad ROOH + R^* \tag{1.3}$$

Termination:

$$ROO^* + R^* \quad \rightarrow \quad ROOR^1 R^* + R^* \quad \rightarrow \quad RR^1 \tag{1.4}$$

(R= lipid alkyl, R^*= lipid radical, ^{1} = Non-radical species)

In the initiation step the hydrogen atom of fatty acids or acylglyceroles is removed. The energy required for such cleavage is dependent on the hydrogen atoms position within the fatty acid, and can be facilitated by metal catalysts, heat, and light. Lipid alkyl radicals react with atmospheric triplet oxygen ($^3O_2$) and form lipid peroxy radicals (ROO^*), these are reactive as well. The lipid peroxy radical will then, through hydrogen abstraction, remove a hydrogen atom from other fatty acids forming hydroperoxides ($ROOH$) and another lipid radical. Due to the abundance of radicals present, and how they influence the oxidation reactions, the process has been named the free radical chain reaction, figure 1.5. When finally two radicals react with each other, termination of the chain reaction occurs [Choe and Min, 2006].

Photosensitized oxidation and activation of singlet oxygen

When marine oils contain photosensitizers (pigments such as porphyrin or riboflavin) and undergo light exposure, atmospheric triplet oxygen ($^3O_2$) can be converted into it's active form - singlet oxygen ($^1O_2$). The photosensitizers absorb light rapidly becoming excited. The return to ground-state is preferable and the photosensitizer emits the extra energy as light, heat, or

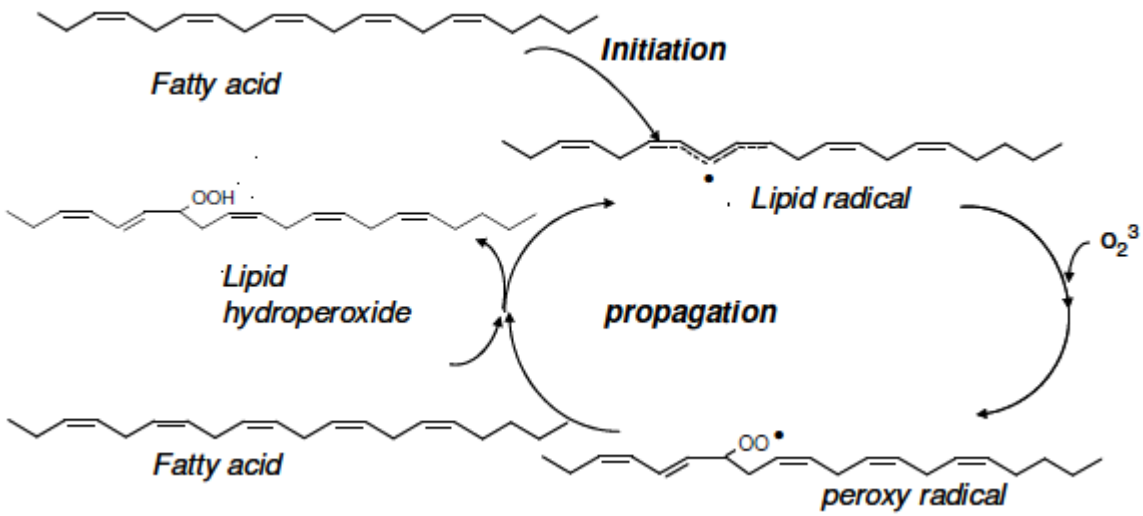

Figure 1.5: *An illustration of fatty acid autoxidation, the free radical chain reaction.* ***Initiation:*** *the formation of lipid radicals due to interaction with activated oxygen species (such as OH^*, O_2^* etc.), by transition metals, by photosensitizers, or by thermal cleavage.* ***Propagation:*** *Lipid radicals that are formed react with atmospheric triplet oxygen ($^3O_2$), resulting in peroxy radicals, which in turn act as radicals and react with another unsaturated fatty acid. The chain of autoxidation is thereby propagated. Adopted from Aursand et al.* [Aursand et al., 2011].

converts other non-radicalized molecules to radicals by activation (mainly $^3O_2$). The electron configuration of $^1O_2$ differs slightly from that of $^3O_2$. The 2pπ antibonding orbitals in the MO diagram 1.6, show that one has two paired electrons, and the other is empty. In comparison, the MO diagram of $^3O_2$ (figure 1.4) contains one electron in each antibonding orbital. This makes $^1O_2$ electrophilic and non-radical. The electrophilic $^1O_2$ is highly reactive with compounds containing high electron densities, such as the double bonds of unsaturated fatty acids [Choe and Min, 2006].

Figure 1.6: *Electronic configuration of 2pπ antibonding orbital of singlet oxygen, adopted from Choe et al.* [Choe and Min, 2006].

Enzymatic oxidation

Enzymatic oxidation depends on enzymes acting as catalysts in the oxidation reactions. The presence of endogenous enzymes in rest raw materials is obvious, with viscera containing the most part, however the circulatory system also contains some enzymes that can initiate or catalyze lipid oxidation. There are several mechanisms connected to distinct groups of enzymes. Lipases and phospholipases (viscera) catalyze the cleavage of fatty acids from the triacylglyceride, leading to the formation of free fatty acids. FFA in general are low-molecular-weight compounds that are volatile and unstable, resulting in high susceptibility to oxidation. Lipoxygenases are iron-containing enzymes that catalyze the addition of O_2 to a fatty acid, resulting in the formation of lipid hydroperoxides (primary oxidation products). Myeloperoxidase can initiate lipid oxidation when hydrogen peroxide and halogens such as bromine or iodine are present. These are critical during the processing of rest raw materials due to the interaction between atmospheric air (oxygen), blood, and marine lipids [Aursand et al., 2011].

1.7.2 Mechanisms of oxidation; secondary oxidation products

Primary oxidation products decompose to secondary oxidation products in the presence of transition metals and high temperatures. Homolytic cleavage between the oxygen atom and the oxygen bond on hydroperoxides (primary oxidation products) leads to the formation of alkoxy radicals. β-scission (figure 1.7 of the carbon-carbon bond in the alkoxy radical produces intermediary compounds leading to the formation of mostly low-molecular-weight volatile substances such as aldehydes, ketones, esters, alcohols, short-chain hydrocarbons, and acids [Choe and Min, 2006]. Because of these unstable and volatile substances, secondary oxidation products are responsible for the off-flavors and smells that we associate with rancidity in oils. Different fatty acids produce multiple, differing hydroperoxides, and hydroperoxides can decompose into a variety of secondary oxidation products [Choe and Min, 2006].

- Volatile substances: Short-chain cleavage products that are responsible for the characteristic "fishy smell" of oxidized marine oils. Mostly connected to free aldehydic groups.
- Non-volatile substances: Substances that do not contribute to sensory input (smell and taste free), and therefore do not have an influence on the sensory quality of marine oils.

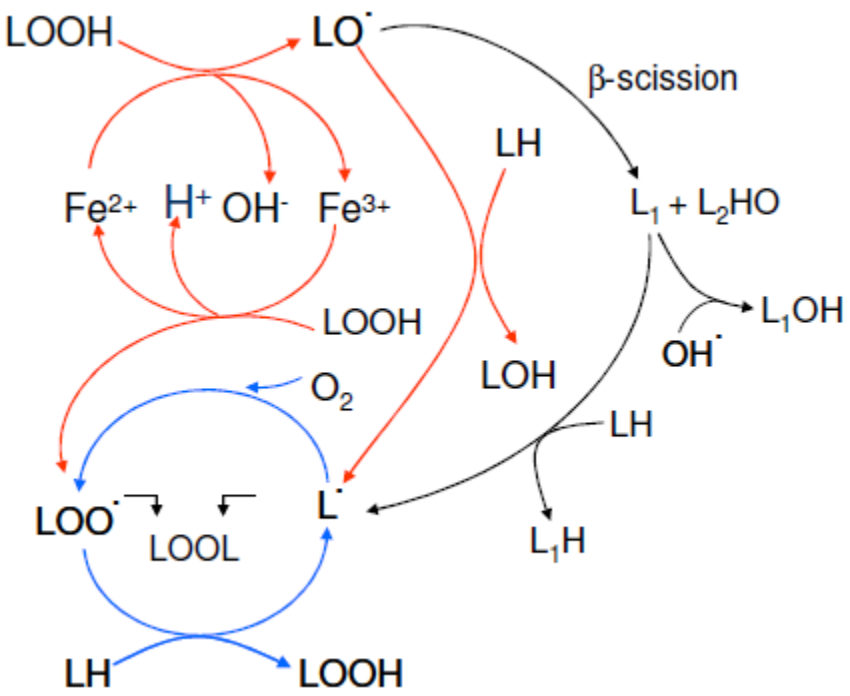

Figure 1.7: *The decomposition of lipid hydroperoxides (**LOOH**) to alkoxy radicals (**LO**) and peroxy radicals (**LOO**) through β-scission pathway, and other redox cycling pathways for further propagation of lipid peroxidation, modified from Kristinova et al.* [Kristinova et al., 2009].

Other reactions resulting in secondary oxidation products:

- Fatty acid radicals (R^*) can react with hydrogen-(H^+) and hydroxyl-(OH^-) radicals to produce olefins and alcohols respectively [Choe and Min, 2006].
- Alkoxy radicals (RO^*) can cleave hydrogen from other molecules and form fatty acid alcohols (ROH) [Aursand et al., 2011].
- Peroxy radicals (ROO^*) can react with the adjacent double bonds on the same fatty acid and form bicyclic endoperoxides [Aursand et al., 2011].

1.7.3 The state of oxidation

The TOTOX value entails using the sum of primary oxidation products, expressed as PV, and the sum of secondary oxidation products, expressed as AV to evaluate an oils quality state. It has been shown that a one unit increase in PV, has lead to an approximate two unit increase in AV, therefore describing equation 1.5 [Aursand et al., 2011].

$$2 \times PV + AV \tag{1.5}$$

The TOTOX value is considered to be a good indicator of an oils past history, combining evidence about its past quality (in the form of AV), and its present state quality (as reflected in the PV), see figure 1.8. It has been traditionally used in the trading of fish oil, however, despite the practical advantages, the value does not have a scientifically sound basis. It combines parameters of different dimensions/units, which makes the value empirical [Akoh and Min, 2008].

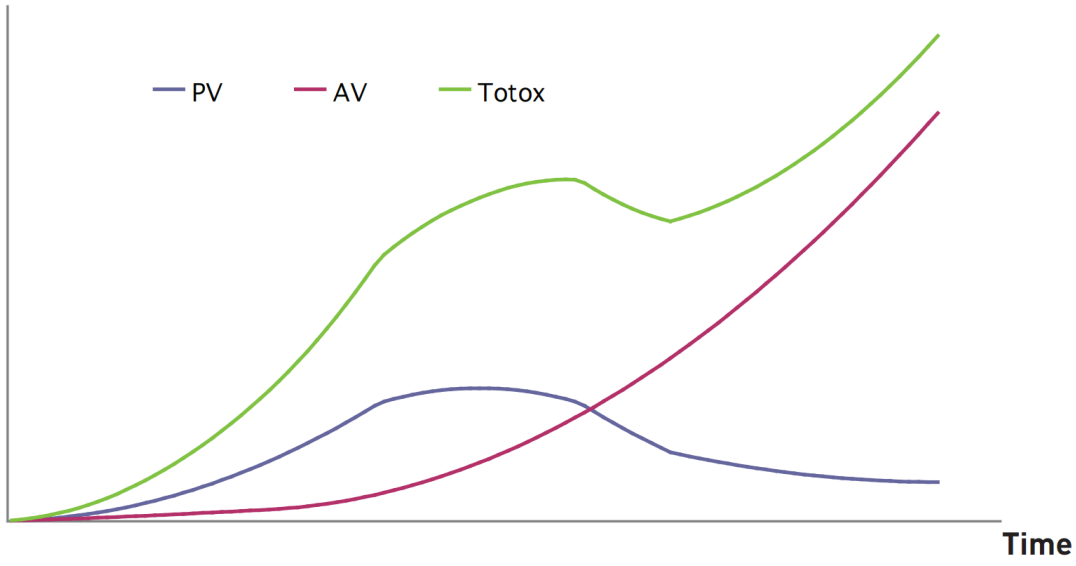

Figure 1.8: *The oxidation of an oil over time as measured by peroxide value (PV), anisidine value (AV) and TOTOX value. The chart gives a snapshot of the oxidation history, and current state of the oil up to that point in time.*

1.7.4 Oxidative stability

Oxidative stability of a marine oil is crucial for the shelf life and quality of the product. Oxidative stability measurements determine how resilient an oil is to being artificially oxidized, using accelerated methods of oxidization, such as high temperature, abundance of oxygen, and/or transition metals, this is an alternative to the long storage time needed in order for the (an industrial perspective) reactions of oxidation in an oil to occur naturally. The oxidation resilience of an oil is directly correlated to the shelf life of the final product, and is therefore extremely important to maintain. Figure 1.9 shows an ideal thermogram of an oil and the corresponding oxidation products produced. This can give an idea of how an oil might react under oxidative conditions, however there is still some debate on whether these types of accelerated-oxidation analyses can give a skewed picture of the way an oil might behave under normal conditions and circumstances. The main argument being that the methods do not, and can not, simulate oxidation under "normal" conditions, due to the accelerated time frame [Frankel, 1993].

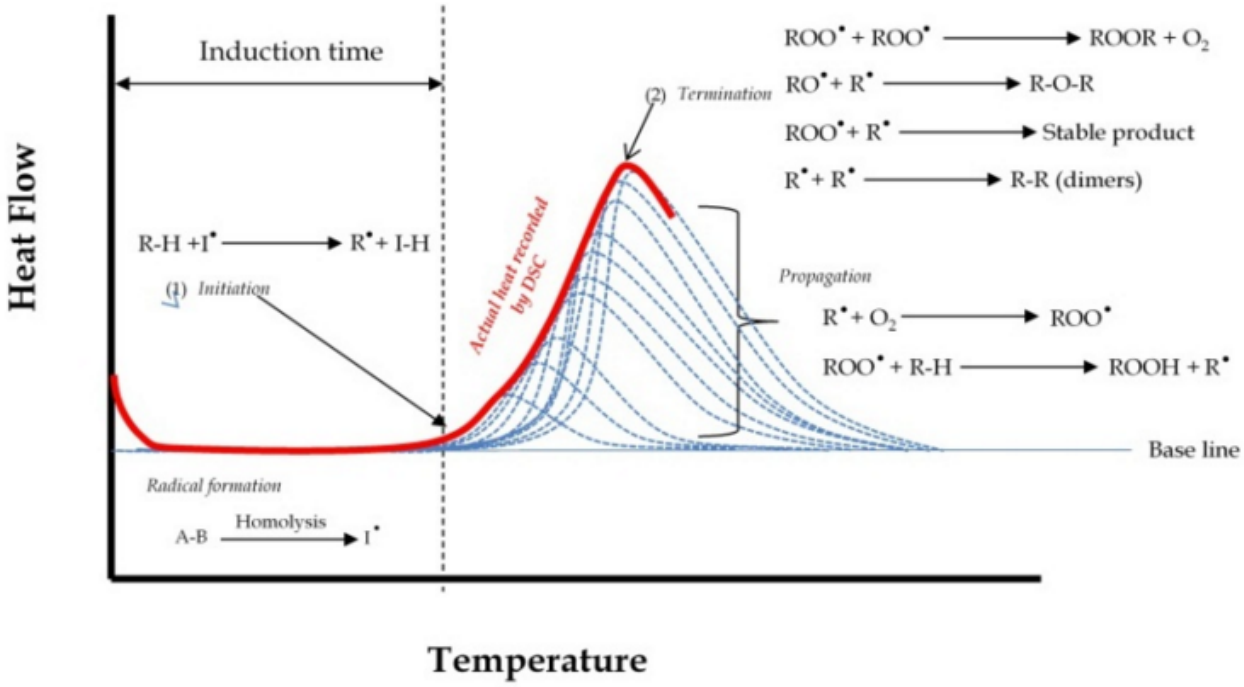

Figure 1.9: *An ideal thermogram showing the non-isothermic oxidation of a general oil,* ***DSC****: Differential Scanning Calorimetry, adopted from Saldaña et al.* [Saldaña and Martínez-Monteagudo, 2013].

1.8 Pro-oxidants

Pro-oxidants are compounds or factors that influence or increase the rate of oxidation. As mentioned earlier singlet oxygen is a type of pro-oxidant, and so is the enzyme family of lipogenases. Both having direct interaction with unsaturated fatty acids and forming lipid hydroperoxides [Aursand et al., 2011].

1. **Singlet oxygen** (light induced oxidation):

    - Contains an empty 2pπ antibonding orbital, which makes the molecule electrophilic. Seeking high electron densities such as the double bonds of unsaturated fatty acids.
    - Spin conservation is retained and the reaction is thermodynamically favorable. Forming hydroperoxides 1500 times faster than atmospheric triplet oxygen.
    - Is able to react with either carbon atom within a double bond, potentially resulting in the ability to produce four different hydroperoxide isomers [Choe and Min, 2006].

2. **Lipogenase** (enzymatic oxidation):

    - An enzyme family containing iron.
    - Catalyzes the formation of lipid hydroperoxides.
    - Associated with the circulatory system, found in blood and organs such as gills etc. [Aursand et al., 2011].

3. **Transition metals** (autoxidation):

    - Reduces the stability of food and biological tissue by lowering the activation energy of the initiation step of autoxidation, and by degrading hydroperoxides into free radicals.
    - Leads to the formation of alkoxy (RO^*), hydroxy (OH^*) and peroxy (ROO^*) radicals.
    - Examples: Copper and iron are predominant in food [Choe and Min, 2006].

1.9 Antioxidants

Antioxidants are compounds that hinder or inhibit the oxidative processes that edible oils go through. Thus giving resilience against oxidation and the impairment of quality [Aursand et al., 2011]. Natural occurring oils contain antioxidants such as tocopherols, carotenoids, phenolic compounds, tocotrienols, and sterols. These compounds can hinder the oxidative process by slowing down the oxidation rate, or by extending the induction period of oxidation. Primary antioxidants are called "free radical scavengers (FRS)" because of their ability to react and non-radicalize lipid peroxy radicals or lipid alkyl radicals. They can also quench singlet oxygen molecules, rendering them inactive (triplet oxygen). They are also capable of controlling transition metals, and inactivate photo-sensitizers [Choe and Min, 2006]. Antioxidants can be divided into three groups; dependent on their main method of action, however some antioxidants can have to differing methods of action.

- **Scavengers:** Compounds that donate a proton (H^+) to reduce free radicals from an active oxidized state [Fennema et al., 2007].
- **Quenchers:** Compounds that absorb the excess energy from "active" singlet oxygen, and transfer it to triplet oxygen, without being chemically altered.
- **Chelators:** Compounds that bind metallic ions (transition metals) and form complexes that are insoluble (precipitation) [Aursand et al., 2011].

1.9.1 Primary antioxidants - free radical scavengers

The free radical scavengers (FRS) inhibit or slow down the oxidation process by donating a proton or an electron, see equations 1.6 to 1.8.

$$\text{Peroxy radicals} \quad (LOO^*) + \text{Antioxidant} \quad (AH) \quad \rightarrow \quad LOOH + A^* \tag{1.6}$$

$$\text{Alkoxy radicals} \quad (LO^*) + AH \quad \rightarrow \quad LOH + A^* \tag{1.7}$$

$$\text{Fatty acid radicals} \quad (L^*) + AH \quad \rightarrow \quad LH + A^* \tag{1.8}$$

Antioxidants will not be consumed during the reaction, there will therefore not be a net increase in the amount of radicals. (A^*) is a radical antioxidant that is produced after the reaction with a lipid peroxy radical or lipid alkyl radical, however it has a lower energy than the lipid radicals due to its resonance structure, figure 1.10 [Choe and Min, 2006]. Every FRS molecule can potentially terminate two distinct free radicals, see equations 1.9 to 1.12. (A^*) can be terminated by the following reactions to a non-radical species [Fennema et al., 2007].

$$LOO^* + A^* \rightarrow LOOA \tag{1.9}$$

$$LO^* + A^* \rightarrow LO \tag{1.10}$$

$$L^* + A^* \rightarrow LA \tag{1.11}$$

$$A^* + A^* \rightarrow AA \tag{1.12}$$

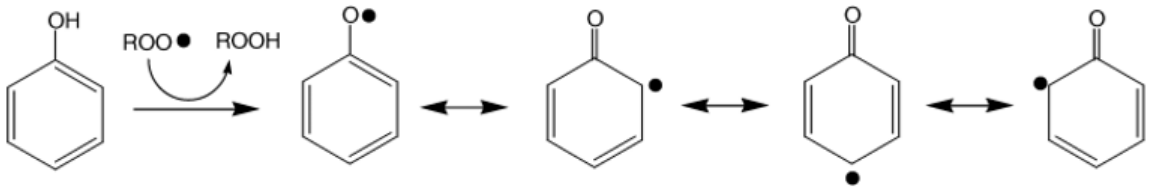

Figure 1.10: *An illustration of the stabilizing resonance structure of antioxidant radicals, adopted from Choe et al.* [Choe and Min, 2006].

Some examples of free radical scavenging antioxidants that are naturally occurring are tocopherols and plant polyphenols. Synthetic compounds such as *butyl*-hydroxytoluene and *butyl*-hydroxyanisole are widely used. The effectiveness of an FRS is reliant on it's ability to donate protons (H^+). FRS' need to have low reduction potentials to ensure that they reduce the free radicals [Aursand et al., 2011].

1.9.2 Secondary antioxidants - quenchers and chelators

Chelators inhibit and hinder the formation of lipid radicals, and the propagation of hydro peroxides by controlling the metal ions that initiate the reactions. They form insoluble metal com-

plexes, which sterically hinder the reaction between metal and lipid. However this may influence the bio-availability of minerals (iron) within the product. Naturally occurring examples such as citric acid and ethylenediaminetetraacetic acid (EDTA) are normally water soluble, however there are some that are hydrophobic. The effectivity of chelators is dependent upon the pH, due to the fact that they need to be in an ionized form before they can react. The presence of other ions will lower their affinity to the substrate, and the presence of other reducing agents can lead to the liberation of the metal ion(s) from the chelator [Aursand et al., 2011].

Quenchers hinder and inhibit activated singlet oxygen from reacting with fatty acids to produce radicals. Quenchers are often fat-soluble. Chemical quenching depends on the deactivation of singlet oxygen by the transfer of energy from the singlet oxygen molecule to a triplet oxygen molecule. The remaining energized state of the quencher is released as heat to the environment, therefore returning the quencher to its initial state. Quenchers such as β-caroten, tocopherols, amino acids, and peptides do not alter their chemical structure after participating in the reaction, see equations 1.13 and 1.14 [Aursand et al., 2011].

$$\text{Singlet oxygen}(^1O_2) + {}^1\text{Carotenoid} \quad \rightarrow \quad {}^3\text{Carotenoid} + \text{Triplet oxygen}(^3O_2) \tag{1.13}$$

$$^3\text{Carotenoid} \quad \rightarrow \quad {}^1\text{Carotenoid} + \text{Heat} \tag{1.14}$$

Physical quenching however requires the substrate to have a conjugated system containing nine or more double bonds. Equation 1.15 shows the transfer of energy occurring in the charge transfer complex, resulting also in triplet oxygen [Bortolus et al., 1989].

$$(^1O_2) + {}^1Q \quad \rightarrow \quad [O_2 - Q]^1 \quad \rightarrow \quad [O_2 - Q]^3 \quad \rightarrow \quad {}^3O_2 + {}^1Q \tag{1.15}$$

(Q= quencher)

Antioxidants help keep the oxidative status of an oil low, especially when added after the removal of oxidation products and pro-oxidants in the refining processes within the industry. An effective antioxidant system is based upon a variety of mechanisms, it has to include scavenging, quenching, and chelating, as well as antioxidants with good functional properties. Dif-

fering mechanisms work synergistically to identify and neutralize oxidation products and pro-oxidants.

Chapter 2

Materials and methods

2.1 Overview

Characterization of four different fractions of rest raw material, from North Atlantic mackerel (*Scomber scombrus*), was carried out to determine the chemical composition. The fractions were; heads, backbone and tail, bellyflaps, viscera, and a mix of all four (hereby known as mixture). Further, mixed raw material was stored at two different temperatures (4 °C and 10 °C) from 0 to 120 hours to study the effect of storage on the quality of produced products (oil/protein). Figures 2.2 and 2.3 show flow charts for the experiments carried out in this thesis. Figure 2.2 illustrates the procedures for the compositional determination on the five different fractions of rest raw material, while figure 2.3 gives an overview of the storage experiment.

2.1.1 Preparation of raw material

The determination of composition of the five different mackerel fractions: 5 whole round fish were weighed and filleted, and the fractions were collected and weighed individually for the characterization. Figure 2.1 shows an illustration of the filleting, as well as a description of the procedure. The fractions were as follows; head, backbone and tail (BB + T), bellyflap, viscera, and the fifth and final fraction was a mixture of all four fractions. The fractions were then minced with a food processor (hole diameter 5 mm). Samples were also taken for the determination of total lipid content, utilizing the Bligh & Dyer method [Bligh and Dyer, 1959]. The oil extracted

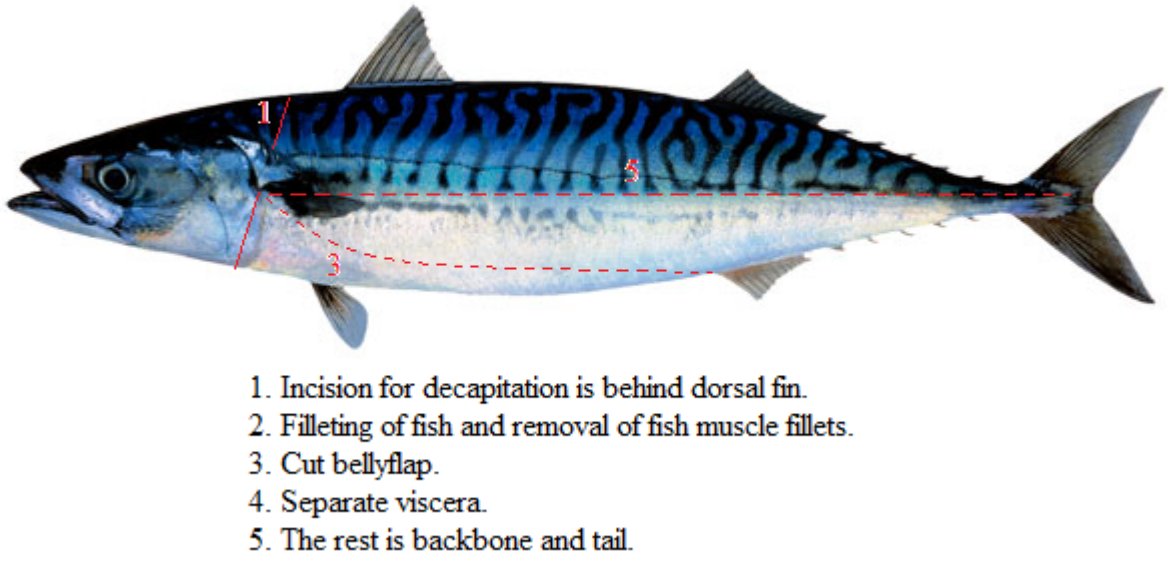

Figure 2.1: *An illustration of a generic North Atlantic mackerel. The red lines indicate incisions for the separation of the different rest raw material fractions, followed by a list explaining the procedure* [Fisheries.no, 2016].

by the Bligh & Dyer method was further analyzed. Determination of free fatty acids, peroxide value, and lipid class analysis were performed, figure 2.2.

For the storage experiments, rest raw material from 20-25 whole round fish (delivered by Pelagia^{TM}) was used. The fish were filleted and all rest raw material produced was minced in a food processor hole diameter 5 mm). The minced rest raw material was then partitioned into air tight zip-lock bags and stored at 4 and 10 °C respectively. Before the heat treatment, the minced rest raw materials were to be stored for 0-120 hours, see figure 2.3. Heat treatment proceeds after the duration of time has passed, 0 hours - immediately after filleting, 24 hours, 48 hours, 72 hours, and 120 hours respectively. Stored minced rest raw material was transferred to centrifugal tubes (50 mL, 16 tubes), rapidly heated up to 95 °C by utilizing a microwave (750-800 W, for approximately 40 seconds ± 5), then placed in a water bath kept at 95 °C for 15 minutes. After the heat treatment, the tubes were centrifuged to separate the different fractions; oil phase, emulsions, water phase, and sediments. The tubes were then frozen at -80 °C. The frozen samples, each containing four different phases were then manually separated using a scalpel, and

weighed individually. The identical phases of all 16 tubes were then mixed, oil with oil, stickwater with stickwater, etc. for further analysis.

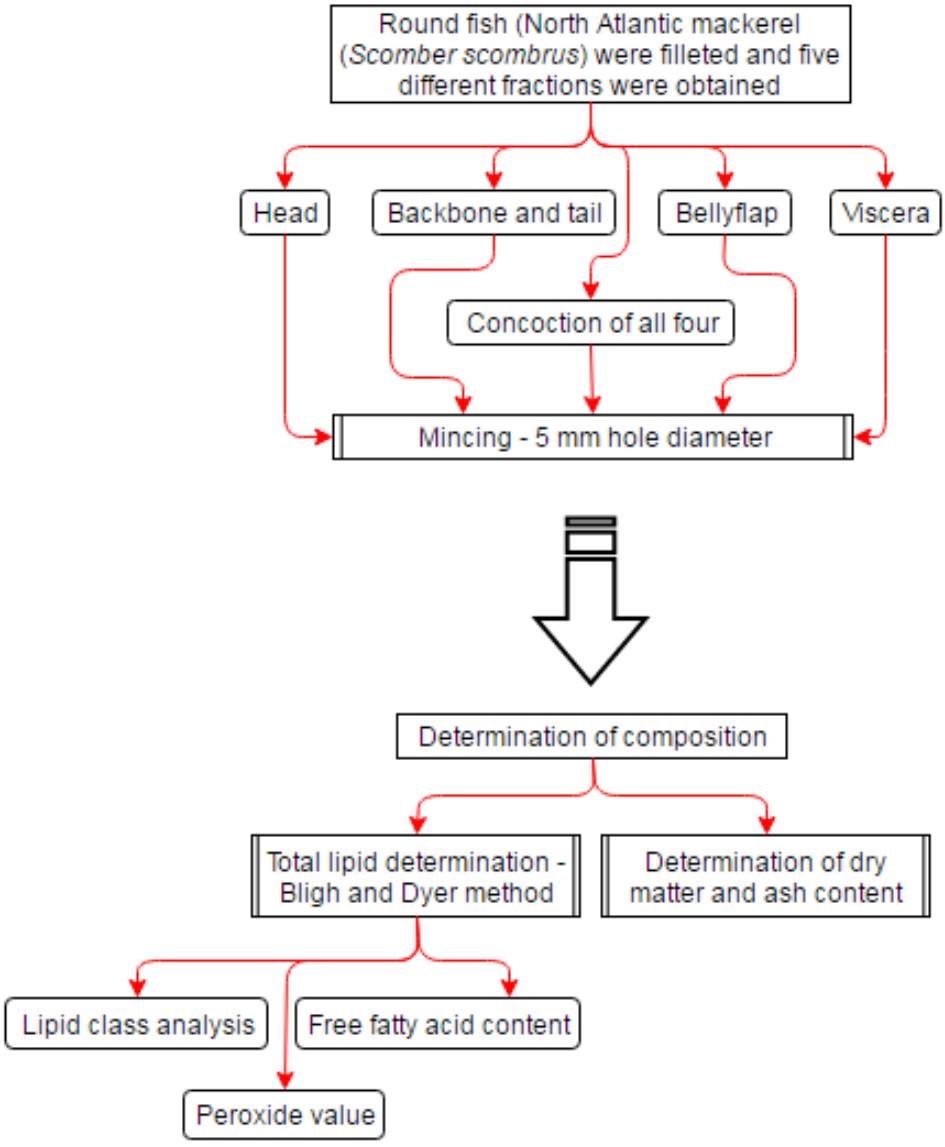

Figure 2.2: *A flow chart showing the procedure for the compositional determination of North Atlantic mackerel (Scomber scombrus) rest raw materials for five different fractions.*

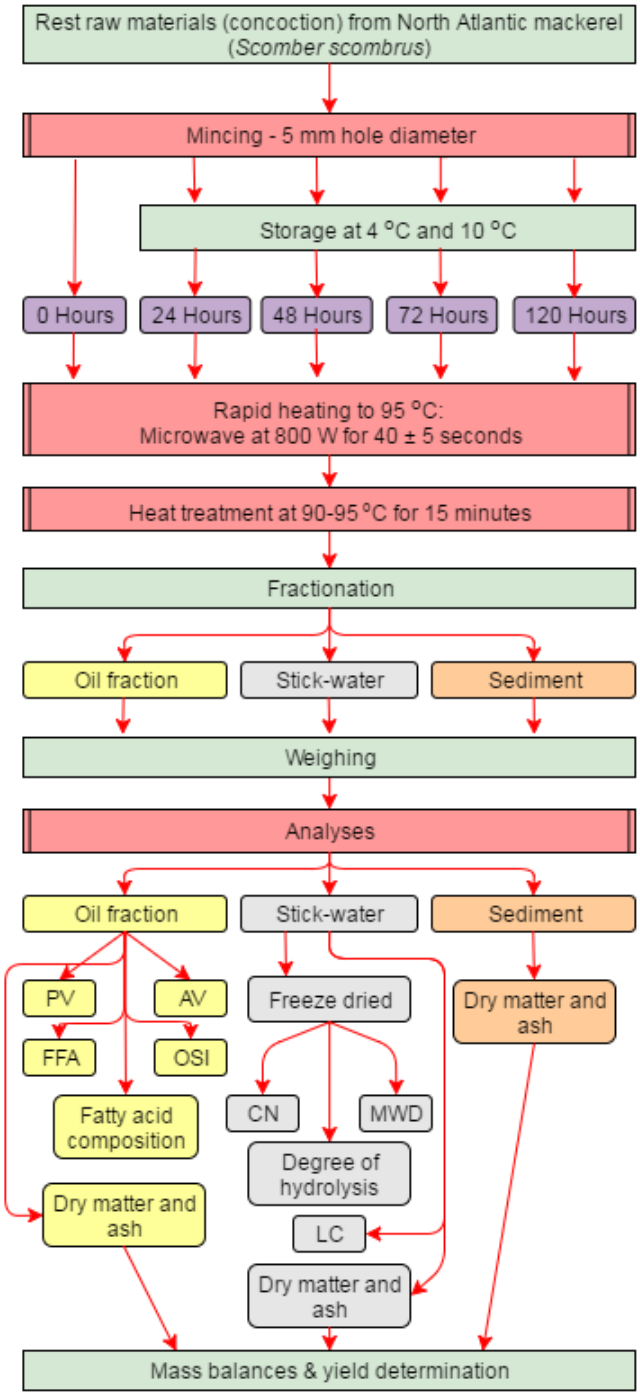

Figure 2.3: *A flow chart showing the procedure and analyses conducted during the storage experiment.* ***Stick-water*** *= the hydrophilic fraction containing water, proteins, and other water soluble content.* ***PV*** *= Peroxide value.* ***AV*** *= Anisidine value.* ***FFA*** *= Free fatty acid content.* ***OSI*** *= Oxidative stability index.* ***CN*** *= Total carbon and nitrogen.* ***MWD*** *= Molecular Weight Distribution.* ***LC*** *= Lipid content.*

2.2 Mass balance

Mass balances were determined for batch nr. 2 to verify the validity of the yield. Equation 2.1 was used to determine if the amount of dry matter in a 100 grams of resource (wet weight) was plausibly equal to the amount of dry weight in the fractions obtained per 100 grams of resource (wet weight).

$$\begin{aligned} \text{Weight}_{\text{resource}} \times (\text{Dry weight \%})_{\text{resource}} &= \text{Weight}_{\text{oil}} \times (\text{Dry weight \%})_{\text{oil}} + \\ &\quad \text{Weight}_{\text{emulsion}} \times (\text{Dry weight \%})_{\text{emulsion}} + \\ &\quad \text{Weight}_{\text{stickwater}} \times (\text{Dry weight \%})_{\text{stickwater}} + \\ &\quad \text{Weight}_{\text{sediment}} \times (\text{Dry weight \%})_{\text{sediment}} \end{aligned} \tag{2.1}$$

2.3 Methods of analysis

2.3.1 Dry matter and ash content

The dry matter content was determined by drying the sample at 105 °C until sample mass was constant. Ash content was measured according to the method by AOAC [AOAC, 1990]. Approximately 2 grams of sample was transferred to crucibles, and placed in a drying cabinet for 24 hours at 105 °C, and then transferred to a the desiccator to cool, the samples were then weighed to determine the dry matter content. Thereafter the samples were placed in a muffle furnace at 600 °C for 6 hours, transferred to a desiccator to cool before weighing to determine ash content.

2.3.2 The determination of total lipid content

A modified version of the Bligh and Dyer method [Bligh and Dyer, 1959] was utilized for the extraction and determination of total lipid content of raw mackerel fractions, see figure 2.2. This method is based on the physical properties between hydrophilic and hydrophobic molecules. This method is also scalable, and therefore can be used when there are small amounts of sample. The micro-B&D method is used in this thesis when the lipid content of the freeze dried stickwater is determined.

PRINCIPLE

Wet tissue is homogenized with the addition of a mixture of methanol and chloroform. The homogenization produces a miscible system between the water and the fat in the tissue. Dilution by water and chloroform separates the mixture into a hydrophilic (methanolic layer) and a hydrophobic phase (chloroformic layer). The chloroformic layer would then contain all lipids, while the methanolic layer would contain all the non-lipids. By isolating the chloroformic layer, the lipid can be extracted.

Analytical procedure

The total lipid content for the characterization of rest raw material, was determined using the Bligh and Dyer method [Bligh and Dyer, 1959]. Approximately 5 grams of wet sample was weighed precisely to a thousandth of a gram (mg). The weight was noted, and the sample was homogenized with 16 mL of distilled water, 20 mL of chloroform, and 40 mL of methanol for 2 minutes. 20 mL of chloroform were then added, and the sample was homogenized (ULTRA TURRAX®) for 40 seconds. 20 mL of distilled water was added, and the sample was additionally homogenized for another 40 seconds. The homogenized sample was then centrifuged at 4000 × g for 15 minutes. Two phases were formed; the chloroform (hydrophobic) phase, and the water (hydrophilic) phase. This method relies on the fact that all lipids are contained within the chloroform phase because of their hydrophobic nature. 2 mL of the chloroform phase was pipetted into glass tubes (conical bottom, Pyrex®). The chloroform was then evaporated utilizing a heating block (60 °C) with the addition of N_2 for approximately 20-30 minutes. The samples were then cooled, weighed, and the total amount of lipids was calculated from equation 2.2. The homogenization step was performed in ice to minimize evaporation and increase precision.

$$\text{Total lipid content in \%} = \frac{\text{Weight of lipid in tube [g]} \times \text{Volume of chloroform layer [mL]}}{\text{Volume of chloroform utilized in tube [mL]} \times \text{Mass of sample [g]}} \tag{2.2}$$

The total lipid content for in the storage experiment was determined by means of weight, after heat treatment, and separation.

The total lipid content in stickwater was determined using the micro Bligh and Dyer method, which is a scaled down version of the original [Bligh and Dyer, 1959]. Samples of freeze dried stickwater were weighed into pre-weighed kimax® tubes. Sample size was between 10 - 50 mg. 0.8 mL of distilled water, 2.0 mL of methanol (CH_3OH), and 1.0 mL of chloroform ($CHCl_3$) were added to the tube. The tube was then homogenized with an ULTRA TURRAX® for 1 minute. 1.0 mL of chloroform was added, and the tube was homogenized again for 20 seconds. Subsequently, 1.0 mL of distilled water was added, and the tube homogenized for another 20 seconds. The tube was then centrifuged for 10 minutes at 4000 rpm. 0.5 mL of the top-phase of the tube (containing the chloroform and any dissolved lipids) was then transferred to another pre-weighed tube. The tube containing the 0.5 mL of top-phase was then placed in a heating block at 60 °C with nitrogen (N_2) insufflation to evaporate the chloroform. The tube was then transferred to an desiccator before weighing. This method is only different to the original in the amount of solvents, and the amount of time under homogenization. Equation 2.2 is utilized to calculate the lipid content, using mg instead of grams for the "wight of lipid in tube".

2.3.3 Lipid class analysis

The IATROSCAN MK-VI combines the techniques of thin layer chromatography (TLC), an established technique for the separation of organic compounds, with a flame ionization detector (FID). It utilizes specially designed Chromarods® (quartz rod coated with a thin layer of silica or alumina on which the sample is dissolved and separated). This method separates non-polar lipids, and calculates the amount of free fatty acids, cholesterol, cholesterol esters, triacylglycereides, and phospholipids.

PRINCIPLE

The sample dissolved on the Chromarod® is burned by the FID flame at a constant speed. The substances of interest (lipids) are ionized by the energy of the hydrogen flame. The ions generated are then effected by an electric field applied at the poles of the FID. The ions will therefore induce an electric current with an intensity proportional to the amount of each substance entering the flame. This is measured by a detector. The SES-i-ChromStar® software is used to produce graphs and curves of the substances detected.

Analytical procedure

A neutral solvent system was made; Hexane:Diethyl ether:Formic acid with the ratio of 85 mL: 15 mL: 40 μL respectively. A Whatman® filter paper (approximately 25 cm × 25 cm) was placed in the elution chamber. An elution chamber is a closed container, the purpose of which is to enclose the media used as well as the mobile phase to maintain a constant environment in the vapor phase. Use grease on the edges of the chamber for a tight fit. The Chromarod® S-III rods need to be activated before usage, by running a blank scan, and making sure there are no local maximums on the charts developed from the blanks. The samples were applied onto the rods by the use of a 10 μL Hamilton needle. The needle applies the sample while being in contact with the rods, and being flushed slightly by N_2. The rods were then put in a chamber containing a saturated $NaCl$ solution for 8 minutes. The rods are then transferred to the elution chamber containing the solvent system, for 27 minutes (maximum 30 min). The rods are then removed from the elution chamber and left to evaporate under a hood for a few minutes before they are put into a drying oven at 100 °C for 5 minutes. The rods are then run through the Iatroscan TLC-FID instrument, and the classes present in the oil were determined.

The oxidation state of an oil is defined as the sum of all oxidation products that are present. The oxidative state and stability are of extreme importance and are crucial for the quality and shelf life of the product. It is however difficult to measure the exact oxidation state, due to the abundance of different oxidation products. Most methods that are developed were developed for animal and plant lipids, which are considered to be "simple" fats compared to marine lipids. Marine lipids are highly unstable, resulting in deterioration of quality (oxidation) during testing, which leads to uncertainty and impreciseness within results. Many of the methods are also reliant on large sample sizes; this can be problematic, as one normally deals with small amounts of marine oils.

2.3.4 The determination of free fatty acid content

Free fatty acid (FFA) content in oil samples was determined according to a procedure proposed by Bernardez et al. [Bernárdez et al., 2005]. The method is adapted by the use of iso-octane as a solvent for lipids, instead of cyclohexane. Each sample was measured in four parallels

(n=4). Standard curve was prepared with oleic acid standard (0 – 20 μmol), and was used for FFA content calculation. The results are expressed as % of oleic acid ± standard deviation/error [Bernárdez et al., 2005]. Each sample was measured in four parallels (n=4).

PRINCIPLE

Acidic groups in free fatty acids (FFA) react with cupric acetate-pyridine reagent, forming stable cupric soaps (complexes) of blue colour, which absorb at 715 nm in a spectrophotometer.

Analytical procedure

The determination of the free fatty acid content was done with the method described by [Bernárdez et al., 2005]. The oleic acid (18:1 n-9) standard was prepared by dissolving 0.2825 g of oleic acid in 10 mL of iso-octane (0.1 M). Procedure: 0.2 g of oil were weighed (with 0.0001 g precision) into a short Kimax® tube. 5 mL of iso-octane were added and the solution was vortexed shortly. 0.5 mL of cupric acetate-pyridine aqueous reagent was added, and the tube closed tightly with a screw cap. The mixture was then vortexed for 30 seconds and centrifuged at 2000 × g for 5 minutes. The upper layer of the mixture was then transferred to a glass cuvette, and the absorbance was measured with a spectrophotometer at 715 nm against iso-octane. A standard curve was prepared from the oleic acid standard. The calculation of FFA content as % FFA (as oleic acid) in the sample was extrapolated from the oleic acid standard curve, and by utilizing equation 2.3. Four replicates were run per sample, and two parallels per concentration were run for the oleic acid standard curve.

$$\text{FFA \%} = \frac{(Abs - B) \times 282.46}{A \times m \times 10000} \tag{2.3}$$

- **Abs** is the absorbance of the sample.
- **B** is the y-intercept of the linear regression performed on the oleic acid standard curve - absorbance of the blank.
- **282.46** is the molar weight of oleic acid [g/mol].
- **A** is the slope of the linear regression [1/μmol].
- **m** is the mass of the sample [g].
- **10 000** is the conversion of units to get percentages [%].

2.3.5 Primary oxidation products and the peroxide value

A modified version of the AOCS Official Method Cd 8b-90 [AOCS, 2013] was utilised (ISO 3960 [ISO, 2001]) to determine the peroxide value of the oil sample. An iodometric endpoint titration was conducted using the Automatic Potentiometric Titrator. Instead of using starch as iodine determinant (visual blue complex), the auto-titrator uses an electrode to measure the charge of the solution, graphing the use of titrant against the charge (eV), and determining the inflection point (equivalence point). All substances that were determined are in terms of milliequivalents (mEq) of peroxide per 1000 grams of oil sample. The substances are generally assumed to be peroxides or other similar products of lipid oxidation. This method is highly empirical, and any variation in the test procedure may result in erratic results. This method gives erratic results at peroxide values around 70 [AOCS, 2013]. Each sample was measured in four replicates (n=4).

PRINCIPLE

A saturated potassium iodide solution (KI) reacts with the hydroperoxides within the oil sample at a stoichiometric rate. The liberated iodide ion is then titrated with a sodium thiosulphate solution ($Na_2S_2O_6$). The titrator's output is the amount of titrant used in mL.

Analytical procedure

AOCS official method Cd 8b-90. The peroxide value determination involves a two-step redox-reaction 2.4 and 2.5 [ISO, 2001, AOCS, 2013].

1. The reaction of a peroxide group with an excess of iodide ions:

$$\text{R-O-O-R} + 2I^- + 2H^+ \quad \rightarrow \quad 2ROH + I_2 \tag{2.4}$$

2. The titration of Iodine with Sodium thiosulphate ($Na_2S_2O_3$) solution (0.1 M):

$$I_2 + 2S_2O_3^{2-} \quad \rightarrow \quad 2I^- + S_4O_6^{2-} \tag{2.5}$$

The titration is carried out to the detection of an inflection point on the auto-generated graph. The original method is scalable, therefore the modified version uses half the amount

of sample and reagents. Approximately 2 grams of oil was added to a beaker. The oil is then diluted with 25 mL of iso-octane/acetic acid solution with a ratio of 2:3 respectively. 0.25 mL (250 μL) of a saturated potassium iodide solution is then added to the mix and stirred for 1 minute. Distilled water is then added (30 mL), and the sample run under the auto-titrator. A generalized titration curve is shown in figure 2.4. Four parallels were run per sample. Calculations were done according to equation 2.6.

$$\text{PV} = \frac{Concentration_{(Na_2S_2O_3)} \times (\text{Titration of sample} - \text{Titration of blank}) \times 1000}{\text{Weight of sample}} \quad (2.6)$$

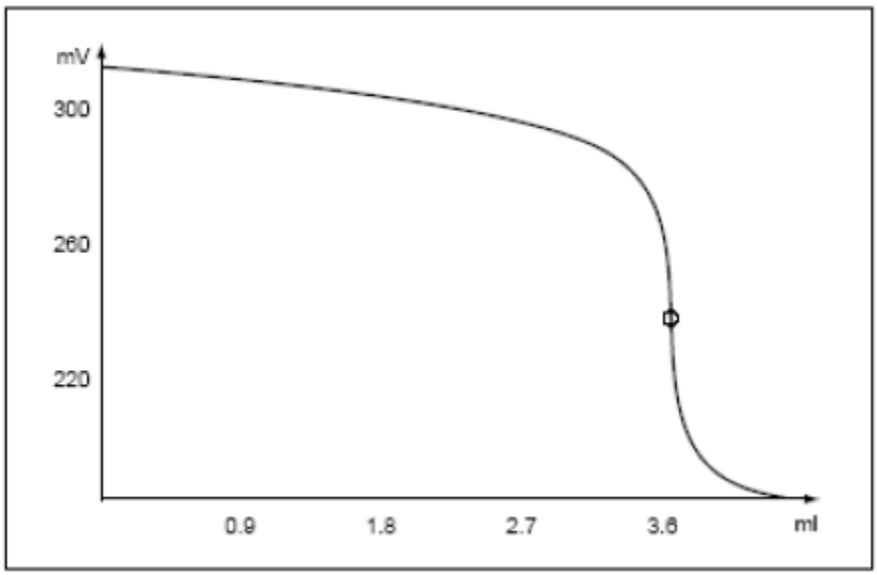

Figure 2.4: *A general curve showing a peroxide value titration. The inflection point is marked.* ***x-axis:*** *volume [mL] of titrant used.* ***y-axis:*** *Electrical potential measured [mV] due to ions in the solution* [ISO, 2001]

.

2.3.6 Secondary oxidation products and the *para*-Anisidine value

The determination was done using the AOCS Official method Cd 18-90 [AOCS, 2013] for the determination of the *p*-Anisidine value. This measures the amount of secondary oxidation products (rancidity) produced from the degradation of lipid-peroxides in the oil sample. By defi-

nition, the *p*-Anisidine value is defined as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1.00 g of oil (sample) of a mixture of solvent (2,2,4-Trimethylpentane, also known as iso-octane) and reagent (*p*-Anisidine) [AOCS, 2013]. Each sample was measured in triplicates (n=3).

PRINCIPLE

This method determines the amount of "core" aldehydes (principally 2-alkenals and 2,4-dienals) in animal and vegetable fats and oils. In an acetic acid solution, the aldehydic compounds (in the oil) react with the *p*-anisidin reagent, and produce a complex in which the solution turns slightly yellow, which enables the measurement of absorbance with a spectrophotometer at the wavelength of 350 nm.

Analytical procedure

The determination of *para*-Anisidine value was done by the AOCS official method Cd 18-90 [AOCS, 2013]. This method utilizes a spectrophotometer with the usage of glass cuvettes. Approximately 2 grams of oil were added to a 25 mL volumetric flask, and the oil was then diluted with iso-octane (Trimethylpentane) to the 25 mL mark. Then the mixture was mixed thoroughly. Using glass cuvettes, 2.5 mL of the sample solution were added to a glass cuvette (S). 2.5 mL of iso-octane were then added to another cuvette (B). The absorbance of cuvettes S and B were measured at 350 nm, and the result was written down as S_1 and B_1. Then 0.5 mL of the *para*-Anisidine reagent (0.25 g *p*-Anisidine dissolved in 100 mL 100 % acetic acid) was added to both cuvettes. After 10 minutes, another measurement of absorbance (S_2 and B_2) was run. Three parallels were run per sample. Equation 2.7 was used to calculate the *p*-Anisidine value of the oil.

$$25 \times \frac{1.2 \times \big((S_2 - B_2) - (S_1 - B_1)\big)}{m} \tag{2.7}$$

- $\boldsymbol{B_1}$ and $\boldsymbol{B_2}$ is the blank before and after the addition of the *p*-Anisidine reagent respectively.
- $\boldsymbol{S_1}$ and $\boldsymbol{S_2}$ is the sample before and after addition of the *p*-Anisidine reagent respectively.
- **m** is the sample mass [g].

2.3.7 The Oxidative Stability Index (OSI)

The AOCS Official Method Cd 12b-92 was used to determine the oxidative stability of the oil produced. This method utilizes the (OSI instrument). An oils resistance to oxidation is highly dependent on the degree of unsaturation, and whether the oil is natural or contains added preservatives such as antioxidants. The oxidation process tends to start slowly, until the resistance towards oxidation is overcome, wherein the process starts accelerating and becomes very rapid. The amount of time needed for the oil to reach the boundary into which it rapidly starts oxidizing is the measurement of the resistance to oxidation. This measurement is commonly referred to as the induction period [AOCS, 2013]. Each sample was measured in four parallels (n=4).

PRINCIPLE

The OSI instrument determines the induction period by blowing a stream of purified air into a sample of oil, which is contained within an isothermic bath. The outflow of air from the sample is then bubbled through a vessel containing deionized water. The deionized water vessel contains an electrode, measuring and monitoring the conductivity of the water solution. During the airs journey through the oil sample, it will transport volatile organic acids into the deionized water, increasing its conductivity as oxidation proceeds. The instrument is connected to a computer, monitoring the conductivity as a function of time, thus measuring the induction period. The oil stability index (OSI) is defined as the point of maximum change of the rate of oxidation, or mathematically as the inflection point on the graph (i.e. the maximum of the second derivative of the conductivity with respect to time) [AOCS, 2013].

Analytical procedure

By utilizing the Omnion OSI software, the instruments conductivity channels are displayed. 50 mL of deionized water is added to a 50 mL polycarbonate tube. The conductivity sensor is then lowered into the water tube in tandem with a 9 inch pasteur pipette, and locked into place by a rubber cork. The water tube, containing the conductivity sensor and the pipette is placed within one of the channel holds on the side of the OSI instrument. The conductivity sensor is then connected to the computer (data analyzer). Another 50 mL glass tube is used for the aeration of the oil sample. 5 g $\pm$ 0.2 g of oil sample is added to the empty 50 mL glass tube, including two

9 inch pasteur pipettes, and locked into place by a rubber cork. Connect one pasteur pipette by a hose to the air intake, while the second pipette gets connected to the water tube, so that the air can flow through the oil tube, interact with oxidation products, and transport them into the water tube, ultimately changing its conductivity. A psi pressure of 5.5 was used, and the pipette from the air intake was firmly placed under the surface of the oil, to ensure that bubbling occurs, in order to accelerate oxidation of the oil sample.

2.3.8 Determination of fatty acid composition by gas chromatography

The preparation stage consists of the methylation of test samples (oil). The lipids need to be methylated into methyl ester derivatives of their respective fatty acids, to measure the amounts of different types of fatty acids by means of chromatography, in this case, gas chromatography.

PRINCIPLE

Methylation of the lipids was done by transesterification utilizing the acidic catalyst boron trifluoride in methanol ($BF_3 - CH_3OH$, 12 - 14 % w/v). The acid-catalyzed method methylates the lipids by heating the samples containing large amounts of excess anhydrous methanol in the presence of an acidic reagent, formula 2.8.

$$RCOOH + CH_3OH \quad \rightleftharpoons \quad RCOOCH_3 + H_2O \qquad \text{with} \quad H^+ \quad \text{acting as a catalyst} \tag{2.8}$$

Analytical procedure

Methylation of lipids, preparation for GC

Two replicates of 10 mg of fat were weighed into kimax® tubes and the weight was noted. 1 mL of chloroform (w./ 1 mg of standard) was added to each tube. The tubes were then vortexed and the chloroform was evaporated by heating the samples with a heating block (60 °C, with nitrogen (N_2) flush. 1 mL of 0.5 M NaOH dissolved in methanol was added, the tube was corked, and put in a heat block (100 °C) for 15 minutes. The tube was left to cool down to room temperature before adding 2 mL of boron trifluoride-methanol ($BF_3 - CH_3OH$), and then placed in a

heating block at 100 °C for 5 minutes. The tube was again cooled to room temperature before adding 1 mL of hexane (C_6H_14), and placed in a heating block at 100 °C for 1 minute. The rube was cooled, 1 mL of hexane, and 2 mL of saturated NaCl solution were added. The tube was then vortexed and centrifuged at 3000 rpm for 3 minutes. The top phase (hexane phase) was then removed and stored in another tube. Another addition of 1 mL of hexane was done, and the tube was vortexed and centrifuged at 300 rpm for 3 minutes. The top phase was removed and added to the prior top phase. The third and final addition of 2 mL of hexane was done, and the tube was vortexed and centrifuged again at 3000 rpm for 3 minutes, before removing the top phase, and transferring it to the prior two top phases. A sample of the top phase tube was then transferred to a GC-tube for further analysis.

The GC program is provided in the table below, 2.1:

Table 2.1: GC program for the determination of fatty acid composition. **Column** - Agilent CP7713: 265 °C: 25 m × 250 μm × 0.2 μm, Inlet: Helium, Front detector: FID

	Rate [^{0}C/min]:	Value [^{0}C]:	Hold time [min]:	Run time [min]:
Initial		80	1	1
Ramp 1	25	180	8	13
Ramp 2	2.5	205	2	25
Ramp 3	2.5	215	5	34

FID detector	
Heater:	250 ^{0}C
H_2 flow:	35 mL/min
Air flow:	350 mL/min
Makeup flow (He):	0.3 mL/min

2.3.9 Total protein determination by method of total nitrogen

The Carlo Erba NA-1500 CN elemental analyzer was used to determine the total nitrogen (N) content in the freeze dried stick-water samples.

PRINCIPLE

Solid samples (powder) are weighed in tin capsules (tin is of importance for the right combustion in the elemental analyzer) and loaded into an automatic sampler. The tin cups are

then dropped in a tube where in the presence of external oxygen, flash combustion occurs at a temperature of 1800 °C. The gaseous combustion products N_2, NO_x, H_2O, O_2,and CO_2 are carried by the helium as carrier gas through a column filled with chrome- and cobalt-oxide, and from there to a copper-column where nitrogen oxides are reduced to elementary nitrogen, and O_2 to CuO. Water is absorbed in another column. The remaining gasses are introduced in a gas chromatograph-oven, where N_2 and CO_2 are separated on a Poropak QS-column. The flow along a thermal conductivity detector (TCD) which produces an electrical signal proportional to the concentration of nitrogen and carbon.

Analytical procedure

Freeze dried stickwater (powder) samples were weighed into tin containers (approximately between 1.5-3 mg), and thereafter run through a CN elemental analyzer by Engineer Marte Schei (Department of Processing Technology at SINTEF Fisheries and Aquaculture AS). Samples were run in triplicates.

2.3.10 Molecular weight distribution

Utilizing the High Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS) method, determination of the molecular weight distribution was done on the different protein polymers present stickwater, separated from mackerel rest raw material.

PRINCIPLE

Polymers (proteins in this case) are built up of smaller monomers and are influenced my the molecular weight, molecular size and structure, and the total molecular weight distribution of the constituent molecules. Being a liquid chromatography method combined with a mass spectrometer, it is similar to other methods in that separation of the different substances present is possible because the different compounds (proteins, peptides, etc.) have different affinities for the "mobile phase" (the moving fluid) and the "stationary phase" (the porous solid). However further analysis of the mass of the substances results in the measurement of the molecular weight distribution.

Analytical procedure

Dry powders of stickwater were dissolved in 30 % acetonitrile/0.1 % trifluoroacetic acid (TFA) in water solution to a concentration of about 10 mg/mL. The samples were then diluted in water (1:10) to a final concentration of 1 mg/mL. The samples were analyzed on a Hitachi HPLC with a UV detector at 214 nm, using a Superdex® peptide 10/300 column. The run was isocratic with 30 % acetonitrile, 0.1 % TFA in water, at 0.3 mL/min. The sample volume was 20 μL. The tests were conducted at room temperature. The standards used were cytochrome C (12327 Da), aprotinin (6511 Da), insulin A (2532 Da), leucine enkephaline (555.6 Da), Val-Tyr-Val (379.5 Da) and Gly-Tyr (238.2 Da). The regression line for the standards was $R^2 = 0.960$. The chromatogram integration was done manually. All samples were analyzed in triplicates.

The molecular weight was divided into 11 intervals, as shown in figure 3.20 in chapter 3.4.4; below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20000 Da and above 20 000 Da.

2.3.11 Formol Titration

Formol titration is a method involving the use of formaldehyde to discern the activity of proteolytic hydrolysis that has taken place in a sample. The method determines the degree of hydrolysis (DH) within a sample, and is based on the method of Sørensen et al. [Sørensen, 1907]. The method itself was evaluated and verified by Taylor et al. [Taylor, 1957], and is now a widely used method for the determination of proteolytic hydrolysis.

PRINCIPLE

A method of titration based on the reactiveness of amino groups within amino acids, towards the addition of formaldehyde to the neutral solution; the formaldehyde reacts with the NH_3^+ group, liberating an equivalent quantity of H^+, which is then estimated by an acid-base titration with $NaOH$.

Analytical procedure

A slight modification of the 1957 Taylor et al. [Taylor, 1957] method was used due to the fact that the stickwater present was already freeze dried into a powder. The method suggests using 1.5

grams of sample, the modified version uses 0.3 grams of dry powder. Procedure: 0.3 grams of sample powder was weighed out into a beaker, and 50 mL of distilled water was added. 0.1 M of $NaOH$ was added to the solution until the pH (utilizing a pH-electrode) was 7.0. Formaldehyde 10 mL was added, and 5 minutes of wait time are necessary for the reaction to occur sufficiently. the solution is then titrated to a pH of 8.5 with a 0.1 M $NaOH$, and the amount of titrant used was noted. Calculation of the percentage of free amino groups was done by using equation 2.9. However for the calculation of hydrolysis degree, equation 2.10 was used.

$$\text{Free amino groups \%} = \frac{A \times B \times 14.007 \times 100}{C \times 1000} \tag{2.9}$$

- A is the volume [mL] of titrant ($NaOH$) used during the titration.
- B is the concentration of the titrant (0.1 M $NaOH$).
- C is the mass of the sample [g].

Degree of hydrolysis (% DH) is obtained by dividing by the total amount of nitrogen, a result found by CN analysis. Equation 2.9 was used for the calculation.

$$\text{Degree of hydrolysis \%} = \frac{D \times 100}{E} \tag{2.10}$$

- D is the percentage of free amino groups, see equation 2.7.
- E is the total percentage of nitrogen.

2.4 Summary

THE OIL PHASE

To keep oxidation to a minimum, the oil from the 16 tubes of each time and temperature parallel were filtered through glass wool, partitioned into 50 mL tubes for the different analyses that were planned, and then stored at -80 °C. The oil was then only thawed once for the analyses. The analyses that were done on the oil phase are listed below.

- Free fatty acid determination
- Peroxide value

- *para*-Anisidine value
- Oxidative stability index
- Fatty acid composition
- Dry matter and ash content

THE EMULSION PHASE

The emulsion phase was not discernible to the naked eye, and therefore not possible to cut out using a scalpel, meaning that the content was negligible.

THE WATER PHASE - STICKWATER

The stickwater was filtered through glass wool, partitioned into 50 mL tubes, and stored at -80 °C. The analyses that were done on the stickwater phase are listed below.

- C.N. - total carbon and nitrogen determination
- MWD - molecular weight distribution
- Formol titration - determination of degree of hydrolysis
- Dry matter and ash content

THE SEDIMENT PHASE

The sediment phase was stored at -20 °C, and analyzed for water content (dry matter) and inorganic minerals (ash), for the calculation of mass balance. Table 2.2 gives an overview of the analyses run throughout this thesis.

Table 2.2: Analytical methods used in; the characterization of rest raw material, and the storage experiment. **rrm** - Rest raw material, **O** - Oil, **SW** - Stickwater, **SL** - Sludge. Adopted from [Carvajal, 2013]

Determined parameter	Fraction	Rest raw material characterization	Storage experiment	Method
Composition				
Moisture and ash	rrm, O, SW, SL	X	X	Moisture: drying at 105 ^{0}C until sample mass is constant, Ash: according to the method by AOAC (AOAC, 1990).
Total lipids	rrm, SW	X	X	According to the method of Bligh and Dyer (Bligh and Dyer, 1959).
Protein content	rrm, SW	X$^{\Delta}$	X	Total nitrogen (N) determined by CHN-S/N elemental analyzer 1106 (Costech Instruments). Crude protein estimated by multiplying total N by a factor of 6.25 (5.82 and 4.94 were also determined), nitrogen-to-protein conversion factors, suggested by (Gnaiger, 1984, Sosulski and Imafidon, 1990), and (Salo-väänänen and Koivistoinen, 1996).
Lipid classes	rrm	X		Lipid classes were determined by thin-layer chromatography Iatroscan® system (TLC-FID analyzer TH-10 MK-VI) according to the method of Fraser et al. (Fraser et al., 1985) as described by Slizyte et al. (Slizyte et al., 2005).
Fatty acid composition	O		X	Methylation of the lipids to methyl esters was performed according to AOCS method CE 2-66 (AOCS, 2013). Fatty acid composition was determined by gas chromatography of methyl esters as described by Dauksas et al. (Dauksas et al.,2005)
Oil quality and stability				
Peroxide value (PV)	O	X	X	Determined according to AOCS Official method Cd 8b-90 (AOCS, 2013).
Anisidine value (AV)	O		X	Determined according to AOCS Official method Cd 18-90 (AOCS, 2013).
Free fatty acid content (FFA)	O	X	X	Determined according to Bernárdez et al.(Bernárdez et al., 2005), based on AOCS Official method Ca 5a-40 (AOCS, 2013).
Oxidative stability index (OSI)	O		X	Determined according to AOCS Official method Cd 12b-92 (AOCS, 2013).
Protein quality and properties				
Degree of hydrolysis (DH)	SW		X	Evaluated as the proportion (%) of α-amino nitrogen with respect to total N in the sample according to Taylor (Taylor, 1957). Modified: sample size was 0.3 g instead of 1.5 g.
Molecular weight distribution (MWD)	SW		X	Utilizing High Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS) method.

[AOAC, 1990, Bligh and Dyer, 1959, Gnaiger, 1984, Sosulski and Imafidon, 1990, Salo-väänänen and Koivistoinen, 1996, Fraser et al., 1985, Slizyte et al., 2005, Dauksas et al., 2005, Bernárdez et al., 2005, Taylor, 1957]

Chapter 3

Results and Discussion

Characterization and composition of five different fractions from North Atlantic mackerel (*Scomber scombrus*) were determined, as described in chapter 2. The goal was to determine the amount and composition of the different fractions that mackerel consists of. Knowledge on the amount and composition will enable informed decisions towards what fraction is utilized, and why. The composition was determined for mackerel rest raw material consisting of: head, backbone and tail, bellyflap, viscera, and a mixture of all four. The quality of the product was judged based on lipid content, protein content, and oil quality (oxidation status). Figure 2.2 in chapter 2.1 shows a complete overview of the compositional determination.

In addition, a storage experiment was conducted, where a mixture (unsorted) of mackerel rest raw material was stored at two different temperatures, 4 °C and 10 °C, for different amounts of time (0 hours, 24, 48, 72, 120). The raw material was then heat treated at 95 °C for 15 min in order to produce oil and protein fractions. The yield, as well as the composition and quality of the oil and protein fractions were determined by different analyses. The oil content was run through a different variety of oxidation measurements. While the protein content was tested for degree of hydrolysis. Figure 2.3 in chapter 2.1 shows a complete overview of the storage experiment, and the different analyses conducted.

All results are given as mean values of "n" number of replicates. Charts include Standard Error of Mean (SEM) as calculation of deviation. Tables include the standard deviation (SD) as calculation of deviation. The raw data of all results is found under their respective appendices, at the back of the book.

3.1 Characterization of North Atlantic mackerel rest raw material

(Figure 2.1 in chapter 2.1 presents the different body parts chosen and excised in the characterization of the rest raw material.)

Whole round fish and their respective fractions (head, bellyflap, backbone & tail, viscera, and fillet) were weighed individually to attain the amount of rest raw material fractions in mackerel. Figure 3.1 shows the rest raw material fractions in proportion to full body mass. The average weight of the five fish was 421 grams.

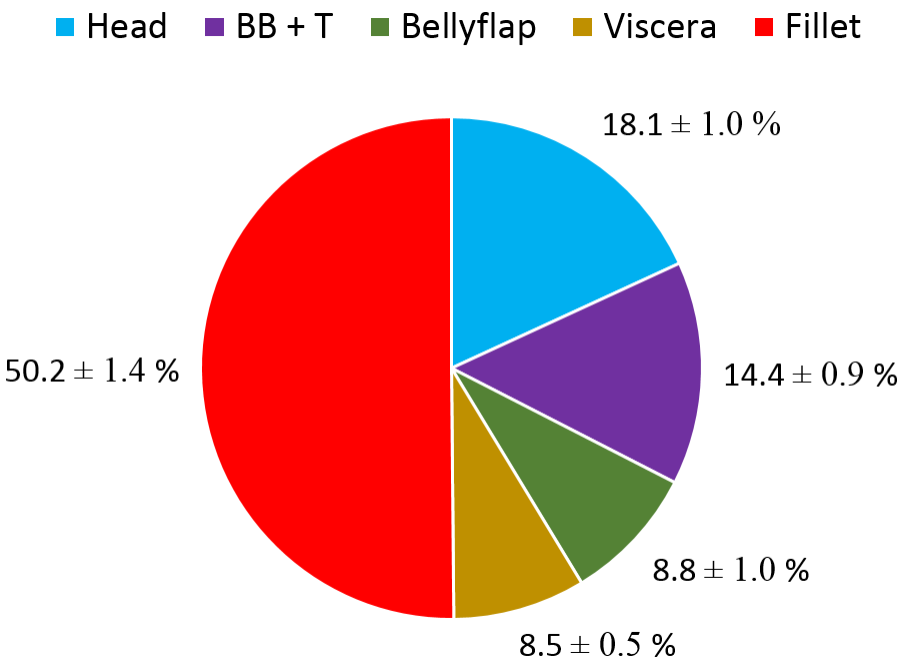

Figure 3.1: *Pie chart showing the mean percentage of the rest raw material fractions present in mackerel.* ***BB + T*** *- Backbone & tail.*

As shown, the rest raw material fractions constitute approximately 50 % of the fish, a potentially valuable resource that can be transformed into useful products.

3.2 Chemical composition of rest raw material

The composition of the raw material (North Atlantic mackerel (*Scomber scombrus*)) throughout the season differs during the natural cycle, the life stage at which the fish was caught (maturity), as well as the geographical location of the catch [Wallace, 1991]. Keeping that in mind, the determination of composition of the mackerel fractions may differ based on when and where the raw material was captured. In this thesis the seasonal variation was not studied, consequently such findings will not be reported.

The determination of composition for the five different fractions of rest raw material, showed some major differences between the different types of body parts making up the rest raw material. Constituting mostly in oil content and quality, water content, organic matter (dry matter), and elemental minerals (ash). These five different fractions have different compositional profiles, and it is therefore extremely important to characterize this before industrial utilization. The choice of rest raw material fraction depends highly on the product that is to be produced. Figure 3.2 shows the dry matter and ash content in the different fractions.

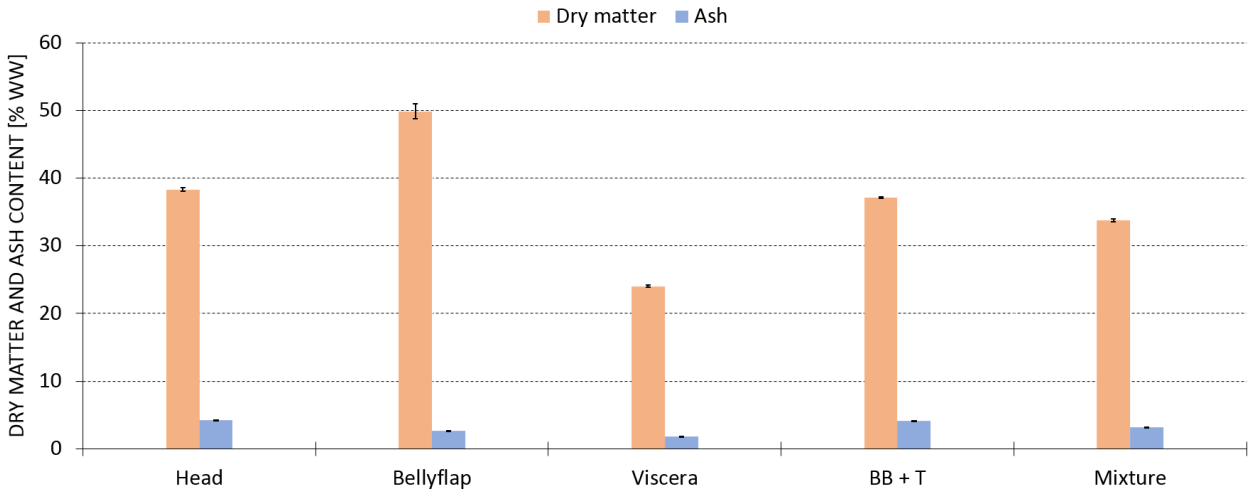

Figure 3.2: *Dry matter and ash content in rest raw material fractions. The values are given as mean percentages of dry matter/ash of a rest raw material fraction.* ***BB + T*** *- Backbone and tail. Samples were run in duplicates (n = 2).*

The head and backbone & tail regions have a high ash content due to the presence of bones and bone like materials. Moisture content appears to be relatively low in the bellyflap region compared to other fractions, this is a result of the large amount of lipids in this fraction, as shown

below. The viscera contains large amounts of moisture and low amounts of ash, as expected, due to the lack of bones and mineral-like materials in general. The mixture fraction illustrates an average, since it is a blend of all four rest raw material fractions.

The total lipid content of the different fractions of rest raw material is presented in figure 3.3.

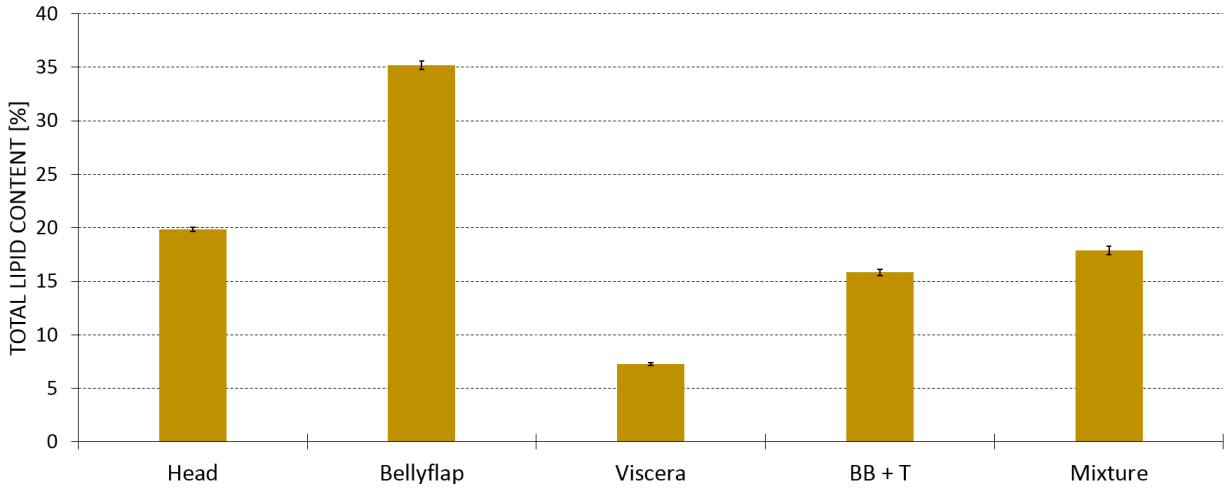

Figure 3.3: *Total lipid content in rest raw material fractions.* ***BB + T*** *- Backbone & tail. Samples were run in four replicates (n = 4).*

The lipid content ranged from 7 to 35 % in the rest raw material, viscera had the lowest amount, 7 % lipids, while the bellyflap had the highest, 35 % lipids. The head and backbone & tail fractions contained 20 and 16 % respectively, while the mixture contained around 18 %. These results give a good indicator of how the different fractions can be utilized as a resource for potential oil production. The head and backbone & tail fractions contained good amounts of lipid due to protease activity loosening collagen matrices, leading to weakened cells (less thermally stabile), releasing lipids. However, even if the fraction with the highest content of lipids is the bellyflap, it still is industrially hard to use, due to the small percentage of mass that each bellyflap constitutes per fish. Figure 3.1 shows that the average bellyflap is 9 % of total fish weight, and 18 % of total rest raw material weight.

Lipid class analysis (figure 3.4) was run to profile the oil extracted, see figure 3.14 for fatty acid composition. The analyses give insight into the different types of lipids present in the oil.

The theoretical protein content was calculated as the remaining fraction not analyzed, figure 3.1 shows the protein content, the lipid content, as well as the ash content present in the rest raw

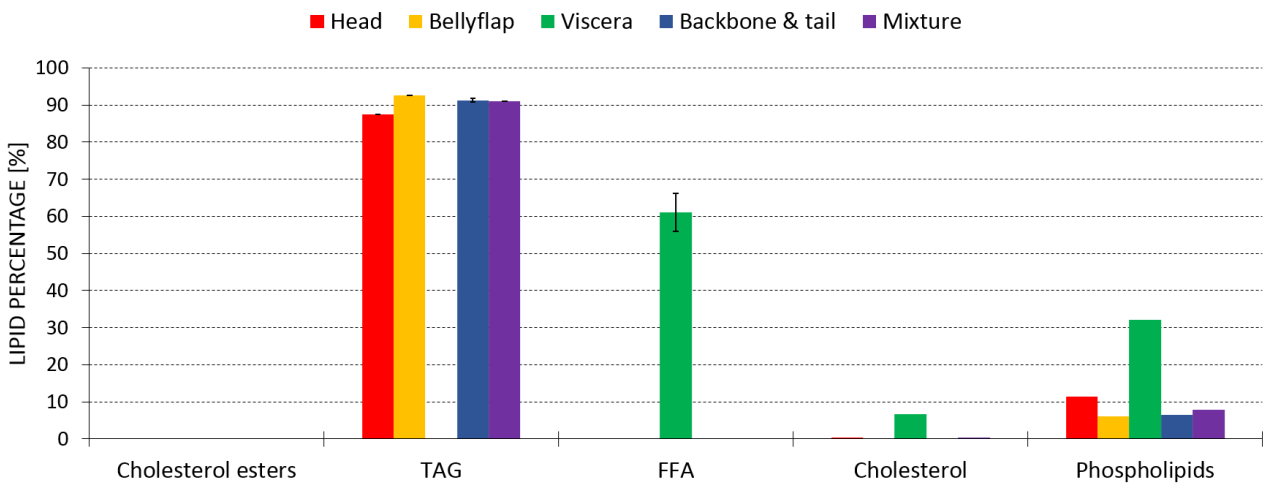

Figure 3.4: *Lipid class analysis on the different fractions obtained from mackerel.* ***BB + T*** *stands for backbone and tail.*

material fractions.

Table 3.1: *Theoretical protein content, lipid content, and ash content in the different rest raw material fractions. Values are given as g/100 g raw material (WW).* ***BB + T*** *- Backbone and tail,* ***PrC*** *- Protein content.*

	Lipid [g/100g rm (WW)]	Water [g/100g rm (WW)]	Ash [g/100g rm (WW)]	Protein [g/100g rm (WW)]
Head	19.9	61.7	4.2	14.3
Bellyflap	35.2	50.1	2.7	12.0
Viscera	7.3	76.0	1.8	15.0
BB + T	15.8	62.9	4.1	17.2
Mixture	17.9	66.3	3.2	12.7

The theoretical protein content ranged between 12.0-17.2, where the highest belonged to the backbone & tail fraction. This is explained by the amount of bone and bone-like materials present, which is mainly composed of protein and ash. The viscera and head fractions had 15.0 % and 14.3 % of theoretical protein respectively. The bellyflap fraction contained the least amount of protein, 12.0 %, which is expected due to the distinctly high content of lipids.

3.3 Quality of oil extracted from the rest raw material

The quality of the oil extracted from the different rest raw material fractions was evaluated by the determination of free fatty acid content and peroxide value (PV). The content of FFA (expressed as % oleic acid) and PV as a function of rest raw material fractions, is shown in figures 3.5 and 3.6, respectively.

The % FFA content is a shelf life indicator test, and the % FFA is also a quality parameter for hydrolytic rancidity, and is therefore used to indicate oil quality.

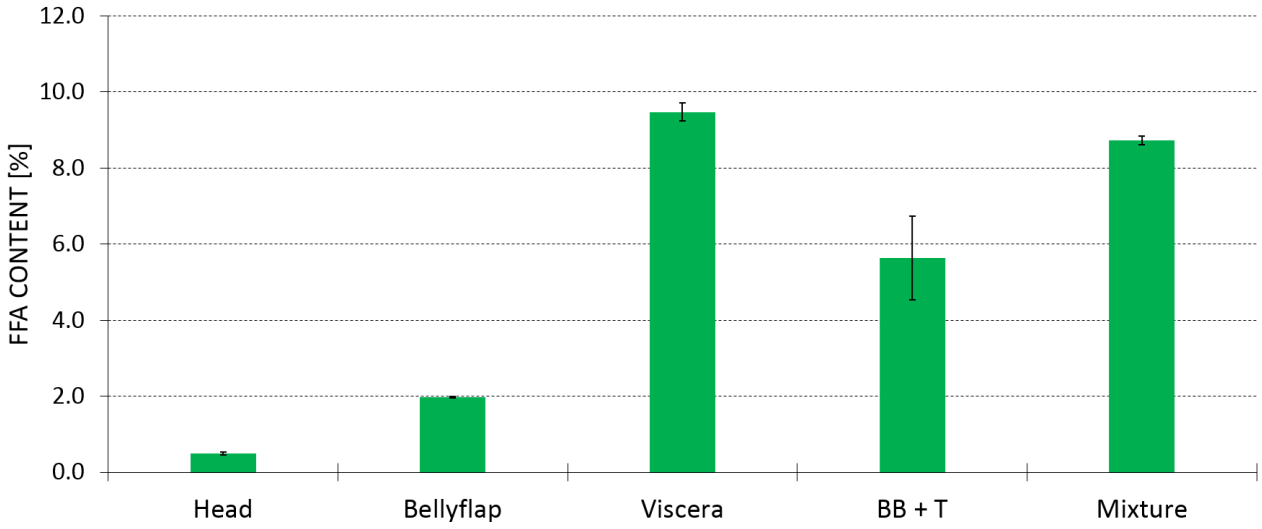

Figure 3.5: *Free fatty acid content (expressed as % oleic acid) in oil extracted from mackerel rest raw material fractions.*

As expected, fractions containing high concentrations of endogenous enzymes have higher FFA values, i.e. viscera has the highest value at 9.5 %. Viscera is also included in the mixture fraction, which has influenced the FFA content negatively. The lowest value of % FFA belonged to the head fraction, this may be due to lack of digestional enzymes, such as lipases, that degrade lipids.

Formation of peroxides is the first sign of rancidity in an oil. PV analysis is used in both shelf life studies, and quality measurements to measure the amount of primary oxidation products present, which can indicate initiation of the autoxidation process in lipids. The PV value measures the present quality of the oil, and in tandem with the AV value, can provide a qualitative "status" of the oil by calculating the TOTOX value, referring to chapter 1.7.3 for definition of

TOTOX.

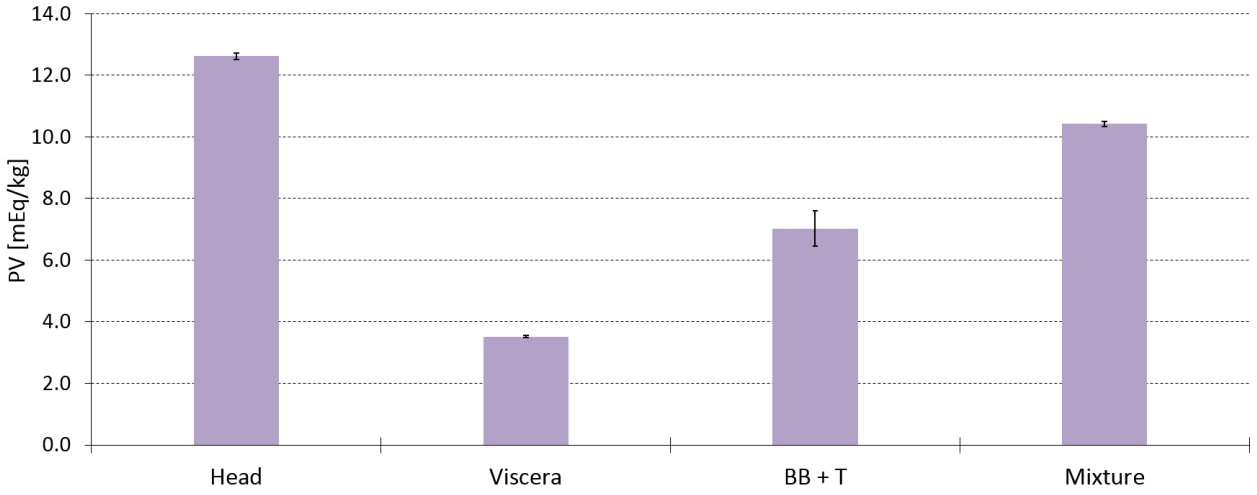

Figure 3.6: *PV measurement of oil extracted from rest raw material fractions. Values are expressed as mEq of oxygen per kg of lipid.*

Unfortunately, the bellyflap fraction "the most important and interesting, due to its high lipid content" - was not analyzed by PV measurement. This was a result of a lack of oil extracted from this fraction.

The characterization of the rest raw material was done on frozen fish that have been thawed more than once. It should be noted that this may influence the quality parameters with respect to FFA and PV values. Oxidation may still occur during freezing [Aursand et al., 2011, Fennema et al., 2007]. The characterization was also performed on mackerel fish captured during the fall season (august-october), which may have a different chemical composition with regards to lipid, protein, and ash content. In comparison, the rest raw material ordered for the storage experiment was captured in January [Wallace, 1991, Falch et al., 2006].

The decision to use the mixture as the product for the storage experiment was based on the industrial need. The utilization of a mixture of all rest raw material fractions is more beneficial and practical, than to separate the rest raw material into different fractions, industrially speaking. Therefore the rest raw material bulk was utilized in its entirety, meaning that the storage experiment was done on with a mixture of heads, bellyflaps, viscera, and backbones & tails.

3.4 The storage experiment

The results of the storage experiment are presented below. The oil and stickwater fractions were analyzed to determine chemical composition, quantity and quality, while only the determination of water and ash content was run on the sludge fraction. The quantity and quality of both the lipid and protein products extracted from the raw material were analyzed by a variety of analyses. Table 2.2 in chapter 2.4 gives an overview of all analyses that were conducted to quantify the composition, quantity, and quality of the lipids and proteins extracted.

To the best of my knowledge, very few studies have looked at the possibility of producing oil and protein products from mackerel rest raw material. This is reflected in the amount of research papers that are published on the subject. Therefore, further in this thesis, most comparisons to earlier work will be made to results on the closely related marine species family of herrings (*Clupeidae*).

3.4.1 Effect of storage time on quantitative yield

Thermal treatment was conducted on the mixture of rest raw material that was either stored at 4 or 10 °C for 0-120 hours. It should be noted that the initial heating of the samples was done with a microwave at 800 watts for 40 ± 5 seconds. This was done to avoid the raw material gradually heating up to 95 °C, which can activate certain endogenous enzymes at their optimal temperature, resulting in lower oil or protein quality. Therefore the heating step can be neglected as a potential source of higher endogenous enzyme activity. The post thermal treatment yield was determined for all replicates of crude oil, stickwater, and sludge, as a function of storage time. Sampling was conducted at 0 h, 24 h, 48 h, 72 h, and 120 h of storage. Mean values of "product" yield, e.g. crude oil, stickwater, or sludge, are given in figures 3.7, 3.8, and 3.9 as g product (dry weight) per 100 g raw material (wet weight). The dry matter content of the crude oil is assumed to be 100 %.

Figure 3.7 shows that the crude oil yield increases by approximately 25-30 % in the first 24 hours, regardless of storage temperature. However, in comparison to the rest of the storage period 24 h - 120 h, the yield remains relatively consistent and unchanged. This correlates well with the decrease in sludge yield, based on the tandem increase in both oil and stickwater yields,

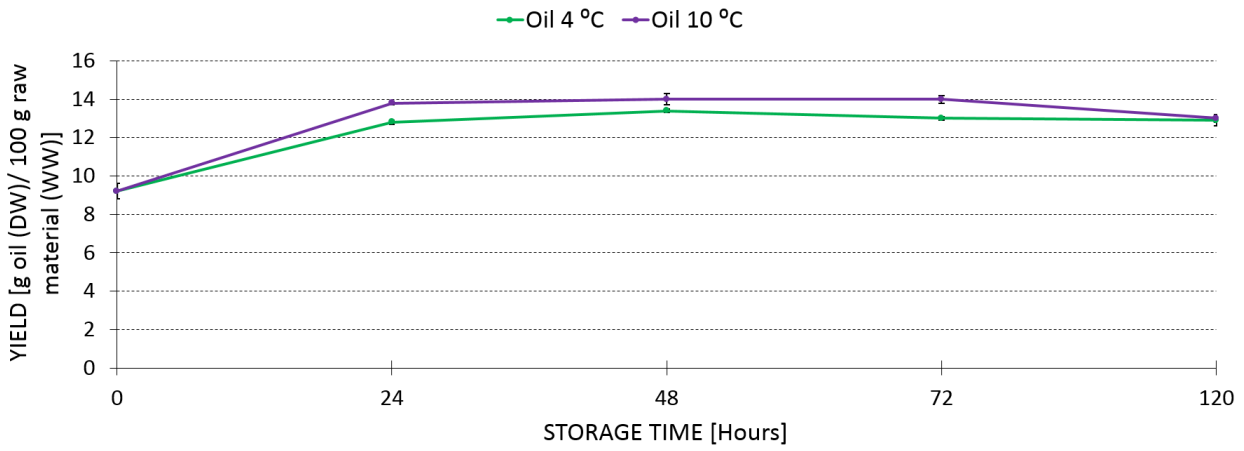

Figure 3.7: *Post thermal treatment yield of crude oil given as g (DW) / 100 g raw material (WW) as a function of storage time. Extracted from raw material stored at 4 and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h, and 120 h of storage. 16 replicates (n = 16)$^{\gamma}$.*

γ - Except storage period 120 h, where the number of replicates for storage of raw material at 4 and 10 °C were 14 (n = 14) and 13 (n = 13) respectively.

as seen in figures 3.7 and 3.8. The temperature difference during storage also plays a minor role in the activity of the endogenous enzymes present in the raw material. This is shown by the slight increase in crude oil yield across the storage periods. Storage at 10 °C results in more solubilized raw material, due to the increased activity of the endogenous enzymes as a result of temperature increase 1.4.2.

The yield of stickwater shows similar trends to the yield of crude oil, increasing in the first 24 h, figure 3.8. However the stickwater yield decreases after 48 h , and retains a consistent value after 72 h and 120 h. The temperature of storage does not have a large influence on the yield of stickwater, and the value remains relatively stable. At 120 h, the chart shows a decrease in yield for the stickwater stored at 10 °C, and an increase in the stickwater yield that was stored at 4 °C. This may be due to the increased degradation of proteins, as a result of higher temperature. Increase in degradation may result in an increase in emulsification/insolubility of proteins, leading to an increase in sludge yield.

Figure 3.9 presents the yield of the sludge fraction. The decrease in sludge yield in the first 24 h might possibly be correlated with the simultaneous increase in both oil and stickwater yields. This may be caused by endogenous enzyme activity, and an increase in solubility of proteins and

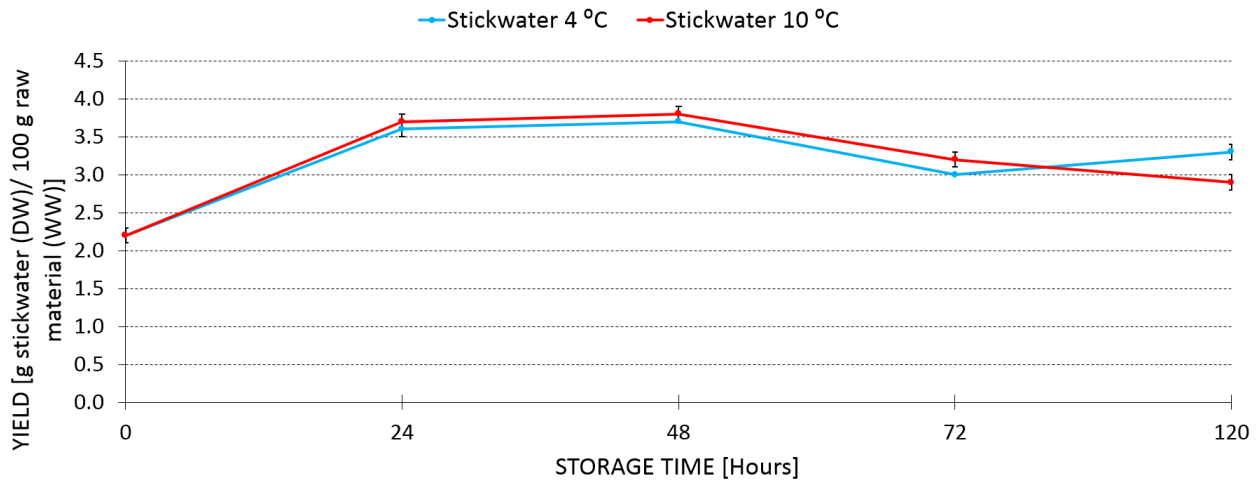

Figure 3.8: *Post thermal treatment yield of stickwater given as g (DW) / 100 g raw material (WW) as a function of storage time. Extracted from raw material stored at 4 and 10 °C. Sampling was done after 24 h, 48 h, 72 h, and 120 h of storage. 16 replicates (n = 16)$^{\gamma}$.*

γ - Except storage period 120 h, where the number of replicates for storage of raw material at 4 and 10 °C were 14 (n = 14) and 13 (n = 13) respectively.

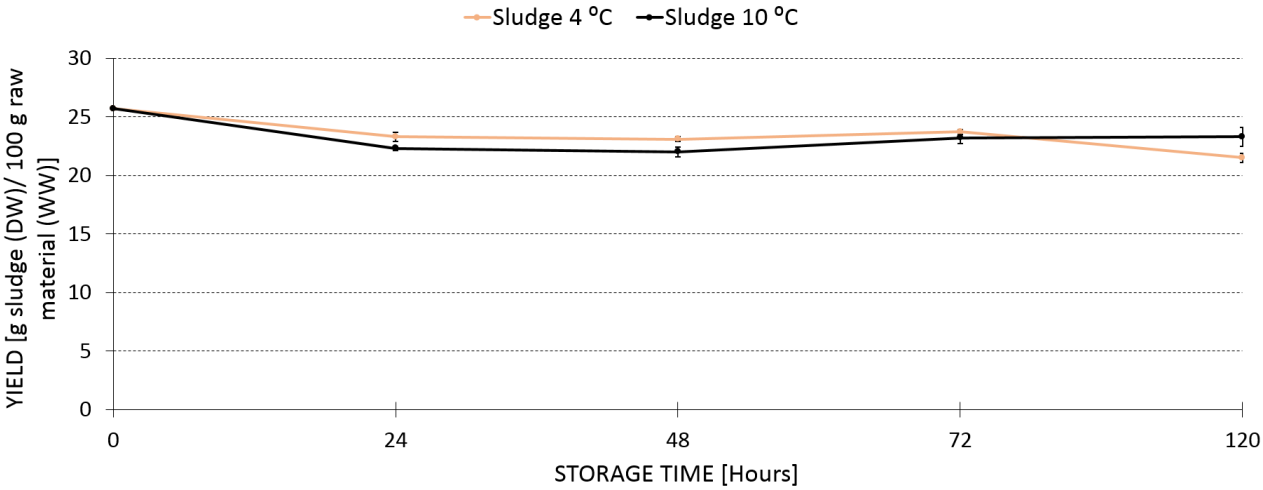

Figure 3.9: *Post thermal treatment yield of sludge given as g (DW) / 100 g raw material (WW) as a function of storage time. Extracted from raw material stored at 4 and 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h, and 120 h of storage. 16 replicates (n = 16)$^{\gamma}$.*

γ - Except storage period 120 h, where the number of replicates for storage of raw material at 4 and 10 °C were 14 (n = 14) and 13 (n = 13) respectively.

release of lipids from the sludge phase. Storage at 10 °C results in more solubilized raw material, due to the increased activity of the endogenous enzymes.

The sludge yield may also increase due to higher solubilization of the raw material. This may explain the change in sludge yield after 48 h. The increase in sludge yield may be explained by increases in the emulsifying properties of both water soluble and non-water-soluble proteins, where the increase in emulsifying ability may result in the incorporation of more protein and lipids into the sludge fraction.

In conclusion, all yields of crude oil, stickwater, and sludge were influenced by storage time and storage temperature. Storage at 10 °C resulted in higher yields of crude oil and stickwater, in comparison to storage at 4 °C. The yield of the sludge fraction was observed to decrease simultaneously when the stickwater and oil fractions increased. The crude oil and stickwater yields increase rapidly in the early stages of storage, while the sludge yield decreases. After 24 h of storage, the yield of both crude oil and stickwater remains relatively constant, as does the sludge yield. In general, storage time had a stronger impact than storage temperature on the yield of crude oil, stickwater, and sludge.

3.4.2 Effect of storage on quality parameters of crude oil

Free fatty acid content in crude oil

Free fatty acid content (% FFA) is an important quality parameter for crude marine oils. The levels of FFA in the crude oil extracted by thermal treatment are presented in figure 3.10 as a function of storage time. The raw data is found in appendix J.

Figure 3.10 shows that the % FFA is influenced by storage time, especially for the raw material that was stored at 10 °C. % FFA increases with increasing storage time of raw material. This is due to the activity of endogenous lipases, which are temperature dependent enzymes that degrade lipids (referring back to chapter 1.4.2). % FFA increases rapidly after 48 h due to increased lipid degradation by endogenous lipases [Carvajal, 2013]. The increase was larger for raw material stored at 10 °C, as opposed to 4 °C, due to the temperature dependence of the endogenous lipases. Storage at 10 °C for 72 h lead to an increase of % FFA from 0.5 to 4.6 %, while Carvajal [Carvajal, 2013] reported 0.3-3.4 % for the same storage conditions of herring rest raw material

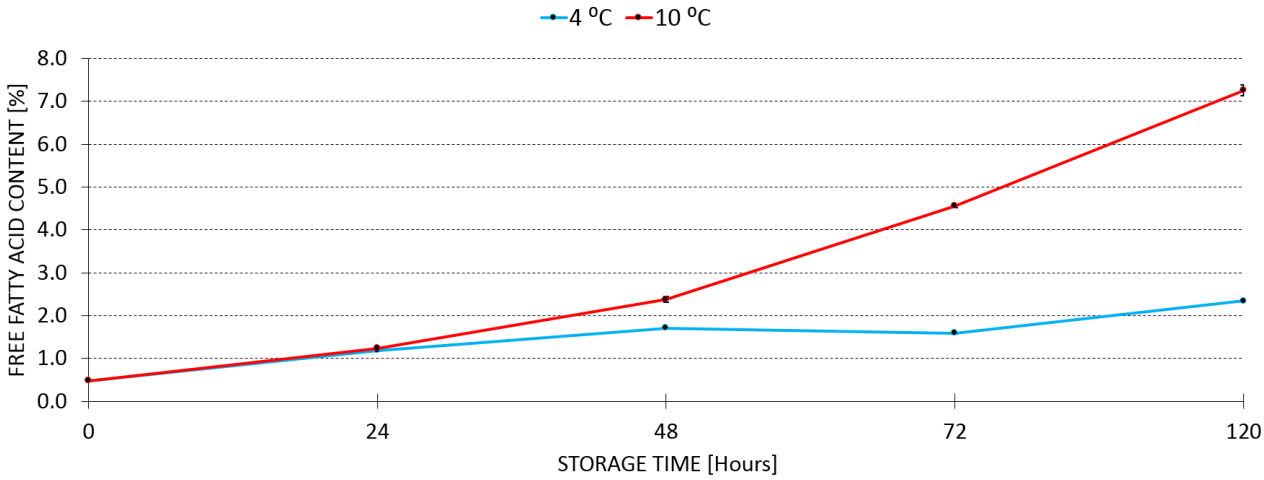

Figure 3.10: *Free fatty acid content (% FFA) of crude oil extracted from raw material stored at 4 and 10 °C, as a function of storage time. Sampling was done after 24 h, 48 h, 72 h, and 120 h of storage. Values are given as a mean of four replicates (n = 4).*

(including viscera).

The crude oil from fresh raw material (0 hours of storage) in this thesis had a mean % FFA content of 0.5 ± 0.02 %. In comparison, Aidos et al. [Aidos et al., 2002] reported that the % FFA of crude herring oil produced by thermal treatment at 95 °C from fresh raw material (including viscera) was 0.60 ± 0.01 %. Findings by Carvajal et al. [Carvajal, 2013] indicated that the % FFA levels were lower in crude oil produced from rest raw materials (including viscera) of Norwegian spring spawning (NSS) herring, when utilizing thermal treatment as opposed to enzymatic hydrolysis. % FFA was 0.15 ± 0.02 % after thermal treatment (95 °C), and 0.4 ± 0.05 % after enzymatic hydrolysis with commercial enzymes (bromelain and papain). This difference may be due to the fact that while enzymatic hydrolysis utilizes a lower temperature than thermal treatment, the process involves stirring (aeration) the minced raw material in the presence of oxygen and for lengthy time periods. Carvajal [Carvajal, 2013] conducted the research industrially through a pilot plant, where the processing of the rest raw material was optimal and effective. The raw materials composition may have also influenced the difference in FFA values, herring captured during the January/February period has a lower percentage of lipid [Carvajal, 2013]. Other reasons pertaining to lower FFA content by thermal treatment may be related to the temperature of denaturation of endogenous enzymes. Endogenous enzymes in enzymatic hydrolysis are still active up to 60 °C. Inactivation of lipases occurs first above 50 °C,

and Søvik et al. [Søvik and Rustad, 2005] found that cod fish lipases, needed heating for 10 min at temperatures above 60 °C for their inactivation.

The raw material stored at 4 °C was relatively stable, with a change in % FFA content of only 0.5 ± 0.02 % to 2.3 ± 0.01 % occurring during 120 hours of storage. However, in comparison, a change of 0.5 ± 0.02 % to 7.2 ± 0.25 % occurred during 120 h of storage at 10 °C. Nonetheless, these values are still acceptable and are edging the recommended limit of 5-7 %, that was proposed by Bimbo [Bimbo, 1998] for crude marine oils intended for human consumption. These values however, do not take into consideration the conditions of storage and how the raw material is treated along the production chain.

Research by Aidos et al. [Aidos et al., 2002] has presented findings on the quality of herring oil as a function of storage time, but not considered if the freshness of the raw material itself plays a crucial role. The study reported that the crude oil maintained a good quality over extended periods of storage (<155 days, <50 °C), but concluded that the storage temperature had a large impact on the stability of the oil, in terms of PV and AV. FFA content was relatively stable and consistent throughout, which was suggested to be a result of the inactivation of lipases and phospholipases during thermal treatment, leading to little to no enzymatic hydrolysis occurring. As mentioned in 1.6, one of the main factors influencing the formation of FFA is the presence of lipases and phospholipases, while microbial activity is regarded as less influential.

Few studies have utilized thermal treatment as the sole method to extract crude oils and proteins in comparison to enzymatic hydrolysis. However Wu et al. [Wu and Bechtel, 2008] found that the quality of crude oil produced by thermal treatment (15 min at 95 °C) of salmon heads and viscera stored at 15 °C for up to 4 days, remained higher than anticipated, based on previous work. Wu et al. [Wu and Bechtel, 2008] concluded that the oil still was a good source of high quality LC-PUFAs. % FFA remained below 7 % and the natural antioxidants present in the oil were 25 % of the initial value.

In general the % FFA values were affected more by the storage temperature, than the storage time, indicating that the quality and the freshness of the raw material is heavily influenced by the temperature. This, in tandem with other findings may support the concept of transporting raw material from remote geographical locations to central hubs for processing. However, standardization of storage conditions must be well in place, to ensure that the raw material retains

its high quality and can be used for human consumption. The palatability of the produced oil, i.e. the raw material stored for more than 72 hours had an unpleasant odor, and was relatively dark in color in comparison to what oil normally looks like. This means that organoleptic properties are highly influenced by the content of FFA.

Oxidation status of lipids in crude oil

The oxidation status of the crude oil extracted from mackerel rest raw material is given as peroxide value (PV), *para*-anisidine value (AV), and cooperatively calculates the total oxidation status (TOTOX = 2 × PV + AV). Table 3.2 presents the oxidation status of the crude oil extracted from fresh raw material (0 hours of storage).

Table 3.2: The oxidation status of crude oil extracted from fresh raw material (0 hours of storage). Values of PV, AV, and TOTOX are presented with their respective deviations.

	PV [mEq/kg]$^{\pi}$	AV [-]$^{\rho}$	TOTOX [-]$^{\pi}$
Crude oil, 0 hours of storage	5.3 ± 0.4	1.0 ± 0.2	11.6 ± 0.4

- π - expressed as mEq/kg lipid.
- ρ - expressed as anisidine value units.

Carvajal [Carvajal, 2013] reported that the PV value of crude oil extracted by thermal treatment at 90 °C, for NSS herring rest raw material (including viscera), was 4.6 ± 0.2 mEq/kg. The PV value obtained in this thesis supports Carvajal's findings, across species. The TOTOX value in table 3.2 is well within the recommended limit of 26 (the value is specified for refined oils). It is standardized by the National Science Foundation, the American National Standards Institute, and the Global organization for EPA and DHA omega-3s [NSF, 2011, GOED, 2012]. This indicates that the oil extracted is of good quality. Figures 3.11 and 3.12 show the obtained results for PV, AV, and TOTOX calculation for the storage experiment at temperature 4 and 10 °C respectively.

Figure 3.11 shows expected trend development between the PV and AV values when the raw material was stored at 4 °C, referring to figure 1.8 in chapter 1.7.3. The early stages of storage initiate oxidation, which manifests in the formation of peroxides and the increase in PV value.

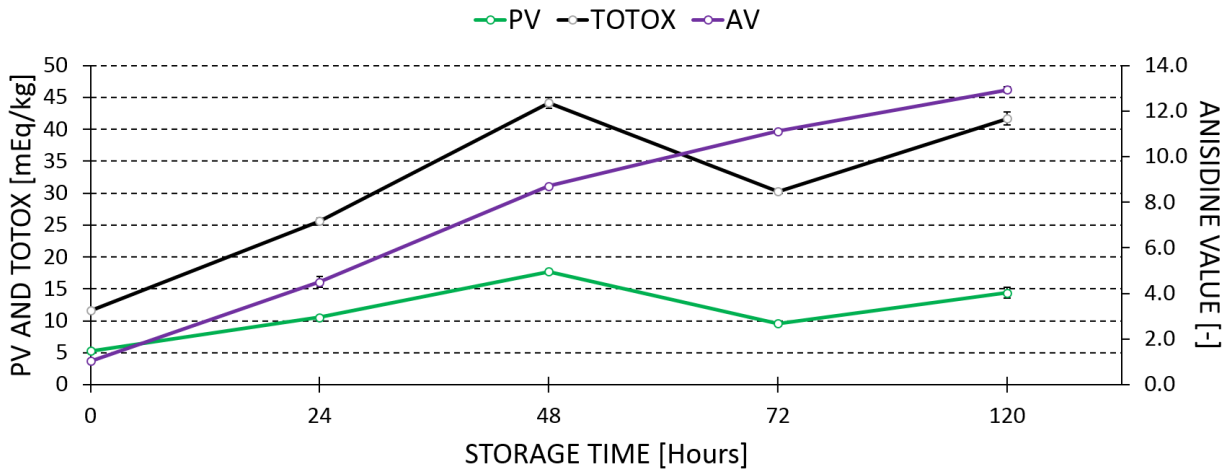

Figure 3.11: *PV, AV, and TOTOX values are given as a function of storage time for crude oil extracted from raw material (stored at 4 °C) by thermal treatment (90 °C, 15 min). Sampling was done after 24 h, 48 h, 72 h, and 120 h of storage. Values are given as a mean of 3 replicates of PV, AV, and TOTOX respectively (n = 3).*

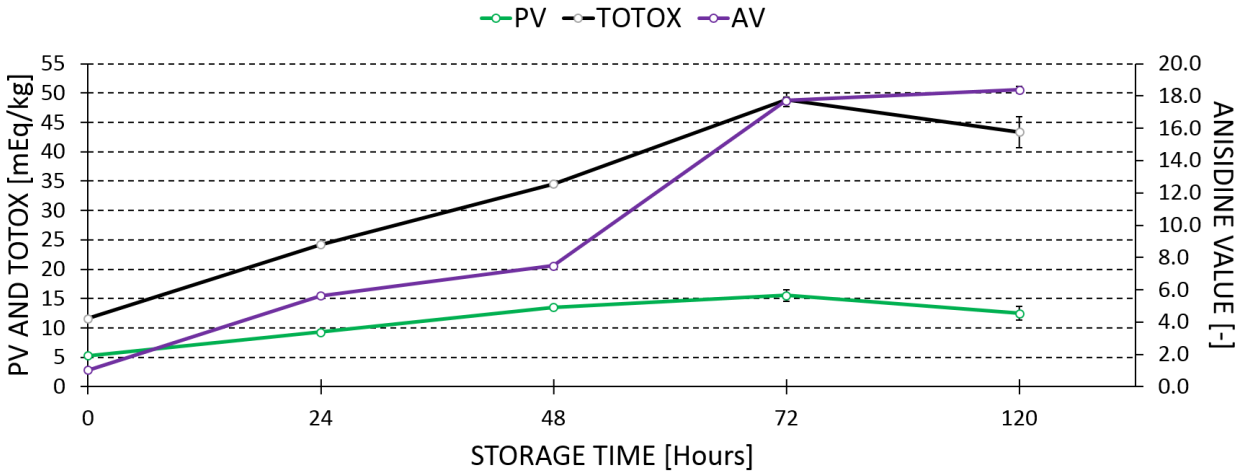

Figure 3.12: *PV, AV, and TOTOX values are given as a function of storage time for crude oil extracted from raw material (stored at 10 °C) by thermal treatment (90 °C, 15 min). Sampling was done after 24 h, 48 h, 72 h, and 120 h of storage. Values are given as a mean of 3 replicates of PV, AV, and TOTOX respectively (n = 3).*

The peroxides are unstable and degrade to form secondary oxidation products in the mid-stages of storage, resulting in an increased AV value. Figure 3.12, raw material stored at 10 °C, shows that the PV and AV values do not have the same "expected" trend. The PV value decreases later in the storage period (after 72 h), even though a drastic increase in the AV value is observed between 48 and 72 hours, which does not match with the raw material stored at 4 °C. This may

be due to the increased activity of the endogenous enzymes (higher temperature), leading to higher amounts of peroxides, which rapidly increase the AV value.

In comparison, Aidos et al. [Aidos et al., 2002] reported values for the oxidation of crude oil extracted from herring rest raw materials (including viscera) using thermal treatment (90 °C), to be PV 0.7 ± 0.2 mEq/kg lipid, and AV 0.4 ± 0.06. Aidos' PV value, in comparison to this project, table 3.2, is extremely low. This is believed to be due to the freshness of the raw material utilized. The raw material obtained for this project was caught and delivered (approximately 24-72 h after capture) to the lab, upon which filleting took place (approximately 3 hours), and the raw material was partitioned and stored (mark 0 hours in storage experiment). This may induce higher PV values due to contact with atmospheric air during transport, and contact with blood during filleting.

Carvajal et al. [Carvajal, 2013] reported that the AV value of crude oil extracted from NSS herring rest raw material after thermal treatment and storage at 10 °C for 72 hours, increased from 0.7 to 19.0. This is supported by the results obtained in this thesis, AV increased from 1.0 to 17.7 in the span of 72 h when stored at 10 °C. Drastic AV value changes indicate improper storage of the raw material, resulting in a decrease in quality.

Overall, the results obtained show that the rest raw material used has great potential. All values of oxidation of the crude oil extracted in this thesis are well under the maximum recommended values of PV and AV for crude oils intended for human consumption. Standardization of quality parameters exists [NSF, 2011, GOED, 2012]. Recommended values of PV are reported to be between 3-20 mEq/kg lipid, and AV between 4-60 [Hamm, 2009]. The PV obtained for this thesis varied between 5.3-17.7 ± 0.9 mEq/kg lipid, and the AV ranged between 1.0-18.4 ± 0.2.

Crude oil oxidation stability

The oxidative stability of the crude oil was evaluated utilizing the oxidative stability instrument, to determine the oils oxidative stability index (OSI). The OSI was determined by continuous measurement of the conductivity (increases indicate presence of oxidation products), while the oil was introduced to oxygen and high temperature, 70 °C (accelerated oxidation). Figure 3.13 shows an overview of the results obtained. Values are means of 4 replicates. The OSI-time (indicating the time it takes to reach the "inflection point" (marked in figure 3.13) for the production

of oxidation products) values are given in tables 3.3 and 3.4.

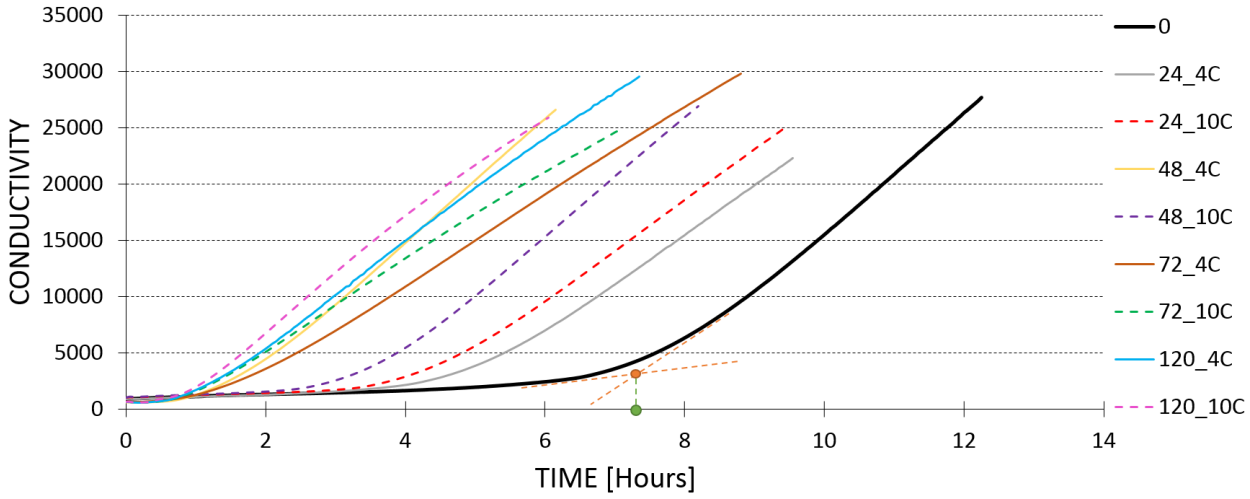

Figure 3.13: *OSI analysis, conductivity as a function of time for crude oil extracted from raw material, stored at 4 and 10 °C, accelerated oxidation at 70 °C. An illustration of how inflection point is determined is done on the black curve line. Values are given as a mean of 4 replicates (n = 4).* ***Straight lines*** *- raw material stored at 4 °C, except 0 (fresh raw material, 0 hours storage),* ***Stippled lines*** *- raw material stored at 10 °C.*

Table 3.3: The OSI time with deviation, for the oxidized crude oil extracted from mackerel rest raw material stored at 4 °C. Values are given as a mean of 4 replicates. Nomenclature; ex. 24_4C is the crude oil extracted from rest raw material that was stored at 4 °C for 24 hours.

Sample	OSI time [Hours]	SD	SEM
0 (not stored)	7.4	0.4	0.2
24_4C	5.4	0.5	0.3
48_4C	1.7	0.2	0.1
72_4C	2.4	0.1	0.0
120_4C	1.5	0.2	0.1

Figure 3.13 shows that the crude oil produced from fresh (0 hours of storage) raw material was the most stable in terms of oxidation, with an OSI time of 7.4 ± 0.4 hours to reach the inflection point. The OSI-time decreased consistently with the increase in storage time, as shown in tables 3.3 and 3.4. However, table 3.3 shows that the OSI-time value for crude oil stored at 4 °C for 48 hours is much lower than the expected value, which is supposed to be between the values of 24 h and 72 h, between 5.4-2.4 hours. It is difficult to explain the lower stability (low OSI-time) in the crude oil stored at 4 °C for 48 h, compared to crude oil stored at 10 °C for 48

Table 3.4: The OSI time with deviation, for the oxidized crude oil extracted from mackerel rest raw material stored at 10 °C. Values are given as a mean of 4 replicates. Nomenclature; ex. 72_10C is the crude oil extracted from rest raw material that was stored at 10 °C for 72 hours.

Sample	OSI time [Hours]	SD	SEM
0 (not stored)	7.4	0.1	0.1
24_10C	4.7	0.3	0.2
48_10C	3.7	0.2	0.1
72_10C	1.6	0.4	0.2
120_10C	1.3	0.3	0.2

h, since one would expect a decrease in stability with increased storage temperature. Another explanation might be that the analysis of OSI time for 48 h was flawed. Decreases in stability with increases in storage temperature can be explained by the increase in lipid oxidation rate as temperature increases [Carvajal, 2013]. The formation of peroxides may lead to decomposition towards lipid radicals, resulting in the formation of secondary oxidation products, which shorten the induction period.

Except for storage period 48_4C, all other values satisfy that stability decreases as storage temperature increases, as well as that stability decreases with increased storage time, due to the high production of oxidation products (increased PV and AV).

Fatty acid composition in crude oil

The fatty acid profile of the crude oil was evaluated by gas chromatography, figure 3.14 gives an overview of fatty acid composition of the crude oil extracted during the storage experiment. The blue series designates the crude oil extracted from raw material stored at 4 °C, while the red series designates the crude oil extracted from raw material stored at 10 °C, both series increase in color shade to indicate increasing storage time. The fresh raw material (0 hours of storage) is designated by the black series.

Figure 3.14 shows that the crude oil contains a broad spectrum of fatty acids. No consistent trends show differences between storage time or temperature, as well as little to no difference between fresh and stored raw material. However, as given in table 3.5, the amount of the most abundant fatty acids indicate high amounts of omega-3 PUFA's. EPA 8.1 %, DHA 10.6 %, and Cetoleic acid with the highest amount at 13.7 %. As reported by Ruyter et al. [Ruyter, 2016],

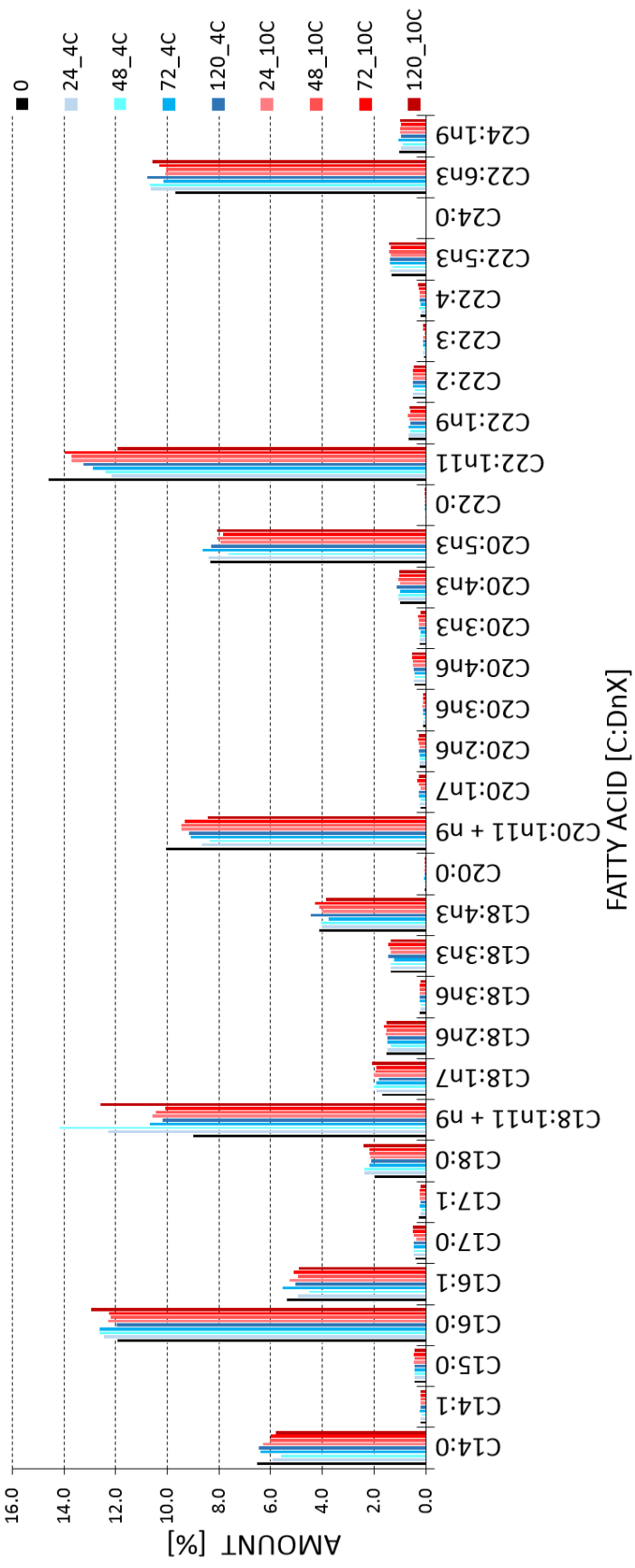

Figure 3.14: *Fatty acid profile for crude oil extracted during the storage experiment. Values are given as means (%) of 2 replicates (n = 2).* ***C*** *- number of carbon atoms,* ***D*** *- number of double bonds,* ***nX*** *- stands for the n - x nomenclature system.* ***Black series*** *- fresh raw material (0 hours of storage),* ***Blue series*** *- raw material stored at 4 °C,* ***Red series*** *- raw material stored at 10 °C.*

cetoleic acid from herring oils, has been shown to stimulate cells to convert short omega-3 fatty acids into the healthier omega LC-PUFA's. This was conducted both as cell based experiments on liver cells *in vitro*, and as a feed experiment carried out on salmon. Cetoleic acid added to salmon feed, caused the salmon to store 10 % more marine omega-3 fatty acids (EPA and DHA) in the body than they do otherwise . The effect on humans is still unknown, but the study suggests that consumption of herring and/or fish species with high amounts of cetoleic acid, may influence the amount of stored healthy marine omega-3 acids in the body [Ruyter, 2016].

Table 3.5: Fatty acid profile, containing the fatty acids that contribute the most to the total lipid content. Total values of unknown$^{\pi}$, saturated, mono-unsaturated, poly-saturated, and omega-3 fatty acids are given as well. Values are given as means of 2 replicates.

Fatty acid lipid number [C:D]:	n – x:	Trivial name:	Amount [%]:
14:0	-	Myristic acid	~ 6.0
16:0	-	Palmitic acid	~ 12.2
16:1	n - 7	Palmitoleic acid	~ 5.3
18:1	n - 9, n - 11	Oleic acid	~ 10.6
18:4	n - 3	Stearidonic acid	~ 4.1
20:1	n - 11, n - 9	Gadoleic acid, Eicosenoic acid	~ 9.5
20:5	n - 3	Eicosapentaenoic acid (EPA, Timnodonic acid)	~ 8.1
22:1	n - 11	Cetoleic acid	~ 13.7
22:6	n - 3	Docosahexaenoic acid (DHA)	~ 10.6
Sum of unknown fatty acids$^{\pi}$		~ 5.8 %$^{\pi}$	
Sum of saturated fatty acids		~ 21.7 %	
Sum of mono-unsaturated FA's		~ 42.7 %	
Sum of poly-unsaturated FA's		~ 29.9 %	
Sum of omega-3 FA's		~ 26.6 %	

π - fatty acids that were not identified by GC analysis, due to lack of standards.

3.4.3 Chemical composition of stickwater

Freeze dried stickwater samples were analyzed for protein and lipid content to have a complete perspective of the composition.

Protein content in stickwater

The protein content was determined by means of total nitrogen (N) measurement with CN elemental analyzer. The nitrogen percentage is then multiplied by a specific factor to determine the protein content. A conversion factor of 6.25 is the most widely used for protein determination

on the basis of % nitrogen. However, some suggest that this factor is not suitable for all protein sources, due to the large variation in amino acid composition [Falch et al., 2009]. Gnaiger et al. [Gnaiger, 1984], as well as Sosulski et al. [Sosulski and Imafidon, 1990] have both shown that a conversion factor of 5.82 may be more correct for raw material from fish. An interesting study by Salo-väänänen et al. [Salo-väänänen and Koivistoinen, 1996] has also reported the use of the factor 4.94 for the determination of protein content. The reason being that fish raw material can contain significant amounts of nitrogen from other sources than amino acids, peptides, or proteins, including but not limited to; free nucleotides, urea, nucleic acids, and trimethylamine N-oxide (TMAO). Protein content presented in this thesis will be calculated by the traditional factor of 6.25, unless otherwise stated.

Figure 3.15 presents the protein content as a function of storage time of raw material.

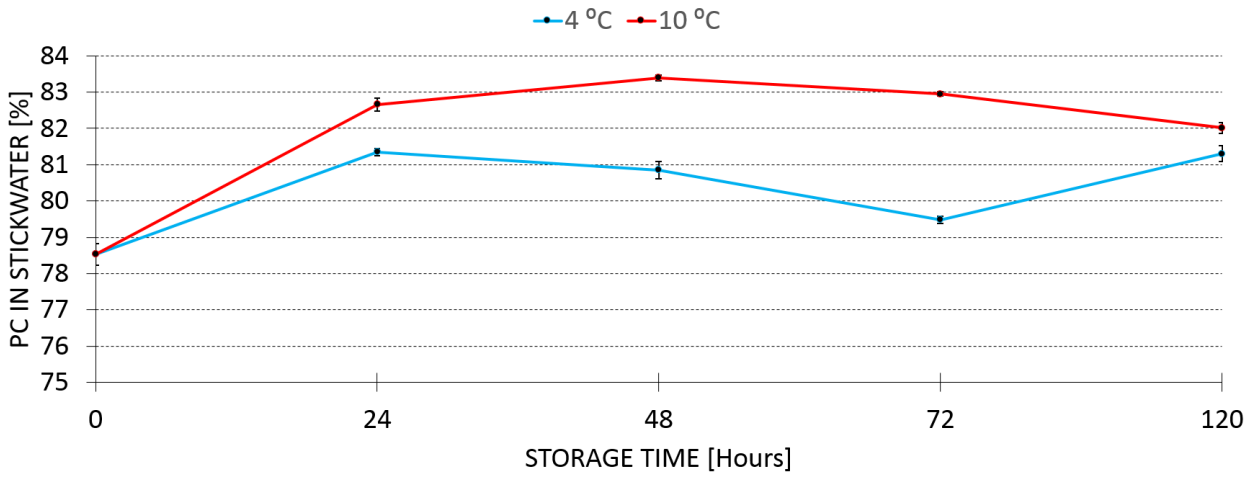

Figure 3.15: *Protein content of freeze dried stickwater as a function of storage time of raw material. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. Values are given as a mean of 4 replicates (n = 4).* ***PC*** *- Protein content*

The protein content ranged from 78.5 ± 0.6 % to 83.4 ± 0.2 %, and the values suggest that the storage temperature influenced the protein content in stickwater significantly. Raw material stored at 10 °C contains higher values of protein content than raw material stored at 4 °C. This was expected, a higher rate of degradation by endogenous enzymes solubilizes more proteins. Figure 3.15 also shows the importance of the effect that endogenous hydrolysis has on protein content. Protein content increases consistently in the first 24 hours of endogenous hydrolysis, irrespective of storage temperature. However, the values also suggest a stagnation or decrease

in protein content after 24 h and 48 h, for raw material stored at 4 and 10 °C respectively. This may be due to higher degradation, which possibly results in the incorporation of lipids into the stickwater fraction, due to increased emulsifying abilities. This may also influence the products quality with respect to purity and stability. After 48 hours of storage, the decrease in protein content may also be contributed to the formation of volatile amines, since the freeze dried powders began developing pungent odors after 48 h of storage. Increased storage time may also effectively solubilize impurities such as skin, bone, or cartilage, resulting in their incorporation.

For further illustration, figure 3.16 and 3.17 present the protein content calculated with factors 5.82 and 4.94 in comparison to 6.25.

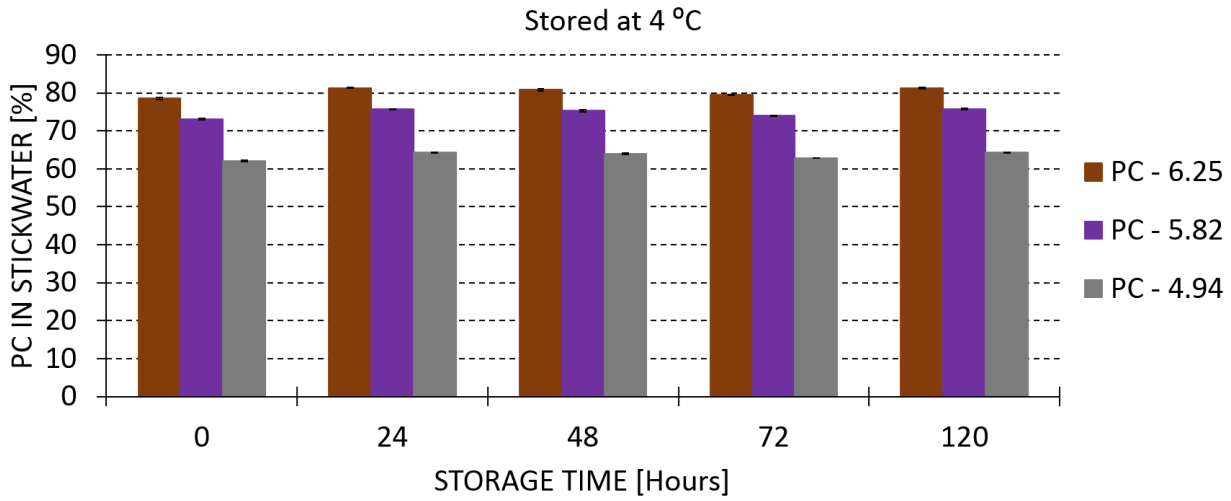

Figure 3.16: *Protein content of freeze dried stickwater as a function of storage time of raw material (stored at 4 °C). Calculated with factors 6.25, 5.82, and 4.94. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. Values are given as a mean of 4 replicates (n = 4).* ***PC*** *- Protein content*

Protein content in stickwater ranged between 78.5-83.4 %, 73.1-77.7 %, and 62.1-65.9, values were calculated from factors 6.25, 5.82, and 4.94 respectively. A previous study by Carvajal [Carvajal, 2013] reported that the protein content from NSS herring rest raw material (FPH, enzymatic hydrolysis with papain and bromelain) was 79.0 ± 0.5 %, utilizing a conversion factor of 5.82. In comparison, the endogenous hydrolysis of rest raw material from mackerel lead to a protein content of 73.1-77.7 % in stickwater, post thermal treatment (95 °C, 15 min).

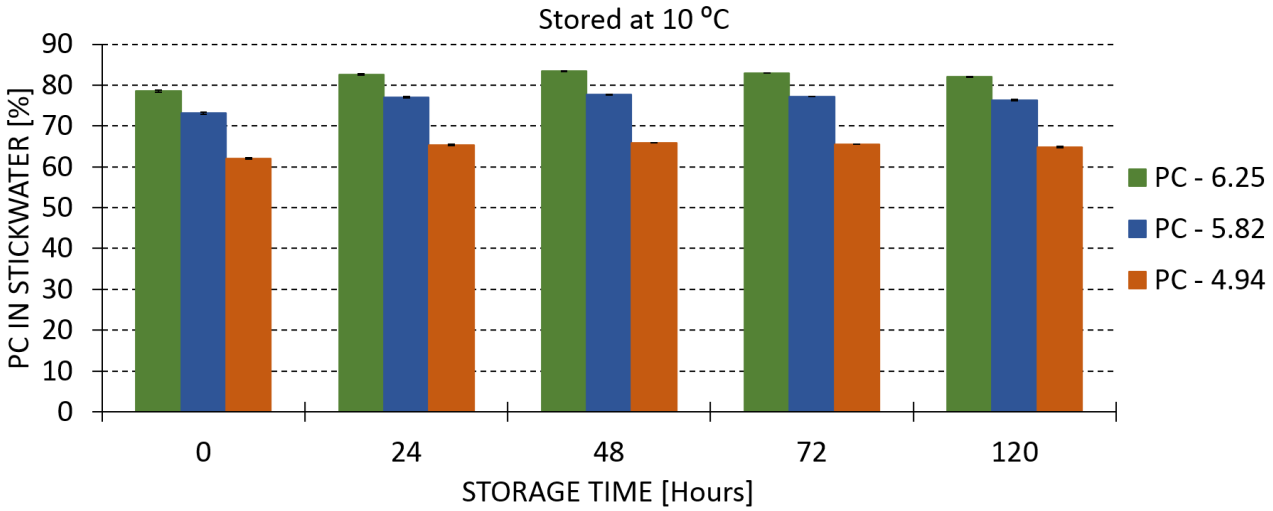

Figure 3.17: *Protein content of freeze dried stickwater as a function of storage time of raw material (stored at 10 °C). Calculated with factors 6.25, 5.82, and 4.94. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. Values are given as a mean of 4 replicates (n = 4).* ***PC*** *- Protein content*

Lipid content in stickwater

The lipid content in stickwater was determined as a function of storage time, figure 3.18. Lipid content ranged between 0.4 ± 0.02 % - 4.5 ± 0.2 %. Figure 3.18 quite clearly shows a relationship between lipid content and storage time, as well as the difference that storage temperature contributes. Lipid content in stickwater increases steadily with storage time, subsequently, the raw material stored at 10 °C has a significantly higher lipid content in the stickwater in comparison to raw material stored at 4 °C. It could be postulated that a relationship is apparent between the degree of hydrolysis and lipid content, as figures 3.18 and 3.19 show similar trends for each storage temperature.

Higher temperature of storage may possibly lead to more comprehensive degradation of fat cells, which in turn may release mono, di -glycerides, and FFA, acting as emulsifiers. This may influence the lipid content to increase, as mentioned earlier. A more extensive degradation of protein, may result in better emulsifying properties, ultimately increasing the amount of lipids bound in the stickwater.

Lipid content values of 0.5 % or higher, have been suggested to be too high for protein hydrolysates, in order to prevent further alterations in the lipids during storage [Spinelli et al., 1972]. Only the lipid content of the fresh raw material (0 hours of storage) is below the 0.5 % limit,

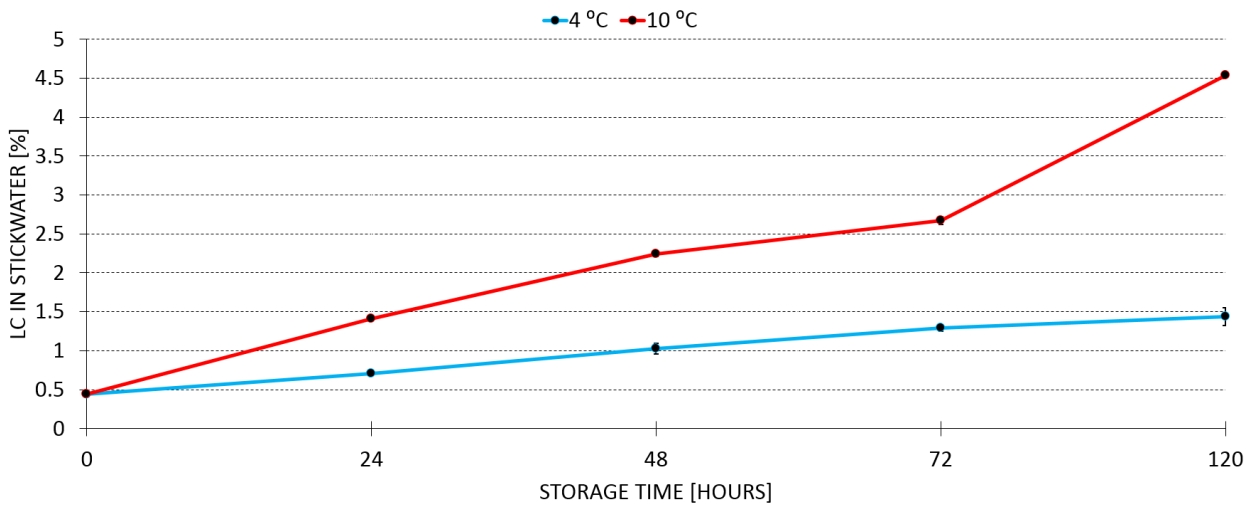

Figure 3.18: *Lipid content of freeze dried stickwater as a function of storage time of raw material. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. Values are given as a mean of 4 replicates (n = 4).* ***LC*** *- Lipid content*

which indicates that the lipid content of stickwater (24 h - 120 h) in general was too high. This may be due to the inadequate separation and filtration methods used. A better centrifuge might have separated the stickwater fraction better, subsequently filtration through glass wool is not optimal, since it does not prohibit or impede hydrophobic molecules. Industrially speaking, a better solution would be filtration based on hydrophobic/hydrophilic properties, as well as larger and more effective centrifuges. NSS herring hydrolysates contained 1.7 ± 0.6 % lipids according to Carvajal et al. [Carvajal, 2013]. This value is higher than what was found in the stickwater of mackerel rest raw material stored at 4 °C for up to 120 hours, however it is lower than values pertaining to raw material stored at 10 °C for more than 24 hours.

3.4.4 Effect of storage on quality parameters of stickwater protein

Protein degree of hydrolysis

The degree of hydrolysis for stickwater extracted from mackerel rest raw material stored at 4 and 10 °C is presented in figure 3.19. The % DH ranged between 17.7 ± 0.1 % and 27.8 ± 0.4 % for raw material stored at 4 °C and between 17.7 ± 0.1 % and 36.6 ± 0.1 % for raw material stored at 10 °C.

Figure 3.19 shows that the degree of hydrolysis increases consistently with storage time. Raw

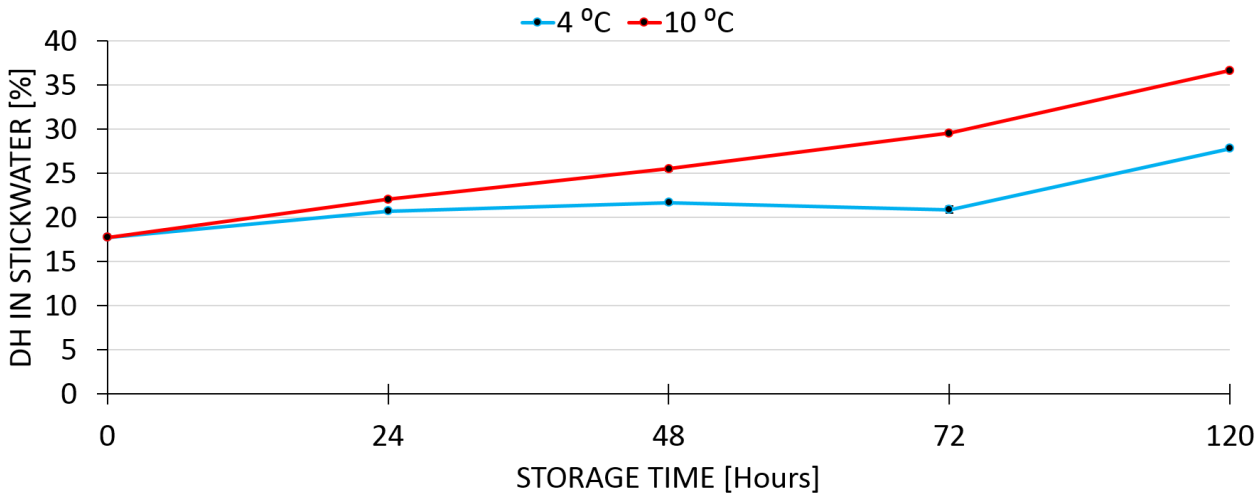

Figure 3.19: *Degree of hydrolysis as a function of storage time for stickwater extracted from mackerel rest raw material. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. Values are given as a mean of 3 replicates (n = 3).* ***DH*** *- Degree of hydrolysis*

material stored at higher temperature has higher % DH. There may be a correlation between the consistent increase in degree of hydrolysis and lipid content, as mentioned before, figure 3.18. However, by definition, it is also expected that the degree of hydrolysis also increases as hydrolysis proceeds, meaning that increased storage time in the presence of endogenous enzymes, will lead to higher degrees of hydrolysis.

Molecular weight distribution of protein content

The molecular weight distribution can influence protein quality, which is often measured by bioavailability, functional and organoleptic properties. The molecular distribution of the proteins and peptides present may also affect the color of the protein product through the Maillard reaction. Figure 3.21 and 3.22 present two chromatograms of the molecular weight distribution analysis for the storage experiment at both 4 and 10 °C. The chromatograms are given in milli absorbance units (mAU) as a function of retention time. The column used, Superdex® peptide 10/300, is recommended for the separation of peptide fractions in the range of 100-7 000 Da, which may seemingly influence some of the results, since four of the intervals of mass for the molecular weight distribution are above the 7 000 Da mark. The absorption at 214 nm may also contribute to higher retention peaks, due to the absorption of all structures other than peptides,

amino acids, and proteins, that absorb at 214 nm. However, the results below seem to be relatively consistent throughout the storage experiment. The molecular weight was divided into 11 intervals, as shown in figure 3.20; below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20000 Da and above 20 000 Da.

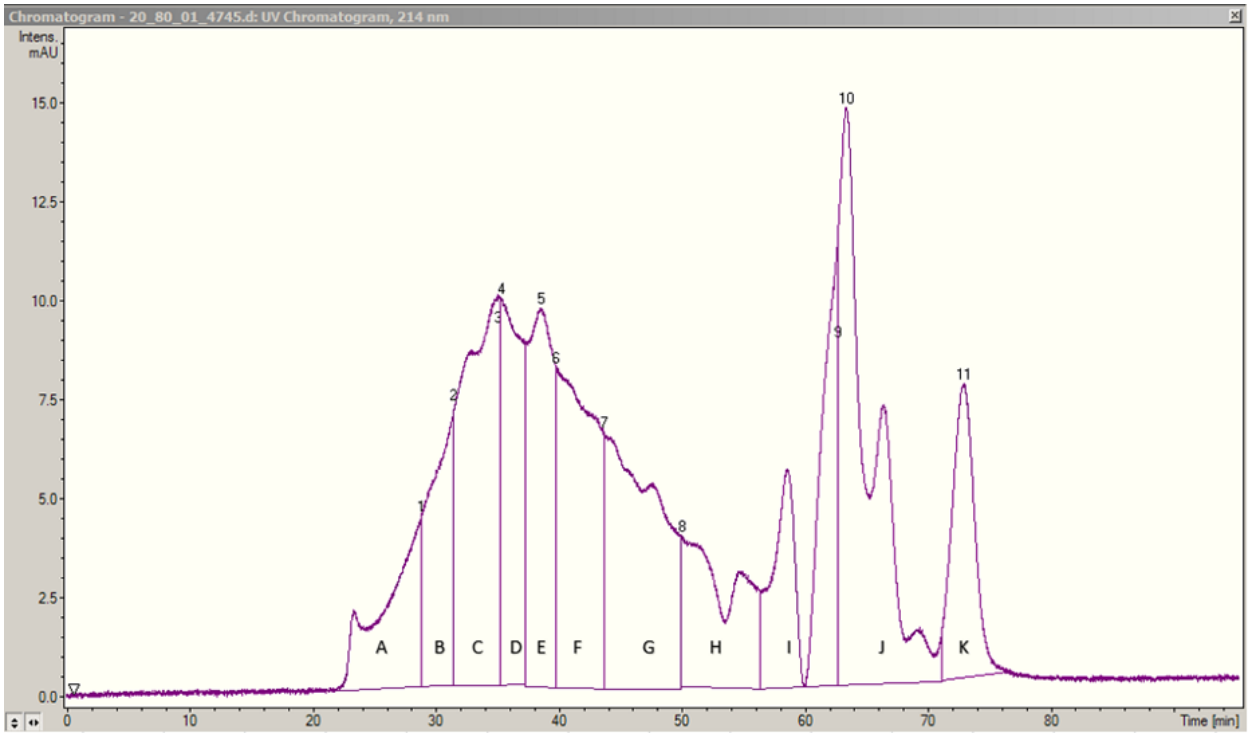

Figure 3.20: Molecular weight distribution intervals. **Standards** - cytochrome C (12327 Da), aprotinin (6511 Da), insulin A (2532 Da), leucine enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da) and Gly-Tyr (238.2 Da).

As shown in figure 3.21, the larger the molecule the less retention time required for it to be registered by the detector. The molecular weight distribution of freeze dried stickwater from mackerel rest raw materials, stored at 4 °C, changes considerably when subjected to increasing periods of storage. The distribution of molecular mass shifts towards the right, meaning that the proteins and peptides present are being hydrolyzed and broken down into smaller molecules. This appears to be consistent. Increases in storage time shift the concentration of molecules from the left to the right i.e. bigger molecules are becoming smaller. There are two distinct "high concentration" peaks present in the raw material that is not stored (0 hours of storage), the

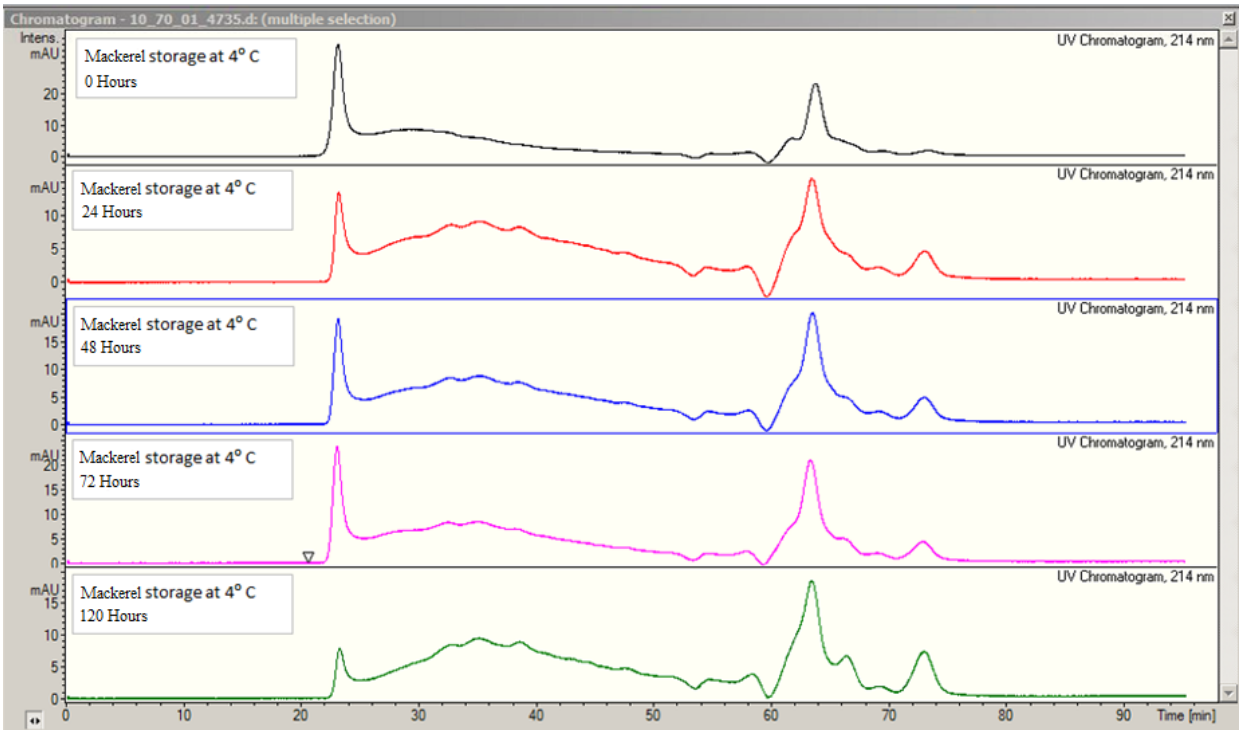

Figure 3.21: Molecular weight distribution of peptides in stickwater throughout storage at 4 °C. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. All samples were analyzed in triplicates (n = 3). **Standards** - cytochrome C (12327 Da), aprotinin (6511 Da), insulin A (2532 Da), leucine enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da) and Gly-Tyr (238.2 Da).

first one for molecules larger than 20 000 Da, and the second for molecules of approximate size 500 Da. There is as well a mid-range concentration of molecules at sizes between 6 000-20 000 Da. These intervals change somewhat drastically as storage time increases and the endogenous enzymes break down the larger molecules into smaller ones. The largest difference is observed in the first peak (> 20 000 Da) where the percentage of molecules over 20 000 Da in size, decreases from 29.9 % to 9.0 % during the 120 hour storage period. The fraction of molecules 6 000-8 000 Da increases from 4.3 % to 8.0 %, 4 000-6 000 Da 4.7-9.1 % 2 000-4 000 5.2-11.1 %, and < 200 Da from 1.6-5.5 %. All values and their respective deviations are given in table 3.6.

As shown in table 3.6, loss of concentration at any size, will increase the concentration of molecules in sizes below that size, i.e. when molecules above 20 000 Da get broken down, the % loss is observed in other fractions as % gain, meaning that bigger molecules are digested and cleaved into smaller ones. Some anomalies may occur. The concentrations of the top three intervals, >20 000 Da, 15 000-20 000 Da, and 10 000-15 000 Da, have increased from 24 h to 48 h

Table 3.6: Molecular weight distribution (%) for stickwater from mackerel rest raw material stored at 4 °C. Values and their deviations (SD) are given as means of 3 replicates.

Stored at 4 °C	0 hours		24 hours		48 hours		72 hours		120 hours
>20 000 Da	29.9±0.56	→	13.3±0.52	→	15.5±0.71	→	18.5±1.30	→	9.0±0.15
15000-20000 Da	8.8±0.12	→	6.4±0.36	→	6.7±0.25	→	6.7±0.30	→	5.6±0.00
10000-15000 Da	10.5±0.10	→	11.7±0.44	→	11.8±0.46	→	11.6±0.17	→	11.8±0.00
8000-10000 Da	4.3±0.06	→	6.2±0.06	→	6.1±0.12	→	5.8±0.10	→	6.3±0.06
6000-8000 Da	4.3±0.06	→	7.7±0.06	→	7.2±0.17	→	6.6±0.06	→	8.0±0.12
4000-6000 Da	4.7±0.15	→	9.3±0.20	→	8.3±0.15	→	7.4±0.36	→	9.1±0.15
2000-4000 Da	5.2±0.06	→	11.6±0.68	→	10.0±0.10	→	8.3±0.67	→	11.1±0.10
1000-2000 Da	3.4±0.35	→	7.0±0.93	→	5.7±0.21	→	4.7±0.47	→	6.8±0.12
500-1000 Da	7.1±0.12	→	8.0±0.25	→	8.2±0.32	→	9.3±0.42	→	9.9±0.36
200-500 Da	20.2±0.40	→	14.9±0.62	→	17.3±0.46	→	17.6±0.06	→	16.9±0.45
<200 Da	1.6±0.12	→	3.6±0.12	→	3.3±0.06	→	3.5±0.35	→	5.5±0.00

Red arrows - Indication of concentration loss. **Green arrows** - Indication of concentration gain.

of storage, this may indicate that larger molecules (»20 000 Da) have had "enough" time to be broken down to smaller sized molecules of around 10 000-20 000 Da. Overall, these values give a good indication of the molecular distribution status, before and after each consecutive storage period at 4 °C. Big sized molecules decrease drastically in concentration from 0 to 24 hours, they increase from 24 h to 72 h, and subsequently decrease again from 72 h to 120 h. Mid-range sized molecules increase in concentration in the first 24 h, they decrease from 24 h to 72 h, and subsequently increase again from 72 h to 120 h. The mid-range sized molecules 1 000-10 000 Da increase in concentration in the first 24 h, however, prolonged time under endogenous hydrolysis leads to concentration loss between 24 h and 72 h, ultimately reverting back to concentration gain after 120 h.

Figure 3.22 is similar to the figure above, however everything has happened quicker and more drastically. The fact that the storage was done at 10 °C, as opposed to 4 °C, has influenced the activity of the endogenous enzymes (proteases in particular), and increased the rate of hydrolysis. This has resulted in large differences in the molecular weight distribution as storage time increases. Similarly to the rest raw material stored at 4 °C, the first peak (>20 000 Da) has changed the most, decreasing from 29.9 % to 2.8 % in 120 hours. Other fractions with drastic changes in concentration are 15 000-20 000 Da 8.8-4.0 %, 6 000-8 000 Da 4.3-8.8 %, 4 000-6 000 Da 4.7-10.8 %, 2 000-4 000 Da 5.2-13.5 % 200-500 Da 20.2-14.9 %, <200 Da 1.6-6.9 %. All values

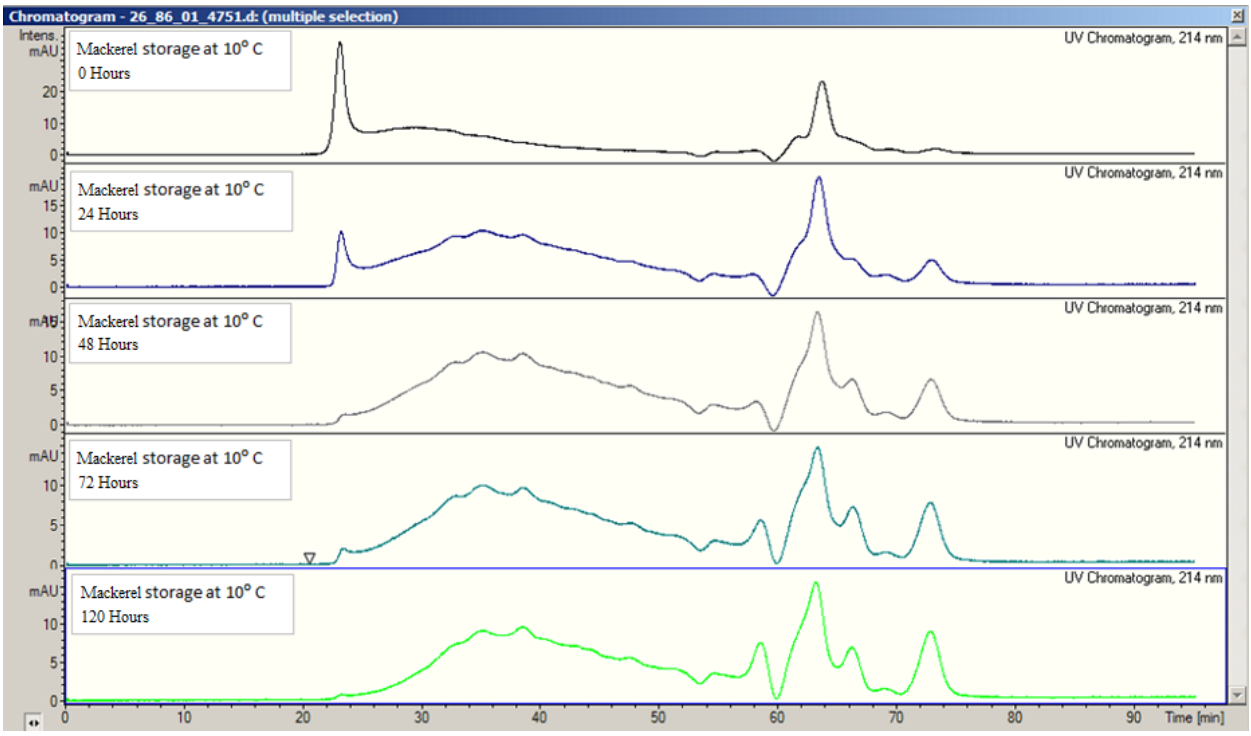

Figure 3.22: Molecular weight distribution of peptides in stickwater throughout storage at 10 °C. Sampling was done after 0 hours, 24 h, 48 h, 72 h, and 120 h. All samples were analyzed in triplicates (n = 3). **Standards** - cytochrome C (12327 Da), aprotinin (6511 Da), insulin A (2532 Da), leucine enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da) and Gly-Tyr (238.2 Da).

and their respective deviations are given in table 3.7.

Table 3.7: Molecular weight distribution (%) for stickwater from mackerel rest raw material stored at 10 °C. Values and their deviations are given as means of 3 replicates.

Stored at 10 ^0C	0 hours		24 hours		48 hours		72 hours		120 hours
>20 000 Da	29.9±0.56	→	10.6±0.40	→	5.5±0.06	→	5.4±0.15	→	2.8±0.17
15000-20000 Da	8.8±0.12	→	6.0±0.06	→	5.6±0.06	→	5.5±0.12	→	4.0±0.15
10000-15000 Da	10.5±0.1	→	12.2±0.10	→	12.4±0.12	→	12.4±0.12	→	10.9±0.35
8000-10000 Da	4.3±0.06	→	6.8±0.15	→	6.9±0.26	→	6.8±0.00	→	6.5±0.10
6000-8000 Da	4.3±0.06	→	8.4±0.15	→	9.2±0.1	→	8.7±0.00	→	8.8±0.15
4000-6000 Da	4.7±0.15	→	9.8±0.15	→	11.1±0.25	→	10.4±0.10	→	10.8±0.25
2000-4000 Da	5.2±0.06	→	11.4±0.35	→	13.2±0.10	→	12.2±0.17	→	13.5±0.32
1000-2000 Da	3.4±0.35	→	6.3±0.53	→	7.4±0.06	→	6.9±0.06	→	8.6±0.10
500-1000 Da	7.1±0.12	→	8.3±0.25	→	9.0±0.30	→	10.6±0.46	→	12.2±0.42
200-500 Da	20.2±0.40	→	16.8±0.42	→	15.5±0.75	→	15.1±0.50	→	14.9±0.67
<200 Da	1.6±0.12	→	3.4±0.10	→	4.7±0.12	→	5.8±0.12	→	6.9±0.20

Red arrows - Indication of concentration loss. **Green arrows** - Indication of concentration gain.

Table 3.7 shows clear indication that higher temperature during storage results in higher degree of hydrolysis. Cleavage occurs more rapidly and at higher levels when the raw material is stored at 10 °C as opposed to 4 °C. It should be noted that the high activity of the proteases (endogenous enzymes) may have contributed to the lack of increase in concentration for big sized molecules ranging 10 000-20 000 Da, between 24 h and 72 h, as seen in table 3.6. During the whole 120 h storage period, these large molecules do not increase in concentration. The same thing happens for molecules ranging in size between 200-500 Da. Overall, the higher temperature has lead to more digested proteins and peptides, increasing their respective bioavailability.

The next four figures, 3.23, 3.24, 3.25, 3.26, present a more detailed and chronological description of the changes occurring in the molecular weight distribution between the fresh raw material (0 hours of storage), the storage period, and the temperature at which the raw material was stored.

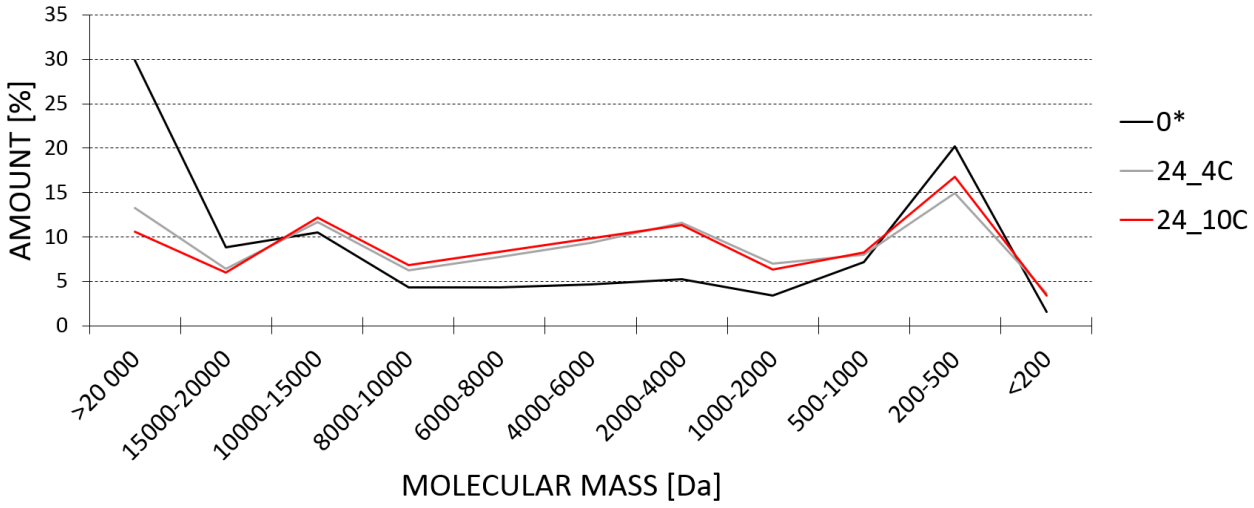

Figure 3.23: Molecular weight distribution of peptides in stickwater stored for 24 hours at 4 and 10 °C. All samples were analyzed in triplicates (n = 3). **Mass intervals** - from right to left, below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20 000 Da and above 20 000 Da.

After 24 hours of storage, figure 3.23 shows that the fresh raw material (0 hours of storage) has already started to degrade. There is not a large difference between storage at 4 or 10 °C for 24 h, however it is clear that large molecules have been digested more at 10 °C, while the mid-sized molecules have increased in concentration at 10 °C.

Subsequently, 48 hours of storage, figure 3.24 shows that the raw material stored at 10 °C

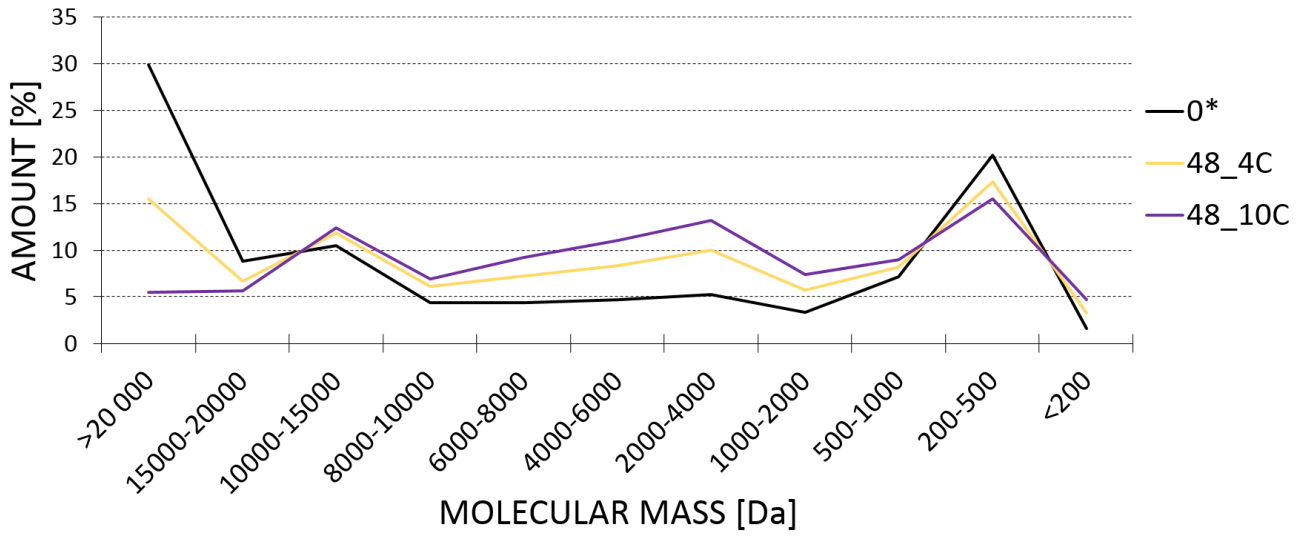

Figure 3.24: Molecular weight distribution of peptides in stickwater stored for 48 hours at 4 and 10 °C. All samples were analyzed in triplicates (n = 3). **Mass intervals** - from right to left, below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20 000 Da and above 20 000 Da.

is digested even further, which is expected. The difference between the raw material stored at 10 °C as opposed to 4 °C, is now increasing at certain molecular masses. A difference of 10 % decrease in concentration is observed between large molecules stored at 10 °C in comparison to 4 °C. The difference in mid-sized molecules, 1 000-10 000 Da, ranges from 1-3 %.

After 72 hours of storage, figure 3.25, some trends are prominent, and the process of digestion (figures 3.23, 3.24, 3.25) is visibly shifting from left to right, leading to higher concentrations amongst low-mass molecules.

At 120 hours, figure 3.26, the molecular weight distribution is highly influenced by both storage temperature and time. Any rest raw material intended for further processing must be stored at temperatures and for periods that are optimal for the final product's yield, quality, and safety.

It should be mentioned that the molecular weight distribution, as well as the degree of hydrolysis, are both influenced by the types of enzymes present in the raw material. Identification of the endogenous exo-proteases and endo-proteases will provide further information on the influence the endogenous enzymes have on the protein cleavage. Overall, the results given above give great insight in the digestion of mackerel proteins, peptides, and amino acids, which should influence further decisions about protein or oil production.

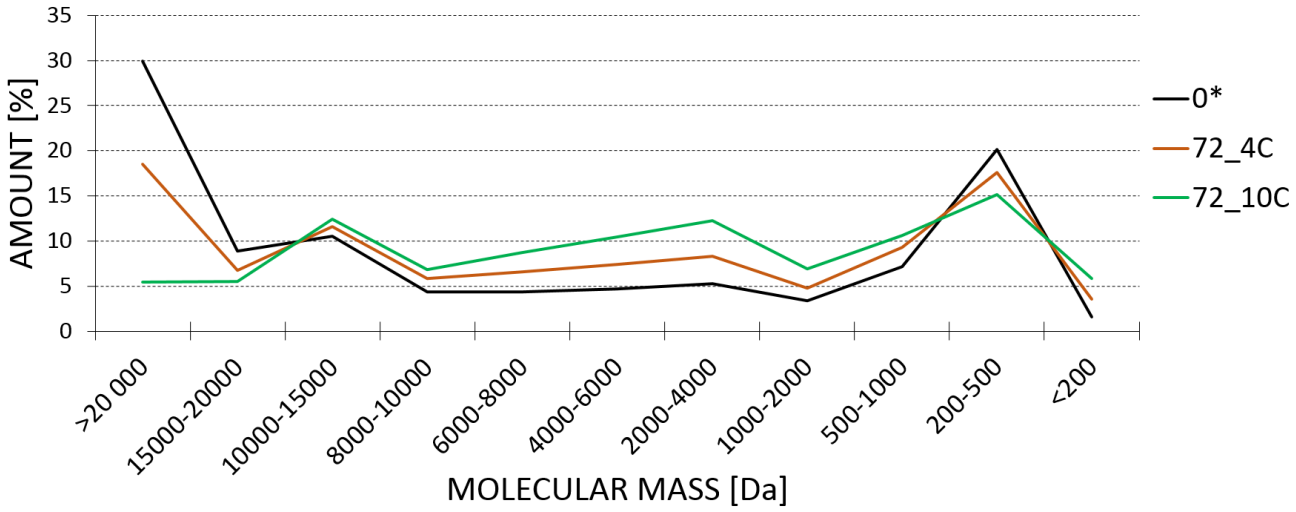

Figure 3.25: Molecular weight distribution of peptides in stickwater stored for 72 hours at 4 and 10 °C. All samples were analyzed in triplicates (n = 3). **Mass intervals** - from right to left, below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20 000 Da and above 20 000 Da.

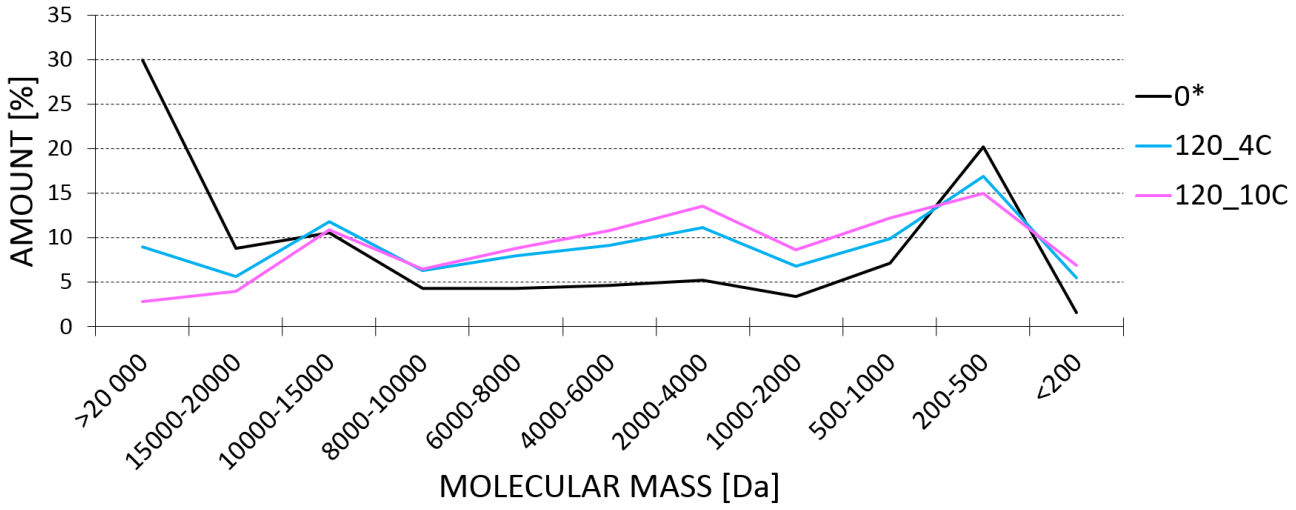

Figure 3.26: Molecular weight distribution of peptides in stickwater stored for 120 hours at 4 and 10 °C. All samples were analyzed in triplicates (n = 3). **Mass intervals** - from right to left, below 200 Da, 200-500 Da, 500-1 000 Da, 1 000-2 000 Da, 2 000-4 000 Da, 4 000-6 000 Da, 6 000-8 000 Da, 8 000-10 000 Da, 10 000-15 000 Da, 15 000-20 000 Da and above 20 000 Da.

3.4.5 Shelf-life of crude oil and protein

The quality measurements for crude oil suggest the use of low temperatures during transport and storage of mackerel rest raw material, this is to maintain high quality resources for further

production of high quality crude oil. The crude oil quantity and quality are highly influenced by the storage time and temperature. In general, crude oil production by the utilization of mackerel rest raw material should be done after 24 hours of storage (endogenous hydrolysis). This leads to higher oil yield, without influencing the quality significantly. However, use of lower storage temperature will result in a more oxidative stable crude oil.

The quality measurements for protein extracted from stickwater support the suggestion of utilizing low temperature during storage and transportation of rest raw material. For the production of protein, it is recommended storing the raw material for 24 h (endogenous hydrolysis), to increase yield, at 4 °C to reduce lipid content in the stickwater fraction.

It should be mentioned that after thermal treatment at 95 °C0, microorganisms will not be present in the crude oil or stickwater fractions. This is helpful in hindering further spoilage and degradation.

These suggestions only pertain to the use of thermal treatment as the main method of extraction.

Chapter 4

Conclusion

This study has shown that the utilization of North Atlantic mackerel (*Scomber scombrus*) rest raw material for the production of crude oil and protein is possible. The rest raw material contained 17.9 ± 0.4 % lipids, 12.7 ± 0.6 % protein, and 3.2 ± 0.01 % ash. Rest raw material stored for 24 h underwent endogenous hydrolysis, which gave good yields, both in amount of oil (lipids) and stickwater (protein). Storage of rest raw material at 10 °C gave higher yield in the crude oil fraction, however, did not influence the yield of stickwater significantly. The yield of both oil and stickwater fractions was increased in the first 24 hours of storage, irrespective of storage temperature. The yield of oil remained consistent between 24-120 h, however the yield of stickwater decreased slightly after 48 h, due to possible increase in the incorporation of lipids into the stickwater fraction. Protein content in the stickwater fraction increased after 24 h of storage, due to prolonged endogenous hydrolysis. The protein content was also influenced by the storage temperature, where rest raw material stored at 10 °C resulted in a higher content of protein in comparison to rest raw material stored at 4 °C. However, irrespective of storage temperature, the amount of protein within the stickwater fraction decreased after 48 h of storage. This might be correlated to the increase in degree of hydrolysis, which increased emulifying ability, resulting in the incorporation of lipids in the stickwater fraction.

The quality of the crude oil was high and may be used in food, pharmaceutical, or cosmetic industries. The quality parameters e.g. peroxide value (PV), anisidine value (AV), and amount of free fatty acids (% FFA) were well under the recommended limits for crude oil intended for human consumption, as long as the rest raw material was stored for 24 hours or less, irrespective

of storage temperature. However, storage at higher temperature lead to higher FFA values. The OSI time indicated that increases in storage time and temperature lead to decreased oxidative stability. The storage conditions did not significantly influence the fatty acid composition of the crude oil. The crude oil produced from fresh raw material had % FFA 0.5 ± 0.02, PV 5.3 ± 0.4 mEq/kg lipid, AV 1.0 ± 0.2, TOTOX 11.6 ± 0.3, and OSI 7.4 ± 0.1 hours.

The molecular weight distribution was closely related to the storage conditions. Increases in both storage time and temperature resulted in increased endogenous hydrolysis, which in turn increased the degree of hydrolysis, and how the molecular weight distribution behaved. Increased storage time resulted in large molecules being digested by exo- and endo-proteases to form mid-sized and small sized peptides and amino acids. Increased temperature resulted also in an increase in the rate of endogenous hydrolysis, which in turn lead to the rapid digestion and cleavage of most large molecules, ultimately forming higher concentrations of mid-sized and small molecules. The degree of hydrolysis increased with storage time and temperature, the value ranging between 17.7 ± 0.1 % and 27.8 ± 0.2 % for rest raw material stored at 4 °C, and between 17.7 ± 0.1 % and 36.6 ± 0.1 % for rest raw material stored at 10 °C.

The results of this thesis indicate that the protein content is well suited for applications in the pharmaceutical industry and nutritional additives (nutraceuticals), as opposed to the food industry. This is due to the high amount of small sized peptide fractions, which are beneficial for pharmaceuticals, higher bioavailability. For use in the food industry a lower degree of hydrolysis is needed, due to loss of functional properties such as foaming, gelling etc.. There are additionally some issues with the organoleptic properties of the protein produced. Undesired smell and color are hard to incorporate into foodstuffs. The crude oil produced had also some issues with regards to color and smell, however this is easily managed by refining and polishing the oil.

In conclusion, further study is needed to identify the process that mediates the incorporation of lipids into the protein fraction. As well as look into the issues regarding organoleptic properties. Currently, the best option seems to be the production of crude oil from mackerel rest raw material.

Chapter 5

Future work

This thesis has shown that North Atlantic mackerel rest raw material has the potential to produce valuable oil and protein fractions. Thermal treatment, in tandem with endogenous hydrolysis, produced high quality oil and protein fractions. Further study of the endogenous enzymes present in mackerel rest raw material are needed to fully control the endogenous hydrolysis process, to design the final product for its desired application area.

The results in this thesis are promising, however before designing an industrial process for the production of crude oil, or indeed protein, some challenges should be looked further into. A study utilizing commercial enzymes and the use of enzymatic hydrolysis should be investigated and compared to the results in this thesis. Since a lot of mackerel is frozen during the year, a study to determine oil and protein quality after freezing/thawing would improve understanding of mackerel resources. The thermal treatment process did not vary in temperature usage, this needs to be optimized in accordance with type of hydrolysis, endogenous vs. exogenous. Possible mineral extraction from the sludge fraction should be studied (high yield), extraction of calcium (Ca) or collagen proteins from the sludge fraction may be another possibility to exploit.

For further production of omega-3 rich oil intended for human consumption, a refining process is needed to convert the crude oil into refined oil. For the intention of protein production, focus on minimizing the lipid content, as well as identifying the processes that induce foul smell and dark color. Further detailed analysis of the types of proteins, peptides, and amino acids present in the protein fraction should be studied to determine the best application of the fi-

nal product, whether it be in the food, pharmaceutical, or cosmetics industry. Furthermore, characterization of lipase and proteolytic activity in mackerel during different stages of seasonal change and fish maturity will provide insight into the activities of different endogenous enzymes, thereby leading to increased control and predictability of the endogenous hydrolytic process.

Bibliography

[Abeywardena and Head, 2001] Abeywardena, M. Y. and Head, R. J. (2001). Longchain n-3 polyunsaturated fatty acids and blood vessel function. *Cardiovascular Research*, 52(3):361–371. ISSN: 0008-6363, DOI: 10.1016/S0008-6363(01)00406-0, URL: http://cardiovascres.oxfordjournals.org/content/52/3/361.

[Aidos et al., 2002] Aidos, I., Lourenclo, S., Van Der Padt, A., Luten, J., and Boom, R. (2002). Stability of crude herring oil produced from fresh byproducts: Influence of temperature during storage. *Journal of Food Science*, 67(9):3314–3320. ISSN: 1750-3841, URL: http://dx.doi.org/10.1111/j.1365-2621.2002.tb09585.x, DOI: 10.1111/j.1365-2621.2002.tb09585.x.

[Akoh and Min, 2008] Akoh, C. and Min, D. (2008). *Food Lipids: Chemistry, Nutrition, and Biotechnology, Third Edition*. Food Science and Technology. Taylor & Francis. ISBN: 9781420046649, URL: https://books.google.no/books?id=sPglndmgXU8C.

[Anderson and Ma, 2009] Anderson, B. M. and Ma, D. W. (2009). Are all n-3 polyunsaturated fatty acids created equal? *Lipids in Health and Disease*, 8:33. ISSN: 1476-511x, DOI: 10.1186/1476-511x-8-33.

[AOAC, 1990] AOAC (1990). *Official methods of analysis of AOAC International*. AOAC International. ISSN: 1080-0344, URL: https://books.google.no/books?id=sPglndmgXU8C.

[AOCS, 2013] AOCS (2013). *Official methods and recommended practices of the AOCS*. AOCS Press, Urbana, Ill., 6th edition. American Oil Chemist's Society ISBN: 978-1-893997-74-5.

[Aursand et al., 2011] Aursand, M., Mozuraityte, R., Hamre, K., Knutsen, H., Maage, A., and Arukwe, A. (2011). Description of the processes in the value chain and risk as-

sessment of decomposition substances and oxidation products in fish oils. pages 73–118. Norwegian Scientific Committee for Food Safety - ISBN: 978-82-8259-035-8 URL: http://www.vkm.no/dav/4be9bee090.pdf.

[Bang et al., 1971] Bang, H. O., Dyerberg, J., and Nielsen, A. B. (1971). Plasma lipid and lipoprotein pattern in greenlandic west-coast eskimos. *The Lancet*, 297(7710):1143–1146. ISSN: 0140-6736, DOI: 10.1016/S0140-6736(71)91658-8, URL: http://dx.doi.org/10.1016/S0140-6736(71)91658-8.

[Baybutt et al., 2002] Baybutt, R. C., Rosales, C., Brady, H., and Molteni, A. (2002). Dietary fish oil protects against lung and liver inflammation and fibrosis in monocrotaline treated rats. *Toxicology*, 175(1–3):1–13. ISSN: 0300-483X,DOI: http://dx.doi.org/10.1016/S0300-483X(02)00063-X,URL: http://www.sciencedirect.com/science/article/pii/S0300483X0200063X.

[Bernárdez et al., 2005] Bernárdez, M., Pastoriza, L., Sampedro, G., Herrera, J. J. R., and Cabo, M. L. (2005). Modified method for the analysis of free fatty acids in fish. *Journal of Agricultural and Food Chemistry*, 53(6):1903–1906. PMID: 15769111, URL: http://dx.doi.org/10.1021/jf040282c.

[Bimbo, 1998] Bimbo, A. P. (1998). Guidelines for characterizing food-grade fish oil. *International news on fats, oils and related materials*, 9(5):473–483.

[Bligh and Dyer, 1959] Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8):911–917. DOI: 10.1139/o59-099, URL: http://dx.doi.org/10.1139/o59-099, PMID: 13671378.

[Bortolus et al., 1989] Bortolus, P., Monti, S., Albini, A., Fasani, E., and Pietra, S. (1989). Physical quenching and chemical reaction of singlet molecular oxygen with azo dyes. *The Journal of Organic Chemistry*, 54(3):534–540. DOI: 10.1021/jo00264a006, URL: http://dx.doi.org/10.1021/jo00264a006.

[Carvajal, 2013] Carvajal, A. K. (2013). *Utilization of By-Products from Norwegian Spring Spawning Herring for Human Consumption*. PhD thesis, Norwegian University of Science and Technology, Department of Biotechnology.

[Choe and Min, 2006] Choe, E. and Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety*, 5(4):169–186. ISSN: 1541-4337, DOI: 10.1111/j.1541-4337.2006.00009.x, URL: http://dx.doi.org/10.1111/j.1541-4337.2006.00009.x.

[Csapó et al., 2008] Csapó, J., Csapó-Kiss, Z., Albert, C., and Lóki, K. (2008). Hydrolysis of proteins performed at high temperatures and for short times with reduced racemization, in order to determine the enantiomers of d-and l-amino acids. *Acta Univ. Sapientiae Alimentaria*, 1:31–48.

[Dauksas et al., 2005] Dauksas, E., Falch, E., Slizyte, R., and Rustad, T. (2005). Composition of fatty acids and lipid classes in bulk products generated during enzymic hydrolysis of cod (gadus morhua) by-products. *Process Biochemistry*, 40(8):2659–2670. ISSN: 1359-5113, URL: http://www.sciencedirect.com/science/article/pii/S0032959205000336.

[Falch et al., 2009] Falch, E., Overrein, I., Solberg, C., and Slizyte, R. (2009). *Handbook of Seafood and Seafood Products Analysis*, chapter 16: Composition and calories. CRC Press. ISBN: 978-1-4200-4633-5.

[Falch et al., 2006] Falch, E., Rustad, T., and Aursand, M. (2006). By-products from gadiform species as raw material for production of marine lipids as ingredients in food or feed. *Process Biochemistry*, 41(3):666–674. ISSN: 1359-5113, URL: http://www.sciencedirect.com/science/article/pii/S1359511305003545.

[FAO, 2015] FAO (2011–2015). *Fisheries and aquaculture software - FishStatJ - software for fishery statistical time series*. Rome. FAO - Food and Agriculture Organization of the United Nations - Fisheries and Aquaculture Department [online].

[Fennema et al., 2007] Fennema, O. R., Damodaran, S., and Parkin, K. L. (2007). *Fennema's Food Chemistry*. CRC Press, 4th edition. ISBN: 9780849392726.

[Fisheries.no, 2016] Fisheries.no (2016). Atlantic mackerel (scomber scombrus). http://www.fisheries.no/ecosystems-and-stocks/marine_stocks/fish_stocks/atlantic_mackerel/#.VxOZofl96Ul. Online, Accessed 17.04.2016.

[Frankel, 1993] Frankel, E. (1993). In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends in Food Science & Technology*, 4(7):220–225. ISSN: 0924-2244, DOI: http://dx.doi.org/10.1016/0924-2244(93)90155-4, URL: http://www.sciencedirect.com/science/article/pii/0924224493901554.

[Frankel, 2014] Frankel, E. (2014). *Lipid Oxidation*. Oily Press Lipid Library Series. Elsevier Science. ISBN: 9780857097927, URL: https://books.google.no/books?id=1eFQBAAAQBAJ.

[Fraser et al., 1985] Fraser, A., Tocher, D., and Sargent, J. (1985). Thin-layer chromatography — flame ionization detection and the quantitation of marine neutral lipids and phospholipids. *Journal of Experimental Marine Biology and Ecology*, 88(1):91–99. ISSN: 0022-0981, URL: http://www.sciencedirect.com/science/article/pii/0022098185902047.

[Freemantle, 1995] Freemantle, M. (1995). *Chemistry in action*. Macmillan.

[Gnaiger, 1984] Gnaiger, E.and Bitterlich, G. (1984). Proximate biochemical composition and caloric content calculated from elemental chn analysis: a stoichiometric concept. *Oecologia*, 62(3):289–298. ISSN: 1432-1939, URL: http://dx.doi.org/10.1007/BF00384259, DOI: 10.1007/BF00384259.

[GOED, 2012] GOED (2012). Goed voluntary monograph (v. 4). Global Organization for EPA and DHA omega-3s.

[Gunstone, 1996] Gunstone, F. (1996). Fatty acids — nomenclature, structure, isolation and structure determination, biosynthesis and chemical synthesis. In *Fatty Acid and Lipid Chemistry*, pages 1–34. Springer US. ISBN: 978-1-4613-6852-6.

[Gurr, 1999] Gurr, M. (1999). *Lipids in Nutrition and Health: A Reappraisal*. Oily Press Lipid Library Series. Elsevier Science. ISBN: 9780857097989, URL: https://books.google.no/books?id=IeiiAgAAQBAJ.

[Haag, 2003] Haag, M. (2003). Essential fatty acids and the brain. *Can J Psychiatry*, 48:195–203.

[Hamm, 2009] Hamm, W. (2009). Processing of fish oil. *fish oils, Rossell, B., Ed. Blackwell Publishing Ltd*, pages 81–98.

[Hardinger, 2015] Hardinger, S. A. (2015). Illustrated glossary of organic chemistry. URL: http://www.chem.ucla.edu/harding/IGOC/P/peptidebond.html. Department of Chemistry & Biochemistry, UCLA.

[ISO, 2001] ISO (2001). Animal and vegetable fats and oils – determination of peroxide value. International Organization for Standardization - ISO 3960:2001.

[IUPAC, 1978] IUPAC (1978). The nomenclature of lipids (recommendations 1976). iupac-iub commission on biochemical nomenclature. ISSN: 0022-2275.

[Kim and Wijesekara, 2010] Kim, S.-K. and Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, 2(1):1–9. ISSN: 1756-4646, DOI: http://dx.doi.org/10.1016/j.jff.2010.01.003, URL: http://www.sciencedirect.com/science/article/pii/S1756464610000046.

[Kris-Etherton et al., 2002] Kris-Etherton, P. M., Harris, W. S., Appel, L. J., and the Nutrition Committee (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*, 106(21):2747–2757. URL:http://circ.ahajournals.org/content/106/21/2747.short.

[Kristinova et al., 2009] Kristinova, V., Mozuraityte, R., Storro, I., and Rustad, T. (2009). Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. *Journal of Agricultural and Food Chemistry*, 57(21):10377–10385. ISSN: 0021-8561, DOI: 10.1021/jf901072t.

[Kristinsson and Rasco, 2000] Kristinsson, H. G. and Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40(1):43–81. ISSN: 1040-8398.

[Lehninger et al., 2013] Lehninger, A. L., Cox, M. M., and Nelson, D. L. (2013). *Lehninger principles of biochemistry*. W.H. Freeman, New York, USA, 6th edition. ISBN: 9781464109621 1464109621 9781429234146 1429234148.

[McClements and Decker, 2000] McClements, D. and Decker, E. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science*, 65(8):1270–1282. ISSN: 1750-3841, DOI: 10.1111/j.1365-2621.2000.tb10596.x, URL: http://dx.doi.org/10.1111/j.1365-2621.2000.tb10596.x.

[NDH, 2015] NDH (2015). Næringsstoffanbefalinger – energi, karbohydrater, fett, protein, vitaminer, mineraler. Norwegian Directorate of Health, URL: https://helsedirektoratet.no/folkehelse/kosthold-og-ernering/neringsstoffanbefalinger-energi-karbohydrater-fett-protein-vitaminer-mineraler, accessed 24.4.2016.

[NSF, 2011] NSF (2011). Dietary supplements. National Science Foundation / American National Standards Institute, designated as an NSF/ANSI 173 – 2010 standard.

[Phleger et al., 2002] Phleger, C. F., Nelson, M. M., Mooney, B. D., and Nichols, P. D. (2002). Interannual and between species comparison of the lipids, fatty acids and sterols of antarctic krill from the us amlr elephant island survey area. *Comparative Biochemistry and Physiology: B Biochemistry and Molecular Biology*, 131(4):733–747. ISSN: 1096-4959.

[Richardsen et al., 2015] Richardsen, R., Nystøyl, R., Strandheim, G., and Andrea, V. (2015). Analyse marint restråstoff, 2014. Technical report, SINTEF Fiskeri og havbruk AS and Kontali Analyse AS. Written in Norwegian.

[Rigaudy and Klesney, 1979] Rigaudy, J. and Klesney, A. P. (1979). *Nomenclature of organic chemistry : Sections A,B,C,D,E,F and H*. Pergamon, Oxford, 4th edition. ISBN 0080223699.

[RUBIN, 2000] RUBIN (2000). *Biprodukter fra fiskerinæringen - fra utkast til inntekt*. Skipnes Offsettrykkeri AS, Trondheim. Resirkulering og utnyttelse av organiske biprodukter i Norge - ISBN: 82-993089-1-7.

[Rustad, 2006] Rustad, T. (2006). Physical and chemical properties of protein seafood by-products. In Shahidi, F., editor, *Maximising the Value of Marine By-Products*, pages 3–21. Woodhead Publishing, 2006, illustrated edition. ISBN: 9781845692087.

[Rustad et al., 2011] Rustad, T., Storrø, I., and Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. *International Journal of Food Science & Technology*, 46(10):2001–2014. ISSN: 1365-2621, DOI: 10.1111/j.1365-2621.2011.02736.x, URL: http://dx.doi.org/10.1111/j.1365-2621.2011.02736.x.

[Rutledge et al., 2010] Rutledge, J. C., Ng, K. F., Aung, H. H., and Wilson, D. W. (2010). Role of triglyceride-rich lipoproteins in diabetic nephropathy. *Toxicology*, 6(6):361–370. ISSN: 1759-5061, URL: http://dx.doi.org/10.1038/nrneph.2010.59.

[Ruyter, 2016] Ruyter, B. (2016). Herring is better for you than we thought. `http://nofima.no/en/nyhet/2016/01/herring-is-better-for-you-than-we-thought/`. Food research institute - Nofima, Acessed 13.5.2016, Journalist Reidun Lilleholt, Kraugerud.

[Saldaña and Martínez-Monteagudo, 2013] Saldaña, M. and Martínez-Monteagudo, S., editors (2013). *Applications of Calorimetry in a Wide Context - Differential Scanning Calorimetry, Isothermal Titration Calorimetry and Microcalorimetry*, chapter 19. InTech, 1 edition. DOI: 10.5772/54486, ISBN: 978-953-51-0947-1.

[Salo-väänänen and Koivistoinen, 1996] Salo-väänänen, P. P. and Koivistoinen, P. E. (1996). Determination of protein in foods: comparison of net protein and crude protein (n × 6.25) values. *Food Chemistry*, 57(1):27–31. The Second International Food Data Base Conference: Food Composition Research - The Broader Context, ISSN: 0308-8146.

[Shriver, 2014] Shriver, D. (2014). *Inorganic Chemistry*. Oily Press Lipid Library Series. W. H. Freeman and Co., New York, NY, 6th edition. ISBN: 9781429299060.

[Slizyte et al., 2005] Slizyte, R., Dauksas, E., Falch, E., Storrø, I., and Rustad, T. (2005). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (gadus morhua) by-products. *Process Biochemistry*, 40(3–4):1415–1424. ISSN: 1359-5113, URL: http://www.sciencedirect.com/science/article/pii/S0032959204002584.

[Sosulski and Imafidon, 1990] Sosulski, F. W. and Imafidon, G. I. (1990). Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. *Journal of Agricultural and Food Chemistry*, 38(6):1351–1356. URL: http://dx.doi.org/10.1021/jf00096a011, DOI: 10.1021/jf00096a011.

[Spinelli et al., 1972] Spinelli, J., Koury, B., and Miller, R. (1972). Approaches to the utilization of fish for the preparation of protein isolates. *Journal of Food Science*, 37(4):599–603. ISSN: 1750-3841, URL: http://dx.doi.org/10.1111/j.1365-2621.1972.tb02702.x, DOI: 10.1111/j.1365-2621.1972.tb02702.x.

[Sørensen, 1907] Sørensen, S. (1907). Formol titration: an evaluation of its various modifications. *Biochemistry*, 7:45.

[Søvik and Rustad, 2005] Søvik, S. L. and Rustad, T. (2005). Effect of season and fishing ground on the activity of lipases in byproducts from cod (gadus morhua). *{LWT} - Food Science and Technology*, 38(8):867–876. ISSN: 0023-6438, URL: http://www.sciencedirect.com/science/article/pii/S0023643804002774, DOI: http://dx.doi.org/10.1016/j.lwt.2004.09.013.

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

[Wallace, 1991] Wallace, P. (1991). *Seasonal variation in fat content of mackerel (Scomber scombrus L.) caught in the western English Channel*. Citeseer.

[Wilailuk et al., 2007] Wilailuk, C., Ryan J., E., D. Julian, M., and Eric A., D. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition*, 47(3):299–317. PMID: 17453926, DOI: 10.1080/10408390600754248, URL: http://dx.doi.org/10.1080/10408390600754248.

[Wu and Bechtel, 2008] Wu, T. H. and Bechtel, P. J. (2008). Salmon by-product storage and oil extraction. *Food Chemistry*, 111(4):868–871. ISSN: 0308-8146,

URL: http://www.sciencedirect.com/science/article/pii/S0308814608005165, DOI: http://dx.doi.org/10.1016/j.foodchem.2008.04.064.

Appendix A

Characterization of raw material

Raw data for the characterization of fractions in mackerel rest raw material. Table A.1 presents the raw data for the mean weight of head, bellyflap, BB + T, and viscera in mackerel rest raw material.

Table A.1: Raw data for the characterization of fractions in mackerel rest raw material. Mean values and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

	Round weight [g]:	Head [g]:	BB + T [g]:	Bellyflap [g]:	Viscera [g]:
Fish 1	356.42	66.62	52.39	27.14	27.59
Fish 2	385.01	70.4	50.99	35.15	35.06
Fish 3	322.39	57.07	49.93	24.39	27.77
Fish 4	453.55	88.83	68.16	44.12	37.2
Fish 5	585.95	96.47	78.74	58.15	52.84
Means:	**420.664**	**75.878**	**60.042**	**37.79**	**36.092**

Percentages [%]:	**Head [%]:**	**BB + T [%]:**	**Bellyflap [%]:**	**Viscera [%]:**	**Totals:**
Fish 1	18.69	14.70	7.61	7.74	48.75
Fish 2	18.29	13.24	9.13	9.11	49.76
Fish 3	17.70	15.49	7.57	8.61	49.37
Fish 4	19.59	15.03	9.73	8.20	52.54
Fish 5	16.46	13.44	9.92	9.02	48.84
Means:	**18.15**	**14.38**	**8.79**	**8.54**	**49.85**
SD	**1.0**	**0.9**	**1.0**	**0.5**	**1.4**
SEM	**0.5**	**0.4**	**0.5**	**0.2**	**0.6**

Appendix B

Dry matter and ash content, rm characterization

Dry matter and ash samples rom the mackerel rest raw material were analyzed in order to determine what the raw material was composed of. Table B.1 presents the raw data of dry matter and ash content.

Table B.1: Raw data for the characterization of dry matter and ash content in mackerel rest raw material. Mean values and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Crucible [g]:	Crucible + sample [g]:	Wet sample [g]:	Crucible + dry matter [g]:	Dry matter [g]:	Dry matter percentage [%]:	Mean dry matter percentage [%]:	SD	SEM	Crucible + ash [g]:	Ash [g]:	Ash percentage [%]:	Mean ash percentage [%]:	SD	SEM
Head`	25.0149	27.8471	2.8322	26.0892	1.0743	37.9				25.1379	0.1230	4.3			
Head``	21.8456	24.6670	2.8214	22.9379	1.0923	38.7	38.3	0.4	0.3	21.9603	0.1147	4.1	4.2	0.1	0.1
Bellyflap`	17.9680	20.1226	2.1546	19.0752	1.1072	51.4				18.0266	0.0586	2.7			
Bellyflap``	17.0211	19.3292	2.3081	18.1371	1.1160	48.4	49.9	1.5	1.0	17.0809	0.0598	2.6	2.7	0.1	0.0
Viscera`	17.4490	19.5800	2.1310	17.9658	0.5168	24.3				17.4868	0.0378	1.8			
Viscera``	17.3823	19.5283	2.1460	17.8931	0.5108	23.8	24.0	0.2	0.2	17.4208	0.0385	1.8	1.8	0.0	0.0
BB+T`	18.5319	20.7487	2.2168	19.3592	0.8273	37.3				18.6256	0.0937	4.2			
BB+T``	17.6182	20.5508	2.9326	18.7021	1.0839	37.0	37.1	0.2	0.1	17.7350	0.1168	4.0	4.1	0.1	0.1
Mixture`	17.9601	21.5110	3.5509	19.1478	1.1877	33.4				18.0717	0.1116	3.1			
Mixture``	16.6167	19.5103	2.8936	17.6006	0.9839	34.0	33.7	0.3	0.2	16.7083	0.0916	3.2	3.2	0.0	0.0

Appendix C

Total lipid content, rm characterization

The determination of total lipid content, in the raw material, was done by the Bligh & Dyer method. Table C.1 presents the raw data for the calculation of total lipid content in mackerel rest raw material. Calculations were done by utilizing equation C.1.

$$\text{Total lipid content in \%} = \frac{\text{Weight of lipid in tube [g]} \times \text{Volume of chloroform layer [mL]}}{\text{Volume of chloroform utilized in tube [mL]} \times \text{Mass of sample [g]}} \tag{C.1}$$

Table C.1: Raw data for the calculation of total lipid content for the characterization of mackerel rest raw material. Lipid content percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Mass [g]:	Tube [g]:	Tube + lipid extracted [g]:	Lipid [g]:	Total lipid [g/100g]:	Mean [g/100g]:	SD	SEM
Head`	10.6000	12.1567	12.2606	0.1039	19.6038	19.9	0.4	0.2
Head``	10.1500	12.2411	12.3434	0.1023	20.1576			
Head```	10.0000	12.4121	12.5086	0.0965	19.3000			
Head`````	9.8700	12.5008	12.6012	0.1004	20.3445			
Bellyflap`	10.1800	12.2292	12.4038	0.1746	34.3026	35.2	0.8	0.4
Bellyflap``	11.1200	12.3432	12.5408	0.1976	35.5396			
Bellyflap```	10.2900	12.2905	12.4683	0.1778	34.5578			
Bellyflap`````	10.3400	12.2002	12.3881	0.1879	36.3443			
Viscera`	10.3500	12.3457	12.3825	0.0368	7.1111	7.3	0.3	0.1
Viscera``	9.8800	12.4168	12.4513	0.0345	6.9838			
Viscera```	9.9100	12.1193	12.1559	0.0366	7.3865			
Viscera`````	9.4800	12.2129	12.2492	0.0363	7.6582			
BB+T`	11.2500	12.2924	12.3782	0.0858	15.2533	15.8	0.6	0.3
BB+T``	10.3100	12.4013	12.4808	0.0795	15.4219			
BB+T```	9.6000	12.2577	12.3339	0.0762	15.8750			
BB+T`````	9.9500	12.2506	12.3343	0.0837	16.8241			
Mixture`	10.3800	12.3048	12.3935	0.0887	17.0906	17.9	0.8	0.4
Mixture``	10.3300	12.2551	12.3433	0.0882	17.0765			
Mixture```	10.4200	12.4785	12.5762	0.0977	18.7524			
Mixture`````	9.6500	12.1722	12.2617	0.0895	18.5492			

Appendix D

Lipid class analysis, rm characterization

Lipid class analysis was done on the oil extracted to determine the majority of lipids present in the mackerel rest raw material. The analysis was done by TLC-FID. Table D.1 presents the raw data of the lipid class analysis.

Table D.1: Raw data for the characterization of lipids in the oil extracted from mackerel rest raw material. Lipid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Head:	**1**	**2**	**3**	**4**	**Mean [%]:**	**SD**	**SEM**
Cholesterol esters	N/A	N/A	1.4330	N/A			
TAG	80.3510	91.7020	90.4180	N/A	87.5	5.1	2.9
FFA	N/A	N/A	N/A	N/A			
Cholesterol	0.5560	0.2240	0.4170	N/A	0.4	0.1	0.1
PL	19.0920	7.9240	7.1210	N/A	11.4	5.5	3.2
Bellyflap:	**1**	**2**	**3**	**4**			
Cholesterol esters	N/A	N/A	N/A	N/A			
TAG	87.5560	95.1030	94.2740	93.7570	92.7	3.0	1.5
FFA	0.2070	N/A	N/A	N/A			
Cholesterol	0.1360	N/A	0.1410	0.1760	0.2	0.0	0.0
PL	11.9480	4.8980	1.2780	6.0670	6.0	3.8	1.9
Viscera:	**1**	**2**	**3**	**4**			
Cholesterol esters	N/A	N/A	N/A	N/A			
TAG	N/A	N/A	N/A	N/A			
FFA	44.0520	64.1710	66.4690	69.5330	61.1	10.0	5.0
Cholesterol	6.2220	6.6150	6.6590	7.4090	6.7	0.4	0.2
PL	49.7260	29.2140	26.6720	23.0580	32.2	10.4	5.2
BB + T:	**1**	**2**	**3**	**4**			
Cholesterol esters	N/A	N/A	N/A	N/A			
TAG	90.9010	93.4520	89.1390	91.7430	91.3	1.6	0.8
FFA	N/A	N/A	N/A	N/A			
Cholesterol	0.4780	N/A	N/A	0.9440			
PL	7.7130	6.5480	6.3520	5.4200	6.5	0.8	0.4
Mixture:	**1**	**2**	**3**	**4**			
Cholesterol esters	N/A	N/A	N/A	N/A			
TAG	89.6390	89.3320	92.0780	92.8140	91.0	1.5	0.8
FFA	N/A	N/A	N/A	N/A			
Cholesterol	0.3680	0.3232	0.3820	0.4190	0.4	0.0	0.0
PL	8.6360	9.8863	6.7300	5.7920	7.8	1.6	0.8

Appendix E

Free fatty acid content (FFA), rm characterization

The determination of FFA content in the oil extracted from mackerel rest raw material was done by a potentiometric titration according to [Bernárdez et al., 2005]. Table E.1 presents the raw data for the calculation of FFA content in the oil. The standard curve (oleic acid) is also provided, see figure E.1. Calculations were done by utilizing equation E.1.

Standard curve:							
Oleic acid [µmol]:	**0.0**	**2.5**	**5.0**	**7.5**	**10.0**	**15.0**	**20.0**
Absorbance:	0.000	0.042	0.094	0.150	0.198	0.312	0.405
	0.002	N/A	0.094	0.151	0.194	0.297	0.392
	0.004						
	0.002						
Mean:	0.002	0.042	0.094	0.151	0.196	0.305	0.399
Oleic acid atomic mass:	282.46 g/mol						

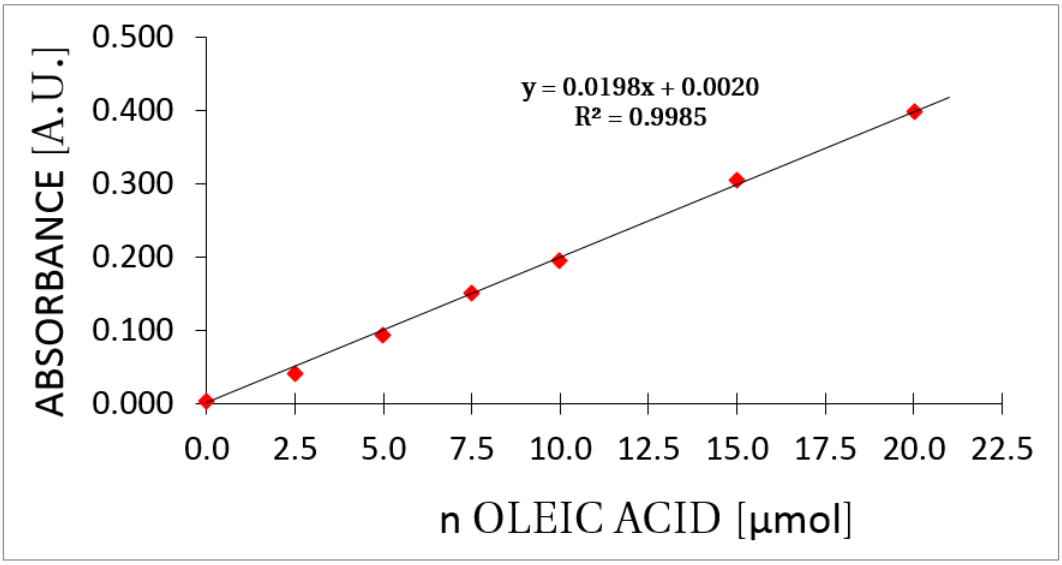

Figure E.1: *Oleic acid standard curve for the calculation of FFA content in oil extracted from mackerel rest raw material.*

$$\text{FFA \%} = \frac{(Abs - B) \times 282.46}{A \times m \times 10000} \tag{E.1}$$

- Abs is the absorbance of the sample.
- B is the y-intercept of the linear regression performed on the oleic acid standard curve - absorbance of the blank.
- 282.46 is the molar weight of oleic acid [g/mol].
- A is the slope of the linear regression [$1/\mu$mol].
- m is the mass of the sample [g].
- 10000 is the conversion of units to get percentages [%].

Table E.1: Raw data for the calculation of FFA content in oil extracted from mackerel rest raw material. FFA percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Slope of standard curve: 0.0198			Intercept of standard curve: 0.0020					
Sample:	Mass [g]:	Abs [A.U.]:	n sample FFA [µmol]	FFA mass [mg]:	FFA content [mg/g]:	FFA content [%]:	Results:	
Head`	0.1291	0.053	2.551	0.72	5.58	0.56	**Mean:**	0.49
Head``	0.1510	0.054	2.626	0.74	4.91	0.49	**SD:**	0.05
Head```	0.1136	0.036	1.722	0.49	4.28	0.43	**n**	3
							SEM:	0.03
Bellyflap`	0.0795	0.116	5.732	1.62	20.37	2.04	**Mean**	1.97
Bellyflap``	0.0947	0.132	6.566	1.85	19.58	1.96	**SD:**	0.05
Bellyflap```	0.0792	0.109	5.404	1.53	19.27	1.93	**n**	3
							SEM:	0.03
Viscera`	0.1111	0.750	37.798	10.68	96.10	9.61	**Mean**	9.47
Viscera``	0.1384	0.964	48.561	13.72	99.11	9.91	**SD:**	0.42
Viscera```	0.1535	0.960	48.384	13.67	89.03	8.90	**n**	3
							SEM:	0.24
BB + T `	0.1160	0.320	16.071	4.54	39.13	3.91	**Mean**	5.64
BB + T ``	0.1328	0.440	22.141	6.25	47.09	4.71	**SD:**	1.91
BB + T ```	0.1671	0.974	49.111	13.87	83.02	8.30	**n**	3
							SEM:	1.10
Mixture`	0.1261	0.770	38.803	10.96	86.92	8.69	**Mean**	8.72
Mixture``	0.1604	1.012	51.000	14.41	89.81	8.98	**SD:**	0.20
Mixture```	0.1687	1.007	50.747	14.33	84.97	8.50	**n**	3
							SEM:	0.11

Appendix F

Peroxide value determination (PV), rm characterization

The determination of primary oxidation products (peroxide value) in the oil extracted from mackerel rest raw material was done with the AOCS official method Cd 8b-90. Table F.1 presents the raw data for the calculation of PV content in the oil. Calculations were done by utilizing equation F.1.

$$\text{PV} = \frac{Concentration_{(Na_2S_2O_3)} \times (\text{Titration of sample} - \text{Titration of blank}) \times 1000}{\text{Weight of sample}} \quad \text{(F.1)}$$

Table F.1: Raw data for the calculation of PV content in oil extracted from mackerel rest raw material. PV percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sodium Thiosulphate (titrant) standardization					
$Na_2S_2O_3$ concentration	0.0101 mol/L				
Blanks:	**Consumption [mL]:**				
1	0.0942	**Mean:**	0.0878		
2	0.0789	**SD:**	0.0136		
3	0.0715	**n**	4		
4	0.1066				
Sample:	**Mass [g]:**	**Consumption [mL]:**	**PV [mEq/kg]:**	**Results:**	
Head`	2.0042	2.5653	12.4520	**Mean:**	12.6
Head``	2.1026	2.7553	12.7795	**SD:**	0.2
				n	2
				SEM:	0.1
Viscera`	0.6253	0.30889	3.5619	**Mean:**	3.5
Viscera``	0.6761	0.3201	3.4613	**SD:**	0.1
				n	2
				SEM:	0.0
BB + T`	2.0203	1.333	6.2086	**Mean:**	7.0
BB + T``	1.3688	1.1514	7.8272	**SD:**	0.8
				n	2
				SEM:	0.6
Mixture`	2.0306	2.2107	10.5310	**Mean:**	10.4
Mixture``	1.8642	1.9964	10.3131	**SD:**	0.1
				n	2
				SEM:	0.1

Appendix G

Yield, storage experiment

Tables G.1-G.9 present the yield of the oil, stickwater, and sludge fractions extracted from mackerel rest raw material by means of thermal treatment. Each table represents raw material that has been stored for a distinct amount of time at a certain temperature, excluding the first table, G.1, which is fresh raw material (0 hours stored).

Table G.1: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from fresh mackerel rest raw material (0 hours of storage). Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 0*	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	46.48	3.48	7.49	9.55	20.55	28.76	61.88
2	45.44	3.11	6.84	8.96	19.72	27.97	61.55
3	43.34	3.61	8.33	7.92	18.27	26.97	62.23
4	41.51	4.46	10.74	10.02	24.14	25.24	60.80
5	42.69	4.61	10.80	11.44	26.80	25.85	60.55
6	44.51	3.83	8.60	11.44	25.70	27.35	61.45
7	41.19	4.62	11.22	11.40	27.68	24.20	58.75
8	46.62	3.28	7.04	10.36	22.22	28.02	60.10
9	43.49	3.88	8.92	10.27	23.61	27.25	62.66
10	43.48	4.74	10.90	11.02	25.34	27.23	62.63
11	42.08	4.58	10.88	10.97	26.07	25.82	61.36
12	45.67	3.48	7.62	8.94	19.58	28.38	62.14
13	43.42	4.26	9.81	11.40	26.26	26.58	61.22
14	44.79	4.19	9.35	11.86	26.48	27.25	60.84
15	43.39	4.48	10.32	11.61	26.76	26.32	60.66
16	41.48	3.71	8.94	11.01	26.54	25.17	60.68
		Mean [%]:	9.2	Mean [%]:	24.1	Mean [%]:	61.2
		SD:	1.4	SD:	3.0	SD:	1.0
		SEM:	0.4	SEM:	0.7	SEM:	0.2

Table G.2: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 4 °C for 24 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 24_4C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	40.47	5.52	13.64	13.70	33.85	20.94	51.74
2	41.34	5.25	12.70	12.47	30.16	20.64	49.93
3	37.63	4.96	13.18	13.40	35.61	18.75	49.83
4	41.75	5.45	13.05	12.70	30.42	19.97	47.83
5	40.03	5.39	13.46	13.31	33.25	21.08	52.66
6	41.57	4.81	11.57	13.53	32.55	21.54	51.82
7	41.24	5.31	12.88	13.54	32.83	22.12	53.64
8	37.62	4.76	12.65	12.50	33.23	19.73	52.45
9	40.53	5.19	12.81	11.83	29.19	23.31	57.51
10	40.05	5.04	12.58	12.96	32.36	21.61	53.96
11	37.27	4.83	12.96	11.33	30.40	20.82	55.86
12	41.04	5.05	12.31	13.85	33.75	21.79	53.09
13	40.18	5.02	12.49	13.39	33.33	21.02	52.31
14	42.38	5.34	12.60	10.05	23.71	26.36	62.20
15	37.35	4.77	12.77	10.60	28.38	21.58	57.78
16	38.84	4.95	12.74	11.68	30.07	21.78	56.08
		Mean [%]:	12.8	Mean [%]:	31.4	Mean [%]:	53.7
		SD:	0.5	SD:	2.8	SD:	3.4
		SEM:	0.1	SEM:	0.7	SEM:	0.9

Table G.3: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 10 °C for 24 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 24_10C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	44.34	5.76	12.99	13.27	29.93	23.56	53.13
2	43.83	5.81	13.26	14.80	33.77	22.46	51.24
3	44.22	5.93	13.41	14.38	32.52	22.57	51.04
4	41.25	5.55	13.45	14.14	34.28	20.86	50.57
5	43.72	5.96	13.63	14.70	33.62	22.68	51.88
6	38.88	5.51	14.17	12.44	32.00	20.53	52.80
7	41.15	5.74	13.95	13.95	33.90	21.08	51.23
8	43.55	6.14	14.10	14.62	33.57	22.23	51.04
9	41.65	5.70	13.69	12.67	30.42	23.03	55.29
10	40.40	5.67	14.03	12.09	29.93	22.38	55.40
11	38.59	5.35	13.86	12.04	31.20	20.98	54.37
12	39.72	5.58	14.05	12.38	31.17	21.53	54.20
13	38.89	5.20	13.37	11.53	29.65	21.91	56.34
14	38.42	5.35	13.93	11.17	29.07	21.64	56.32
15	39.37	5.63	14.30	11.74	29.82	21.63	54.94
16	39.23	5.53	14.10	11.37	28.98	22.15	56.46
		Mean [%]:	13.8	Mean [%]:	31.5	Mean [%]:	53.5
		SD:	0.4	SD:	1.8	SD:	2.1
		SEM:	0.1	SEM:	0.5	SEM:	0.5

Table G.4: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 4 °C for 48 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 48_4C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	38.33	5.35	13.96	12.13	31.65	20.44	53.33
2	39.99	5.64	14.10	13.01	32.53	21.00	52.51
3	39.61	5.53	13.96	12.71	32.09	20.95	52.89
4	41.01	5.57	13.58	12.98	31.65	21.32	51.99
5	39.34	5.55	14.11	12.83	32.61	20.64	52.47
6	40.00	5.22	13.05	13.01	32.53	21.29	53.23
7	39.84	5.45	13.68	13.38	33.58	20.55	51.58
8	37.52	5.10	13.59	12.05	32.12	20.04	53.41
9	39.60	5.23	13.21	12.23	30.88	21.88	55.25
10	42.06	6.14	14.60	13.02	30.96	22.70	53.97
11	38.44	5.08	13.22	13.00	33.82	20.10	52.29
12	39.28	5.08	12.93	11.89	30.27	21.86	55.65
13	39.93	5.14	12.87	13.59	34.03	20.94	52.44
14	40.33	5.16	12.79	12.46	30.90	22.43	55.62
15	40.09	5.14	12.82	13.87	34.60	22.61	56.40
16	42.15	5.33	12.65	12.12	28.75	22.53	53.45
		Mean [%]:	13.4	Mean [%]:	32.1	Mean [%]:	53.5
		SD:	0.6	SD:	1.5	SD:	1.4
		SEM:	0.1	SEM:	0.4	SEM:	0.4

Table G.5: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 10 °C for 48 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 48_10C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	39.49	5.71	14.46	9.95	25.20	22.50	56.98
2	40.74	5.52	13.55	9.85	24.18	24.98	61.32
3	41.16	4.78	11.61	11.76	28.57	19.25	46.77
4	42.00	5.52	13.14	11.25	26.79	21.52	51.24
5	41.77	5.61	13.43	12.23	29.28	22.16	53.05
6	39.44	5.52	14.00	10.29	26.09	21.64	54.87
7	37.91	5.34	14.09	12.52	33.03	18.29	48.25
8	38.13	5.90	15.47	11.17	29.29	20.83	54.63
9	39.65	5.68	14.33	11.10	27.99	21.69	54.70
10	39.74	6.22	15.65	12.09	30.42	21.01	52.87
11	38.45	5.60	14.56	12.12	31.52	20.42	53.11
12	38.05	5.63	14.80	11.42	30.01	20.25	53.22
13	39.56	5.89	14.89	8.82	22.30	24.53	62.01
14	40.28	5.51	13.68	12.34	30.64	21.14	52.48
15	38.91	5.46	14.03	12.05	30.97	20.46	52.58
16	41.36	5.12	12.38	10.89	26.33	22.12	53.48
		Mean [%]:	**14.0**	**Mean [%]:**	**28.3**	**Mean [%]:**	**53.8**
		SD:	**1.0**	**SD:**	**2.8**	**SD:**	**3.8**
		SEM:	**0.3**	**SEM:**	**0.7**	**SEM:**	**0.9**

Table G.6: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 4 °C for 72 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 72_4C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	39.54	5.64	14.26	10.93	27.64	22.83	57.74
2	40.38	5.10	12.63	11.26	27.89	21.86	54.14
3	40.24	6.12	15.21	12.08	30.02	21.76	54.08
4	40.09	5.59	13.94	12.50	31.18	21.75	54.25
5	37.99	5.39	14.19	11.55	30.40	20.91	55.04
6	41.28	5.72	13.86	12.40	30.04	23.00	55.72
7	40.78	5.79	14.20	11.57	28.37	22.69	55.64
8	40.32	5.68	14.09	11.74	29.12	22.70	56.30
9	39.80	5.42	13.62	10.74	26.98	23.02	57.84
10	40.24	5.78	14.36	10.99	27.31	23.34	58.00
11	42.33	6.06	14.32	11.59	27.38	24.52	57.93
12	39.69	5.42	13.66	10.81	27.24	23.22	58.50
13	38.04	5.18	13.62	9.67	25.42	22.90	60.20
14	37.99	5.15	13.56	10.05	26.45	22.32	58.75
15	39.15	5.40	13.79	10.53	26.90	23.09	58.98
16	37.59	5.24	13.94	9.97	26.52	22.23	59.14
		Mean [%]:	14.0	Mean [%]:	28.1	Mean [%]:	57.0
		SD:	0.5	SD:	1.6	SD:	1.9
		SEM:	0.1	SEM:	0.4	SEM:	0.5

Table G.7: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 10 °C for 72 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 72_10C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	39.76	5.37	13.51	10.50	26.41	21.24	53.42
2	38.18	5.10	13.36	12.13	31.77	18.24	47.77
3	40.75	5.69	13.96	10.40	25.52	24.24	59.48
4	42.72	4.82	11.28	10.92	25.56	20.88	48.88
5	41.44	5.55	13.39	8.93	21.55	26.61	64.21
6	37.93	5.35	14.10	10.10	26.63	22.17	58.45
7	39.50	5.21	13.19	11.77	29.80	19.29	48.84
8	39.22	5.05	12.88	11.21	28.58	19.73	50.31
9	38.73	5.12	13.22	11.37	29.36	20.77	53.63
10	39.39	5.22	13.25	9.61	24.40	22.47	57.04
11	38.64	5.08	13.15	10.82	28.00	20.90	54.09
12	40.59	4.93	12.15	9.58	23.60	21.25	52.35
13	40.15	5.17	12.88	8.34	20.77	24.43	60.85
14	38.87	5.20	13.38	10.29	26.47	22.67	58.32
15	40.17	5.30	13.19	7.65	19.04	26.00	64.72
16	41.96	4.87	11.61	8.86	21.12	24.07	57.36
		Mean [%]:	13.0	Mean [%]:	25.5	Mean [%]:	55.6
		SD:	0.7	SD:	3.5	SD:	5.1
		SEM:	0.2	SEM:	0.9	SEM:	1.3

Table G.8: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 4 °C for 120 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 120_4C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	37.63	5.28	14.03	12.53	33.30	18.39	48.87
2	38.97	4.25	10.91	12.71	32.61	19.40	49.78
3	40.38	4.07	10.08	8.69	21.52	19.64	48.64
4	39.05	5.26	13.47	11.46	29.35	19.41	49.71
5	40.23	5.78	14.37	12.44	30.92	21.22	52.75
6	38.09	5.03	13.21	12.29	32.27	18.99	49.86
7	38.08	5.64	14.81	13.15	34.53	18.97	49.82
8	38.49	5.39	14.00	13.34	34.66	18.88	49.05
9	39.45	4.74	12.02	11.42	28.95	22.23	56.35
10	38.96	4.65	11.94	10.92	28.03	21.26	54.57
11	37.96	5.00	13.17	11.33	29.85	19.90	52.42
12	41.75	5.40	12.93	12.53	30.01	22.98	55.04
13	40.25	5.13	12.75	12.55	31.18	20.78	51.63
14	41.96	5.15	12.27	9.61	22.90	26.47	63.08
		Mean [%]:	**12.9**	**Mean [%]:**	**30.0**	**Mean [%]:**	**52.3**
		SD:	**1.3**	**SD:**	**3.7**	**SD:**	**3.8**
		SEM:	**0.3**	**SEM:**	**1.0**	**SEM:**	**1.0**

Table G.9: Raw data for the calculation of yield of extracted oil, stickwater, and sludge fractions from mackerel rest raw material stored at 10 °C for 120 h. Values include the mean, SD, and SEM.

Before thermal treatment:		After thermal treatment and separation:					
Sample: 120_10C	Raw material [g]:	Oil [g]:	Oil [%]:	Stickwater [g]:	Stickwater [%]:	Sludge [g]:	Sludge [%]:
1	39.52	4.93	12.47	9.99	25.28	17.74	44.89
2	41.08	5.01	12.20	8.67	21.11	19.86	48.34
3	39.90	5.39	13.51	10.31	25.84	18.89	47.34
4	40.62	5.61	13.81	7.64	18.81	25.38	62.48
5	42.28	5.32	12.58	8.36	19.77	23.66	55.96
6	40.11	5.15	12.84	8.73	21.77	22.22	55.40
7	42.19	5.19	12.30	9.74	23.09	21.37	50.65
8	40.19	5.81	14.46	10.56	26.28	21.97	54.67
9	42.44	5.07	11.95	8.89	20.95	20.94	49.34
10	39.57	5.56	14.05	11.91	30.10	20.47	51.73
11	38.78	5.24	13.51	7.09	18.28	25.27	65.16
12	39.13	5.15	13.16	8.51	21.75	22.77	58.19
13	42.88	5.13	11.96	8.10	18.89	29.24	68.19
		Mean [%]:	**13.0**	**Mean [%]:**	**22.5**	**Mean [%]:**	**54.8**
		SD:	**0.8**	**SD:**	**3.4**	**SD:**	**6.9**
		SEM:	**0.2**	**SEM:**	**0.9**	**SEM:**	**1.9**

Appendix H

Mass balance calculations

Samples from each extracted fraction (e.g. oil, stickwater, and sludge) were dried to determine the total yield of the products, on a dry matter basis. The determination of mass balances on a dry matter basis was done to verify that the yield percentages were reasonable. The dry matter raw data used to calculate the mass balance based on dry matter basis are given in appendix I.

The calculation of mass balances (conservation of mass) for the storage experiment is presented in the table below, H.1.

Table H.1: Raw data for the calculation of mass balance for yield fractions from mackerel rest raw material. **WW** - Wet weight, **DW** - Dry weight, **rm** - Raw material.

	0*	**24_4C**	**24_10C**	**48_4C**	**48_10C**	**72_4C**	**72_10C**	**120_4C**	**120_10C**
Oil:									
Total [g]:	4.02	5.1	5.65	5.36	5.6	5.5	5.2	5.1	5.27
g WW/100g rm (WW)	9.19	12.77	13.76	13.44	14	14	13	12.8	12.97
%DW	100	100	100	100	100	100	100	100	100
Yield [g DW/100g rm(WW)]	9.19	12.77	13.76	13.44	14	14	13	12.8	12.97
Stickwater:									
Total [g]:	10.51	12.55	12.96	12.77	11.2	11.1	10.2	11.8	9.1
g WW/100g rm (WW)	24.04	31.42	31.54	32.04	28.2	28.1	25.5	29.93	22.41
%DW	9.3	11.6	11.7	11.5	13.3	10.7	12.7	11.1	13
Yield [g DW/100g rm(WW)]	2.24	3.64	3.69	3.68	3.8	3	3.23	3.32	2.91
Sludge:									
Total [g]:	26.77	21.44	21.95	21.33	21.42	22.63	22.19	20.61	22.29
g WW/100g rm (WW)	61.23	53.66	53.44	53.53	53.84	56.99	55.64	52.34	54.81
%DW	41.9	43.4	41.7	43.2	40.9	41.5	41.7	41.2	42.5
Yield [g DW/100g rm(WW)]	25.66	23.29	22.28	23.13	22.02	23.65	23.2	21.56	23.29
Sum total [g]:	41.3	39.09	40.56	39.46	38.22	39.23	37.59	37.51	36.66
Sum DW [g/100g rm (WW)]	37.09	39.7	39.73	40.25	39.82	40.65	39.43	37.68	39.17
DW of rm [g/100g rm (WW)]	38.7	36.6	37.38	36.55	37.77	37.03	34.57	38.06	37.28
Difference (DW of rm – sum DW)	1.61	-3.1	-2.35	-3.7	-2.05	-3.62	-4.86	0.38	-1.89

Appendix I

Dry matter content of rm, storage experiment

Samples from all fractions pertaining to the storage experiment were dried to determine the dry matter and ash content. Subsequently used for the verification of mass balances on dry basis, and to calculate the yield of the oil and stickwater phases in dry weight per 100 grams of raw material (wet weight). The mass balance calculations can be found in appendix H.

I.1 Stored at 4 °C

The raw data and the calculated dry weight percentage with standard deviation (SD) and standard error of mean (SEM) for samples stored for 0 hours, 24 hours, 48 hours, 72 hours, and 120 hours at 4 °C, are presented in table I.1.

I.2 Stored at 10 °C

The raw data and the calculated dry weight percentage with standard deviation (SD) and standard error of mean (SEM) for 0 hours, 24 hours, 48 hours, 72 hours, and 120 hours at 10 °C, are presented in table I.2.

Table I.1: Raw data and the calculated dry weight percentage for mackerel rest raw material stored at 4 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h, and 120 h.

Sample:		Crucible [g]:	Crucible + sample [g]:	Wet sample [g]:	Crucible + dry matter [g]:	Dry matter [g]:	Dry matter percentage [%]:	Mean [%]:	SD	SEM	Crucible + ash [g]:	Ash [g]:	Ash percentage [%]:	Mean [%]:	SD	SEM
0*																
	1	19.4112	21.7094	2.2982	20.3046	0.8934	38.9	38.7	1.7	1.0	19.4997	0.0885	3.9	4.1	0.2	0.1
	2	18.8904	20.9125	2.0221	19.7129	0.8225	40.7				18.9779	0.0875	4.3			
	3	19.035	21.1623	2.1273	19.8128	0.7778	36.6				19.1205	0.0855	4.0			
24_4C																
	1	21.3941	23.4996	2.1055	22.1236	0.7295	34.6	36.6	2.1	1.2	21.4483	0.0542	2.6	2.7	0.2	0.1
	2	22.334	24.5171	2.1831	23.1135	0.7795	35.7				22.3914	0.0574	2.6			
	3	24.4303	26.6925	2.2622	25.3226	0.8923	39.4				24.4976	0.0673	3.0			
48_4C																
	1	17.8118	20.0224	2.2106	18.6156	0.8038	36.4	36.5	1.1	0.6	17.8746	0.0628	2.8	2.9	0.1	0.1
	2	16.3401	18.5403	2.2002	17.1161	0.776	35.3				16.4028	0.0627	2.8			
	3	21.6793	24.0624	2.3831	22.585	0.9057	38.0				21.7536	0.0743	3.1			
72_4C																
	1	29.2234	31.3162	2.0928	29.9845	0.7611	36.4	37.0	1.6	0.9	29.2846	0.0612	2.9	3.0	0.1	0.0
	2	27.7623	29.901	2.1387	28.6004	0.8381	39.2				27.8272	0.0649	3.0			
	3	32.2377	34.6353	2.3976	33.0898	0.8521	35.5				32.3072	0.0695	2.9			
120_4C																
	1	18.7439	21.1388	2.3949	19.6065	0.8626	36.0	38.1	1.4	0.8	18.8333	0.0894	3.7	3.6	0.5	0.3
	2	18.286	20.8526	2.5666	19.2871	1.0011	39.0				18.3907	0.1047	4.1			
	3	19.1729	21.1519	1.979	19.948	0.7751	39.2				19.2305	0.0576	2.9			

Table I.2: Raw data and the calculated dry weight percentage for mackerel rest raw material stored at 10 °C. Sampling was done after 0 h, 24 h, 48 h, 72 h, and 120 h.

Sample:	Crucible [g]:	Crucible + sample [g]:	Wet sample [g]:	Crucible + dry matter [g]:	Dry matter [g]:	Dry matter percentage [%]:	Mean [%]:	SD	SEM	Crucible + ash [g]:	Ash [g]:	Ash percentage [%]:	Mean [%]:	SD	SEM
0*															
1	19.4112	21.7094	2.2982	20.3046	0.8934	38.9	38.7	1.7	1.0	19.4997	0.0885	3.9	4.1	0.2	0.1
2	18.8904	20.9125	2.0221	19.7129	0.8225	40.7				18.9779	0.0875	4.3			
3	19.035	21.1623	2.1273	19.8128	0.7778	36.6				19.1205	0.0855	4.0			
24_10C															
1	19.0733	21.2157	2.1424	19.8963	0.823	38.4	37.4	0.9	0.5	19.1409	0.0676	3.2	3.0	0.3	0.2
2	23.1457	25.4233	2.2776	23.9984	0.8527	37.4				23.2212	0.0755	3.3			
3	22.1559	24.5842	2.4283	23.0368	0.8809	36.3				22.2196	0.0637	2.6			
48_10C															
1	25.8447	28.1304	2.2857	26.728	0.8833	38.6	37.8	0.8	0.5	25.9070	0.0623	2.7	2.8	0.3	0.2
2	25.9333	27.8836	1.9503	26.6496	0.7163	36.7				25.9956	0.0623	3.2			
3	13.1746	15.2585	2.0839	13.9651	0.7905	37.9				13.2252	0.0506	2.4			
72_10C															
1	28.2012	30.1351	1.9339	28.9059	0.7047	36.4	34.6	1.3	0.8	28.2600	0.0588	3.0	2.7	0.3	0.2
2	29.8883	31.9783	2.09	30.585	0.6967	33.3				29.9383	0.0500	2.4			
3	30.5334	32.8397	2.3063	31.3159	0.7825	33.9				30.5924	0.0590	2.6			
120_10C															
1	30.9363	33.0844	2.1481	31.7461	0.8098	37.7	37.3	0.6	0.3	31.0085	0.0722	3.4	3.6	0.2	0.1
2	27.231	29.6898	2.4588	28.1568	0.9258	37.7				27.3261	0.0951	3.9			
3	22.6054	24.9135	2.3081	23.4477	0.8423	36.5				22.6873	0.0819	3.5			

Appendix J

Free fatty acid content (FFA), storage experiment

The determination of FFA content in the oil extracted from mackerel rest raw material was done by a potentiometric titration according to [Bernárdez et al., 2005]. Table J.1 presents the raw data for the calculation of FFA content in the oil. The standard curve (oleic acid) is also provided, see figure J.1. Calculations were done by utilizing equation J.1.

Standard curve:							
Oleic acid [µmol]:	**0.0**	**2.5**	**5.0**	**7.5**	**10.0**	**15.0**	**20.0**
Absorbance:	0.000	0.042	0.094	0.153	0.198	0.312	0.405
	0.002	N/A	0.097	0.151	0.198	0.292	0.399
	0.003						
	0.002						
Mean:	0.002	0.042	0.096	0.152	0.198	0.302	0.402
Oleic acid atomic mass:	282.46 g/mol						

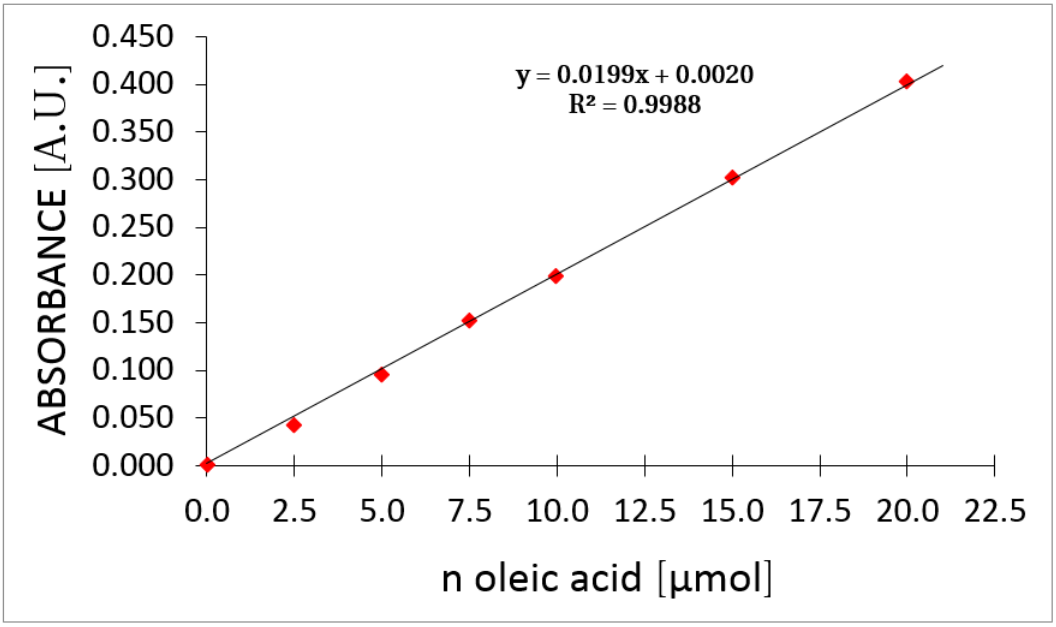

Figure J.1: *Oleic acid standard curve for the calculation of FFA content in oil extracted from mackerel rest raw material.*

$$\text{FFA \%} = \frac{(Abs - B) \times 282.46}{A \times m \times 10000} \tag{J.1}$$

- Abs is the absorbance of the sample.
- B is the y-intercept of the linear regression performed on the oleic acid standard curve - absorbance of the blank.
- 282.46 is the molar weight of oleic acid [g/mol].
- A is the slope of the linear regression [$1/\mu$mol].
- m is the mass of the sample [g].
- 10000 is the conversion of units to get percentages [%].

Table J.1: Raw data for the calculation of FFA content in oil extracted from mackerel rest raw material. FFA percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Slope of standard curve: 0.0199		**Intercept of standard curve: 0.0020**						
Sample:	**Mass [g]:**	**Abs [A.U.]:**	**n sample FFA [µmol]**	**FFA mass [mg]:**	**FFA content [mg/g]:**	**FFA content [%]:**	**Results:**	
0* `	0.1897	0.070	3.3970	0.9595	5.0581	0.5	**Mean:**	0.5
0* ``	0.1930	0.063	3.0854	0.8715	4.5156	0.5	**SD:**	0.0
0* ```	0.1920	0.066	3.2161	0.9084	4.7313	0.5	**n**	4
0* ````	0.1947	0.066	3.2010	0.9042	4.6438	0.5	**SEM:**	0.0
24_4C `	0.1964	0.167	8.2814	2.3392	11.9102	1.2	**Mean**	1.2
24_4C ``	0.1944	0.156	7.7437	2.1873	11.2515	1.1	**SD:**	0.0
24_4C ```	0.1845	0.166	8.2211	2.3221	12.5861	1.3	**n**	4
24_4C ````	0.1975	0.163	8.0905	2.2852	11.5708	1.2	**SEM:**	0.0
24_10C `	0.2012	0.174	8.6181	2.4343	12.0987	1.2	**Mean**	1.2
24_10C ``	0.1900	0.165	8.1709	2.3079	12.1470	1.2	**SD:**	0.0
24_10C ```	0.1959	0.179	8.9146	2.5180	12.8535	1.3	**n**	4
24_10C ````	0.1876	0.163	8.1055	2.2895	12.2041	1.2	**SEM:**	0.0
48_4C `	0.1923	0.224	11.1759	3.1567	16.4157	1.6	**Mean**	1.7
48_4C ``	0.1940	0.241	12.0251	3.3966	17.5083	1.8	**SD:**	0.0
48_4C ```	0.1877	0.223	11.1005	3.1354	16.7046	1.7	**n**	4
48_4C ````	0.1918	0.237	11.8040	3.3342	17.3835	1.7	**SEM:**	0.0
48_10C `	0.1880	0.342	17.0804	4.8245	25.6624	2.6	**Mean**	2.4
48_10C ``	0.1804	0.287	14.3065	4.0410	22.4004	2.2	**SD:**	0.1
48_10C ```	0.1910	0.306	15.2663	4.3121	22.5766	2.3	**n**	4
48_10C ````	0.1821	0.311	15.5025	4.3788	24.0463	2.4	**SEM:**	0.1
72_4C `	0.1955	0.214	10.6734	3.0148	15.4210	1.5	**Mean**	1.6
72_4C ``	0.1929	0.225	11.2060	3.1653	16.4088	1.6	**SD:**	0.0
72_4C ```	0.1956	0.216	10.7387	3.0333	15.5074	1.6	**n**	4
72_4C ````	0.1843	0.209	10.4070	2.9396	15.9499	1.6	**SEM:**	0.0
72_10C `	0.1828	0.599	29.9950	8.4724	46.3478	4.6	**Mean**	4.5
72_10C ``	0.1951	0.613	30.6935	8.6697	44.4371	4.4	**SD:**	0.1
72_10C ```	0.1822	0.589	29.4975	8.3319	45.7292	4.6	**n**	4
72_10C ````	0.1890	0.600	30.0352	8.4837	44.8875	4.5	**SEM:**	0.0
120_4C `	0.1985	0.325	16.2412	4.5875	23.1108	2.3	**Mean**	2.3
120_4C ``	0.1985	0.329	16.4171	4.6372	23.3611	2.3	**SD:**	0.0
120_4C ```	0.1913	0.318	15.8643	4.4810	23.4241	2.3	**n**	4
120_4C ````	0.1987	0.331	16.5176	4.6656	23.4804	2.3	**SEM:**	0.0
120_10C `	0.1892	1.016	50.9497	14.3913	76.0638	7.6	**Mean**	7.2
120_10C ``	0.1857	0.930	46.6482	13.1763	70.9546	7.1	**SD:**	0.3
120_10C ```	0.1872	0.958	48.0603	13.5751	72.5166	7.3	**n**	4
120_10C ````	0.1963	0.960	48.1558	13.6021	69.2923	6.9	**SEM:**	0.1

Appendix K

Peroxide value determination (PV), storage experiment

The determination of primary oxidation products (peroxide value) in the oil extracted from mackerel rest raw material was done with the AOCS official method Cd 8b-90. Table K.1 presents the raw data for the calculation of PV content in the oil. Calculations were done by utilizing equation K.1.

$$\text{PV} = \frac{Concentration_{(Na_2S_2O_3)} \times (\text{Titration of sample} - \text{Titration of blank}) \times 1000}{\text{Weight of sample}} \quad \text{(K.1)}$$

Table K.1: Raw data for the calculation of PV content in oil extracted from mackerel rest raw material. PV percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sodium Thiosulphate (titrant) standardization					
$Na_2S_2O_3$					
concentration	0.0101 mol/L				
Blanks:	**Consumption [mL]:**		**Consumption [mL]:**	**Results:**	
1	0.1089	**6**	0.1590	**Mean:**	0.1
2	0.1196	**7**	0.1438	**SD:**	0.0
3	0.1257	**8**	0.1743	**n**	10
4	0.1484	**9**	0.1461		
5	0.1565	**10**	0.1406		

Sample:	**Mass [g]:**	**Consumption [mL]:**	**PV [mEq/kg]:**	**Results:**	
0*`	2.0475	1.1348	4.9	**Mean:**	5.3
0*``	2.0095	1.1801	5.3	**SD:**	0.4
0*```	2.1006	1.1627	4.9	**n**	4
0*`````	2.0397	1.3498	6.0	**SEM:**	0.2
24_4C`	2.0550	2.1525	9.9	**Mean:**	10.5
24_4C``	2.0226	2.2461	10.5	**SD:**	0.5
24_4C```	2.0307	2.2548	10.5	**n**	4
24_4C`````	2.0225	2.3834	11.2	**SEM:**	0.2
24_10C`	2.0070	1.9914	9.3	**Mean:**	9.3
24_10C``	2.0464	1.9961	9.2	**SD:**	0.2
24_10C```	2.0196	2.0616	9.6	**n**	4
24_10C`````	2.0043	1.9453	9.1	**SEM:**	0.1
48_4C`	2.0107	3.4975	16.9	**Mean:**	17.7
48_4C``	1.9985	3.8328	18.7	**SD:**	0.7
48_4C```	2.0277	3.7406	17.9	**n**	4
48_4C`````	2.0840	3.7358	17.4	**SEM:**	0.3
48_10C`	2.0083	2.6163	12.5	**Mean:**	13.5
48_10C``	2.0147	2.7598	13.1	**SD:**	0.9
48_10C```	2.0613	2.8721	13.4	**n**	4
48_10C`````	2.0004	3.1149	15.0	**SEM:**	0.5
72_4C`	2.0317	2.0031	9.3	**Mean:**	9.6
72_4C``	2.0205	2.0834	9.7	**SD:**	0.2
72_4C```	2.0379	2.0664	9.6	**n**	4
72_4C`````	2.0221	2.0729	9.7	**SEM:**	0.1
72_10C`	2.0528	3.0194	14.2	**Mean:**	15.6
72_10C``	2.1831	3.0447	13.4	**SD:**	1.9
72_10C```	2.0115	3.3610	16.2	**n**	4
72_10C`````	2.0239	3.8388	18.4	**SEM:**	1.0
120_4C`	2.0676	2.7780	12.9	**Mean:**	14.4
120_4C``	2.0607	2.7710	12.9	**SD:**	1.7
120_4C```	2.0704	3.1807	14.8	**n**	4
120_4C`````	1.3135	2.3368	16.9	**SEM:**	0.8
120_10C`	2.0308	1.9034	8.8	**Mean:**	12.5
120_10C``	2.0123	2.9649	14.2	**SD:**	2.3
120_10C```	2.0022	2.6287	12.6	**n**	4
120_10C`````	2.0315	3.0438	14.4	**SEM:**	1.1

Appendix L

para-anisidine value (AV), storage experiment

The determination of secondary oxidation products (anisidine value) in the oil extracted from mackerel rest raw material was done according to the AOCS official method Cd 18-90. Table L.1 presents the raw data for the calculation of AV content in the oil. Calculations were done by utilizing equation L.1.

$$25 \times \frac{1.2 \times \big((S_2 - B_2) - (S_1 - B_1)\big)}{m} \tag{L.1}$$

- B_1 and B_2 is the blank before and after the addition of the *p*-Anisidine reagent respectively.
- S_1 and S_2 is the sample before and after addition of the *p*-Anisidine reagent respectively.
- m is the sample mass in [g].

Table L.1: Raw data for the calculation of AV content in oil extracted from mackerel rest raw material. AV values and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Mass [g]:	Volume [mL]:	B1	S1	B2	S2	AV	Results:	
0*	2.0489	25	0.0007	0.2529	0.0062	0.3051	1.3	**Mean:**	1.0
	2.1027	25	0.0000	0.2559	0.0048	0.2904	1.0	**SD:**	0.2
	2.1134	25	0.0048	0.2574	0.0118	0.2769	0.8	**n**	3.0
								SEM:	0.1
24_4C	2.0180	25	0.0037	0.6400	0.0076	0.8057	4.0	**Mean:**	4.5
	2.1439	25	0.0149	0.6001	0.0195	0.8328	4.6	**SD:**	0.4
	2.0302	25	0.0077	0.5437	0.0126	0.7953	5.0	**n**	3.0
								SEM:	0.2
24_10C	2.0839	25	0.0004	0.4739	0.0063	0.7953	5.7	**Mean:**	5.6
	2.1236	25	0.0175	0.4884	0.0216	0.8196	5.7	**SD:**	0.1
	2.1108	25	0.0006	0.4719	0.0045	0.7822	5.5	**n**	3.0
								SEM:	0.1
48_4C	2.0215	25	0.0105	0.3857	0.0149	0.9196	8.8	**Mean:**	8.7
	2.0678	25	0.0052	0.3894	0.0092	0.9377	8.8	**SD:**	0.1
	2.1040	25	0.0133	0.3885	0.0175	0.9264	8.5	**n**	3.0
								SEM:	0.1
48_10C	2.1053	25	0.0017	0.7584	0.0046	1.1741	7.7	**Mean:**	7.5
	2.1235	25	0.0206	0.7661	0.0251	1.1765	7.5	**SD:**	0.2
	2.0826	25	0.0057	0.7566	0.0085	1.1410	7.3	**n**	3.0
								SEM:	0.1
72_4C	2.1287	25	0.0052	0.6869	0.0042	1.3590	11.1	**Mean:**	11.1
	2.0395	25	0.0148	0.6519	0.0172	1.3019	11.1	**SD:**	0.0
	2.1436	25	0.0070	0.6820	0.0103	1.3706	11.2	**n**	3.0
								SEM:	0.0
72_10C	2.0176	25	0.0061	1.0345	0.0112	2.0740	17.9	**Mean:**	17.7
	2.0308	25	0.0067	1.0364	0.0022	2.0740	18.0	**SD:**	0.3
	2.3064	25	0.0070	1.1661	0.0107	2.3025	17.2	**n**	3.0
								SEM:	0.2
120_4C	2.1016	25	0.0022	0.8806	0.0016	1.6383	12.9	**Mean:**	12.9
	2.1256	25	0.0011	0.8818	0.0052	1.6760	13.2	**SD:**	0.2
	2.1560	25	0.0010	0.8921	0.0023	1.6605	12.7	**n**	3.0
								SEM:	0.1
120_10C	2.1423	25	0.0012	1.6040	0.0031	2.6387	18.2	**Mean:**	18.4
	2.1308	25	0.0309	1.6107	0.0370	2.6387	18.1	**SD:**	0.4
	2.1729	25	0.0073	1.6176	0.0035	2.7179	18.9	**n**	3.0
								SEM:	0.2

Appendix M

Oxidative stability index (OSI), storage experiment

The raw data for the determination of oxidative stability in oil extracted from mackerel rest raw material was done according to the AOCS Official Method Cd 12b-92. There was an immense amount of raw data collected, which is too large to incorporate in an appendix. Refer to author for data.

Appendix N

Fatty acid composition, storage experiment

The raw data for fatty acid composition of oil extracted from mackerel rest raw material was measured by methylating the lipids, and running a GC analysis. Tables N.1 to N.9 present the raw data including the SD and SEM calculations.

Table N.1: Raw data of fatty acid composition in oil extracted from fresh mackerel rest raw material. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]:					
Sample: 0* (fresh raw material)					
Fatty acid:	**1**	**2**	**Mean**	**SD**	**SEM**
C14:0	6.52	6.53	6.53	0.01	0.01
C14:1	0.22	0.22	0.22	0.00	0.00
C15:0	0.43	0.43	0.43	0.00	0.00
C16:0	11.96	11.95	11.96	0.01	0.01
C16:1	5.40	5.39	5.40	0.01	0.01
C17:0	0.40	0.41	0.41	0.01	0.00
C17:1	0.29	0.30	0.30	0.01	0.01
C18:0	2.01	2.00	2.01	0.01	0.00
C18:1n11 + n9	9.06	8.96	9.01	0.07	0.05
C18:1n7	1.60	1.81	1.71	0.15	0.11
C18:2n6	1.53	1.53	1.53	0.00	0.00
C18:3n6	0.25	0.23	0.24	0.01	0.01
C18:3n3	1.37	1.36	1.37	0.01	0.01
C18:4n3	4.13	4.13	4.13	0.00	0.00
C20:0	0.05	0.04	0.05	0.01	0.01
C20:1n11 + n9	10.17	9.97	10.07	0.14	0.10
C20:1n7	0.23	0.21	0.22	0.01	0.01
C20:2n6	0.24	0.25	0.25	0.01	0.01
C20:3n6	0.11	0.12	0.12	0.01	0.01
C20:4n6	0.46	0.45	0.46	0.01	0.01
C20:3n3	0.24	0.24	0.24	0.00	0.00
C20:4n3	0.99	1.00	1.00	0.01	0.01
C20:5n3	8.35	8.33	8.34	0.01	0.01
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	14.67	14.52	14.60	0.11	0.08
C22:1n9	0.65	0.70	0.68	0.04	0.03
C22:2	0.51	0.51	0.51	0.00	0.00
C22:3	0.09	0.09	0.09	0.00	0.00
C22:4	0.20	0.20	0.20	0.00	0.00
C22:5n3	1.32	1.32	1.32	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	9.73	9.69	9.71	0.03	0.02
C24:1n9	1.04	1.03	1.04	0.01	0.01
Sum	94.24	93.94	94.09		
Sum unknown	5.76	6.06	5.91	0.21	
Sum sat	21.39	21.38	21.39	0.01	
Sum mono	43.33	43.11	43.22	0.16	
Sum poly	29.52	29.45	29.49	0.05	
Sum omega 3	26.13	26.07	26.10	0.04	

Table N.2: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 24 h at 4 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 24_4C Fatty acid:	1	2	Mean	SD	SEM
C14:0	5.96	5.91	5.94	0.04	0.02
C14:1	0.20	0.20	0.20	0.00	0.00
C15:0	0.42	0.42	0.42	0.00	0.00
C16:0	12.45	12.48	12.47	0.02	0.02
C16:1	4.95	4.91	4.93	0.03	0.02
C17:0	0.44	0.45	0.45	0.01	0.01
C17:1	0.20	0.18	0.19	0.01	0.01
C18:0	2.36	2.38	2.37	0.01	0.01
C18:1n11 + n9	12.31	12.28	12.30	0.02	0.02
C18:1n7	1.95	1.89	1.92	0.04	0.03
C18:2n6	1.49	1.46	1.48	0.02	0.02
C18:3n6	0.21	0.21	0.21	0.00	0.00
C18:3n3	1.36	1.35	1.36	0.01	0.01
C18:4n3	4.03	4.01	4.02	0.01	0.01
C20:0	0.01	0.04	0.03	0.02	0.02
C20:1n11+ n9	8.65	8.69	8.67	0.03	0.02
C20:1n7	0.23	0.23	0.23	0.00	0.00
C20:2n6	0.24	0.24	0.24	0.00	0.00
C20:3n6	0.11	0.11	0.11	0.00	0.00
C20:4n6	0.46	0.46	0.46	0.00	0.00
C20:3n3	0.23	0.24	0.24	0.01	0.00
C20:4n3	1.04	1.04	1.04	0.00	0.00
C20:5n3	8.41	8.40	8.41	0.01	0.00
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	12.08	12.23	12.16	0.11	0.08
C22:1n9	0.67	0.63	0.65	0.03	0.02
C22:2	0.50	0.50	0.50	0.00	0.00
C22:3	0.09	0.09	0.09	0.00	0.00
C22:4	0.02	0.29	0.16	0.19	0.14
C22:5n3	1.39	1.39	1.39	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.60	10.70	10.65	0.07	0.05
C24:1n9	0.94	0.97	0.96	0.02	0.02
Sum	94.02	94.40	94.21		
Sum unknown	5.98	5.60	5.79	0.27	
Sum sat	21.66	21.70	21.68	0.03	
Sum mono	42.18	42.21	42.20	0.02	
Sum poly	30.18	30.49	30.34	0.22	
Sum omega 3	27.06	27.13	27.10	0.05	

Table N.3: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 24 h at 10 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 24_10C Fatty acid:	1	2	Mean	SD	SEM
C14:0	6.31	6.27	6.29	0.03	0.02
C14:1	0.21	0.21	0.21	0.00	0.00
C15:0	0.45	0.45	0.45	0.00	0.00
C16:0	12.32	12.27	12.30	0.04	0.03
C16:1	5.27	5.27	5.27	0.00	0.00
C17:0	0.31	0.39	0.35	0.06	0.04
C17:1	0.23	0.23	0.23	0.00	0.00
C18:0	2.13	2.13	2.13	0.00	0.00
C18:1n11 + n9	10.51	10.61	10.56	0.07	0.05
C18:1n7	2.00	1.99	2.00	0.01	0.01
C18:2n6	1.53	1.54	1.54	0.01	0.01
C18:3n6	0.22	0.22	0.22	0.00	0.00
C18:3n3	1.35	1.35	1.35	0.00	0.00
C18:4n3	4.02	4.04	4.03	0.01	0.01
C20:0	0.05	0.05	0.05	0.00	0.00
C20:1n11+ n9	9.49	9.45	9.47	0.03	0.02
C20:1n7	0.20	0.19	0.20	0.01	0.01
C20:2n6	0.24	0.24	0.24	0.00	0.00
C20:3n6	0.12	0.12	0.12	0.00	0.00
C20:4n6	0.51	0.51	0.51	0.00	0.00
C20:3n3	0.25	0.25	0.25	0.00	0.00
C20:4n3	0.99	1.00	1.00	0.01	0.01
C20:5n3	7.92	7.96	7.94	0.03	0.02
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	13.73	13.66	13.70	0.05	0.04
C22:1n9	0.67	0.61	0.64	0.04	0.03
C22:2	0.48	0.48	0.48	0.00	0.00
C22:3	0.09	0.09	0.09	0.00	0.00
C22:4	0.23	0.23	0.23	0.00	0.00
C22:5n3	1.33	1.34	1.34	0.01	0.01
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.03	10.14	10.09	0.08	0.06
C24:1n9	0.98	0.97	0.98	0.01	0.01
Sum	94.19	94.28	94.24		
Sum unknown	5.81	5.72	5.76	0.06	
Sum sat	21.59	21.58	21.59	0.01	
Sum mono	43.29	43.19	43.24	0.07	
Sum poly	29.31	29.51	29.41	0.14	
Sum omega 3	25.89	26.08	25.99	0.13	

Table N.4: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 48 h at 4 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 48_4C Fatty acid:	1	2	Mean	SD	SEM
C14:0	5.65	5.61	5.63	0.03	0.02
C14:1	0.19	0.19	0.19	0.00	0.00
C15:0	0.45	0.44	0.45	0.01	0.01
C16:0	12.83	12.44	12.64	0.28	0.20
C16:1	4.54	4.55	4.55	0.01	0.00
C17:0	0.48	0.49	0.49	0.01	0.01
C17:1	0.17	0.17	0.17	0.00	0.00
C18:0	2.40	2.39	2.40	0.01	0.00
C18:1n11 + n9	14.16	14.19	14.18	0.02	0.01
C18:1n7	2.02	2.00	2.01	0.01	0.01
C18:2n6	1.38	1.38	1.38	0.00	0.00
C18:3n6	0.20	0.21	0.21	0.01	0.00
C18:3n3	1.36	1.37	1.37	0.01	0.01
C18:4n3	4.07	4.09	4.08	0.01	0.01
C20:0	0.06	0.06	0.06	0.00	0.00
C20:1n11+ n9	8.40	8.39	8.40	0.01	0.00
C20:1n7	0.20	0.20	0.20	0.00	0.00
C20:2n6	0.24	0.24	0.24	0.00	0.00
C20:3n6	0.09	0.09	0.09	0.00	0.00
C20:4n6	0.44	0.44	0.44	0.00	0.00
C20:3n3	0.25	0.26	0.26	0.01	0.01
C20:4n3	1.06	1.07	1.07	0.01	0.01
C20:5n3	7.79	7.52	7.66	0.19	0.14
C22:0	0.01	0.01	0.01	0.00	0.00
C22:1n11	12.40	12.41	12.41	0.01	0.00
C22:1n9	0.62	0.60	0.61	0.01	0.01
C22:2	0.44	0.44	0.44	0.00	0.00
C22:3	0.07	0.07	0.07	0.00	0.00
C22:4	0.28	0.29	0.29	0.01	0.00
C22:5n3	1.31	1.31	1.31	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.66	10.72	10.69	0.04	0.03
C24:1n9	0.90	0.89	0.90	0.01	0.01
Sum	95.12	94.53	94.83		
Sum unknown	4.88	5.47	5.17	0.42	
Sum sat	21.88	21.44	21.66	0.31	
Sum mono	43.60	43.59	43.60	0.01	
Sum poly	29.64	29.50	29.57	0.10	
Sum omega 3	26.50	26.34	26.42	0.11	

Table N.5: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 48 h at 10 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]:					
Sample: 48_10C					
Fatty acid:	**1**	**2**	**Mean**	**SD**	**SEM**
C14:0	6.05	6.04	6.05	0.01	0.00
C14:1	0.20	0.20	0.20	0.00	0.00
C15:0	0.44	0.44	0.44	0.00	0.00
C16:0	12.20	12.20	12.20	0.00	0.00
C16:1	5.03	4.84	4.94	0.13	0.10
C17:0	0.45	0.44	0.45	0.01	0.01
C17:1	0.22	0.23	0.23	0.01	0.01
C18:0	2.19	2.19	2.19	0.00	0.00
C18:1n11 + n9	10.51	10.41	10.46	0.07	0.05
C18:1n7	1.89	1.99	1.94	0.07	0.05
C18:2n6	1.52	1.53	1.53	0.01	0.01
C18:3n6	0.22	0.22	0.22	0.00	0.00
C18:3n3	1.40	1.40	1.40	0.00	0.00
C18:4n3	4.11	4.11	4.11	0.00	0.00
C20:0	0.04	0.04	0.04	0.00	0.00
C20:1n11+ n9	9.47	9.44	9.46	0.02	0.02
C20:1n7	0.25	0.25	0.25	0.00	0.00
C20:2n6	0.26	0.26	0.26	0.00	0.00
C20:3n6	0.11	0.11	0.11	0.00	0.00
C20:4n6	0.50	0.50	0.50	0.00	0.00
C20:3n3	0.26	0.26	0.26	0.00	0.00
C20:4n3	1.06	1.07	1.07	0.01	0.01
C20:5n3	8.07	8.06	8.07	0.01	0.00
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	13.74	13.70	13.72	0.03	0.02
C22:1n9	0.67	0.69	0.68	0.01	0.01
C22:2	0.48	0.48	0.48	0.00	0.00
C22:3	0.09	0.00	0.05	0.06	0.05
C22:4	0.24	0.24	0.24	0.00	0.00
C22:5n3	1.40	1.41	1.41	0.01	0.01
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.07	10.05	10.06	0.01	0.01
C24:1n9	0.99	0.98	0.99	0.01	0.01
Sum	94.15	93.80	93.98		
Sum unknown	5.85	6.20	6.03	0.25	
Sum sat	21.39	21.37	21.38	0.01	
Sum mono	42.97	42.73	42.85	0.17	
Sum poly	29.79	29.70	29.75	0.06	
Sum omega 3	26.37	26.36	26.37	0.01	

Table N.6: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 72 h at 4 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 72_4C Fatty acid:	1	2	Mean	SD	SEM
C14:0	6.38	6.38	6.38	0.00	0.00
C14:1	0.23	0.23	0.23	0.00	0.00
C15:0	0.43	0.43	0.43	0.00	0.00
C16:0	12.63	12.60	12.62	0.02	0.02
C16:1	5.54	5.55	5.55	0.01	0.00
C17:0	0.46	0.47	0.47	0.01	0.00
C17:1	0.24	0.24	0.24	0.00	0.00
C18:0	2.18	2.18	2.18	0.00	0.00
C18:1n11+n9	10.66	10.70	10.68	0.03	0.02
C18:1n7	1.94	1.90	1.92	0.03	0.02
C18:2n6	1.48	1.47	1.48	0.01	0.01
C18:3n6	0.23	0.23	0.23	0.00	0.00
C18:3n3	1.23	1.24	1.24	0.01	0.01
C18:4n3	3.74	3.75	3.75	0.01	0.00
C20:0	0.05	0.05	0.05	0.00	0.00
C20:1n11+n9	9.10	9.08	9.09	0.01	0.01
C20:1n7	0.25	0.26	0.26	0.01	0.01
C20:2n6	0.23	0.23	0.23	0.00	0.00
C20:3n6	0.11	0.11	0.11	0.00	0.00
C20:4n6	0.44	0.44	0.44	0.00	0.00
C20:3n3	0.21	0.21	0.21	0.00	0.00
C20:4n3	0.98	0.98	0.98	0.00	0.00
C20:5n3	8.61	8.64	8.63	0.02	0.02
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	12.88	12.86	12.87	0.01	0.01
C22:1n9	0.67	0.65	0.66	0.01	0.01
C22:2	0.49	0.49	0.49	0.00	0.00
C22:3	0.09	0.08	0.09	0.01	0.01
C22:4	0.21	0.21	0.21	0.00	0.00
C22:5n3	1.38	1.39	1.39	0.01	0.01
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.13	10.15	10.14	0.01	0.01
C24:1n9	1.04	1.05	1.05	0.01	0.01
Sum	94.26	94.27	94.27		
Sum unknown	5.74	5.73	5.74	0.01	
Sum sat	22.15	22.13	22.14	0.01	
Sum mono	42.55	42.52	42.54	0.02	
Sum poly	29.56	29.62	29.59	0.04	
Sum omega 3	26.28	26.36	26.32	0.06	

Table N.7: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 72 h at 10 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 72_10C Fatty acid:	1	2	Mean	SD	SEM
C14:0	6.01	5.99	6.00	0.01	0.01
C14:1	0.21	0.20	0.21	0.01	0.00
C15:0	0.47	0.45	0.46	0.01	0.01
C16:0	12.23	12.25	12.24	0.01	0.01
C16:1	5.11	5.11	5.11	0.00	0.00
C17:0	0.48	0.50	0.49	0.01	0.01
C17:1	0.22	0.21	0.22	0.01	0.01
C18:0	2.17	2.18	2.18	0.01	0.01
C18:1n11 + n9	10.07	10.12	10.10	0.04	0.02
C18:1n7	1.95	1.89	1.92	0.04	0.03
C18:2n6	1.59	1.61	1.60	0.01	0.01
C18:3n6	0.23	0.22	0.23	0.01	0.01
C18:3n3	1.45	1.44	1.45	0.01	0.01
C18:4n3	4.29	4.27	4.28	0.01	0.01
C20:0	0.04	0.01	0.03	0.02	0.02
C20:1n11+ n9	9.31	9.33	9.32	0.01	0.01
C20:1n7	0.32	0.31	0.32	0.01	0.01
C20:2n6	0.28	0.28	0.28	0.00	0.00
C20:3n6	0.11	0.10	0.11	0.01	0.01
C20:4n6	0.52	0.52	0.52	0.00	0.00
C20:3n3	0.30	0.30	0.30	0.00	0.00
C20:4n3	1.03	1.03	1.03	0.00	0.00
C20:5n3	7.85	7.84	7.85	0.01	0.00
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	13.91	14.02	13.97	0.08	0.05
C22:1n9	0.61	0.59	0.60	0.01	0.01
C22:2	0.48	0.48	0.48	0.00	0.00
C22:3	0.10	0.10	0.10	0.00	0.00
C22:4	0.26	0.26	0.26	0.00	0.00
C22:5n3	1.36	1.36	1.36	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.29	10.34	10.32	0.04	0.03
C24:1n9	0.93	0.95	0.94	0.01	0.01
Sum	94.20	94.28	94.24		
Sum unknown	5.80	5.72	5.76	0.06	
Sum sat	21.42	21.40	21.41	0.01	
Sum mono	42.64	42.73	42.69	0.06	
Sum poly	30.14	30.15	30.15	0.01	
Sum omega 3	26.57	26.58	26.58	0.01	

Table N.8: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 120 h at 4 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 120_4C Fatty acid:	1	2	Mean	SD	SEM
C14:0	6.44	6.47	6.46	0.02	0.01
C14:1	0.21	0.21	0.21	0.00	0.00
C15:0	0.43	0.43	0.43	0.00	0.00
C16:0	12.00	11.90	11.95	0.07	0.05
C16:1	5.03	5.03	5.03	0.00	0.00
C17:0	0.47	0.45	0.46	0.01	0.01
C17:1	0.20	0.20	0.20	0.00	0.00
C18:0	2.12	2.12	2.12	0.00	0.00
C18:1n11 + n9	10.21	10.16	10.19	0.04	0.03
C18:1n7	1.80	1.83	1.82	0.02	0.02
C18:2n6	1.50	1.49	1.50	0.01	0.01
C18:3n6	0.24	0.23	0.24	0.01	0.00
C18:3n3	1.45	1.45	1.45	0.00	0.00
C18:4n3	4.44	4.44	4.44	0.00	0.00
C20:0	0.03	0.03	0.03	0.00	0.00
C20:1n11+ n9	9.14	9.17	9.16	0.02	0.01
C20:1n7	0.27	0.26	0.27	0.01	0.01
C20:2n6	0.25	0.25	0.25	0.00	0.00
C20:3n6	0.11	0.11	0.11	0.00	0.00
C20:4n6	0.40	0.49	0.45	0.06	0.05
C20:3n3	0.26	0.26	0.26	0.00	0.00
C20:4n3	1.11	1.11	1.11	0.00	0.00
C20:5n3	8.31	8.29	8.30	0.01	0.01
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	13.23	13.24	13.24	0.01	0.00
C22:1n9	0.56	0.60	0.58	0.03	0.02
C22:2	0.50	0.50	0.50	0.00	0.00
C22:3	0.10	0.10	0.10	0.00	0.00
C22:4	0.24	0.24	0.24	0.00	0.00
C22:5n3	1.38	1.37	1.38	0.01	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.78	10.75	10.77	0.02	0.01
C24:1n9	0.96	0.95	0.96	0.01	0.01
Sum	94.19	94.15	94.17		
Sum unknown	5.81	5.85	5.83	0.03	
Sum sat	21.51	21.42	21.47	0.06	
Sum mono	41.61	41.65	41.63	0.03	
Sum poly	31.07	31.08	31.08	0.01	
Sum omega 3	27.73	27.67	27.70	0.04	

Table N.9: Raw data of fatty acid composition in oil extracted from mackerel rest raw material stored for 120 h at 10 °C. Fatty acid percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Fatty acid composition [%]: Sample: 120_10C Fatty acid:	1	2	Mean	SD	SEM
C14:0	5.80	5.77	5.79	0.02	0.02
C14:1	0.19	0.19	0.19	0.00	0.00
C15:0	0.44	0.44	0.44	0.00	0.00
C16:0	12.96	12.94	12.95	0.01	0.01
C16:1	4.90	4.89	4.90	0.01	0.01
C17:0	0.49	0.50	0.50	0.01	0.01
C17:1	0.19	0.19	0.19	0.00	0.00
C18:0	2.42	2.42	2.42	0.00	0.00
C18:1n11 + n9	12.56	12.59	12.58	0.02	0.01
C18:1n7	2.09	2.05	2.07	0.03	0.02
C18:2n6	1.49	1.51	1.50	0.01	0.01
C18:3n6	0.20	0.20	0.20	0.00	0.00
C18:3n3	1.35	1.35	1.35	0.00	0.00
C18:4n3	3.85	3.83	3.84	0.01	0.01
C20:0	0.01	0.01	0.01	0.00	0.00
C20:1n11+ n9	8.44	8.41	8.43	0.02	0.01
C20:1n7	0.26	0.28	0.27	0.01	0.01
C20:2n6	0.25	0.25	0.25	0.00	0.00
C20:3n6	0.10	0.10	0.10	0.00	0.00
C20:4n6	0.53	0.53	0.53	0.00	0.00
C20:3n3	0.20	0.19	0.20	0.01	0.01
C20:4n3	1.03	1.03	1.03	0.00	0.00
C20:5n3	8.07	8.05	8.06	0.01	0.01
C22:0	0.02	0.02	0.02	0.00	0.00
C22:1n11	11.92	11.90	11.91	0.01	0.01
C22:1n9	0.62	0.65	0.64	0.02	0.02
C22:2	0.45	0.45	0.45	0.00	0.00
C22:3	0.09	0.09	0.09	0.00	0.00
C22:4	0.28	0.28	0.28	0.00	0.00
C22:5n3	1.43	1.43	1.43	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00
C22:6n3	10.59	10.56	10.58	0.02	0.01
C24:1n9	0.98	1.00	0.99	0.01	0.01
Sum	94.20	94.10	94.15		
Sum unknown	5.80	5.90	5.85	0.07	
Sum sat	22.14	22.10	22.12	0.03	
Sum mono	42.15	42.15	42.15	0.00	
Sum poly	29.91	29.85	29.88	0.04	
Sum omega 3	26.52	26.44	26.48	0.06	

Appendix O

% Total nitrogen (N), storage experiment

The determination of nitrogen content in the stickwater fraction was done by CN analysis. Table O.1 presents the raw data for the analysis of total nitrogen content in the stickwater. Total protein content is calculated in appendix P.

Table O.1: Raw data for the analysis of total nitrogen content in stickwater extracted from mackerel rest raw material. (%) nitrogen content values and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Mass [mg]:	µgN/Capsel	mgN/Capsel	Nitrogen content [%]:	Mean nitrogen [%]:
0'	1.8964	240.4	0.2404	12.68	
0''	1.3426	168.2	0.1682	12.53	12.6
0'''	2.6715	337.0	0.3370	12.62	
0''''	1.6841	209.4	0.2094	12.43	
24_4C'	2.0984	272.3	0.2723	12.98	
24_4C''	1.8199	236.5	0.2365	12.99	13.0
24_4C'''	2.5847	337.5	0.3375	13.06	
24_4C''''	2.6411	344.3	0.3443	13.04	
24_10C'	2.7676	367.5	0.3675	13.28	
24_10C''	2.8855	382.6	0.3826	13.26	13.2
24_10C'''	2.5698	340.0	0.3400	13.23	
24_10C''''	2.1423	281.4	0.2814	13.13	
48_4C'	2.2728	294.7	0.2947	12.97	
48_4C''	2.4884	321.9	0.3219	12.94	12.9
48_4C'''	2.3422	305.0	0.3050	13.02	
48_4C''''	1.7213	220.7	0.2207	12.82	
48_10C'	2.5359	338.6	0.3386	13.35	
48_10C''	3.0398	406.7	0.4067	13.38	13.3
48_10C'''	2.5903	345.4	0.3454	13.33	
48_10C''''	1.9150	254.8	0.2548	13.31	
72_4C'	0.9787	124.0	0.1240	12.67	
72_4C''	1.6811	213.9	0.2139	12.72	12.7
72_4C'''	1.3094	166.8	0.1668	12.73	
72_4C''''	1.1737	149.6	0.1496	12.75	
72_10C'	2.0699	275.2	0.2752	13.30	
72_10C''	2.2933	304.0	0.3040	13.25	13.3
72_10C'''	2.1650	287.5	0.2875	13.28	
72_10C''''	2.3323	309.2	0.3092	13.26	
120_4C'	2.5746	331.9	0.3319	12.89	
120_4C''	2.7010	352.3	0.3523	13.04	13.0
120_4C'''	1.6323	212.9	0.2129	13.05	
120_4C''''	1.6729	218.4	0.2184	13.05	
120_10C'	2.8634	377.9	0.3779	13.20	
120_10C''	1.8725	244.9	0.2449	13.08	13.1
120_10C'''	2.4816	325.9	0.3259	13.13	
120_10C''''	2.2858	299.0	0.2990	13.08	

Appendix P

Protein content in stickwater, storage experiment

The determination of protein content in the stickwater fraction was done by means of total nitrogen (N). Utilizing the factors 6.25, 5.82, and 4.94 for the conversion of % Nitrogen to % protein. Table P.1 presents the raw data for the calculation of protein content in the stickwater. The raw data of total % nitrogen that is needed to calculate the protein content can be found in appendix O.

Table P.1: Raw data for the calculation of protein content in stickwater extracted from mackerel rest raw material. Protein percentages and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Factor 6.25 [%]:	Mean:	SD	SEM	Factor 5.82 [%]:	Mean:	SD	SEM	Factor 4.94 [%]:	Mean:	SD	SEM
0'	79.2				73.8				62.6			
0''	78.3	78.5	0.6	0.3	72.9	73.1	0.5	0.3	61.9	62.1	0.5	0.2
0'''	78.9				73.4				62.3			
0''''	77.7				72.4				61.4			
24_4C'	81.1				75.5				64.1			
24_4C''	81.2	81.4	0.2	0.1	75.6	75.8	0.2	0.1	64.2	64.3	0.2	0.1
24_4C'''	81.6				76.0				64.5			
24_4C''''	81.5				75.9				64.4			
24_10C'	83.0				77.3				65.6			
24_10C''	82.9	82.7	0.3	0.2	77.2	77.0	0.3	0.2	65.5	65.3	0.3	0.1
24_10C'''	82.7				77.0				65.4			
24_10C''''	82.1				76.4				64.9			
48_4C'	81.0				75.5				64.1			
48_4C''	80.8	80.9	0.5	0.2	75.3	75.3	0.4	0.2	63.9	63.9	0.4	0.2
48_4C'''	81.4				75.8				64.3			
48_4C''''	80.1				74.6				63.3			
48_10C'	83.5				77.7				66.0			
48_10C''	83.6	83.4	0.2	0.1	77.9	77.7	0.2	0.1	66.1	65.9	0.1	0.1
48_10C'''	83.3				77.6				65.9			
48_10C''''	83.2				77.4				65.7			
72_4C'	79.2				73.7				62.6			
72_4C''	79.5	79.5	0.2	0.1	74.0	74.0	0.2	0.1	62.9	62.8	0.2	0.1
72_4C'''	79.6				74.1				62.9			
72_4C''''	79.7				74.2				63.0			
72_10C'	83.1				77.4				65.7			
72_10C''	82.8	83.0	0.1	0.1	77.1	77.2	0.1	0.0	65.5	65.6	0.1	0.0
72_10C'''	83.0				77.3				65.6			
72_10C''''	82.9				77.2				65.5			
120_4C'	80.6				75.0				63.7			
120_4C''	81.5	81.3	0.4	0.2	75.9	75.7	0.4	0.2	64.4	64.3	0.3	0.2
120_4C'''	81.5				75.9				64.4			
120_4C''''	81.6				76.0				64.5			
120_10C'	82.5				76.8				65.2			
120_10C''	81.8	82.0	0.3	0.1	76.1	76.4	0.3	0.1	64.6	64.8	0.2	0.1
120_10C'''	82.1				76.4				64.9			
120_10C''''	81.8				76.1				64.6			

Appendix Q

Degree of hydrolysis, storage experiment

The determination of % DH content in the stickwater fraction was done by formol titration [Taylor, 1957]. Table Q.1 presents the raw data for the degree of hydrolysis in the stickwater. The raw data of total % nitrogen that is needed to calculate the degree of hydrolysis, can be found in appendix O.

Table Q.1: Raw data for the calculation of degree of hydrolysis in stickwater extracted from mackerel rest raw material. (%) degree of hydrolysis values and their respective standard deviations (SD), and the standard error of the mean (SEM) are given.

Sample:	Mass [g]:	Start pH:	NaOH [mL]:	Free amino groups [%]:	Degree of hydrolysis [%]:	Mean:	SD	SEM
0'	0.28	6.480	4.50	2.3	17.9			
0''	0.35	6.560	5.63	2.2	17.8	17.7	0.1	0.1
0'''	0.34	6.402	5.40	2.2	17.5			
24_4C'	0.37	6.582	7.19	2.7	21.1			
24_4C''	0.34	6.492	6.44	2.6	20.3	20.7	0.3	0.2
24_4C'''	0.32	6.598	6.20	2.7	20.8			
24_10C'	0.36	6.621	7.59	2.9	22.2			
24_10C''	0.35	6.593	7.31	2.9	22.1	22.1	0.2	0.1
24_10C'''	0.36	6.592	7.39	2.9	21.8			
48_4C'	0.32	6.662	6.39	2.8	21.5			
48_4C''	0.33	6.562	6.43	2.8	21.4	21.7	0.3	0.2
48_4C'''	0.33	6.610	6.76	2.9	22.2			
48_10C'	0.32	6.509	7.54	3.3	25.1			
48_10C''	0.34	6.358	8.45	3.5	26.1	25.5	0.4	0.2
48_10C'''	0.35	6.509	8.37	3.4	25.4			
72_4C'	0.32	6.452	5.85	2.5	19.9			
72_4C''	0.37	6.402	7.04	2.7	21.2	20.9	0.7	0.4
72_4C'''	0.34	6.462	6.56	2.7	21.6			
72_10C'	0.37	6.561	10.32	3.9	29.6			
72_10C''	0.35	6.491	9.76	3.9	29.6	29.6	0.1	0.0
72_10C'''	0.34	6.489	9.47	3.9	29.5			
120_4C'	0.37	6.561	9.50	3.6	28.0			
120_4C''	0.32	6.571	8.47	3.7	28.2	27.8	0.4	0.2
120_4C'''	0.34	6.663	8.66	3.6	27.3			
120_10C'	0.36	6.514	12.34	4.8	36.5			
120_10C''	0.35	6.511	12.17	4.8	36.8	36.6	0.1	0.1
120_10C'''	0.35	6.555	12.00	4.8	36.7			