NTNU

Norwegian University of
Science and Technology

In Vitro Antioxidant Properties of Cod (*Gadus morhua*) Head Hydrolysates and Ultrafiltration Fractions

Marte Vilde Monslaup

Chemical Engineering and Biotechnology
Submission date: June 2018
Supervisor: Turid Rustad, IBT

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Abstract

An increasing world population calls for the development of sustainable primary processing of renewable biological resources. In Norway, there is a potential for increased utilization of white-fish. Hydrolysed cod heads may serve as a source of high-quality proteins that can be used for human consumption and create added-value to the Norwegian fish industry. In the HEADS UP project, the goal has been to develop technology for producing cod head protein hydrolysates (CPH) for human consumption. Studies have shown that hydrolysates produced from fish rest-raw materials exhibit desirable bioactivities, including antioxidant activity. Proteins with antioxidant activity can be applied in foods to prevent lipid peroxidation or as dietary additives to reduce the damage of oxidative stress in the human body.

The aim of this study was to investigate *in vitro* antioxidant properties of CPH samples derived from the HEADS UP project. The hydrolysates were fractioned through a 4 kDa UF (ultrafiltration) membrane. The permeate (<4 kDa) and retentate (>4 kDa) were collected. The molecular weight distributions of the peptides, and the total and free amino acid content and compositions of the CPH, permeates, and retentates were determined. The *in vitro* antioxidant properties were evaluated using four different methods. These assays included the ABTS (2,-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity, FC (Folin-Ciocalteu) reducing capacity, and iron chelating activity. Moreover, the reduction of lipid oxidation in a liposome system induced with the pro-oxidant iron was investigated in the original CPH.

Overall, the results from this study suggest that the CPH and UF fractions exhibited *in vitro* antioxidant properties. Both the CPH and fractionated hydrolysates demonstrated strong *in vitro* iron chelating activity. The crude CPH was found to exhibit the highest ABTS radical scavenging activity and showed promising effects in the reduction of OUR (oxygen uptake rate) in an iron-induced liposome system. The FC antioxidant activity in the permeate were found to be significantly higher than in the retentate, suggesting a higher content of phenolic compounds in this fraction. The fractionation of hydrolysates based on molecular weights did not significantly change *in vitro* antioxidant activities compared to crude CPH. Gel filtration of <4 kDa and >4 kDa fractions showed that the hydrolysate samples were not completely separated by UF. The degree of hydrolysis was found to be negatively correlated with the amount of water used for hydrolysis, suggesting that decreasing the amount of water added, increased the activity of hydrolysis. Based on analysis of the amino acid content, the hydrolysates were found to be of high nutritional value, as all samples contained sufficient amounts of essential amino acids.

Sammendrag

Ettersom verdens befolkning øker blir behovet for bærekraftig utnyttelse av fornybare biologiske resursser større. Det er et potensiale for økt utnyttelse av hvitfisk i Norge. Hydrolyserte torskehoder kan være en kilde til proteiner av høy kvalitet. Disse proteinene kan brukes til humant konsum og dermed gi økt verdiskapning i norsk fiskeindustri. I prosjektet HEADS UP har produksjonsbetingelser for hydrolyse av torskehoder til humant konsum blitt identifisert. Proteiner fra torskehoder har potensiale som bruk i ingredienser i mat, konserveringsmiddel, eller som helsekost. Proteinene er av høy næringsverdi og innehar ønskede bioaktiviteter, inkludert antioksidantaktivitet.

Målet med denne oppgaven var å undersøke *in vitro* antioksidative egenskaper i torskehodehydrolysater fra HEADS UP. Hydrolysatene ble fraksjonert ved bruk av en ultrafiltreringsmembran med cut-off på 4 kDa. Molekylvektsfordelingen og aminosyreinnhold og sammensetning i det samlede retentatet (>4 kDa) og permeatet (<4 kDa), samt i de originale hydrolysatene ble bestemt. Fire metoder ble benyttet for å undersøke antioksidant aktivitet *in vitro*. I analysene ble inhibering av ABTS radikaler, redusering av FC-reagensen, og chelatering av jern i hydrolysatene og UF fraksjonene undersøkt. I tillegg ble reduseringen av oksidasjon av fett i et jern-indusert liposomsystem studert i de originale hydrolysatene.

Fra analysene ble det målt *in vitro* antioksidantaktivitet i torskehodehydrolysatene og UF fraksjonene. Samtlige torskehodehydrolysater, retentater, og permeater målte god evne til å chelatere jern. Den høyeste inhiberingen av ABTS radikaler ble målt i de originale hydrolysatene. Disse ble også funnet til å ha god effekt på reduksjon av oksygenopptak i liposomer. Det ble ikke målt en signifikant forandring i antioksidative egenskaper som følge av UF fraksjonering av hydrolysatene fra ABTS analysen og jernchelateringsevnen. Fra FC analysen ble det målt signifikant høyere antioksidant aktivitet i permeatene sammenlignet med retentatene. Molekylvektsfordelingen av fraksjonene viste at disse ikke ble fullstendig separert av UF-membranen. Det ble funnet en negativ korrelasjon mellom hydrolysegrad og mengde vann tilsatt under hydrolysen, noe som tyder på høyere hydrolyseaktivitet ved mindre tilsatt vann. De originale hydrolysatene var av høy næringsverdi da de inneholdt tilstrekkelige mengder essensielle aminosyrer.

Preface

This master thesis is part of the M.Eng.programme Industrial Chemistry and Biotechnology. The work was carried out at the Department of Biotechnology and Food Chemistry at the Norwegian University of Science and Technology. The work was conducted as a part of the project HEADS UP which is lead by SINTEF Ocean and Fjordlaks, funded by FHF.

I would like to thank my supervisor, Professor Turid Rustad, for guidance and support throughout the process. I would also like to thank Margrethe Fossheim Ohnstad, whom I was fortunate enough to collaborate with in the laboratory. I would like to extend my gratitude to Veronica Hjellnes and Siri Stavrum, not only for their practical help in the Food Chemistry lab, but also for their patience with all the master students.

Lastly, I would like to give a special mention to my friend, Sigrid Husebø Øygard, for her valuable input and proof-reading skills.

Trondheim, June 2018

Marte Vilde Monslaup

Table of Contents

Abstract i

Sammendrag i

Preface ii

List of Tables viii

List of Figures x

Abbreviations xi

1 Introduction 1
1.1 Aim of the thesis . . . 2

2 Background 3
2.1 Marine rest-raw materials . . . 3
2.1.1 Cod heads as rest-raw materials . . . 4
2.2 Fish Protein Hydrolysates . . . 5
2.2.1 Enzymatic hydrolysis . . . 5
2.2.2 The hydrolysis process . . . 5
2.3 Properties of FPH: bioactive, physiochemical, and nutritional . . . 7
2.3.1 Bioactive properties . . . 8
2.3.2 Physiochemical properties . . . 8
2.3.3 Nutritional properties . . . 9
2.4 Current challenges in the applications of FPH . . . 9
2.5 Oxidation . . . 10
2.5.1 Oxidation of lipids . . . 10
2.6 Antioxidants . . . 12
2.6.1 Synthetic and natural antioxidants . . . 13
2.7 Fish peptides as antioxidants . . . 13

2.7.1 Molecular weight and peptide chain length . . . 14
2.7.2 Amino acid composition . . . 14
2.8 *In vitro* antioxidant activity . . . 15
2.8.1 ET-based assays . . . 15
2.8.2 Liposomes as model systems . . . 17
2.9 Findings from the HEADS UP pilot project . . . 18

3 Materials and Methods **21**
3.1 Preparation of hydrolysates . . . 22
3.1.1 Determination of DH . . . 22
3.2 Storage of samples . . . 23
3.3 Fractionation of hydrolysates by ultrafiltration . . . 23
3.4 Determination of protein content by the Lowry Method . . . 23
3.5 Molecular weight distribution of peptides . . . 23
3.6 Total amino acids . . . 24
3.7 Free amino acids . . . 25
3.8 *In vitro* Antioxidant activity assays . . . 25
3.8.1 Preparation of samples . . . 26
3.8.2 ABTS radical scavenging activity . . . 26
3.8.3 Folin-Ciocalteu reducing capacity . . . 26
3.8.4 Iron (Fe^{2+}) chelating activity . . . 27
3.9 Antioxidant activity in a liposome model system . . . 28
3.10 Statistical Analysis . . . 30
3.10.1 ANOVA . . . 30
3.10.2 PCA . . . 30

4 Results and Discussion **31**
4.1 Degree of hydrolysis . . . 31
4.2 Protein solubility . . . 32
4.3 Amino acid content and composition . . . 33
4.3.1 Total amino acids . . . 33
4.3.2 Free amino acids . . . 35
4.4 Molecular weight distributions . . . 37
4.5 Antioxidant activity of CPH and UF fractions . . . 39
4.5.1 ABTS radical scavenging activity . . . 39
4.5.2 Folin-Ciocalteu reducing capacity . . . 41
4.5.3 Iron (Fe^{2+}) chelating activity . . . 42
4.6 Reduction of OUR in liposome system . . . 43
4.7 Principal component analysis . . . 45
4.7.1 CPH . . . 45
4.7.2 CPH and <4 kDa fraction . . . 49

5 Conclusions **51**
5.1 Further work . . . 52

Bibliography **53**

A Raw materials **61**

B Lowry Method - Soluble Protein **63**

C HPLC - TAA and FAA **65**
C.1 TAA 65
C.2 FAA 69

D Chromatograms of Standard Compounds **73**

E Antioxidant Assays - Raw Data and Calculations **75**
E.1 ABTS radical scavenging activity 75
E.2 Folin-Ciocalteu reducing capacity 78
E.3 Iron chelating activity 80

F Oxygen Uptake in Liposomes **81**

G ACE-inhibition Activity **83**

H Principal Component Analysis **85**

List of Tables

2.1 Chemical composition of cod heads (Stoknes and Økland (2002)) 5
2.2 ROS scavenging-based and red-ox potential assays (Zhong, 2015). 16

3.1 Description of experimental conditions in the hydrolysis of pilot CPH samples ($a.$) stickwater from production of fish oil). 22
3.2 List of name, producer and catalog number of compounds used in the antioxidant assays 25
3.3 Composition of the solutions used in the iron chelating assay. 27

4.1 Degree of hydrolysis (n=2, $\pm SD$) 31
4.2 Total amino acid content in CPH samples (n=6, $\pm$ SD) 33
4.3 Average hydrophobic (HAA), aromatic (AAA), and essential (EAA) amino acid fractions in hydrolysates. 35
4.4 Total free amino acid content in CPH samples (n = 3 $\pm$ SD) 35

A.1 pH of samples at a concentration of 10 mg/mL 62

B.1 Protein concentrations determined by the Lowry method. 64

C.1 HPLC raw data for CPH sample T1. 65
C.2 Calculated total amino acid composition and content for CPH sample T1. 66
C.3 Fractions (%), average and SD of TAA in sample T1. n=7 67
C.4 Fractions (%), average and SD of TAA in sample T5. n=7 67
C.5 Fractions (%), average and SD of TAA in sample T9. n=7 67
C.6 Fractions (%), average and SD of TAA in sample T13. n=7 68
C.7 TAA (mg/g CPG) in samples T1, T5, T9, and T13. n=7 68
C.8 Free amino acid composition expressed as the weight percentage of total free amino acids of CPH samples T1, T5, T9, and T13 $\pm SD$, n=3. For sample T13, n=1. 69
C.9 FAA (mg/g CPH) of crude samples. 70
C.10 FAA compositions (%) of <4 kDa fractions. 70

C.11 FAA (mg/g soluble protein) of <4 kDa samples. 70
C.12 FAA compositions (%) of >4 kDa fractions. 71
C.13 FAA compositions (mg/g soluble protein) of >4 kDa fractions. 71

D.1 Molecular weights, and elution volume peaks of standards used in gel filtration. 73

E.1 Absorbance read at 734 nm and the averages of three parallels of PG standard and hydrolysate samples measured in ABTS assay. Blue T1-T13 is the absorbance read for permeate, green is absorbance read for retentates. 76
E.2 Absrobance measured, and concentration and equivalent ABTS radical scavenging activity calculated for > 4 kDa fractions (P) and < 4 kDa fractions (R). 77
E.3 Results from ABTS assay of permeates and retentates of samples T1, T5, T9, and T13. n = 8. 77
E.4 Absorbances read at 725 nm and their averages of standard concentrations of PG and hydrolysate samples measured in FC assay. 78
E.5 Absorbances measured, concentrations, and equivalent PG concentrations calculated in FC assay. 79
E.6 Results obtained in the FC assay of permeates and retentates of samples T1, T5, T9, and T13 based on eight experiments. 79
E.7 Absorbance read at 562 nm of control, sample, and protein, calculated as an average of three measurements (denoted as parallels). 80
E.8 Iron chelating activity results of samples T1, T5, T9, and T13 given as percentages. n = 8. 80

F.1 Measured OUR reduction rates following Fe^{2+} addition in liposomes. . . 82

List of Figures

2.1 Applications of rest-raw materials in various productions in Norway in 2016 (adapted from Richardsen et al. (2017)). 4
2.2 General outline of the process of enzymatic hydrolysis of fish rest-raw material to produce FPH (Kristinsson and Rasco (2000)). 6
2.3 Potential bioactive, physiochemical, and nutritional properties of FPH. . . 8
2.4 The reactions of initiation, propagation, and termination in the autoxidation of unsaturated fatty lipids (adapted from Coultate (2009)). 11
2.5 The molecular structure of propyl gallate (Gálico et al., 2015). 13
2.6 Schematic drawing of a liposome and the surrounding arrangement of peptides (adapted from Zhang et al. (2013)) 17

3.1 Flow diagram illustrating methods applied. 21
3.2 Overview of the methods applied to measure antioxidant activity of CPH in a system of liposomes. 28

4.1 Weight fractions (%) of TAA for CPH samples T1, T5, T9, and T13 as found by HPLC. n=6, $\pm SD$. 34
4.2 Weight fractions (%) of FAA in samples T1, T5, T9, and T13 as found by HPLC. n=3, $\pm SD$. 37
4.3 Gel filtration chromatogram showing the molecular weight distributions of crude CPH samples T1, T5, T9, and T13 compared to the peaks of known standards. 38
4.4 Gel filtration chromatogram showing the molecular weight distributions of >4 kDa and <4 kDa CPH fractions of samples T1, T5, T9, and T13 compared to the peaks of known standards. 39
4.5 ABTS radical scavenging activity calculated as equivalents of reference compound PG of crude CPH, >4 kDa, and <4 kDa fractions. n=8, $\pm SD$. . 40
4.6 FC reducing capacity given as PG equivalents of hydrolyates T1, T5, T9, T13 and their <4 kDa and >4 kDa fractions. Values are expressed as the mean $\pm SD$, n=8. 42

4.7 Fe^{2+} chelating activity (%) of hydrolysates T1, T5, T9, T13 and their <4 kDa and >4 kDa fractions. Values are expressed as the mean $\pm SD$, n=8. . 43
4.8 Dissolved oxygen as a function of time measured in a closed system of liposomes induced with Fe^{2+}, with cells containing water or CPH solution. 44
4.9 The reduction of oxygen uptake rate (OUR) by CPH and casein following the addition of iron to liposome system. 45
4.10 Loadings plot showing correlations found from the PCA model applied for all results measured for hydrolysate samples T1, T5, T9, and T13. 46
4.11 Score plot from PCA applied for all results measured for hydrolysate samples T1, T5, T9, and T13. 48
4.12 Loadings plot of PCA on results measured for hydrolysate and <4 kDa fractions showing measured correlations. 49
4.13 Score plot of PCA on results for hydrolysate and <4 kDa fractions (P). . . 50

A.1 Sample of T1 from hydrolysis 04.02.2017, sample of T5 from hydrolysis 11.08.2017 61
A.2 Sample T9 from hydrolysis 31.08.2017, T13 from hydrolysis 25.08.2017. 62
A.3 Samples T1, T5, T9, T13 at a concentration of 10 mg/mL in distilled water. 62

B.1 BSA concentration plotted against absorbance read at 750 nm. By linear regression the equation y = 0.0018x + 0.0215 was found. R^2 = 0.9883. . . 64

D.1 Chromatogram of pepsin, Mw = 34 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU). 73
D.2 Chromatogram of cytochrome C, Mw = 12.3 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU). . . . 74
D.3 Chromatogram of aprotinin, Mw = 6.5 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU). 74
D.4 Chromatogram of aprotinin, Mw = 1.34 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU). 74

E.1 Standard curve of propyl gallate used in the ABTS assay to determine radical scavenging activity. Absorbance read at 734 nm was plotted against concentration (μM) of propyl gallate used. By linear regression the equation y = -0.0074x + 0.5359 was found.R^2 = 0.97727. 76
E.2 Standard curve of propyl gallate used in the Folin-Ciocalteu assay to determine the total phenolic concentration. Absorbance read at 725 nm was plotted against concentration (mM) of propyl gallate used. By linear regression the equation y = 0.1261x - 0.0378 was found.R^2 = 0.99601. . . . 78

F.1 Dissolved oxygen as a function of time. 81
F.2 Dissolved oxygen as a function of time. 82

G.1 IC_{50} measured in hydrolysates and permeates. 83

H.1 Boxplot of normalized data variables in CPH samples. 85
H.2 Boxplot of normalized data from CPH samples and fractions. 86

Abbreviations

ABTS	=	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AC	=	Antioxidant capacity
ACE	=	Angiotensin converting I enzyme
Ala	=	Alanine
ANOVA	=	Analysis of variance
Arg	=	Arginine
Asp	=	Aspartate
CPH	=	Cod protein hydrolysate
Cys	=	Cysteine
Da	=	Dalton
EAA	=	Essential amino acids
ET	=	Electron transfer
FAA	=	Free amino acids
FC	=	Folin-ciocalteau
FPH	=	Fish protein hydrolysate
FPLC	=	Fast protein liquid chromatography
Gln	=	Glutamine
Glu	=	Glutamate
Gly	=	Glycine
HAT	=	Hydrogen atom transfer
His	=	Histidine
HPLC	=	High pressure liquid chromatography
IC_{50}	=	Half maximal inhibition concentration
Ile	=	Isoleucine
Leu	=	Leucine
Met	=	Methionine
M_w	=	Molecular weight
MWCO	=	Molecular weight cut-off
PB	=	Papain-Bromelain
PCA	=	Principal component analysis
PG	=	Propyl gallate

Phe	=	Phenylalanine
Pro	=	Proline
Rcf	=	Relative centrifugal force
ROS	=	Reactive oxygen species
SD	=	Standard deviation
Ser	=	Serine
TAA	=	Total amino acids
TAC	=	Total allowable catches
Thr	=	Threnonie
Trp	=	Tryptophan
Tyr	=	Tyrosine
UF	=	Ultrafiltration
Val	=	Valine

Chapter 1

Introduction

A shift towards bioeconomy has recently become an aspiration for Norway and other governments around the globe. Bioeconomy includes developing sustainable primary processing of food materials, while reducing emissions and environmental impact. As fish resources are limited, optimal utilization of raw materials is of interest to consumers, fishers, and processors (Rustad et al., 2011). Rest-raw material is defined as anything that is not considered as the main product in the application of a raw-material (Richardsen et al., 2017). Rest-raw materials may be converted into high-value products and thus contribute to reducing waste.

In Norway, there is a potential for increased utilization of white fish. An estimated 43 % of the total weight of white fish is regarded as rest-raw materials (Richardsen et al., 2017). In 2016, 178 100 tons of the total 319 000 tons of rest-raw materials from white-fish caught in Norway was not being utilized. Rest-raw materials may be a source of lipids, proteins, and peptides, as well as minerals, vitamins, antioxidants, and enzymes. In recent years there has been an increasing interest in bioactive peptides from natural sources due to low cost of production, and high nutritional and physiological value (Sarmadi and Ismail, 2010). These peptides may be applied as food ingredients or preservatives, dietary supplements, or pharmaceuticals.

The project HEADS UP was launched in late 2016 by FHF (The Norwegian Seafood Research Fund), SINTEF Ocean, and Fjordlaks. The goal of HEADS UP has been to increase the utilization of and to create added value from Atlantic cod (*Gadus morhua*) head rest-raw materials. Except for tongue and cheek medallions, cod heads have not traditionally been considered as a valuable source of food. In the HEADS UP project, proteins from cod heads were produced by enzymatic hydrolysis for human consumption. Hydrolysates produced from thirteen pilot experiments conducted by HEADS UP found that the protein powders contained essential amino acids and were relatively odourless and good-tasting. The protein content was found to be over 80% and to be of superior quality compared to traditional fish meal (Remme, 2017).

Fish protein hydrolysates have been found to exhibit bioactivity, including significant antioxidant activities in different oxidative systems (Girgih et al., 2015a; Halim et al., 2016). The antioxidant properties of proteins and peptides from marine sources depend on their structure and composition. Consequently, the determination of molecular weight distributions and amino acid compositions are useful in the evaluation of antioxidant activity. This project has been a continuation of the Specialization project completed in TBT4500 at NTNU in December 2017 (Monslaup, 2017).

1.1 Aim of the thesis

The primary aim of this study has been to investigate the relationships between molecular characteristics and antioxidant activity of cod head hydrolysates (CPH) derived from the HEADS UP pilot project. In particular, the antioxidant activity of hydrolysate fractions with different molecular sizes (crude, <4 kDa, and >4 kDa) was studied. Secondary aims have been to evaluate the nutritional value of the hydrolysates, and to investigate the relationship between process parameters and antioxidant activity.

Hydrolysate samples were fractioned by UF (ultrafiltration) based on a molecular weight cut-off of 4 kDa. The relationship between structural characteristics, namely molecular size and amino acid composition, and antioxidant activity was studied. The molecular weight distributions of CPH and its UF fractions were determined by gel filtration. Total and/or free amino acid compositions of hydrolysates and fractions were determined by acid hydrolysis and high pressure liquid chromatography (HPLC). The antioxidant activity of the hydrolysates and fractions were then studied using *in vitro* methods.

Three different chemical assays were applied to determine the *in vitro* antioxidant activity in hydrolysates and fractions, measuring the ABTS radical scavenging activity, the FC reducing capacity, and iron chelating activity. In addition, the ability of CPH to prevent Fe^{2+}-induced lipid oxidation in a liposome model system was studied. Analysis of variance (ANOVA) and principal component analysis (PCA) was applied for statistical comparison and to detect any correlations between variables and samples.

Chapter 2

Background

2.1 Marine rest-raw materials

In Europe, the EU set yearly catch limits or total allowable catches (TACs) for commercial fish stocks. The TAC is set to ensure healthy stocks, while still allowing the fish industry to make profits (EU-Commission, 2017). The Norwegian Ministry of Fisheries and Coastal Affairs are responsible for enforcing TAC in Norway. Maximizing the utilization of the whole catch for value-added and ingredients for human consumption will benefit both the companies and the consumers.

Rest-raw materials are also known as by-products but is distinguished from waste in that the materials can be processed and applied for human consumption (Rustad et al., 2011). The term "rest-raw materials" was introduced to avoid the negative connotations that "by-products" have. Marine rest-raw materials include viscera, heads, bone, skin, cut-offs, and by-catch (Rustad et al., 2011). Rest-raw materials can potentially contribute to creating wealth in the fish and aquaculture industry. The marine food ingredient industry is growing in Norway, which is causing an increase in the application of rest-raw materials in production (Richardsen et al., 2017). Nevertheless, fish silage production is still the most common use of by-products in Norway.

A total of 0.91 million tons of rest-raw materials were produced in Norway in 2016, of which 76% was utilized (Richardsen et al., 2017). In figure 2.1 an overview of the applications of these rest-raw materials is shown. The majority of the materials went to the processing of silage (46 %), fish meal and oil production (21%), and oil and protein production for aquaculture (20%). In total, 9% went towards human consumption.

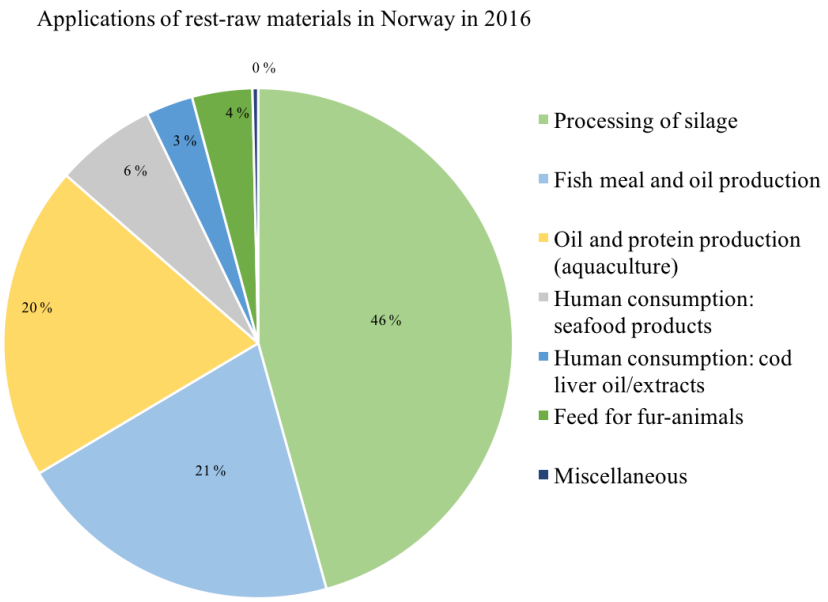

Figure 2.1: Applications of rest-raw materials in various productions in Norway in 2016 (adapted from Richardsen et al. (2017)).

The most abundant source of un-utilized rest-raw materials originates from the heads of white-fish, of which sixty-five thousand tons were not utilized in 2016. Lack of technical solutions and economic rewards are the main reasons for disposing of rest-raw materials, particularly off-shore where only 7% of rest-raw materials from white fish have been utilized (Richardsen et al., 2017).

2.1.1 Cod heads as rest-raw materials

Of the rest-raw materials produced from white fish, cod heads made up 73 000 tons in 2016 (Richardsen et al., 2017). The head of the Atlantic cod make up approximately 17% of its weight, of which 14-15% is protein (Stoknes and Økland, 2002; FHF, 2017). The chemical composition of the cod heads can be found in table 2.1. In previous years, dried heads have been exported to Nigeria. However, the export of dried heads has nearly stopped, and is no longer creating revenues due to unpredictable market demands (FHF, 2017). Other applications include ensilage, reducing the rest-raw material to fish feed, or thermal processing. Thermal processing is mostly reserved for raw materials with a higher fat content (FHF, 2017). Because the heads are lean with a low fat-content, hydrolysis is considered the most viable process option. Hydrolysis, which will be discussed in section 2.2.2, results in three fractions; oil, sludge, and fish protein hydrolysate (FPH).

Table 2.1: Chemical composition of cod heads (Stoknes and Økland (2002))

[%]	Water	Protein	Fat	Ash
Cod heads	79.3	14.2	4.1	6.4

2.2 Fish Protein Hydrolysates

Fish protein hydrolysate are proteins that are enzymatically or chemically broken down into peptides and free amino acids (Kristinsson and Rasco, 2000). Peptides are short protein segments, usually consisting of less than twenty amino acids (Aluko, 2015). FPH may incorporate proteins, peptides and amino acids of a range of molecular sizes.

2.2.1 Enzymatic hydrolysis

Hydrolysis is the cleavage of chemical bonds by addition of water. Chemical hydrolysis involves using strong chemicals and solvents at extreme temperatures and pH. The extreme conditions may destroy some amino acids, thereby decreasing the nutritional value of the hydrolysates. Also, the chemical process can be difficult to control, yielding hydrolysates with varying compositional properties (Kristinsson and Rasco, 2000).

In general, enzymatic hydrolysis is preferred over chemical hydrolysis for making products for human consumption. Enzymatic hydrolysis is based on the use of enzymes to break down the peptide bonds, resulting in smaller peptides. Endogenous enzymes from the viscera and liver, or exogenous, commercial enzymes, may be applied for enzymatic hydrolysis. Enzymes from microbial, plant, and animal sources such as Papain, Alcalase, Bromelain, and Protamex, are often used for hydrolysis of fish proteins (Aspmo et al., 2005). Proteases, or peptidases, are a class of enzymes that cleaves proteins. The class can be divided into two types, exopeptidases or endopeptidases. Exopeptidases can cleave proteins near the N- or C- terminus. Endopeptidases can cleave peptide bonds of non-terminal amino acids. The use of endopeptidases usually result in smaller peptides, while the hydrolysis products of exopeptidases results in a larger concentration of free amino acids (Šližytė et al., 2004b).

2.2.2 The hydrolysis process

The enzymatic hydrolysis process starts with preparing the raw material (e.g. by mincing), and adding water (amount will vary). The main hydrolysis step involves the addition of enzymes at a constant, optimal, temperature. The enzyme is then deactivated at a specified time of hydrolysis, using either heat or chemical inactivation. The remaining suspension contains different particles (lipids, sludge, proteins) that are separated into fractions using separation methods such as centrifugation, sieving, or ultrafiltration (UF). The most commonly used separation method is centrifugation. During centrifugation, several distinct phases are formed. These phases are sludge, the aqueous phase containing hydrolysates, a lipid-protein fraction, occasionally an oil-water emulsion, and oil (Šližytė et al., 2004b). The aim of the process is to recover soluble protein hydrolysates of maximum purity and

yield. Optimal separation will depend on the size, shape, and density of the particles in the mixture, as well as the viscosity of the hydrolysis suspension (Šližytė et al., 2004b). Following separation, the aqueous hydrolysates are either concentrated or dehydrated into a soluble powder. Figure 2.2 shows a general outline of the process of enzymatic hydrolysis. Some of the challenges of using enzymatic hydrolysis to produce high-quality proteins are discussed in section 2.4.

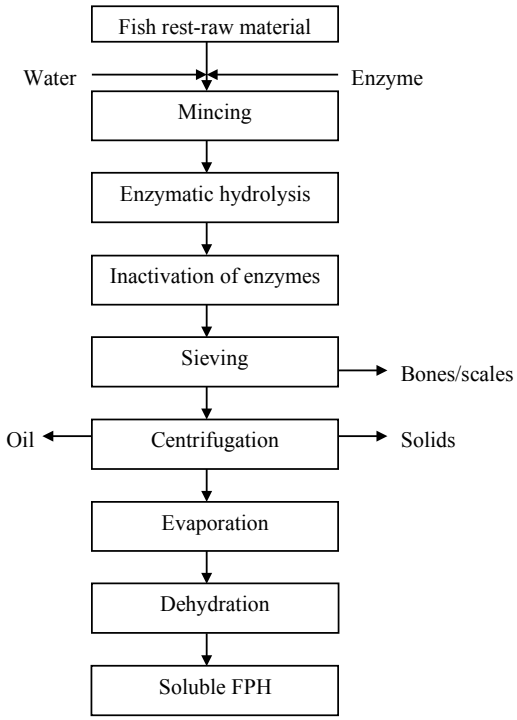

Figure 2.2: General outline of the process of enzymatic hydrolysis of fish rest-raw material to produce FPH (Kristinsson and Rasco (2000)).

Hydrolysis of cod heads

In the master thesis of Meldstad (2015), the effect of raw-material storage conditions on the hydrolysis of cod heads was studied and the composition of protein, lipid, ash, and dry matter content of the raw material and FPH was determined. Results showed that the protein content of the raw material ranged from 12-14%, while the lipid content ranged from 3-4%. Following hydrolysis, the lipid content in the FPH powders was reduced to 0.37-0.71%. The protein concentration in the freeze-dried FPH samples after 60 minutes of hydrolysis ranged from 67.7-88.4%. The highest protein recovery (88.4%) was measured in the hydrolysate from fresh cod heads, compared to the frozen, stored cod heads that were either whole or minced (67.7-71.6%). In the HEADS UP pilot project, fresh cod heads were used in hydrolysis, which yielded a protein content of 84.7 $\pm$ 0.7 % (Remme et al., 2018).

Degree of hydrolysis

The hydrolysates have increased functional value compared to the proteins of the raw material. The hydrolysates consist of peptides with a wide range of molecular weight distributions, depending on the degree of hydrolysis (DH). DH can be defined as the percentage of peptide bonds cleaved by hydrolysis (Adler Nissen, 1979). The degree of hydrolysis describes the extent of hydrolysis, which in turn will determine the characteristics of the hydrolysates. The degree of hydrolysis is an important parameter because the interfacial and surface activities of FPH will depend on the molecular size of the peptides. The relationship between DH and the antioxidant activity of FPH will be discussed in section 2.7.1.

Factors affecting hydrolysates

The yield of FPH from the hydrolysis process depend on the raw-material composition, the type of enzyme, enzyme-raw material compatibility, and the hydrolysis conditions (Šližytė et al., 2004b). Choosing the appropriate enzyme for hydrolysis is crucial because it can influence the peptide profile, depending on specificity and type of enzyme. Broad-spectrum endopepsidases, such as Protamex, Papain, or Bromelain, have been found to be effective in solubilizing fish proteins (Halim et al., 2016). External factors such as pH, temperature, time of hydrolysis, amount of water, and enzyme/raw-material ratio also affect the final peptide profile. The process conditions need to be optimized according to the raw material, the enzymes used, and product specifications (Kristinsson and Rasco, 2000; Najafian and Babji, 2012). In several studies, ultrafiltration (UF), nanofiltration (NF), and gel filtration (GF) have been used to refine the hydrolysates by separation according to the molecular weight of the peptides, the prospective being industrial upgrading of the product (Picot et al., 2010). The aim of filtration is to increase specific activities to produce bioactive or functional ingredients for human or animal consumption (Halim et al., 2016).

2.3 Properties of FPH: bioactive, physiochemical, and nutritional

Functional properties of food proteins refer to "those functional and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation, and consumption" (Kristinsson and Rasco, 2000). Fish muscle contains a variety of proteins and consequently displays a range of functional characteristics. In figure 2.3 a summary of the possible bioactive, physiochemical, and nutritional properties of fish protein hydrolysates is shown.

The compositional characteristics of FPH depend on the physiochemical properties of the original protein, and include amino acid sequences and composition, in addition to molecular size. The molecular weight of peptides has been found to broadly determine bioactivity and function (Kim and Wijesekara, 2010). Peptides with low molecular weights, and thereby short amino acid chains, usually exhibit a higher degree of biological activity (Najafian and Babji, 2012). Peptides of high molecular weights generally exhibit a greater

extent of physiochemical properties, such as foaming and emulsification, water-holding capacity, and the ability to retain and absorb oil (Šližytė et al., 2009).

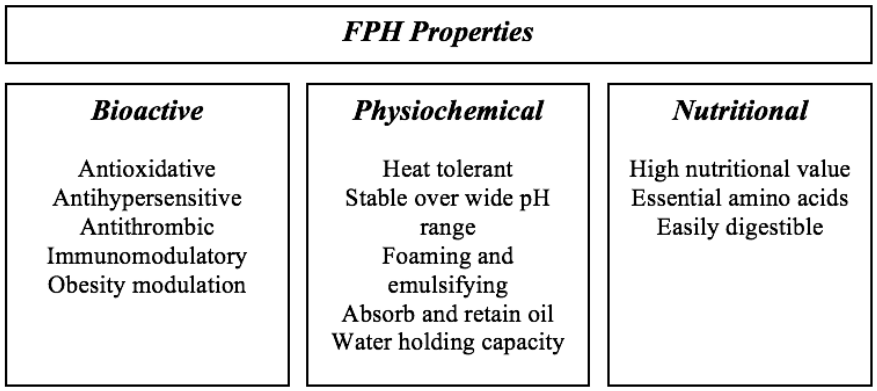

Figure 2.3: Potential bioactive, physiochemical, and nutritional properties of FPH.

2.3.1 Bioactive properties

Bioactive, or biologically active, properties refer to substances having an effect or triggering a response in living tissues (Guaadaoui et al., 2014). Hydrolysates have been found to exhibit bioactive properties such as antioxidative, antihypertensive, antithrombic, immunomodulatory, and obesity modulation (Rustad et al., 2011; SINTEF and Aquaculture, 2015). The antioxidant activity of FPH will be discussed in section 2.7. Besides antioxidant activity, the possible antihypertensive action is the most studied. ACE (angiotensin I converting enzyme) influence blood pressure by turning the inactive ACE I into angiotensin II, a vasoconstrictor contributing to increased blood pressure. *In vitro* studies have shown the inhibition of ACE by fish protein hydrolysates (Jensen et al., 2013; Girgih et al., 2015b). In a study by Girgih et al. (2015b), high *in vitro* ACE-inhibiting activity rates (69-87%) were measured in cod protein hydrolysates. High fractions of aliphatic amino acids such as isoleucine, leucine, alanine, and methionine has been linked to ACE-inhibition activity (Halim et al., 2016).

2.3.2 Physiochemical properties

The physiochemical properties are important to consider for the application of hydrolysates in food products as it will determine the hydrolysate's functionality and quality. Physiochemical, or functional, properties include solubility, water-holding capacity, heat tolerance, pH-stability, foaming and emulsifying properties, and the ability to absorb and retain oil.

Hydrophobicity promotes protein-protein interactions, while ionic interactions promote protein-water solubility. The proteins undergo structural changes during hydrolysis, and the resulting peptides have an increased number of hydrophobic groups exposed at the surface. Also, the hydrophilicity of the peptides is increased as amino and carboxyl groups

are formed at the ends of the peptides when bonds are cleaved, increasing the solubility compared to larger proteins (Kristinsson and Rasco, 2000). FPH has been found to be soluble over a wide range of ionic strengths and pH, and can tolerate strong heat without precipitating (Halim et al., 2016).

Water-holding capacity is a useful property for food applications as functional properties in food systems partially depend on the interactions between protein and water. Increased concentrations of polar groups, such as COOH and NH_2 following enzymatic hydrolysis affect water-holding capacity (Halim et al., 2016). In a study by Šližytė et al. (2009), the water holding capacity of cod-mince increased after addition of FPH from cod backbones by 12% (frozen backbones) and 16% (fresh backbones).

The foaming and emulsifying properties of FPH are related to the surface tension between the hydrophobic and hydrophilic components in food. The hydrophobic and hydrophilic functional groups on the peptides, combined with water-solubility enables the lowering of the interfacial surface tension in foods, thereby stabilizing oil-in-water emulsions (Kristinsson and Rasco, 2000).

The fat-absorbing properties will influence the quality and taste of food products, especially in meat and fish (Kristinsson and Rasco, 2000). The fat-retaining capacity of FPH has been found to be excellent, due to the physical entrapment of oil, and the hydrophobicity on the surface of the peptides (Kinsella, 1981; Kristinsson and Rasco, 2000).

2.3.3 Nutritional properties

Proteins from fish are known to consist of high amounts of essential amino acids, which are a necessary part of any diet. According to FAO/WHO/UNU (1985), if more than 33.9% of the proteins in a compound are made up of essential amino aicds, the compound is considered as being of high nutritional value. FPH's are known to contain all the essential amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In a study by Farvin et al. (2016), cod head hydrolysates were found to contain high amounts of essential amino acids, especially histidine, isoleucine, leucine, and valine. Moreover, because of the water-solubility and small molecular size, fish protein hydrolysates are easily digested.

2.4 Current challenges in the applications of FPH

While FPH contains high-value proteins, there are challenges to overcome before the commercialization of hydrolysates for human consumption. Firstly, reproducible quality and yield is imperative. The hydrolysis process depends on the raw material, the activity and inactivation of endogenous enzymes, type and specificity of the enzymes used for hydrolysis, and the conditions of the hydrolysis process itself (Rustad et al., 2011). The hydrolysis process must therefore be optimized with consideration to both product specifications and raw material composition (FHF, 2017).

A second challenge is that hydrolysis often creates bitter taste and a fish-like smell in the proteins. The bitter taste of FPH is related to the average hydrophobicity of the peptides

and the degree of hydrolysis (Rustad et al., 2011). Hydrophobic amino acids such as valine, leucine, isoleucine, phenylalanine, tyrosine, and tryptophan contribute to the bitter taste of peptides (Coultate, 2009). The taste can be improved by restricting the degree of hydrolysis, by choice of enzymes, or by hydrophobic column separation to remove peptides with bitter-tasting hydrophobic amino acids. However, limiting the degree of hydrolysis may limit the bioactivity of the hydrolysates as it is dependent on the chain length of the peptides. The removal of bitter compounds may also reduce bioactivity because the presence of hydrophobic amino acids is believed to promote antioxidant capacity and other bioactivites (Shahidi, 2015). In addition, debittering the hydrolysates may decrease the nutritional value of the proteins as essential amino acids partially contribute to the bitter taste. Thus, the current methods of debittering the product are counterproductive as the product may lose its intended functional use as antioxidants or antihypertensive agents.

Even if the aforementioned obstacles concerning the use of FPH are overcome, potential health-effects must be documented, including clinical testing, standardization, safety, and quality-control (Rustad et al., 2011). Moreover, the production of FPH must be economically viable, and the market must be ready for the idea of consuming by-products. Nonetheless, the production of FPH can contribute to reducing waste, the peptides have shown promising effects including the regulation of blood pressure and antioxidant activity, and, consumers are showing increased interest in the use of natural ingredients. These factors combined have evoked an extensive amount of research on the potential of FPH.

2.5 Oxidation

To understand the potential antioxidant activity of FPH, it is useful to first look into the mechanisms of oxidation. While oxygen is a fundamental part of the life of aerobic organisms, it is also inherently toxic to those same organisms. Oxidation is coupled with the formation of free radicals and oxidative stress (Dasgupta and Klein, 2014). In foods, especially lipid-rich ones, oxidation reduces the quality through changes in flavour, aroma, colour, and texture. In biological organisms, oxidative stress can accumulate and cause disease. Slowing down the oxidation process in foods is an effective preservation-technique, while including antioxidants in a diet may protect the human body from the negative effects of free radicals.

2.5.1 Oxidation of lipids

As a result of autooxidation, photooxidation, thermal oxidation, and enzymatic oxidation, oxidative rancidity affects fats and lipids, as well as proteins (Zhong, 2015). Of these, autooxidation most commonly lead to oxidative deterioration and damage to biological systems (Zhong, 2015). Autoxidation reactions can be divided into three stages; initiation, propagation, and termination, as shown in figure 2.4 (Coultate, 2009). In figure 2.4, the dot (•) symbolizes the presence of an unpaired electron, and X•denotes a free radical species. Fatty acids are symbolized by R.

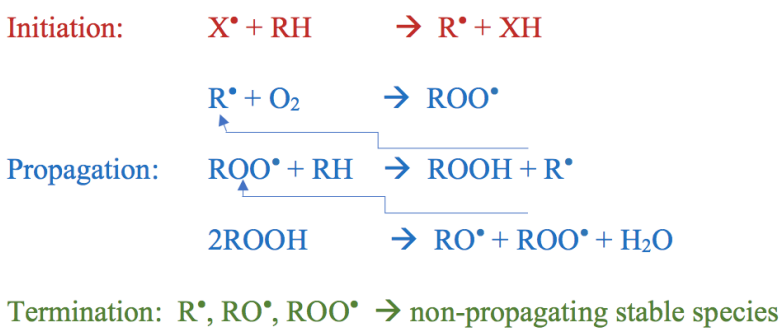

Figure 2.4: The reactions of initiation, propagation, and termination in the autoxidation of unsaturated fatty lipids (adapted from Coultate (2009)).

During initiation highly reactive free radicals are generated. The reactions between molecular oxygen (O_2) and double bonds of lipids are spin-forbidden. Hence, initiators are necessary to either remove an electron from the lipid or to form the highly reactive singlet oxygen by changing the electron spin of the oxygen (Kristinová, 2014; Minotti and Aust, 1992). Known initiators include metals, heat, pre-formed free radicals, or light, resulting in the formation of the lipid alkyl radical, R●(Logan et al., 2013).

Throughout propagation, atmospheric oxygen, O_2, reacts with the lipid alkyl free radical, giving rise to highly reactive peroxy radicals (ROO●). In turn, the peroxy radicals react with fatty acids to produce hydroperoxides (ROOH) and a free radical. The free radical can repeat the process to produce a chain reaction, and the hydroperoxide can break down to generate more free radicals, resulting in an accumulation of free radicals. Termination occurs when radical compounds form stable non-radical end-products. Reactions of termination includes the quenching of individual radicals by radical recombinations, radical transfer, or elimination (Kristinová, 2014).

Tasteless and odorless lipid hydroperoxides (ROOH) are primary products of lipid oxidation. However, when heat, metal ions, or light are present, the ROOH can decompose to compounds with off-odors or off-tastes, thereby creating undesirable changes in a food product (Brewer, 2011). Other undesirable changes resulting from lipid oxidation are changes in colour and texture, and the loss of nutritional value. In marine food products, the oxidation of polyunsaturated essential fatty acids may destroy essential fatty acids, thereby generating toxic compounds that may propose health risks (Turner et al., 2006). The free radicals generated during lipid oxidation may induce oxidation of other compounds present, such as proteins and vitamins, further resulting in the loss of nutritional value in a food product (Choe and Min, 2006; Kristinová, 2014).

2.6 Antioxidants

Antioxidants are molecules that can inhibit or slow down the oxidation of other molecules. The activity of antioxidants depends on the nature of the molecules involved, as well as the system in which they are present (Dasgupta and Klein, 2014). Antioxidants present in foods can either be primary or secondary, depending on their functional mechanism. Some antioxidants exhibit both primary and secondary mechanisms. Primary antioxidants are radical scavengers that can inhibit the initiation or propagation of lipid peroxidation by hydrogen atom transfer (HAT) or electron transfer (ET) (Dasgupta and Klein, 2014). Secondary antioxidants work by promoting the activity of primary antioxidants and by neutralizing the activity of pro-oxidants. Of the methods used to control lipid oxidation, the use of antioxidants is the most efficient and convenient (Zhong, 2015). The different mechanisms of antioxidants relating to lipid oxidation are listed bellow (Brewer (2011)):

1. Scavenging of species that initiate peroxidation.
2. Scavenging of reactive oxygen species (ROS).
3. Quenching of singlet oxygen or free radicals.
4. Breaking the autooxidative chain reaction.
5. Chelating metal ions, rendering them unable to generate reactive species.

Primary antioxidants can scavenge peroxyl radicals (ROO•), free lipid radicals and alkoxy radicals. Radical scavengers react mainly with peroxy radicals as these are long-lived. Although peroxy radicals are not as high in energy as other radical species, antioxidants are often present in small concentrations while highly reactive radicals tend to react with the molecules surrounding their production sites (Damodaran et al., 2008). Phenolic compounds contain a phenol-group, which is an aromatic ring with at least one hydroxyl group. Phenolic compounds are known to be effective radical scavengers and have been applied in food, feed, and cosmetics.

The chelation of pro-oxidant metals is the most important secondary antioxidant mechanism. By inhibiting the activity of catalytic metals, metal chelators can eliminate the initial oxidation step. Metal chelators can decrease the prooxidant activity of metals by occupating the coordination sites of metals, by the formation of insoluble metal complexes, and by affording steric hindrance for metal-lipid interactions (Choe and Min, 2006; Kristinová, 2014).

The intrinsic reactivity toward free radicals and other ROS depend on the chemical structure of the antioxidant. The effectiveness of an antioxidant will also depend on activation energy, the red-ox potential, and volatility and susceptibility. Reaction kinetics plays an important role in the long and short-term protection against oxidation, as well as the rate at which an antioxidant will react with an oxidant (Zhong, 2015). Antioxidants that interfere with the free radical chain reactions are known to be the most effective (Brewer, 2011). The different mechanisms by which the peptides of fish hydrolysates can work as antioxidants are described in section 2.7.

2.6.1 Synthetic and natural antioxidants

Synthetic antioxidants are often used as food preservatives. Examples of synthetic antioxidants include propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and the synthetic chelator ethylenediamine tetraacetic acid (EDTA). The phenolic compound propyl gallate, as shown in figure 2.5, is an ester formed by condensation of gallic acid and propanol. PG is mainly used as an additive in foods containing fats to prevent oxidation (Gálico et al., 2015). Other uses include cosmetics and pharmaceuticals. By trapping peroxy radicals, PG inhibits lipid oxidation. The synthetic PG is often used as a reference compound in spectrophotometric assays used to determine antioxidant activity of other compounds. Although synthetic antioxidants are commonly more potent than those found in nature, they have been reported to be carcinogenic at high concentrations and their use in food products are strictly regulated (Gálico et al., 2015).

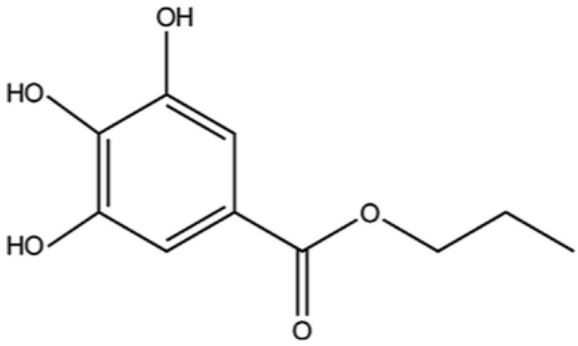

Figure 2.5: The molecular structure of propyl gallate (Gálico et al., 2015).

Natural antioxidants such as herb and tea extracts, vitamin C, and tocopherols have already been commercialized as antioxidants in food systems (Samaranayaka and Li-Chan, 2011). Proteins, peptides, and free amino acids may also serve as natural sources of antioxidants. In general, the antioxidant properties of proteins, peptides, and free amino acids are similar. However, unlike proteins and free amino acids, peptides are soluble in both aqueous and lipid foods. As peptides consist of a chain of amino acids, the antioxidant potency may be additive compared to that of free amino acids. Also, peptides are soluble over a broader range of pH than proteins due to a lower degree of conformational folding, ensuring that the reactive groups of the peptides are accessible for antioxidant reactions (Aluko, 2015). Peptides with antioxidant activity have been sourced from various plant, animal, and fish proteins.

2.7 Fish peptides as antioxidants

Fish peptides are a feasible source of natural antioxidants due to large quantities of fish rest-raw materials being available (Chalamaiah et al., 2012). As previously mentioned, the peptides are considered to be healthy and safe with high antioxidant activity, in addition to being low-cost (Sarmadi and Ismail, 2010). Several studies have indicated that

the short peptide chains and free amino acids of fish peptides can work as antioxidants by scavenging free radicals and reactive oxygen species, by interrupting lipid peroxidation, or by chelating metal ions ((Klompong et al., 2007b; Šližytė et al., 2009; Kim and Wijesekara, 2010; Rustad et al., 2011). The antioxidant activity of fish hydrolysates have been found to depend on peptide-chain lengths, the degree of hydrolysis, hydrophobicity, and the sequence and composition of free amino acids and peptides (Mazorra-Manzano et al., 2018).

2.7.1 Molecular weight and peptide chain length

The functional characteristics of the hydrolysates are affected by the molecular weight (Mw) distribution of its peptides. In several studies, antioxidant peptides have been reported to consist of 2-16 amino acids (Chalamaiah et al., 2012). In general, lower molecular weight FPH's have demonstrated higher radical scavenging activity and reducing power (Halim et al., 2016).

In a study by Intarasirisawat et al. (2012) of hydrolysed Skipjack tuna roe, the metal chelating activity was found to be related to the DH. Metal chelating activity was found to decrease with increasing DH, indicating that when the peptides became too small, they were unable to form stable complexes with metal ions (Aluko, 2015). The mechanism of the reducing power has been found to differ from that of metal chelating activity. The reducing power is dependent on electron donating ability and the hydrophobicity of the peptides (Intarasirisawat et al., 2012; Girgih et al., 2013; Aluko, 2015). Metal chelating activity appears to be affected by the interdependent properties of the amino acids on the peptide chain, especially the presence of negatively charged groups (Aluko, 2015).

Studies of the fractionation of hydrolysates by molecular weight have shown that shorter peptides have higher scavenging activity and lipid peroxidation inhibition. In a study by Farvin et al. (2016), small peptide fractions (M_w <3 kDa) of cod head hydrolysates showed the highest radical scavenging activity, Fe^{2+} chelating activity, and reducing power. The fractions of molecular weights between 3-5 kDa showed greater protection against oxidation in a liposome model system (Farvin et al., 2014). In a study of the fractions of myofibrillar protein hydrolysates from patin, the <3 kDa fractions had stronger reducing power and radical scavenging activity than the <10 kDa fractions (Najafian et al., 2013). However, the antioxidant activity was reduced in the peptide fractions compared to the patin hydrolysates, suggesting a loss of peptide synergistic effect following fractionation (Aluko, 2015).

2.7.2 Amino acid composition

In addition to peptide size, the sequence of amino acids, their composition and hydrophobicity will determine the antioxidant properties of FPH. This includes which reactive groups, such as hydroxyl, carboxyl, and aromatic groups, are present, as well as the sequence of amino acids on the peptide chain (Sarmadi and Ismail, 2010). Many antioxidant peptides have been reported to contain hydrophobic amino acids such as valine and leucine at the N-terminus, while also holding tyrosine, proline, tryptophan, and histidine in their

sequence (Halim et al., 2016).

Tyrosine was suggested to play a significant role in the antioxidant activity of cod head hydrolysates in a study by Farvin et al. (2016). Tyrosine contributes to the scavenging of free radicals as the phenolic lateral chains can act as electron donors, thereby ending the free radical chain reaction (Halim et al., 2016). As aromatic rings are electron dense, aromatic amino acids such as phenylalanine, tryptophan, and tyrosine can contribute to the chelation of pro-oxidant metal ions (Aluko, 2015). Peptide-bound histidine residues have been found to contribute to the scavenging of metal ions, the quenching of active oxygen, and the scavenging of hydroxyl radicals due to the presence of the imidazole group (Aluko, 2015). Moreover, the sulfhur-containing cysteine and methionine can interact with radicals directly as an antioxidant property.

Repeating units of hydrophobic amino acids (Ala, Gly, Leu, Val, and Pro) can increase the peptide-lipid interaction and enhance the scavenging of peroxides and free radicals. Also, repeating units of electron donors on the peptide chains, such as glutamic acid and asparagine, can promote the quenching of free radicals, reduce the oxidative state, and increase the chelating of metal ions (Aluko, 2015).

The antioxidant activity of short peptides is superior to that of free amino acids. However, free amino acids present in FPH may also contribute to antioxidant activity. In a recent study of cod head hydrolysates, it was suggested that both antioxidant peptides and free amino acids in the hydrolysates contributed to antioxidant activity (Farvin et al., 2016). The free amino acids lysine, methionine, and histidine were present in the hydrolysates. The low molecular weight hydrolysate fraction (<3 kDa) had the highest content of free amino acids and showed high radical scavenging and metal chelating activity.

2.8 *In vitro* antioxidant activity

Antioxidant activity can be measured directly or indirectly. Chemical assays based on spectrophotometry are frequently used to measure antioxidant activity indirectly, or *in vitro*. The chemical assays are used to study the intrinsic potential of an antioxidant, the advantages being reproducibility and the simplicity of the method. The measurement of the antioxidant capacity can be divided into electron transfer (ET) and hydrogen atom transfer (HAT).

2.8.1 ET-based assays

Assays based on ET measure the change in absorbance of an oxidant when it is reduced by an antioxidant. Usually, the chemical assay consists of an oxidant, an oxidizable probe, and the antioxidant being studied. The following equation describes the electron transfer reaction (Huang et al., 2005):

$$probe\ (oxidant) + e\ (from\ antioxidant) \rightarrow reduced\ probe + oxidized\ antioxidant \tag{2.1}$$

The antioxidant activity is either expressed as an inhibition against ROS-mediated oxidation or as equivalents of a reference compound, such as PG (Zhong, 2015). All the antioxidant capacity assays used in this master thesis project are ET-based, including the ABTS (2,-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FC (Folin-Ciocalteu) and Fe^{2+}-chelating assays. An overview of the ROS scavenging-based and red-ox potential assays used can be found in table 2.2.

Table 2.2: ROS scavenging-based and red-ox potential assays (Zhong, 2015).

Assay	Activity	Mechanism	Oxidant/probe	Detection
ABTS	ROS scavenging	ET	ABTS radical	spectrophotometry
Folin-Ciocalteu	FC reducing capacity	ET	FC reagent	spectrophotometry
Fe^{2+} chelating	Metal chelating	ET	Ferrozine	spectrophotometry

The ABTS radical assay is one of the most commonly used methods used to measure radical scavenging activity (Nenadis et al., 2004). A spectrophotometer is used to measure the decolorization of the ABTS radical when an antioxidant is added. The ABTS radical is stable over a wide range of pH and is soluble in both aqueous and organic media. It has therefore been used to study the antioxidant activity of many types of biological compounds (Nenadis et al., 2004). It has been reported that phenolic antioxidants that were accepted as being weak radical scavengers were found to be as efficient or more potent than well-known antioxidants (Nenadis et al., 2004). It was therefore concluded that the ABTS assay may provide an indication for the presence of antioxidant compounds, but that any structure-activity relationship cannot be implied.

Phenolic compounds are the most common antioxidants found in nature, including simple phenols and polyphenols. The Folin-Ciocalteu assay is used to measure the total phenolic content (TPC) and is one of the most frequently used methods of quantifying the phenolic content of natural extracts (Tan and Lim, 2015). The FC reagent has a high affinity for the amino acids tyrosine and tryptophan (Berker et al., 2013). The method has the advantage of being fast, simple, sensitive, and do not require advanced equipment. Disadvantages are that the total phenolic content has been reported to be overestimated because the reagent can also react with other reducing compounds such as amino acids and reducing sugars (Tan and Lim, 2015; Shahidi, 2015). While the assay is commonly known as the total phenolic content assay, what is actually measured is a sample's reducing capacity. The measured FC reducing capacity is merely an indication of the TPC (Tan and Lim, 2015)

As previously stated, peptides can act as secondary antioxidant agents by chelating metal ions, thereby reducing the susceptibility of lipids to undergo lipid peroxidation. Ferrous iron is a transition metal with the ability to catalyze lipid oxidation. Hence, the capacity of an antioxidant to chelate Fe^{2+} can be determined by measuring the change in absorbance relating to the inhibition of lipid peroxidation.

The assays discussed above have been criticized for being artificial and biological irrelevant as *in vivo* bioavailbility, stability, and reactivity are not accounted for (Tan and Lim, 2015; Zhong, 2015). ET-based reactions may be slow and prone to interference from trace metal ions or other contaminants. In addition, the methods are not standardized, making comparisons between studies reported in literature challenge. Still, chemical assays are useful as an initial screening and evaluation of novel antioxidant compounds (Zhong, 2015). Two or more assays should be applied to investigate different antioxidant mechanisms. Reports based on ET-based assays alone cannot be extended to represent the overall antioxidant activity of a substance (Tan and Lim, 2015). Antioxidant activity can alternatively be investigated using simple model systems relating to *in vivo* biological systems. The use of liposomes may be an appropriate model system to investigate antioxidants for food (Šližytė et al., 2009).

2.8.2 Liposomes as model systems

The lipid oxidation reactions taking place in food matrices are complicated and difficult to quantify. By using simpler systems, individual reactions can be studied. Lipid oxidation can be monitored by measuring substrate concentrations, oxidants or initiators, intermediates, or final products. This can be quantified as a loss of reactants, oxygen depletion, and formation of primary or secondary oxidation products (Shahidi, 2015). Lipids may exist in the form of small fat droplets dispersed in an aqueous matrix in food products, much like a phospholipid bilayer (Šližytė et al., 2009). A model system of liposomes can therefore be used to investigate the effect of potential antioxidants on lipid oxidation. A liposome is a spherical structure consisting of a phospholipid bilayer encapsulating an aqueous compartment, similar to that found in biological membranes (Kristinová, 2014). A drawing of a liposome and the arrangement of peptides surrounding a liposome is shown in figure 2.6. Peptides may form physical barriers around lipid droplets and block the diffusion of lipid oxidation initiators (Zhang et al., 2013).

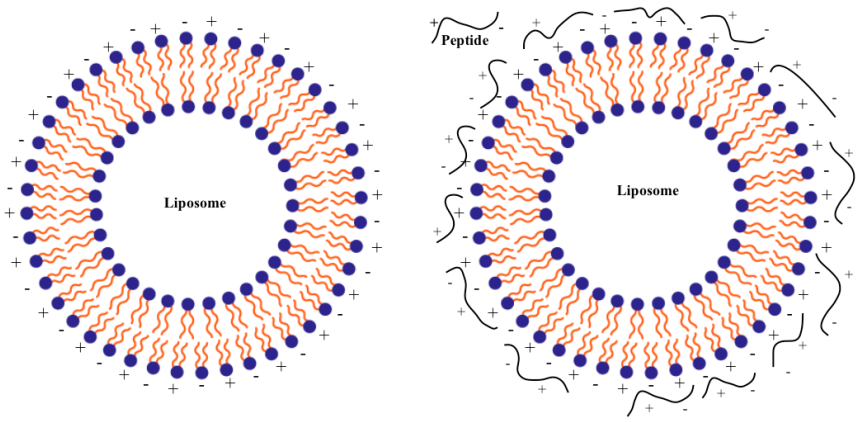

Figure 2.6: Schematic drawing of a liposome and the surrounding arrangement of peptides (adapted from Zhang et al. (2013))

As the direct reaction of lipids with oxygen is spin-forbidden due to the difference in ground states, the reaction must occur via reactions that circumvent the spin barrier (Minotti and Aust, 1992). An initiator, such as iron, can promote these reactions, thereby initiating lipid peroxidation. Iron, a red-ox active metal, is among the most significant prooxidants of lipid oxidation and ubiquitous many in foods (Kristinová, 2014). Mozuraityte et al. (2006b) studied the influence of temperature, pH, Fe^{2+}, and lipid concentration on the pro-oxidant activity of Fe^{2+} in oxidation of cod phospholipids in liposomes. Liposomes were extracted from cod roe phospholipids and the lipid oxidation rate was measured as the consumption of dissolved oxygen in a closed stirred vessel. This method can be applied when measuring the effect of antioxidant compounds, such as FPH, on the lipid oxidation rate.

2.9 Findings from the HEADS UP pilot project

Thirteen pilot experiments had been conducted by late 2017 as part of the HEADS UP project. The aim of the pilot was to investigate how process parameters influenced the yield and quality of proteins. The process parameters included enzymes, amount of water, and addition of non-volatile antioxidants. The enzymes used in the pilot project at Tufjord were either a mixture of Papain and Bromelain or Protamex. The antioxidants sodium sulfite and citric acid were added to two of the hydrolysis samples. In one sample, stickwater from oil production was used in hydrolysis. The following list summarizes the main findings from the pilot project (Remme, 2017):

1. Type of enzyme used had the greatest influence on yield, odour, and taste.
2. Reducing the amount of water used increased the yield of proteins.
3. Yield of dried hydrolysate from raw-material was 10%.
4. Seasonal variations did not influence chemical composition of hydrolysates.
5. Adding non-volatile antioxidants reduced the protein content.
6. Stickwater from oil production should not be used in the hydrolysis.
7. The hydrolysates are best conserved by drying.

The hydrolysates selected for analysis in this project were all produced by the enzyme mix Papain-Bromelain (PB). The average protein content was 84.7%, the fat content ranged from 1.2-3.9%, and the degree of hydrolysis was determined to be 14.3-19.5% (Remme et al., 2018). The protein content was high in all samples, although production of hydrolysate with stickwater reduced the yield of protein (80%). The fat content in the original hydrolysates (4.7-11.2 %) was reduced to 1.2-3.9% by centrifugation. The DH was higher for hydrolysis with Protamex (16.6-18.7 %) compared to Papain-Bromelain. The DH was found to correlate with the reduction of added water (Remme et al., 2018). The molecular weight distributions of the protein and peptides in the hydrolysates showed that the majority (>99%) of the compounds were smaller than 20 kDa, pointing to high

hydrolysis activity (Remme et al., 2018). Most peptides were in the 1-10 kDa molecular weight range (corresponding to 9-90 amino acid residues).

The criteria set for the application of the hydrolysates for human consumption was as follows:

1. High protein content, preferably over 90 %.
2. Low amount of salt and fat.
3. Powders stable for more than one year.
4. Water-solubility.
5. Hydrolysates should be declared as fish/fish powder.

The protein content of the hydrolysates was over 80 %, and the powders were water-soluble. How long the powders are stable for is too early to tell. However, the greatest challenge was said to be the reduction of salt and fat content and improving odour and taste.
’

Chapter 3

Materials and Methods

A sketch of the direction of work taken in this study is found in figure 3.1. The raw material, FPH derived from the HEADS UP project, is from here on referred to as CPH (cod protein hydrolysates). CPH samples were subjected to fractionation by UF with a molecular weight cut-off (MWCO) of 4 kDa. The CPH fractions and crude CPH samples were characterised to find the total/free amino acid compositions, the molecular weight distributions of peptides, and *in vitro* antioxidant activity. Possible correlations between variables and samples were investigated using principal component analysis.

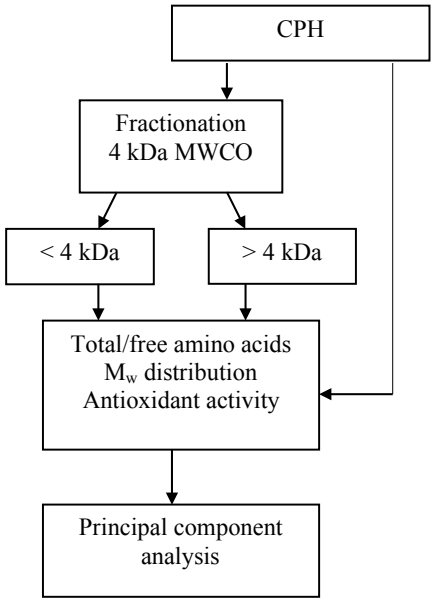

Figure 3.1: Flow diagram illustrating methods applied.

3.1 Preparation of hydrolysates

Hydrolysates derived from cod heads were produced at Tufjord in Northern Norway. Cod heads were first minced and pumped into the hydrolysis tank. Water was added, and the mixture was heated to 50 $^{\circ}C$, followed by the addition of enzymes. Enzymes and slurry were then mixed. After 60 minutes of hydrolysis at 50 $^{\circ}C$, the content was heated to 90 $^{\circ}C$. To inactivate the enzymes, the mixture was kept at 90 $^{\circ}C$ for 10 minutes. A tricanter was used to separate the mixture. The isolated protein hydrolysates were transported frozen to Trondheim where the hydrolysates were freeze-dried into a powder at the food chemistry lab at NTNU.

Thirteen different hydrolysate samples were produced at Tufjord (denoted with the letter T and experimental numbers, *i.e.*, T1-T13) as a part of the HEADS UP pilot experiment. Of these, four samples, T1, T5, T9, and T13, were selected for analysis in the present study. A description of the experimental conditions, including time of catch, amount of water used, type of enzymes, and hydrolysis temperature can be found in table 3.1. In each experiment, 400 kg raw material was used. The amount of water used was either 200, 300, or 400 kg, that is, a raw-material-water ratio of 1:0.5, 1:0.75, and 1:1, respectively. The amount of enzyme used, 0.1 % per kg raw material, was constant.

Table 3.1: Description of experimental conditions in the hydrolysis of pilot CPH samples (*a.*) stickwater from production of fish oil).

Sample	Month of fishing	Water [kg/feed]	Enzyme [$^{\circ}C$]	Hyd. temp. [$^{\circ}C$]
T1	Feb	400	Papain-bromelain	50
T5	March	200	Papain-bromelain	50
T9	March	200[a]	Papain-bromelain	50
T13	May	300	Papain-bromelain + citric acid	50

3.1.1 Determination of DH

The degree of hydrolysis of the samples were determined by formol titration as described by Taylor (1957). Fellow student Ayat Khader Suileman Asfour performed the formol titrations. The analysis was performed in duplicates. The percentage of α-amino nitrogen with respect to the total nitrogen in the sample can be used to evaluate the DH (Taylor, 1957). The formol titration method is based on the reaction between formaldehyde and the amino group of an amino acid at neutral or alkaline pH. A proton is liberated in the reaction, lowering the pKa of the amino acid-formaldehyde complex. The amount of free amino acids present is measured directly as the change in pH of the reaction mixture to the pKa of the amino acid.

3.2 Storage of samples

The CPH samples in their powdered form were stored in plastic zip-lock bags in a freezer (-20 $^{\circ}C$). CPH in solution with water was stored in Eppendorf tubes in a cold room at 2 $^{\circ}C$. Samples were typically kept in the cold room for up to two weeks before new solutions were made. Physical characteristics and pictures of the samples can be found in Appendix A (figures A.1-A.3).

3.3 Fractionation of hydrolysates by ultrafiltration

Ultrafiltration (UF) can be used to refine FPH and obtain peptides of specific molecular sizes. A molecular weigh cut-off, usually smaller than 10 kDa, and often bellow 4 kDa, is used to fractionate the hydrolysates (Picot et al., 2010; Mazorra-Manzano et al., 2018). Specific bioactivies, such as antioxidant activity of the fractionated peptides can then be compared to that of the original hydrolysate.

The hydrolysate samples, T1, T5, T9, and T13 were first dissolved in water at a concentration of 10 mg/mL to a total volume of 150-200 mL. The samples were centrifuged (centrifuge 5804 R, 0033403, Eppendorf) at 4500 rcf (relative centrifugal force) for 15 minutes. The liquid solution was decanted and the residues discarded. Hydrolysate solutions were filtered through a 0.45 μm filter and stored in a freezer (-20 $^{\circ}C$) in Eppendorf tubes prior to UF.

The prepared, filtered solutions were thawed and fractioned by ultrafiltration (Triple System, MMS) at NTNU Kalvskinnet in collaboration with Margrethe Fossheim Ohnstad. A MWCO membrane of 4 kDa was used (NADIR UH004/UH005 P, MICRODYN-NADIR), resulting in two fractions; >4 kDa (retentate) and <4 kDa (permeate). The membrane system consisted of three membrane cells of 28 cm^2. Hydrolysate solution (150-200 mL) was poured into the feed tank and filtered through. A dead volume of 50 mL was left in the feed tank at termination to stop the pumps from drying out. The dead volume is referred to as the retentate or >4 kDa fraction, the filtrate is referred to as the permeate or <4 kDa fraction. UF was performed once for each sample, T1, T5, T9, and T13.

3.4 Determination of protein content by the Lowry Method

As the concentration of proteins in the UF fractions were unknown, the concentration of water-soluble proteins in the crude CPH (10 mg CPH/mL) and the >4 kDa and <4 kDa fractions were determined by the Lowry method (Lowry et al., 1951). A description of the method can be found in Appendix B.

3.5 Molecular weight distribution of peptides

To determine the molecular weight distributions of the peptides in the hydrolysates and UF fractions, size exclusion chromatography with an AktaPurifier FPLC system was used

(GE Healthcare Life Sciences, Uppsala, Sweden). The molecular weight of the different peptides was determined by the retention time while passing through a column. Small peptides had extended retention times, while larger peptides eluted first as they were not retained by the column. The molecular weight distributions were determined in collaboration with Margrethe Fossheim Ohnstad.

Sodium acetate buffer (0.05 M) was prepared, and the pH was adjusted to 5. The solution was filtered through a 0.2 μm filter using suction. Samples in their powdered form (100 mg) were weighed and dissolved in sterilized sodium acetate buffer (4 mL) and filtered through a 0.2μm filter into Eppendorf tubes. The separation was performed using a Superdex Peptide 10/300 GL column, which can detect peptides of a Mw-range of 100-7000 Da. The FPLC machine was started before the computer was started. The flow rate was set at 0.5 mL/min. A syringe with approximately 1 mL of solution, without air, was inserted into the FPLC machine and the full program was run. The measured absorbance at 280 nm was plotted against the retention volume. Due to the relatively low concentration of the UF samples compared to the concentration of 100 mg CPH/4 mL buffer used for the crude samples, the >4 kDa and <4 kDa fractions were directly inserted into the FPLC machine following 0.2 μm filtration.

The molecular weight distributions of each CPH sample and UF fraction was determined once. The chromatograms of the following standards were used for comparison: pepsin (M_w = 34.5 kDa), cytochrome C (M_w = 12.4 kDa), aprotinin (M_w = 6.5 kDa), and vitamin B12 (M_w = 1.3 kDa).

3.6 Total amino acids

The amount of free and total amino acids in the hydrolysates can be determined by high pressure liquid chromatography (HPLC). The different amino acids are separated and detected when injected through a column, allowing the detection of the concentrations of different amino acids.

The total amount of amino acids (TAA) in the FPH was determined by first hydrolysing the samples in acid, before using HPLC to determine amino acid composition, as described by Blackburn (1978). FPH sample (100 mg) was added to 2 mL of 6 mM HCl. The mixture was hydrolysed for 22 hours at 105 °C. The samples were then cooled and transferred into glass beakers using distilled water. The samples were neutralized with NaOH to a pH of 6.5-7.5. The pH was measured using a pH-meter (MP220, 9001, Mettler Toledo). The samples were then filtered through a Whatman glass microfibre filter GF/C using suction, after which the samples were transferred to a volumetric flask (20 mL). Distilled water was added to a volume of 20 mL. The samples were diluted (1:1000) using distilled water. Diluted samples were filtered through a 0.22 μm filter to avoid any contamination. Samples (0.205 mL) were transferred into glass vials. The glass vials were delivered for running on HPLC in three or four parallels to estimate the amount and composition of 17 different amino acids. The amino acids glycine and arginine were determined together due to merging peaks.

The procedure was repeated twice for the four CPH samples, resulting in 7 parallels for inspection. Results are given as the average composition found from the seven parallels run for each sample. The HPLC analysis was run by NTNU employee Siri Stavrum. A Nova-Pak Column Reversed-Phase 4 μm Spherical Silica was used for HPLC. An example of how amino acid fractions were calculated from HPLC chromatograms can be found in Appendix C.1.

3.7 Free amino acids

The determination of the amount and composition of free amino acids (FAA) in the CPH was performed as described by Osnes and Mohr (1985). CPH solution (2 mg/mL, 1 mL) and 10 % sulphosalicylic acid (0.25 mL) was thoroughly mixed in an Eppendorf tube before the samples were left in a cold room (2 °C) for 30 minutes. Three parallels of crude CPH samples T1, T5, T9, one parallel of T13, and three parallels of each UF fraction were prepared. Samples were centrifuged for 15 minutes at 4500 rcf. The liquid solution was decanted and the precipitation discarded. To check that all the protein in the samples had precipitated sulphosalicylic acid (0.25 mL) was added to 1 mL of the supernatant of one of the parallels and mixed as before. Following the complete precipitation of protein, the supernatant was diluted (1:25) using distilled water. The diluted sample was filtered through a 0.22 μm filter. Sample (0.205 mL) was transferred into glass vials, which were delivered for running on HPLC, as previously described.

3.8 *In vitro* Antioxidant activity assays

Three different indirect spectrophotometric assays were used to determine the antioxidant activity of the CPH and the fractions. These were the ABTS radical scavenging assay, the FC reducing capacity assay and, the iron chelating activity assay. A list of name, producer and catalog number of chemicals and compounds used for said assays is found in table 3.2. The synthetic antioxidant propyl gallate was used as a reference compound in the ABTS and FC assays. Absorbance was measured using a spectrophotometer (Genesys 10S UV-Vis, 2L9U235206, Thermo Fischer). All assays were repeated eight times for each CPH, retentate, and permeate sample.

Table 3.2: List of name, producer and catalog number of compounds used in the antioxidant assays

Abreviation	Full Name	Producer	Catalog Number
PG	Propyl gallate	Sigma Aldrich	330-6
ABTS	2,-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	Sigma Aldrich	A1888
FC	Folin and Ciocalteau's phenol reagent	Sigma Aldrich	F9252
Ferrozine	3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine	Fluka	82950

3.8.1 Preparation of samples

Freeze-dried CPH samples were weighed (200 mg) and dissolved in distilled water to a total volume of 10 mL. The samples were then centrifuged (centrifuge 5804 R, 0033403, Eppendorf) at 4500 rcf for 15 minutes. The liquid solution was decanted and the residues discarded. The liquid solution was then appropriately diluted according to the antioxidant assay used. Dilutions are specified for each assay in the following sections. Samples were stored in 15 mL Eppendorf Conical Tubes in a cold room at 2 $^{\circ}C$.

3.8.2 ABTS radical scavenging activity

In the ABTS assay, radical scavenging activity is measured. The blue/green, positively charged, ABTS radical is generated by oxidation with potassium persulfate ($K_2S_2O_8$). The measured decolourization of the ABTS radical quantifies the concentration of radicals reduced by the antioxidant. The reduction is measured as the equivalent of the reduced standard used at a wavelength of 734 nm (Re et al., 1999).

ABTS was performed as described by Nenadis et al. (2004), Nenadis et al. (2007), and, Re et al. (1999). ABTS solution (25 mL, 7 mM) and $K_2S_2O_8$ (440 μL 140 mM) were mixed, covered, and left to react overnight in darkness at room temperature. The reaction mixture was diluted with methanol until the absorbance was 0.75 $\pm$ 0.05 at 734 nm, using water as the reference. A series of 5 dilutions of propyl gallate stock solution were prepared (10-50 μM) and used as standard solutions. ABTS (2 mL) and PG or FPH solution (200 μl) were mixed and incubated for 6 minutes at room temperature. The blank sample was prepared using methanol (80%) instead of sample/PG. The absorbance was read at a wavelength of 734 nm using water as reference. The hydrolysate samples were diluted to a final concentration of 2 mg/mL. The ABTS assay was repeated 8 times for each hydrolysate and each fraction. The radical scavenging activity as measured in the ABTS assay was expressed as PG equivalents. An example of how the ABTS radical scavenging activity was calculated from measured absorbance, as well as the standard curve showing the relationship between PG concentration and absorbance can be found in Appendix E.1.

3.8.3 Folin-Ciocalteu reducing capacity

The total phenolic content is measured in the Folin-Ciocalteu assay. It is based on the oxidation of phenolic compounds in a carbonate solution using the reagent, $3H_20 * P_2O_5 * 13WO_3 * 5MoO_3 * 10H_2$. The Folin-Ciocalteu reagent consists of heteropoly acids, phosphomolybdic and phosphotungstic acids. The molybdenum and tungsten are in the 6^+ oxidation state; these turn blue upon the reduction with reducing agents as a complex between the molybdenum and tungsten forms and the oxidation state changes (Ikawa et al., 2003).

The Folin-Ciocalteu assay was executed as described by Singleton et al. (1999) and Nenadis et al. (2007). A series of four dilutions of propyl gallate stock solutions were made as standards (0.5-2 mM). Distilled water (5 mL), FC-reagent (0.5 mL) and CPH solution (1 mg/mL), peptide fractions (1 mg/mL) or standard solution was mixed. After exactly 3

minutes, 20% Na_2CO_3 (1 mL) was added to all the standard solutions and CPH samples. Distilled water was added (3 mL) and the solution was mixed and left for 1 hour at room temperature. The blank was prepared in the same manner, using 80% methanol instead of sample/PG. The absorbance was measured at a wavelength of 725 nm using water as the reference. The CPH solution was diluted in water to a concentration of 1 mg/mL. A standard curve was plotted of PG concentration (mM) against the absorbance read at 725 nm. The equation found from linear regression of the standard curve was used to find the equivalent PG concentrations of the samples. The raw data, standard curves, and calculations can be found in Appendix E.2.

3.8.4 Iron (Fe^{2+}) chelating activity

Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) is a reagent that can react with divalent iron, forming a stable magenta species (Viollier et al., 2000). Between pH 4 and 9 the magenta Fe(ligand)8z+ species will form completely in aqueous solution (Stookey, 1970). The iron chelating activity can be calculated as a percentage, as shown in equation 3.1. Because the peptides themselves are somewhat turbid when dissolved in water, the absorbance of the peptides in solution is subtracted from the magenta iron-protein solution. $A_{563,sample}$ is the absorbance of the sample. $A_{562,peptide}$ is the absorbance of the peptides in solution without the colour complex.

$$Chelating\ Activity(\%) = (1 - \frac{A_{563,sample} - A_{562,peptide}}{A_{562,control}}) \times 100 \qquad (3.1)$$

The ability of the peptides to chelate Fe^{2+} was determined as described by Klompong et al. (2007b). CPH samples (20 mg/mL) were first centrifuged at 4500 rcf for 10 minutes. The fractions from UF were used directly in analysis. CPH-solution or UF fractions were mixed with distilled water (3.7 mL). $FeCl_2$ (0.1 mL, 2 mM) and ferrozine (0.2 mL, 5 mM) was added to the mixture and left to react for at room temperature for 20 minutes. The absorbance was read at a wavelength of 562 nm, using water as the reference. A control was prepared using the same method but with water instead of the sample. The absrobance of hydrolysate sample without the addition of $FeCl_2$ was used to account for the absorbance of the peptides itself. A summary of how the different solutions were prepared is found in table 3.3. An example of how the metal chelating activity of the samples was calculated from measured absorbance can be found in Appendix E.3.

Table 3.3: Composition of the solutions used in the iron chelating assay.

Solution	Water [mL]	FPH [mL]	$FeCl_2$ [mL]	Ferrozine [mL]	Total Vol. [mL]
Control	4.7	0.0	0.1	0.2	5.0
Peptide	3.8	1.0	0.0	0.2	5.0
Sample	3.7	1.0	0.1	0.2	5.0

3.9 Antioxidant activity in a liposome model system

The initial stage of lipid oxidation can be induced by adding a prooxidant to a lipid system. The rate of lipid oxidation can be monitored by measuring the concentration of dissloved oxygen. Consequently, the effect of an antioxidant on lipid oxidation can be studied as the change in the dissolved oxygen concentration. Figure 3.2 shows a summary of the methods applied to measure antioxidant activity in liposomes.

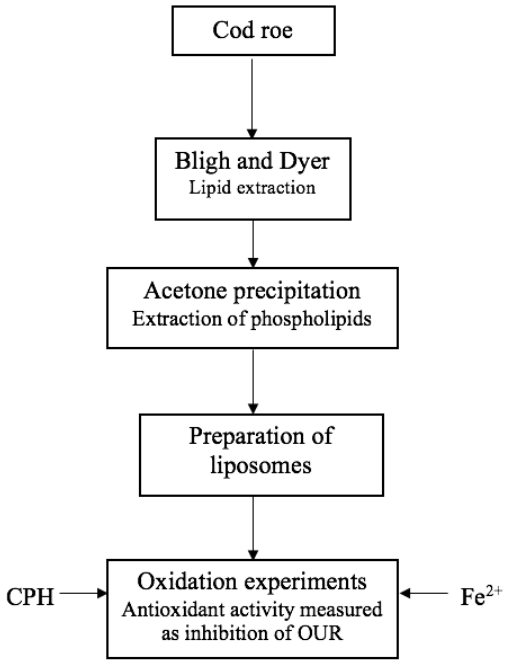

Figure 3.2: Overview of the methods applied to measure antioxidant activity of CPH in a system of liposomes.

Phospholipids were extracted from frozen, thawed cod roe. Measuring the antioxidant activity of CPH using liposomes from phospholipids was performed as described by Mozuraityte et al. (2006b). Lipid extraction was performed as described by Bligh and Dyer (1959). The isolation of phospholipids from total lipids was performed as described by Kates (1986).

Extraction of lipids

Four portions of cod roe (35 g each - 140 g in total) were homogenized with distilled water (25 mL), methanol (100 mL), and chloroform (50 mL) for 2 minutes using an Ultra Turrax homogenizer. The centrifuge tubes were kept on ice. Chloroform (50 mL) was added to the centrifuge tubes, and the mixture was homogenized for 45 seconds. Following homogenization, distilled water (25 mL) was added, and the mixture was homogenized for another 45 seconds. The four mixtures were centrifuged (RC5C, 8505306, Sorvall) at 9682 rcf for 15 minutes.

Aceton precipitation method

The remaining chloroform from the extraction of total lipids was pipetted out, collected, and reduced to approximately 1/3 of the original volume on a rotavapor. The volume left in the round-bottom flask was transferred to a glass flask. Acetone (250 mL) was added, and the mixture was stored at - 20 $^{\circ}C$ overnight.

The next day, the acetone was decanted, and the residue was dissolved in chloroform and transferred to a weighed round-bottom flask. The chloroform was evaporated on a rotor evaporator. The flask and residue were weighed, and the yield was noted. The residue was then dissolved in 25 mL chloroform. The solution was concentrated to approximately 15 mL in a rotor evaporator. Acetone (250 mL) was added, and the solution was stored in a freezer (-20 $^{\circ}C$) overnight.

On the third day, the acetone was decanted the residue left in the flask was dissolved in chloroform. Because the residue was challenging to separate from the acetone, the decanted acetone was centrifuged at 9682 rcf for 15 minutes. The residue was dissolved in chloroform and poured into a round-bottomed flask. The mixture was evaporated. The residue was weighed and dissolved in 15 mL chloroform. The final precipitate (phospholipids) was stored in chloroform at -20 $^{\circ}C$ until needed.

Preparation of liposomes

The preparation of liposomes and oxidation experiments were performed at the laboratory of SINTEF Ocean with the assistance of Revilija Mozuraityte. The preparation of liposomes was performed as described by Mozuraityte et al. (2006b) with some modifications. The chloroform solvent was removed from the phospholipids by gently passing nitrogen gas (99.99%) over the solution. The phospholipids were kept under vacuum for 1 hour in an exicator. The dried film of phospholipids (0.75 g) was dissolved in a pH 5.5 water to a concentration of 3%. The solution was sonicated ten times for 15 seconds with an MSE Ultrasonic Disintegrator Mk2 (MSE Scientific Instruments, Sussex, UK). Each sonication treatment was followed by a 30-s break and cooling in ice to avoid temperature increase.

Oxidation experiments

A close, stirred, water-jacketed cell was used to measure of the rate of lipid oxidation. The concentration of dissolved oxygen by liposomes was measured continuously by a Oxygraph system consisting of a polarographic oxygen electrode (Hansatech Instrument Ltd., Norfolk, UK). A 3% liposome solution in water (pH 5.5) was used in all experiments. All the experiments were performed at 35 $^{\circ}C$. Stock solutions of 15 mM $FeCl_2 \times 4H_2O$ (99.5%, Merck) in 1 M HCl and a working solution of 0.7 mM Fe^{2+} in water (pH 5.5) were prepared.

A concentration of 25 mg/mL CPH was used. First, 0.5 mL water (5.5 pH) was added to the four cells. Then, either 0.5 mL water or protein sample was added, before liposomes (1 mL) were added to the cell. The protein casein was used as a reference compound. Casein is known to absorb iron(II). When measuring dissolved oxygen concentrations, the

background oxygen uptake rate was observed for 4–6 min before Fe^{2+} injection. After injection of Fe^{2+} into the system (20 μl), an initially fast decrease in dissolved oxygen was observed.

The oxygen uptake rate (OUR) of the control (water) following injection of Fe^{2+} was used to find the relative OUR reduction (%) of the protein samples by the following equation:

$$OUR\,reduction\,(\%) = (1 - \frac{rate_{,protein}}{rate_{,control}}) \times 100 \tag{3.2}$$

3.10 Statistical Analysis

The data from the antioxidant assays were analysed by one-way analysis (ANOVA) and principal componant analysis (PCA) using MatLab (MathWorks Inc., USA). Statistical significance was set at a *p*-value <0.05.

3.10.1 ANOVA

One-way ANOVA is an arithmetic method of testing the hypothesis that N population means are equal. ANOVA decomposes a data set into two parts; group means and deviations from group means. The data obtained from antioxidant assays, including ABTS radical scavenging activity, FC reducing capacity, and iron chelating activity, was subjected to ANOVA. The goal of ANOVA was to find any significant differences in measured antioxidant activity between CPH and UF fractions and between the four selected samples, T1, T5, T9, and T13. The data was assumed to be independant and normally distributed.

3.10.2 PCA

Principal component analysis (PCA) is a type of multivariate data analysis that can be used to reduce a mixed data structure into components. In PCA a large set of data is reduced to a smaller set, while still keeping most of the information. The original data set of possibly correlated variables are transformed into a set of two new orthogonal axes called principal components. The first principal component, PCA 1 explains the greatest possible variation in the data, and PCA 2 the second most possible variation (Davies and Fearn, 2005). In PCA, a score plot is generated, allowing the detection of similarities and differences between different samples. Any correlation between variables in the data can be visualized in a loadings plot.

PCA was carried out using MatLab (MathWorks Inc., USA) on the data from the degree of hydrolysis, total and free amino acid composition and content, and antioxidant assays. The ACE-inhibition activity in the hydrolysates as determined by Margrethe Fossheim Ohnstas was also included in PCA. The average values for each variable for each sample was used. As each variable were of different units and numerical sizes, the complete data set was normalized prior to analysis. The normalization of the data is given by equation H.1 found in Appendix H.

Chapter 4

Results and Discussion

The results are presented and discussed separately according to the methods used to characterize hydrolysates and investigate antioxidant activity in sections 4.1-4.6. Possible correlations between variables, namely relationships between structure and antioxidant activity are discussed in light of principal component analysis in section 4.7.

4.1 Degree of hydrolysis

The degree of hydrolysis reflect the number of peptide bonds broken during hydrolysis and can provide information about the average size of the peptides in the hydrolysates. Results of formol titration to determine DH, as given by Ayat Khader Suileman Asfour, are presented in table 4.1. The degree of hydrolysis in samples T1, T5, T9, and T13, ranged from 10.9-15.8 %. At SINTEF Ocean, the DH of the same hydrolysates produced by Papain-Bromelain were determined to be 14.3-19.5% using formol titration performed in duplicates (Remme et al., 2018). The discrepancy in results shows the limitation of few experimental repetitions. The DH of the proteins in the stickwater may have contributed to the higher DH measured in sample T9 (Remme et al., 2018).

Table 4.1: Degree of hydrolysis (n=2, $\pm SD$)

	T1	T5	T9	T13
DH [%]	10.9 ± 0.06	11.5 ± 0.5	15.8 ± 0.9	12.4 ± 0.8

The four hydrolysate samples studied were subjected to similar hydrolysis conditions. The amount of water added differed and may cause the variance in the number of peptide bonds broken during hydrolysis. In the hydrolysis of T1, the ratio of raw material to water was 1:1, while in the hydrolysis of T5 and T9, the ratio was 1:0.5 (table 3.1). From the analysis of all thirteen hydrolysates in the pilot project, a positive correlation was found between the amount of water added and DH (Remme et al., 2018). In contrast, another study of

cod rest-raw hydrolysates found that the DH was not significantly influenced by the ratio of raw material to water added when the ratio was 1:1 or 1:0.75 (Šližytė et al., 2004a).

Previous studies of protein hydrolysates have found the antioxidant activity to be dependent on DH, and that degree of hydrolysis is among the most critical factors to take into account for applications of hydrolysates for human consumption (Klompong et al., 2007a; Mazorra-Manzano et al., 2018). The relationship between DH and results from antioxidant capacity assays will be discussed in section 4.7.

4.2 Protein solubility

During UF, the protein concentrations in the fractions changed as the hydrolysate passed through the membrane. The Lowry method was applied to determine the amount of soluble protein in the CPH samples prior to UF, and in the >4 kDa and <4 kDa fractions following UF. Results are found in Appendix B, table B.1. The initial concentration of CPH in water was 20 mg/mL. The soluble protein concentration in CPH samples ranged from 6.4-10.7 mg/mL, 8.3-11.4 mg/mL in the retentate, and, 1.8-2.2 mg/mL in the permeate. The retentate was concentrated during UF, and the permeate was diluted during UF as water and other small particles passed through the membrane. The Lowry method is commonly used for protein determination. However, compounds other than protein may react with the FC-reagent and the protein content may be overestimated (Mæhre et al., 2018).

The average protein concentration in the studied CPH samples were determined to be 84.7% by SINTEF (Remme et al., 2018). The measured soluble protein content from Lowry was low, at approximately 30-57% of the total weight of the powder. If the total protein content is assumed to be 84.7% in the samples, the relative solubility of the proteins was approximately 35-67%. Similarly, in a study of the *in vitro* antioxidant properties of CPH (<1 kDa), the Lowry protein content was 55% (Girgih et al., 2015a). The presence of polar residues that can form hydrogen bonds with water contributes to solubility in small peptides (Kristinsson and Rasco, 2000). Some hydrophobic interactions can decrease water-solubility by promoting protein-protein interactions. The low concentration of soluble proteins measured in the samples may be related to the hydrophobicity of the proteins. Also, the proteins from the stickwater used in hydrolysis of T9 may have reduced the solubility of the sample.

The hydrolysates showed poor solubility in water at high concentrations. Based on observations, the CPH was soluble at a concentration of 1 mg/mL. However, at a concentration of 10 mg/mL the solution was coloured and precipitates formed. In Appendix A, figure A.3, a concentration of 10 mg CPH/mL is shown. The *in vitro* antioxidant assays are concentration-dependent, and the deviating solubility in water is a source of uncertainty. Moreover, hydrolysate water solubility was set as a requirement for the application of hydrolysates for human consumption (Remme et al., 2018). Solubility is a key characteristic in proteins selected for use in liquid foods and beverages. High solubility increase the potential applications of proteins (Zayas, 1997). Variation in solubility may be related to

the charge of the peptides as pH moves away from the isoelectric point, and the hydrophobicity at the surface of peptides (Halim et al., 2016). Surface hydrophobicity results in decreased water-solubility. Poor water solubility may limit the applications of CPH for human consumption. Still, hydrophobic amino acids in hydrolysates may increase the solubility of peptides in lipids, and thereby contribute to the inhibition of lipid oxidation by enabling antioxidant activity (Farvin et al., 2016).

4.3 Amino acid content and composition

The amino acid composition of protein hydrolysates has been found to influence the bioactivity. To characterize the hydrolysates and evaluate its nutritional properties, the total and free amino acid content and compositions of CPH were determined. In the UF fractions, free amino acid was determined.

4.3.1 Total amino acids

The total amino acid (TAA) content in crude CPH samples are given in figure 4.2. The TAA of the <4 kDa and >4 kda fractions were not determined as the volume of hydrolysate filtrated by UF was not sufficient to perform this analysis. The TAA of crude samples ranged from 506-837 mg/g CPH, with the highest value measured in sample T13. Except for sample T1, the standard deviations were large. In a study of wild cod fillets, the TAA was found to be 995 $\pm$43 mg/g protein (Jensen et al., 2013). Results in table 4.2 are given per grams of CPH powders, which were not 100% protein.

Table 4.2: Total amino acid content in CPH samples (n=6, $\pm$ SD)

	T1	T5	T9	T13
TAA [mg/g CPH powder]	549 ± 16	792 ± 206	504 ± 199	837 ± 233

The total amino acid composition of the different samples are given as weight percentages in figure 4.1. The amino acids Asn, Gln, and Aba, were excluded from the figure as these were only present in trace amounts. A table of all TAA compositions can be found in Appendix C.1 tables C.3-C.6. Analysis showed that all samples had high portions of glycine/arginine, glutamic acid, aspartic acid, alanine, leucine, and lysine. In a report by Farvin et al. (2016), the most abundant amino acids in cod head hydrolysates were Gly, Glu, Lys, and Ala. The same amino acids were reported as the most abundant in cod muscle by Jensen et al. (2013), in addition to Asp and Leu. The amino acid compositions of the four samples were generally similar, with some exceptions. The amount of Gly/Arg ranged from 15.0 - 22.1%, with the highest amount of Gly/Arg (22.1%) found in T13 and the lowest (15.0%) in T9. The amount of Glu was found to be higher in sample T1 at 16.5% compared to 12-4-14.9% for the other samples. Glu is an acidic amino acid that may have the ability to donate excess electrons and act as a metal chelator (Aluko, 2015).

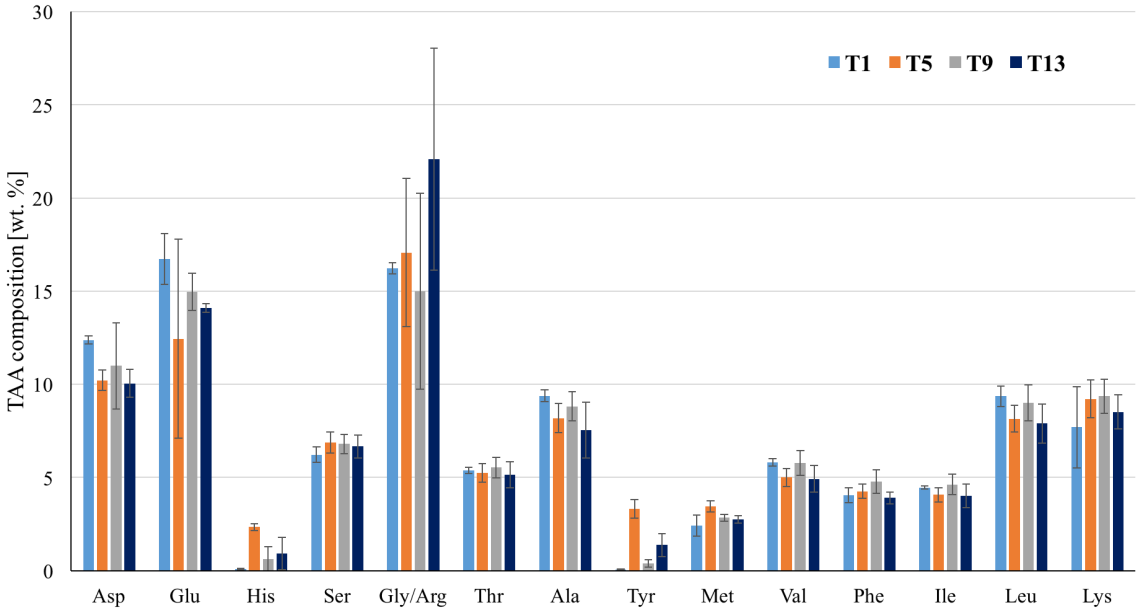

Figure 4.1: Weight fractions (%) of TAA for CPH samples T1, T5, T9, and T13 as found by HPLC. n=6, $\pm SD$.

The results are limited by large standard deviations in the measured amounts of TAA and in amino acid fractions. A large SD was observed for Asp, Glu, and Gly/Arg in particular. The large standard deviations may be a result of problems with the acid hydrolysis used to prepare samples for TAA analysis. This hydrolysis involves many steps, including neutralizing and filtration. Some of the protein may have been lost during analysis, resulting in the discrepancy in the results. The values and fractions presented should be treated as an indication of the protein content and composition in the samples, and not as absolute values.

The fraction of hydrophobic amino acids (HAA - Ala, Tyr, Met, Val, Phe, Ile, and Leu), aromatic amino acid content (AAA - Phe, Tyr, His), and essential amino acids (EAA - His, Thr, Met, Val, Phe, Ile, Leu, and Lys) in the four CPH samples are shown in table 4.3. Hydrophobic and aromatic amino acids are associated with antioxidant activity in fish peptides (Mendis et al., 2005; Aluko, 2015). The AAA content in sample T5 were found to be high (9.9 %) compared to the other samples. Other research has suggested that both tyrosine and histidine contribute to the antioxidant activity of peptides (Halim et al., 2016). The presence of an imidazole ring is attributed to the strong radical scavenging activity of histidine (Samaranayaka and Li-Chan, 2011). Hydrophobic amino acids are reported to be partly responsible for radical-scavenging and inhibition of lipid peroxidation (Mendis et al., 2005). The samples were found to contain high amounts of hydrophobic amino acids (32.4-36.4 %), especially Leu and Ala. In a study of cod protein hydrolysates, the fraction of HAA was found to be 39.6%, including Trp, Cys, and Pro (Girgih et al., 2015a). In this study, the HAA fraction was found to be higher in the smaller peptide fractions (<1 kDa).

If peptides contain high amounts of hydrophobic groups, the solubility of the peptides in a lipid-matrix can be enhanced, thereby improving accessibility to hydrophobic radical species (Qian et al., 2008; Farvin et al., 2016)

Table 4.3: Average hydrophobic (HAA), aromatic (AAA), and essential (EAA) amino acid fractions in hydrolysates.

[wt. %]	T1	T5	T9	T13
HAA	35.5	36.4	36.2	32.4
AAA	4.2	9.9	5.8	6.2
EAA	39.4	41.7	42.5	38.0

According to FAO/WHO/UNU (1985), an essential amino acid fraction exceeding 33.9% of the total amino acid is regarded as a high nutritional value. All four samples were found to contain high EAA fractions at 39.2%, 41.7%, 42.5%, and 38.0% for T1, T5, T9, and T13, respectively. The high protein content in the powders combined with the nutritional value suggests that CPH may be a source of dietary proteins. In a study by Girgih et al. (2015a) of CPH, the EAA content was found to be similar to the current results at 40.1%, including tryptophan. Trp may have been present in the hydrolysates but was destroyed during acid hydrolysis, and was therefore not detected by HPLC column separation. Excluding Trp from the analysis was a limitation as Trp is an essential amino acid. Also, Trp is suggested to be a potent peptide-bound amino acid contributing to antioxidant activity (Aluko, 2015). Alkaline hydrolysis may be used to determine Trp content.

4.3.2 Free amino acids

The amount of total free amino acids (FAA) in CPH samples and UF fractions are given in table 4.4. Of the crude CPH, the highest FAA was found in sample T13 (25.4 mg/g dry CPH) followed by T5 (21.3 mg/ g dry CPH). Sample T1 was found to contain less than half the amount of FAA compared to sample T13 at 11.5 mg/g.

Table 4.4: Total free amino acid content in CPH samples (n = 3 $\pm$ SD)

FAA	Crude [mg/g CPH powder]	>4 kDa [mg/g s.prot]	<4 kDa [mg/g s.prot]
T1	11.5 ± 0.9	2.8 ± 0.12	13.1 ± 0.5
T5	21.3 ± 1.1	8.8 ± 0.14	2.5 ± 0.2
T9	13.3 ± 0.13	4.0 ± 0.04	1.4 ± 0.2
T13	$25.4 \pm n.d.$	11.4 ± 0.11	1.7 ± 0.06

In the master thesis of Meldstad (2015), the total FAA in cod heads hydrolysed for 60 minutes was determined to be 48.4-54.25 mg/g FPH. In a study of the hydrolysates of cod by-products by Šližytė et al. (2005b), the total free amino acid content was found to be 48 mg/g FPH. Compared to 48 mg/g CPH, the total FAA measured in T1, T5, T9, and

T13 were low. The substantial difference in FAA between the four samples is unexpected because the same enzymes were used for hydrolysis and the DH was relatively similar for the samples. The free amino acid content has been found to vary with the type of enzyme used (Šližytė et al., 2005a). The broad-spectrum endopeptidases Papain and Bromelain were used to produce the HEADS UP hydrolysates. Endopeptidases cleave non-terminal peptide bonds, resulting in a smaller portion of free amino acids

In a study of hydrolysates from defatted salmon backbones, the FAA content was found to correlate with the degree of hydrolysis (Šližytė et al., 2016). The lowest DH was measured in sample T1 (table 4.1), which also had the lowest amount of FAA. The same was not observed for sample T9 which also had a relatively low amount of FAA, but the highest DH. However, the aforementioned high DH of sample T9 may be related to the proteins in the stickwater used to produce the samples (Remme et al., 2018).

The FAA content was determined as mg/g CPH for the crude samples, while it was calculated as mg/g soluble protein for the fractions. The FAA content of the CPH and UF fractions are therefore difficult to compare. Between the two UF fractions, the FAA content was lower in the <4 kDa fraction than in the >4 kDa fraction, except for in sample T1. The amount of free amino acids would be expected to be higher in the <4 kDa fraction as free amino acids can pass through the membrane. In a study of cod head hydrolysates, the <3 kDa fraction was found to have the highest content of FAA, compared to >3 kDa and crude samples (Farvin et al., 2016). The chromatograms from HPLC of the amino acids in the UF fractions pointed to errors in peak determination. The errors could stem from proteins not being precipitated during preparations of samples, or from erroneous dilutions. Thus, the fractions of free amino acids in the UF fractions were omitted from the main report. The FAA of these fractions, as given in table 4.2, are likely not representative of the actual FAA content.

The composition of total FAA of the CPH samples are given in figure 4.2, excluding Asn and Aba. The total FAA fractions determined in CPH, >4 kDa, and <4 kDa can be found in table C.8-C.12 in Appendix C.2. The dominating free amino acids in all samples were Leu (11.9-14.7%), Ala (11.1-11.9%), Gly/Arg (10.4-11.7%), and Lys (8.9-10.5%). The compositions were coherent with the results of both Meldstad (2015) and Farvin et al. (2016) in which the most abundant FAA in cod head hydrolysates were found to be leucine, alanine, glycine, arginine, and serine. Overall, the FAA compositions were similar for all samples. The fraction of Leu ranged from 11.9% in sample T1 to 14.7% in sample T13. The hydrophobic amino acid Leu may contribute to antioxidant activity in peptides. Sample T5 was found to contain larger total fractions of Tyr and His than the other samples. The same was not observed in FAA compositions, suggesting T5 may contain more peptide-bound tyrosine and histidine than the other samples.

The fractions of free essential amino acids in FAA, excluding tryptophan, were 49.1%, 54.2%, 51.8%, and 52.8% for samples T1, T5, T9, and T13, respectively. The fraction of EAA was larger than in the total amino acids.

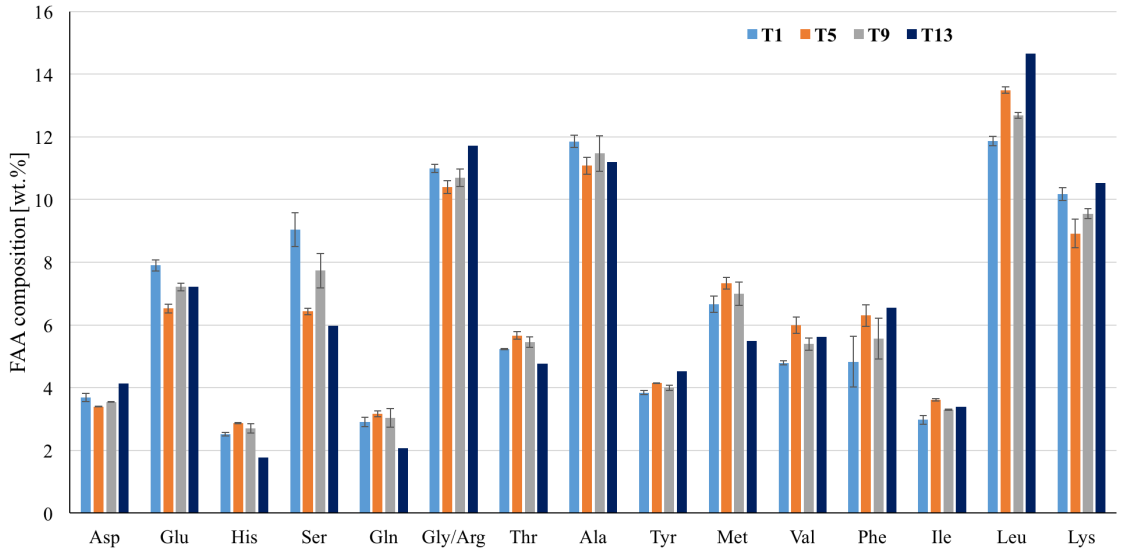

Figure 4.2: Weight fractions (%) of FAA in samples T1, T5, T9, and T13 as found by HPLC. n=3, $\pm SD$.

4.4 Molecular weight distributions

The molecular weight distributions of the peptides in the CPH crude and fractionated samples were determined by gel filtration using an FPLC system. The Mw-distributions of the samples and its UF fractions were determined to characterize the size of the peptides in the hydrolysates and to study the degree of separation performed by the UF membrane. Figure 4.3 shows the molecular weight distribution profile of the peptides for the four crude CPH samples, plotted as absorbance read at 280 nm against elution volume. The size distribution profiles were compared standard compounds of known molecular weights (Pepsin = 34 kDa, Cytochrome C = 12.4 kDa, Aprotinin = 6.5 kDa, Vitamin B12 = 1.3 kDa). The chromatograms of the standards are found in Appendix D. Vertical lines in the figure mark the elution volume of the standards. The column used (Superdex Peptide 10/300 GL) detected peptides with a molecular weight range of 100-7000 Da (approximately 1-64 amino acids).

The majority of the peptides were found to be of molecular weights smaller than 12.4 kDa (Cytochrome C), with closely eluting peptides observed in the 1-12 kDa range. The size distribution profiles of the samples were similar, suggesting the hydrolysates consisted of peptides of similar molecular sizes. The absorbance of the peaks for samples T1 and T5 were weaker than for T9 and T13 in the 1-12 kDa range. In the size distribution profile for sample T9, a distinct peak was observed at approximately 7 mL, suggesting that this sample consisted of some proteins of larger molecular weights than the other samples.

The molecular weight distributions of the samples were compared to known protein standards. However, the weight distributions were not quantified and are based on observations. Several peptides appeared to be outside the validity range of the column. The peaks

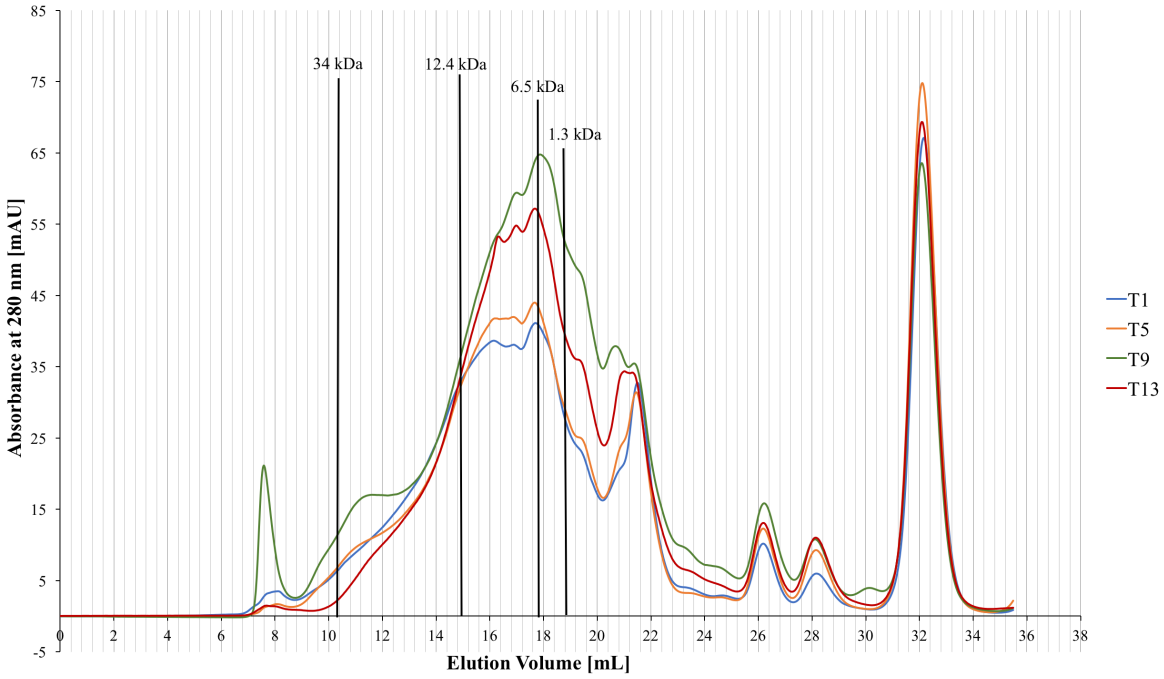

Figure 4.3: Gel filtration chromatogram showing the molecular weight distributions of crude CPH samples T1, T5, T9, and T13 compared to the peaks of known standards.

outside the column range could be either peptides, proteins or amino acids. The peaks detected at elution volumes of 26, 28, and 32 mL could be small water-soluble components or degradation products, causing disturbances in the detection of peptides (Five, 2013).

The molecular weight distributions of the HEADS UP pilot hydrolysates were determined by SINTEF using HPLC and a Superdex 10/300 column (Remme et al., 2018). By SINTEF, the majority (>98%) of the peptides, in all samples, were determined to be smaller than 10 kDa. From figure 4.3 it appears that the hydrolysates may contain a substantial portion of peptides that were larger than 10 kDa. Nevertheless, based on the broad distribution of small peptides (<12 kDa) observed, the hydrolysates may exhibit desirable bioactivities. Furthermore, the high distribution of small peptides suggest efficient hydrolysis of the raw material.

In figure 4.4, size distributions of both the retentate (>4 kDa) and permeate (<4 kDa) of samples T1, T5, T9, and T13 are shown. The majority of the peptides in the permeates were observed to be smaller than 6.5 kDa, as seen by the closely eluting peptides in the figure. A peak at approximately six milliliters was observed for the T1 <4 kDa fraction. Peptides larger than 4 kDa appeared to be present in the permeates. The gel filtration of the retentates appeared to contain a multitude of closely eluting peptides in the 6.5-34 kDa range of high intensity compared to the permeates. The higher intensity of the peaks in the retentate was probably a result of the difference in protein concentration, as found from Lowry-analysis (Appendix B, table B.1).

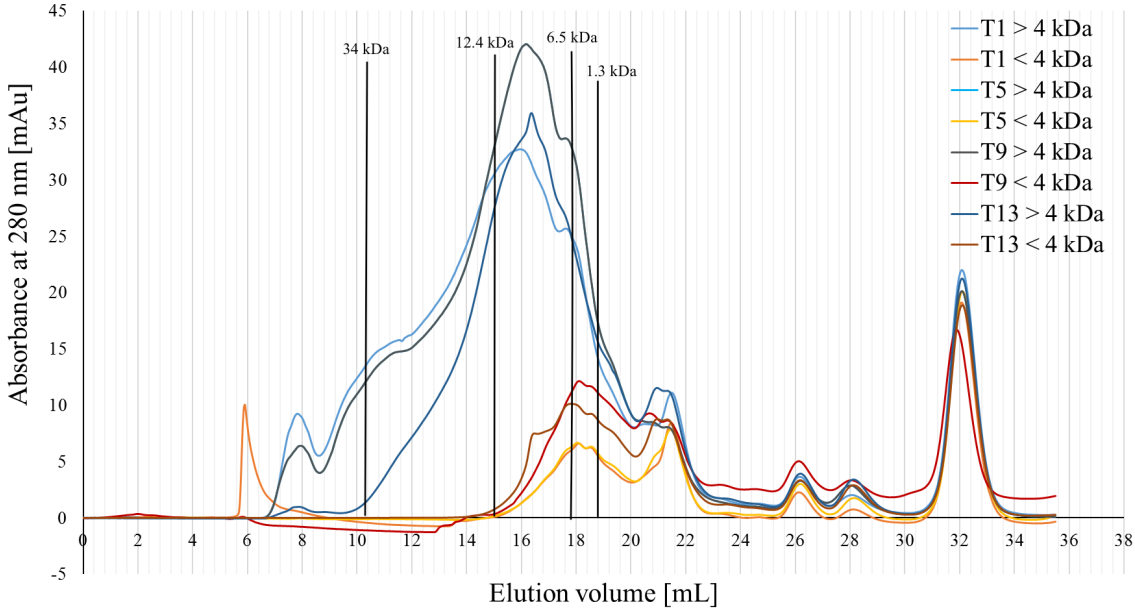

Figure 4.4: Gel filtration chromatogram showing the molecular weight distributions of >4 kDa and <4 kDa CPH fractions of samples T1, T5, T9, and T13 compared to the peaks of known standards.

During UF a "dead-volume" of 50 mL was left in the feed tank at the end of filtration. The hydrolysates were therefore not entirely separated, as the dead volume constituted approximately 1/3 of the initial volume poured into the feed tank. Sample T1 appeared to be insufficiently fractioned compared to the other samples as seen by the peak at an elution volume of *ca* 6 mL. A study of the impact of UF on FPH bioactivities demonstrated that UF membranes did not perform sharp separations (Picot et al., 2010). In particular, the study found that the retentate (>4 kDa) contained a large number of peptides smaller than the MWCO and that referring to the fraction as >4 kDa may be misleading. Figure 4.4 illustrates that, although the peptide profiles were different, the two fractions were incompletely separated by the membrane. The degree of separation following UF was not quantified and is discussed based on the qualitative observations from gel filtration chromatographs.

4.5 Antioxidant activity of CPH and UF fractions

The antioxidant assays were each performed eight times and results were analysed by one-way ANOVA. The data, reported in figures, are given as mean values ± SD. Statistical significance between mean values was set at $p < 0.05$.

4.5.1 ABTS radical scavenging activity

The ABTS is a stable free radical that may accept an electron from a bioactive compound. The ABTS radical scavenging activities, calculated as equivalent concentrations of PG

per grams of soluble protein are given in figure 4.5. An example of how the results were calculated can be found in Appendix E.1. The radical scavenging activity was found to be higher in the original CPH than in the UF fractions for all four samples, although the difference was not statistically significant (p >0.05). The lower radical scavenging activity of UF fractions may be a result of a loss of synergistic effect following fractionation. The results suggest that the hydrolysates contained electron donors that could potentially react with free radicals to convert the radicals to stable end products, thereby interfering with the radical chain reaction.

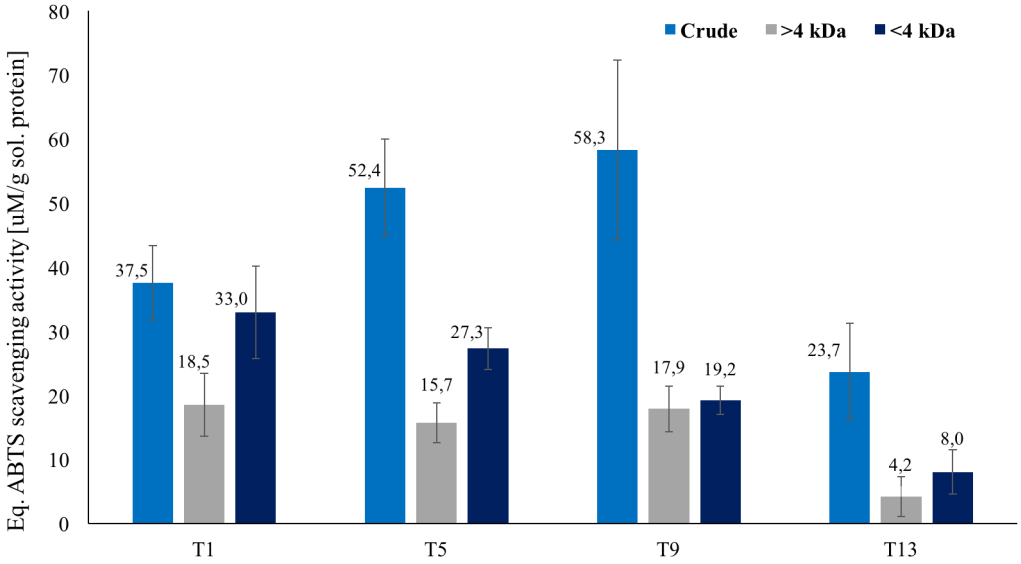

Figure 4.5: ABTS radical scavenging activity calculated as equivalents of reference compound PG of crude CPH, >4 kDa, and <4 kDa fractions. n=8, $\pm SD$.

In contrast to current results, previous studies of the antioxidant activity of cod protein hydrolysates have demonstrated increased radical scavenging activity following UF fractionation (Picot et al., 2010; Farvin et al., 2014; Girgih et al., 2015a). In the mentioned studies, the DPPH assay was used, in which the radical scavenging potential of a substance is measured. Neither the ABTS nor the DPPH assay gives any information about the structure-activity relationship of the antioxidant activity. Hence, the discrepancy of the observations cannot be explained by the ABTS results alone. The calculations of ABTS and FC antioxidant activities are based on the amount of soluble protein measured in CPH and UF fractions by the Lowry method. The concentration of soluble proteins measured are assumed to be accurate. In reality the protein concentrations may differ, in which case the ABTS radical scavenging activity may be higher or lower than what was calculated.

The ABTS antioxidant activity of sample T13 was significantly lower (p <0.05) than in the other samples for all fractions. The low activity of sample T13 was also observed in the experiments conducted during the Specialization project (Monslaup, 2017). During hydrolysis of T13, citric acid was added to the sample. The pH of T13 in water at a con-

centration of 10 mg/mL was low at 4.74, compared to 6.75, 6.73, and 5.94 for T1, T5, and T9, respectively (table A.1). In a study of tea extracts, the ABTS radical scavenging was found to increase with increasing media pH (Muzolf et al., 2008). Also, it has been suggested that the ABTS assay strongly depends on the nature of the compound being studied (Felix et al., 2017). When the pH is below the isoelectric point of a compound, it will be positively charged. If the peptides were positively charged, the ABTS radicals may not be as efficiently reduced, or reaction rates may be slower. The incubation period before end-point determination of ABTS radical scavenging activity was 6 minutes. When pH is lower, a more extended incubation period may be required (Zheng et al., 2016).

A challenge of evaluating the radical scavenging activity of the hydrolysates based on the ABTS assay is that methods are not standardized, nor are the manner in which results are presented. An array of compounds may be used as standards, making it difficult to compare with results reported in the literature. Often limited information as to how the ABTS radical scavenging activity is calculated is given. The ABTS radical scavenging activity of the samples presented in figure 4.5 should only be treated as an indication of antioxidant activity and as a useful way of comparing the activity of the crude CPH with UF fractions.

4.5.2 Folin-Ciocalteu reducing capacity

The total phenolic content (TPC) was estimated by measuring the FC reagent reducing capacity. The presence of phenolic compounds is an indication of antioxidant activity as the phenolic ring can act as a direct radical scavenger by donating hydrogen. The activities, measured as PG equivalents of CPH and >4 kDa and <4 kDa fractions, are given in figure 4.6. An example of how the reducing capacity was calculated is found in Appendix E.2.

The FC antioxidant activity was highest in the <4 kDa fractions (2.34-2.91 mmol PG/g protein). The FC antioxidant activity ranged from 1.47-1.69 mmol PG/g protein for the crude CPH samples, and 0.8-1.05 mmol PG/g protein in the >4 kDa fraction. For samples T1, T5, and T13, the FC reducing capacity of the <4 kDa fraction was significantly larger than the >4 kDa fraction (p <0.05). No significant difference was found between the >4 kDa and crude samples. Results suggest that shorter peptides were responsible for the FC reducing capacity and that the <4 kDa fraction had a higher TPC. In a recent study of UF fractions obtained from *Fucus spiralis* (algae) hydrolysates, the >3 kDa fraction had the highest TPC, or FC reducing capacity (Paiva et al., 2017). It was suggested that fractions of large molecular weights that were high in protein had higher TPC. However, it should be mentioned that the amino acid compositions of the *Fucus spiralis* hydrolysate fractions were dissimilar to CPH. The <3 kDa *Fucus spiralis* fractions contained high amounts of hydrophobic antioxidants (80-90%) and the FC reagent is unable to measure lipophilic antioxidants (Huang et al., 2005).

The measured FC reducing capacity between samples T1, T5, T9, and T13 was similar for each fraction, with none of the differences being significant, suggesting the phenolic concentration in the all samples are similar. Furthermore, process parameters such as the degree of hydrolysis, the raw-material-water ratio, use of stickwater (T9), and addition of

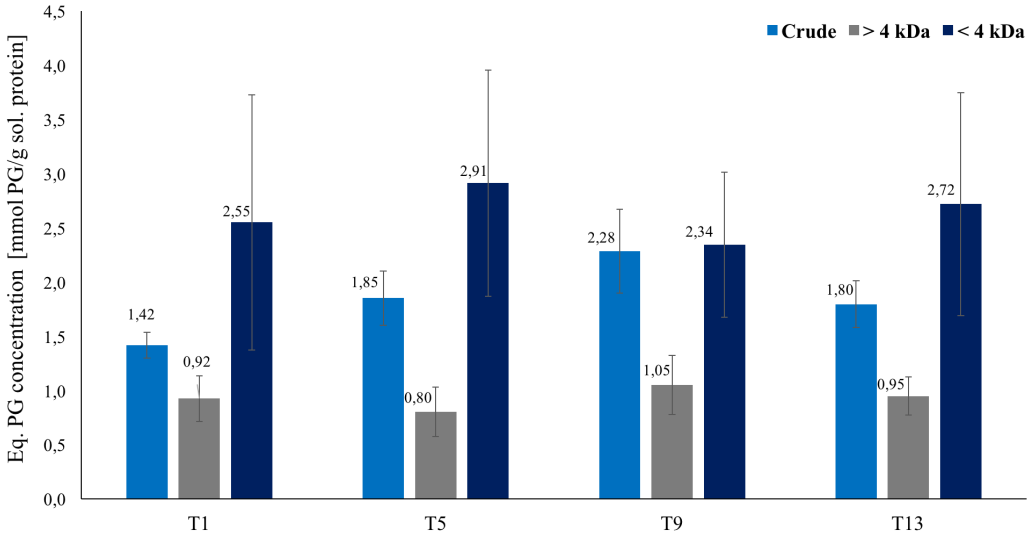

Figure 4.6: FC reducing capacity given as PG equivalents of hydrolyates T1, T5, T9, T13 and their <4 kDa and >4 kDa fractions. Values are expressed as the mean $\pm SD$, n=8.

citric acid (T13) had little effect on the measured FC reducing capacity.

4.5.3 Iron (Fe^{2+}) chelating activity

Transition metals, such as iron, can catalyze the generation of ROS, promoting oxidative damage to cells and in foods. Hence, compounds that can chelate metal ions can reduce the effect of ROS-mediated oxidation. The iron chelating activities of crude hydrolysates and >4 kDa and <4 kDa fractions are shown in figure 4.7. An example of how the chelating activity was measured can be found in Appendix E.3. All samples and fractions showed strong Fe^{2+} chelating activity, indicating that the peptides in the hydrolysates may contribute to the inhibition of lipid oxidation. The activity ranged from 77-90% for the crude samples, 85-94% for the >4 kDa fraction, and 83-94% for the <4 kDa fraction. No significant difference (p >0.05) in iron chelating activity was measured between samples or fractions. Results suggested that the molecular size of the peptides had little influence on the ability of the samples to chelate iron.

From other studies of iron chelating activity in FPH, the chelating activities were expected to be high. In a study of the antioxidant activity in cod head hydrolysates, the iron chelating activity was found to be over 80% in both <3 kDa, 3-5 kDa, and >5 kDa hydrolysate fractions (Farvin et al., 2014). In this study, no significant difference was found between the 3-5 kDa fractions and the >5 kDa fraction. At low concentrations (<1 mg/mL) the chelating activity of the <3 kDa fraction was significantly higher than the other fractions (Farvin et al., 2014). In the present study, the effect of concentration on chelating activity was not investigated. Metal chelating activity has been found to be directly related to peptide concentration (Aluko, 2015). As the concentrations of the <4 kDa fractions

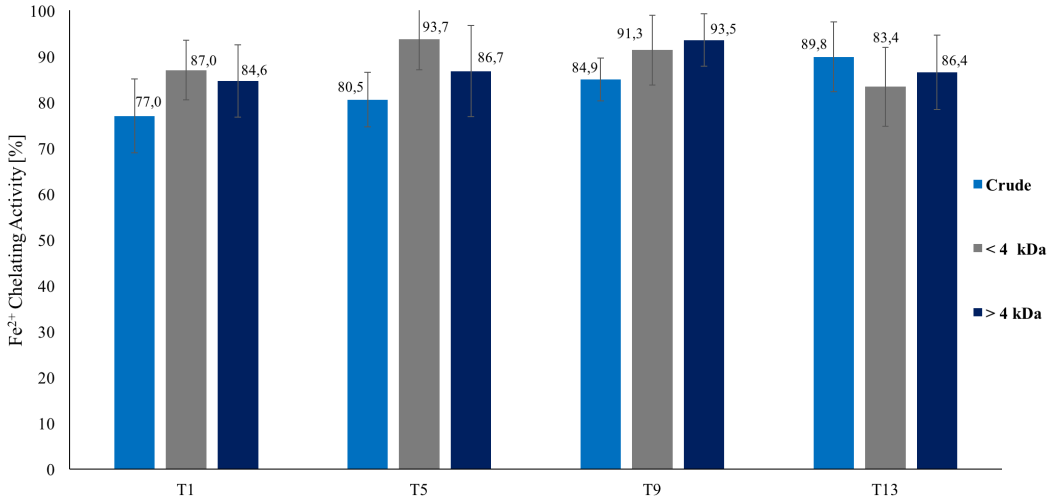

Figure 4.7: Fe^{2+} chelating activity (%) of hydrolysates T1, T5, T9, T13 and their <4 kDa and >4 kDa fractions. Values are expressed as the mean $\pm SD$, n=8.

were lower than the other fractions, smaller peptides may have been more potent metal chelators. In contrast, other studies of FPH's have shown that unfractionated hydrolysates are stronger metal chelators than fractionated FPH, which may be a result of a synergistic effect of different peptides on inhibition of metal catalysed lipid oxidation (Girgih et al., 2015a, 2013). In a study of the bioactivities of defatted salmon backbone hydrolysates, it was found that the iron chelating activity decreased with increasing time of hydrolysis, indicating that larger peptides were better iron chelators than smaller peptides (Šližytė et al., 2016). It suffices to say, factors other than the molecular size of peptides, such as amino acid composition or the presence of specific peptide sequences may have had a greater influence on the metal chelating activity of the hydrolysates.

4.6 Reduction of OUR in liposome system

The antioxidant activity of water-soluble CPH samples in an iron(II)-induced liposome system was investigated. The antioxidant activity was measured as the ability of the protein to inhibit lipid oxidation by reducing the pro-oxidative effect of Fe^{2+}. At a concentration of 25 mg CPH/mL, the oxygen uptake rate (OUR) reduction relative to that of water was calculated. Casein, a protein known to absorb iron, was used as a reference compound. In figure 4.8, the concentration of dissolved oxygen as a function of time is shown. The background oxygen uptake rate was similar in the different cells. After injection of Fe^{2+} into the system an initial drop in oxygen concentration was observed for all samples. The following, linear, oxygen uptake rate was lower when hydrolysate was added to the liposomes compared to water.

The relative oxygen uptake inhibition rate of CPH compared to water is shown in figure

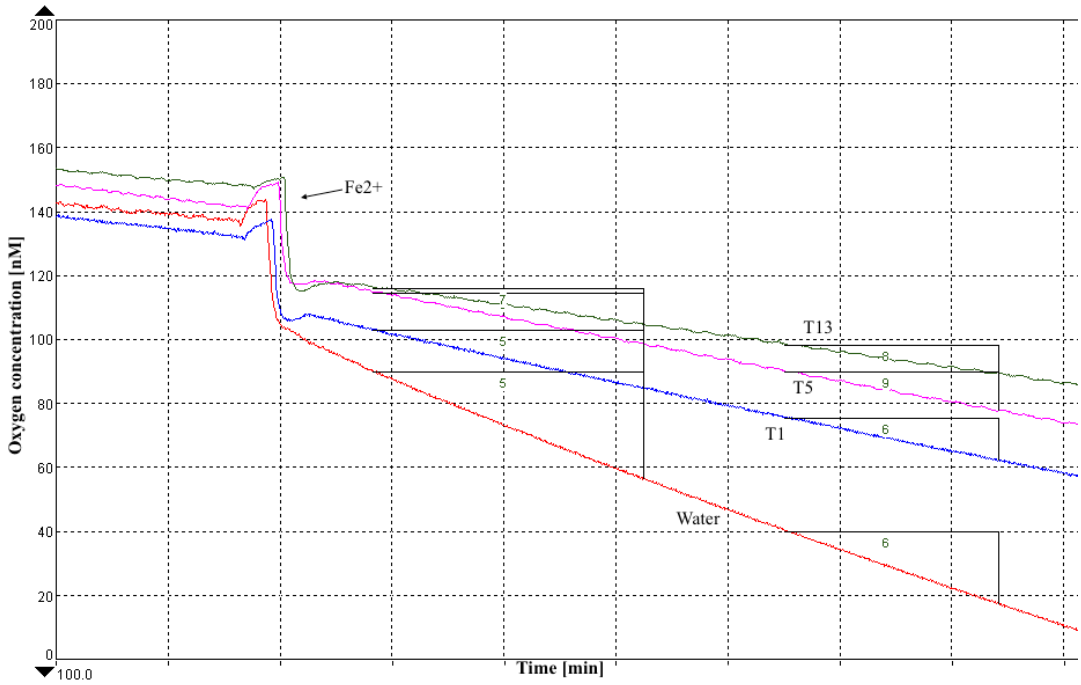

Figure 4.8: Dissolved oxygen as a function of time measured in a closed system of liposomes induced with Fe^{2+}, with cells containing water or CPH solution.

4.9. The raw data used to calculate the OUR reduction activity is found in Appendix F. The addition of CPH reduced the iron-mediated oxygen uptake rate by 25-60%, compared to 78% in casein at the same concentration. The OUR reduction activity indicated the ability of the hydrolysates to inhibit oxidation in a liposome system. In a study of cod backbone hydrolysates, addition of hydrolysate reduced the OUR induced by Fe^{3+} by 60-80% (Šližytė et al., 2009). The antioxidant activity may be a result of the ability of the peptides to chelate iron or scavenge specific free radicals (Šližytė et al., 2009).

Sample T9 showed relatively poor OUR inhibition at 25%, compared to 60% in T13. The inhibition of lipid oxidation is known to be concentration-dependent (Kristinová et al., 2009; Farvin and Jacobsen, 2013). Differences in concentration may partially explain the inhibition of iron mediated oxidation by T9. As observed (figure A.3), and measured from the Lowry method (table B.1), sample T9 had a low concentration of soluble proteins. The low concentration might have influenced the results. Casein dissolved entirely in water, while the hydrolysates were yellow and cloudy with precipitates forming at this concentration. Interestingly, a higher OUR reduction was measured in sample T5 (49.3%) than T1 (44.6 %), despite the lower concentration of water-soluble protein (8.3 vs. 10.67 mg/mL). Other explanations of the difference in OUR reduction activity may be differences in salt concentration of samples. Salts and minerals may have a pro-oxidant effects due to an ability to increase the activity of iron (Mozuraityte et al., 2006a). Salts and minerals from stickwater may have been present in sample T9.

The high OUR reduction activity of proteins in T13 may be attributed to the lower pH of

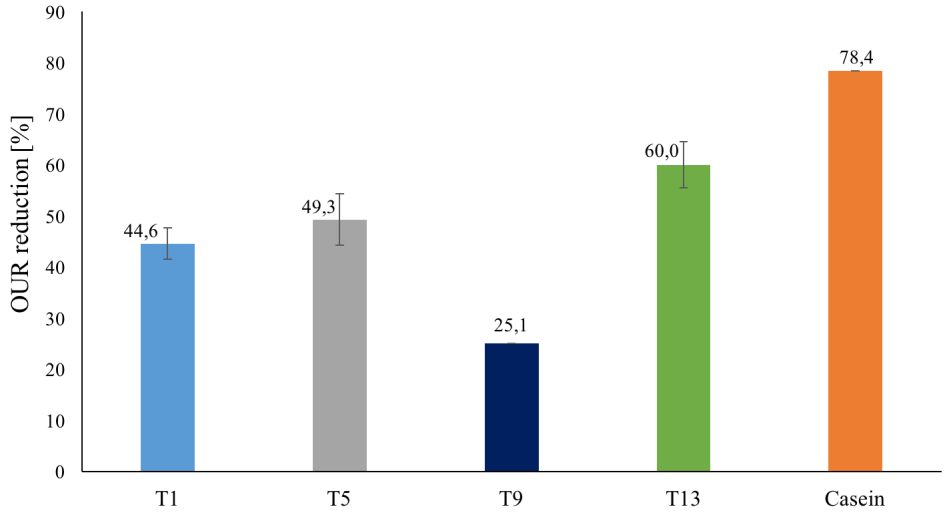

Figure 4.9: The reduction of oxygen uptake rate (OUR) by CPH and casein following the addition of iron to liposome system.

the sample. The pH will influence the oxygen uptake rate (Mozuraityte et al., 2006b). In a study of the pro-oxidant activity of cod phospholipids in liposomes, a maximum oxygen consumption rate was observed between pH 4 and 5 (Mozuraityte et al., 2006b). The pH of the samples differed (table A.1) and the pH in the liposomes should therefore be monitored. In addition, buffer could be used instead of water with adjusted pH.

4.7 Principal component analysis

PCA can be used to extract trends in data sets that contain a large number of variables. The analysis enables the comparison of all data simultaneously, highlighting any associations between samples and experimental methods (Oliveira et al., 2015). PCA was applied twice; once for the data obtained for the four original CPH samples and once for the data obtained for CPH samples and the <4 kDa fractions. Fellow master student Margrethe Fossheim Ohnstad studied the ACE-inhibition activity in the same hydrolysate samples and fractions. Results can be found in figure G.1 in Appendix G. The ACE-inhibition was calculated as half maximal inhibition concentration (IC_{50}). The IC_{50} of crude samples and permeates were included in the principal component analysis to investigate possible correlations to antioxidant activity or amino acid compositions.

4.7.1 CPH

In the PCA for all CPH data, principal component 1 (PCA1) and principal component 2 (PCA2) explained 57 and 27% of the total variance. The loadings plot, illustrated in figure 4.10, shows positive and negative correlations between variables. The variables included

antioxidant capacity, ACE-inhibiting activity, the degree of hydrolysis, water used for hydrolysis, total and free amino acid content, and total amino acid fractions. The further away two variables are diagonally, the more negatively correlated the variables are. Similarly, the closer two variables are in the plot, the more positively correlated they are. The position of the variables in each quadrant is related to the positions of the samples in the score plot (figure 4.11).

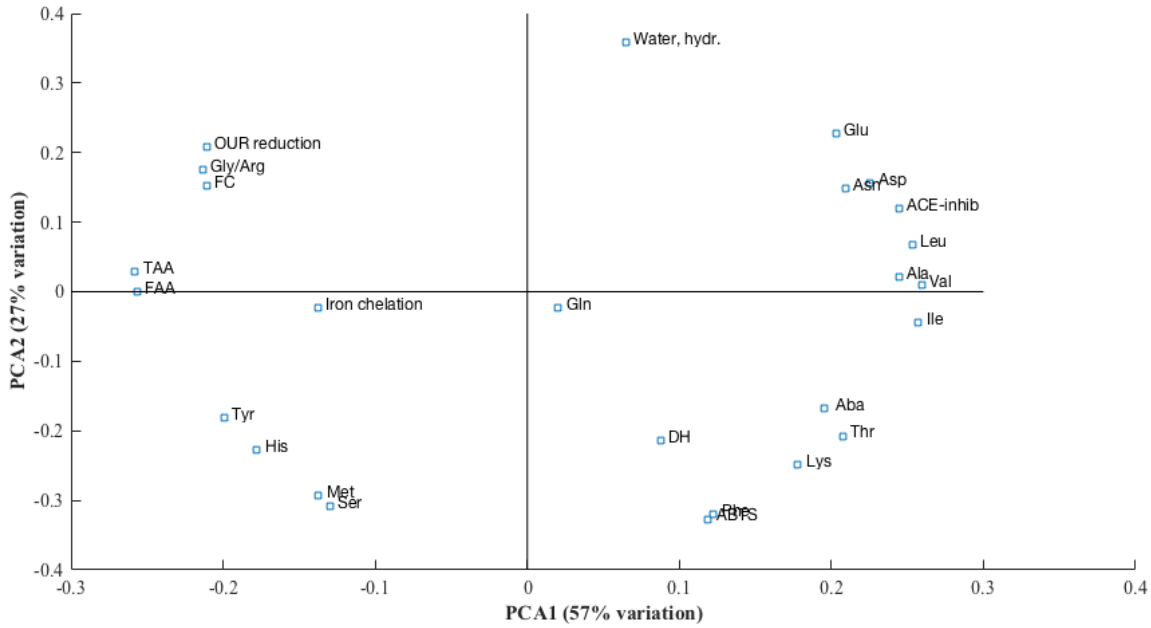

Figure 4.10: Loadings plot showing correlations found from the PCA model applied for all results measured for hydrolysate samples T1, T5, T9, and T13.

PCA1 showed a strong positive correlation between the total and free amino acid content, indicating that when the protein content was high the amount of FAA was also high. ACE-inhibiting activity was positively correlated with the hydrophobic amino acids Leu, Ala, Val, and Ile. This correlation was coherent with other studies that have reported the same hydrophobic amino acids to be involved in the ACE inhibitory activity of fish protein hydrolysates (Halim et al., 2016; Balti et al., 2010). Moreover, Lys and Arg have been found to contribute to ACE inhibitory activity (Amado et al., 2014). No positive correlation between ACE-inhibition and Lys and Arg was measured. Arg was determined together with Gly, and therefore the relationship between Arg and ACE-inhibition was unknown.

ABTS radical scavenging activity was positively correlated with Phe content. The correlation with Phe is possibly explained by the sensitivity of the ABTS radical to phenolic compounds (Felix et al., 2017). The ABTS radical scavenging activity was somewhat correlated with the degree of hydrolysis, indicating that the degree of hydrolysis had some influence on the radical scavenging activity of the hydrolysates. The same observation was made by Šližytė et al. (2009), in which the *in vitro* radical scavenging activity of FPH from cod increased slightly with increasing DH.

The oxygen uptake reduction rate and the FC antioxidant activity were positively correlated, suggesting that the mechanism of OUR reduction was related to the FC reducing capacity. The iron chelating activity was found near the centre of the plot indicating that PCA1 and PCA2 did not explain the variation in this variable. Fe^{2+} chelating activity was found to be similar between the four samples. The ability of the hydrolysates to reduce Fe^{2+}-induced lipid oxidation and the iron chelating activity would be expected to show a positive correlation as these mechanisms are related. However, no such correlation was measured, which may be attributed to the lack of variation in iron chelating activity measured between samples.

ABTS radical scavenging activity was negatively correlated with other measured antioxidant capacities, in particular, oxygen uptake rate (OUR) reduction and FC reducing capacity. Different antioxidant mechanisms were measured in the *in vitro* assays. Results suggested that these mechanisms may be unrelated. Contrary to the current findings, the ABTS and FC antioxidant activity has previously been found to be positively correlated. The ABTS and FC reagents both have a high affinity for phenolic compounds (Everette et al., 2010; Abramovič et al., 2017). This contradiction may require further investigation.

In a study of Sea Cucumber hydrolysates, a strong positive correlation was found between *in vitro* antioxidant activity and ACE-inhibition (Ghanbari et al., 2015). No strong positive or negative correlation was measured between antioxidant activity and ACE-inhibition. The raw-material-water ratio did not appear to have any positive or negative effect on antioxidant or ACE-inhibiting capacity, indicating that this process parameter had little impact on the studied bioactivities.

In the score plot, as seen in figure 4.11, the location of the different samples is associated with the position of variables in the loadings plot. The four samples were spread far apart from both principal component 1 and 2. Sample T1 was somewhat positively correlated with ACE-inhibition, which may have been related to large fractions of amino acids Leu, Ala, Lys, and Glu. Sample T13 correlated positively with FC and OUR reduction, while sample T9 was somewhat positively correlated with ABTS radical scavenging activity. Sample T5 correlated strongly with a high fraction of Tyr, His, Met, and Ser. Tyr and His are amino acids associated with antioxidant activity. However, no positive correlation between the two amino acids and antioxidant activity was observed from PCA. It should be noted that PCA should include five or more samples (Oliveira et al., 2015). Information about the associations between samples may be lost when the sample size is small.

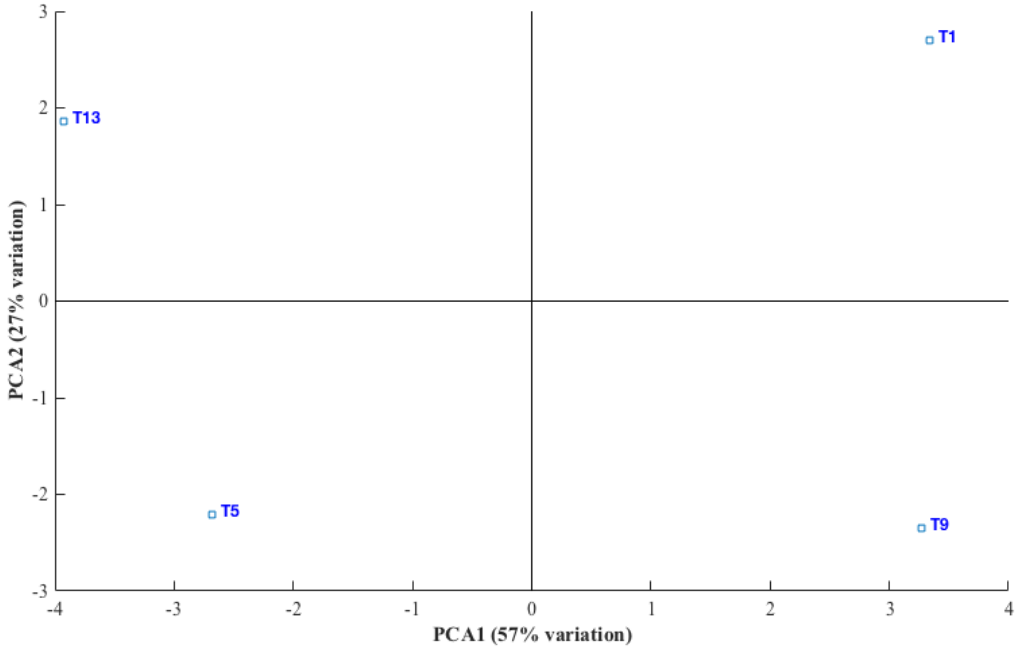

Figure 4.11: Score plot from PCA applied for all results measured for hydrolysate samples T1, T5, T9, and T13.

4.7.2 CPH and <4 kDa fraction

The data obtained for the CPH samples were compared with the data of the <4 kDa fraction to investigate any effect on antioxidant activity following the separation of the hydrolysates by molecular weight. The loadings plot is shown in figure 4.12, while the score plot can be found in figure 4.13. Principal component 1 explained 45% of the variance while principal component 2 explained 26% of the variance. The variables included in the analysis were DH, water added during hydrolysis, the ABTS, FC, and iron chelating antioxidant activity, ACE-inhibition, and FAA content.

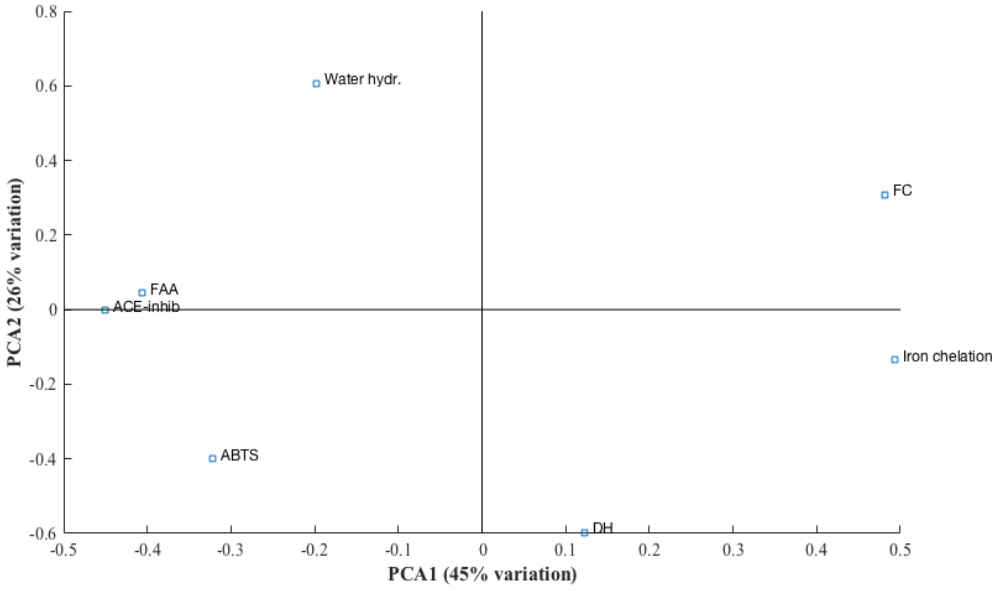

Figure 4.12: Loadings plot of PCA on results measured for hydrolysate and <4 kDa fractions showing measured correlations.

From the loadings plot (figure 4.12), no positive correlation was observed between different antioxidant assays, as was previously observed from figure 4.10. Interestingly, FAA and ACE-inhibition were positively correlated, suggesting that free amino acids may contribute to ACE-inhibition activity. ACE-inhibition was found to correlate positively with the presence of Leu, Ala, and Val (figure 4.10). The CPH samples were found to contain large fractions of free Leu, Ala, and Val (figure 4.2), which may suggest that the mentioned amino acids were involved in the ACE-inhibition activity of the hydrolysates.

From the loadings plot (4.12), a negative correlation between the degree of hydrolysis and the amount of water added during hydrolysis was observed, suggesting that decreasing the amount of water added increased the activity of hydrolysis.

In figure 4.13, the score plot is shown. Permeates (<4 kDa) and CPH samples were not located in the same quadrants of the plot, suggesting that the measured bioactivities of the peptides changed as a result of UF. None of the crude samples were located close to

their <4 kDa fraction in the score plot. Samples T1-P and T13-P were closely located in the score plot, suggesting these samples exhibited similar antioxidant and ACE-inhibiting properties. Sample T5-P correlated positively with iron chelating activity and FC reducing capacity. The score and loadings plots provides evidence to suggest that the <4 kDa fractions exhibited somewhat stronger iron chelating and FC reducing activities than the crude hydrolysates. From one-way ANOVA a significantly higher FC reducing capacity was measured in the <4 kDa fraction compared to the >4 kDa fraction. However, the FC activity of <4 kDa samples was not significantly higher than the crude CPH. The same was found from ANOVA of iron chelating activity, in which no significant differences in iron chelating activity was measured between CPH and its UF fractions. In principal component analysis, the mean data of several variables are put into an algorithm to extract trends and associations in the data set. It should be noted that the positive and negative correlations found are based on the underlying assumption that the data is representative, when the data in reality contains large uncertainties.

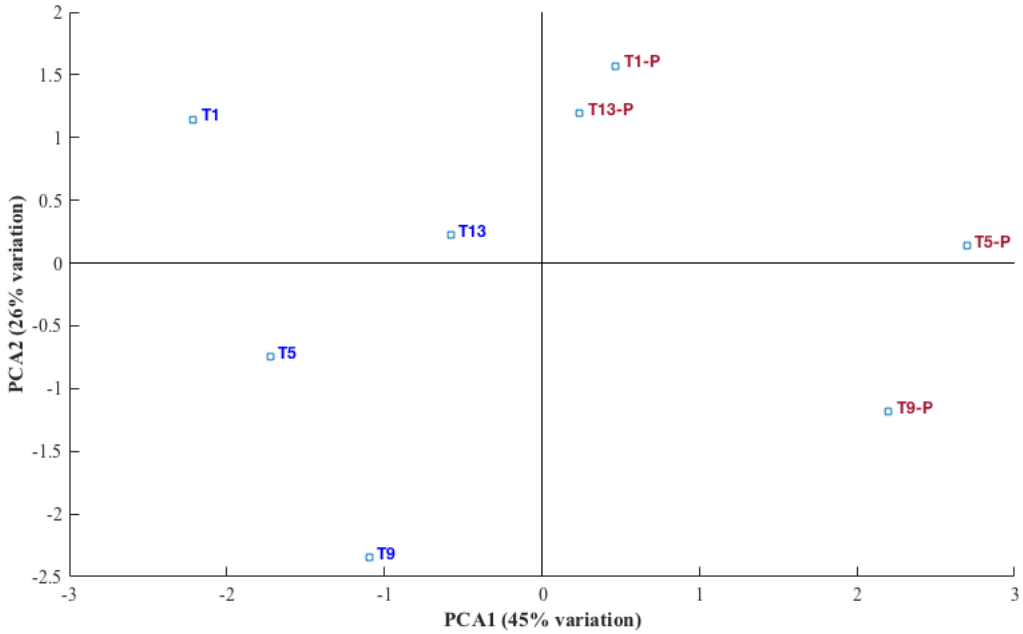

Figure 4.13: Score plot of PCA on results for hydrolysate and <4 kDa fractions (P).

Chapter 5

Conclusions

The relationships between *in vitro* antioxidant activity, molecular weight distributions, and amino acid compositions in hydrolysates derived from cod heads were investigated. Four CPH samples were fractionated by UF using a 4 kDa MWCO. The effect of UF on *in vitro* antioxidant activity was studied and compared to crude samples by statistical analysis. Possible correlations between the measured variables, in addition to process parameters and ACE-inhibition activity, were investigated by PCA.

Overall, the results from the antioxidant assays suggests that CPH and UF fractions exhibited *in vitro* antioxidant properties. In particular, both the CPH and UF fractions exhibited strong iron chelating activity, suggesting that the molecular weight of the peptides was not the determining factor for iron chelating activity. The crude CPH's were found to exhibit the strongest ABTS radical scavenging activity and showed promising effects in the reduction of oxygen uptake rate in a liposome system. The FC antioxidant activity of the <4 kDa fractions were found to be significantly higher than for >4 kDa fractions, suggesting a higher content of phenolic compounds in the <4 kDa fractions. Gel filtration of the permeate and retentate showed that the hydrolysates were not completely separated by molecular weight during UF. The fractionation of hydrolysates based on molecular weights did not significantly change *in vitro* antioxidant activities compared to crude CPH. This suggests that UF fractionation may not be expedient for the purpose of formulating antioxidant foods.

From PCA, the DH was found to be negatively correlated with the amount of water used for hydrolysis, suggesting that decreasing the amount of water added increased the activity of hydrolysis. Except for the FC antioxidant activity and the reduction of oxygen uptake rate in liposomes, the various antioxidant activities in CPH did not correlate, suggesting that the antioxidant mechanisms studied differed. The ACE-inhibition activity was positively correlated with the hydrophobic amino acids, leucine, alalnine, valine, and isoleucine, which was coherent with previous findings. Based on analysis of the amino acid content, the CPH powders were found to be of high nutritional value, as all sam-

ples contained sufficient amounts of essential amino acids. The high protein content in the powders combined with the nutritional value suggests that CPH may be a source of dietary proteins.

5.1 Further work

In order to improve the inaccuracies experienced in the quantification of the protein concentration and consequently the antioxidant activity in the UF fractions, as presented in sections 4.2 and 4.5.1, it would be beneficial to freeze-dry the retentate and permeate. This would enable the analysis of total amino acid content in the fractions, as well as more direct and accurate calculations of the ABTS radical scavenging activity and FC reducing capacity. In this study, the concentration of soluble proteins found in the Lowry method was used to calculate the ABTS and FC antioxidant activity in the CPH and UF fractions. Had the fractions been freeze-dried, these calculations could have been based on the mass of dry powder used in analysis instead of the possibly inaccurate soluble protein concentrations.

As discussed in section 4.2, the water solubility of the samples were observed to be poor at high concentrations (>1 mg/mL). The solubility of CPH powders at different concentrations and pH requires further investigation as poor solubility decrease the potential applications of proteins for human consumption. Moreover, high solubility was set as a product requirement of CPH in the HEADS UP project.

Further studies are required to evaluate the antioxidant activity of CPH in the liposome system. Due to time limitations, UF fractions were not included in the experiments. Moreover, the current results were based on few experiments. The method used to investigate the reduction of OUR in liposomes by hydrolysates could be improved and extended in several ways. Firstly, to study the potency of the antioxidant activity, different hydrolysate concentrations and the effect of pH could be investigated. In addition, other pro-oxidants, such as hemoglobin, could be applied to investigate antioxidant activity further.

The results from the chosen in vitro antioxidant methods points to the applicability of CPH as antioxidants in foods. Further research could focus on characterizing specific antioxidative peptides in the cod head hydrolysates, which could contribute to the understanding of the antioxidant mechanisms. Besides, further studies are required to evaluate the overall antioxidant potential of the hydrolysates in more complex biological systems, including in food systems. *In vivo* studies are necessary to further understand possible health benefits of antioxidants from CPH.

Bibliography

Abramovič, H., Grobin, B., Ulrih, N., Cigić, B., 2017. The methodology applied in dpph, abts and folin-ciocalteau assays has a large influence on the determined antioxidant potential. Acta Chimica Slovenica 64 (2), 491–499.

Adler Nissen, J., 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. Journal of Agricultural and Food Chemistry 27 (6), 1256–1262.

Aluko, R., 2015. 5 - amino acids, peptides, and proteins as antioxidants for food preservation. In: Shahidi, F. (Ed.), Handbook of Antioxidants for Food Preservation. Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 105 – 140.

Amado, I. R., Vázquez, J. A., González, P., Esteban-Fernández, D., Carrera, M., Piñeiro, C., 2014. Identification of the major ace-inhibitory peptides produced by enzymatic hydrolysis of a protein concentrate from cuttlefish wastewater. Marine drugs 12 (3).

Aspmo, S. I., Horn, S. J., Eijsink, V. G. H., 2005. Enzymatic hydrolysis of atlantic cod (gadus morhua l.) viscera. Process Biochemistry 40 (5), 1957 – 1966.

Balti, R., Bougatef, A., El-Hadj Ali, N., Zekri, D., Barkia, A., Nasri, M., 2010. Influence of degree of hydrolysis on functional properties and angiotensin i-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (sepia officinalis) by-products. Journal of the Science of Food and Agriculture 90 (12), 2006–2014.

Berker, K., Olgun, O., Ayca, F., Ozyurt, D., Demirata, B., Apak, R., 2013. Modified folin-ciocalteu antioxidant capacity assay for measuring lipophilic antioxidants. Journal of agricultural and food chemistry 61 (20).

Blackburn, S., 1978. Amino acid determination : methods and techniques, 2nd Edition. Marcel Dekker, New York.

Bligh, E., Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry 37, 911–917.

Brewer, M., 2011. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. Comprehensive Reviews in Food Science and Food Safety 10 (4), 221–247.

Chalamaiah, M., Kumar, B. D., Hemalatha, R., Jyothirmayi, T., 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chemistry 135 (4), 3020 – 3038.

Choe, E., Min, D. B., 2006. Mechanisms and factors for edible oil oxidation. Comprehensive Reviews in Food Science and Food Safety 5 (4), 169–186.

Coultate, T., 2009. Food : the chemistry of its components, 5th Edition. Royal Society of Chemistry, Cambridge.

Damodaran, S., Parkin, K., Fennema, O., 2008. Fennema's food chemistry, 4th Edition. Food science and technology. Taylor Francis, Boca Raton.

Dasgupta, A., Klein, K., 2014. Chapter 1 - introduction to free radicals and the body's antioxidant defense. In: Dasgupta, A., Klein, K. (Eds.), Antioxidants in Food, Vitamins and Supplements. Elsevier, San Diego, pp. 1 – 18.

Davies, A., Fearn, T., 2005. Back to basics: The principles of principal component analysis 16, 20–23.

EU-Commission, 2017. Fishing quotas - managing fisheries. Reviewed 04.04.2018. URL https://ec.europa.eu/fisheries/cfp/fishing_rules/tacs_en

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., Walker, R. B., July 2010. Thorough study of reactivity of various compound classes toward the folin-ciocalteu reagent. Journal of agricultural and food chemistry 58 (14).

FAO/WHO/UNU, 1985. Energy and protein requirements. report of a joint fao/who/unu expert consultation. World Health Organization technical report series 724.

Farvin, K., Andersen, L., Nielsen, H., Jacobsen, C., Jakobsen, G., Johansson, I., Jenssen, F., 2014. Antioxidant activity of cod (gadus morhua) protein hydrolysates: In vitro assays and evaluation in 5 % fish oil-in-water emulsion. Food Chemistry 149, 326–334.

Farvin, K., Andersen, L. L., Otte, J., Nielsen, H. H., Jessen, F., Jacobsen, C., 2016. Antioxidant activity of cod (gadus morhua) protein hydrolysates: Fractionation and characterisation of peptide fractions. Food Chemistry 204, 409–419.

Farvin, K., Jacobsen, C., 2013. Phenolic compounds and antioxidant activities of selected species of seaweeds from danish coast. Food Chemistry 138 (2-3), 1670–1681.

Felix, M., Romero, A., Rustad, T., Guerrero, A., 2017. Rheological properties and antioxidant activity of protein gels-like systems made from crayfish concentrate and hydrolysates. Food and Bioproducts Processing 102, 167–176.

FHF, 2017. Restråstoff: Anvendelser av hoder til humant konsum (heads up), reviewed 28.03.2018.
URL http://www.fhf.no/prosjektdetaljer/?projectNumber=901308

Five, K., 2013. Enzymatic hydrolysis of salmon by-products - effect of process conditions on ace inhibiting activities of fish protein hydrolysates.
URL http://hdl.handle.net/11250/245988

Ghanbari, R., Zarei, M., Ebrahimpour, A., Abdul-Hamid, A., Ismail, A., Saari, N., 2015. Angiotensin-i converting enzyme (ace) inhibitory and anti-oxidant activities of sea cucumber (actinopyga lecanora) hydrolysates. International Journal of Molecular Sciences 16 (12), 28870–28885.
URL http://search.proquest.com/docview/1793003688/

Girgih, A. T., He, R., Hasan, F., Udenigwe, C., Gill, T., Aluko, R. E., 2015a. Evaluation of the in vitro antioxidant properties of a cod (gadus morhua) protein hydrolysate and peptide fractions. Food Chemistry 173, 652–659.

Girgih, A. T., Nwachukwu, I. D., Hasan, F., Fagbemi, T. N., Gill, T., Aluko, R. E., 2015b. Kinetics of the inhibition of renin and angiotensin i-converting enzyme by cod (gadus morhua) protein hydrolysates and their antihypertensive effects in spontaneously hypertensive rats. Food Nutrition Research 59 (1).

Girgih, A. T., Udenigwe, C. C., Hasan, F. M., Gill, T. A., Aluko, R. E., 2013. Antioxidant properties of salmon (salmo salar) protein hydrolysate and peptide fractions isolated by reverse-phase hplc. Food Research International 52 (1), 315–322.

Guaadaoui, A., Benaicha, S., Elmajdoub, N., Bellaoui, M., Hamal, A., 2014. What is a bioactive compound? a combined definition for a preliminary consensus. International Journal of Food Sciences and Nutrition 3 (3), 17–179.

Gálico, D., Nova, C., Guerra, R., Bannach, G., 2015. Thermal and spectroscopic studies of the antioxidant food additive propyl gallate. Food Chemistry 182, 89–94.

Halim, N., Yusof, H., Sarbon, N., 2016. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. Trends in Food Science & Technology 51, 24 – 33.

Huang, D., Ou, B., Prior, R., 2005. The chemistry behind antioxidant capacity assays. Journal Of Agricultural And Food Chemistry 53 (6), 1841–1856.

Ikawa, M., Schaper, T. D., Dollard, C. A., Sasner, J. J., 2003. Utilization of folin-ciocalteu phenol reagent for the detection of certain nitrogen compounds. Journal of agricultural and food chemistry 51 (7).

Intarasirisawat, R., Benjakul, S., Visessanguan, W., Wu, J., 2012. Antioxidative and functional properties of protein hydrolysate from defatted skipjack (katsuwonous pelamis) roe. Food chemistry 135 (4).

Jensen, I., Larsen, R., Rustad, T., Eilertsen, K., 2013. Nutritional content and bioactive properties of wild and farmed cod (gadus morhua) subjected to food preparation. Journal of Food Composition and Analysis 31 (2), 212–216.

Kates, M., 1986. Techniques of lipidology : isolation, analysis and identification of lipids, 2nd Edition. Vol. 3:2 of Laboratory techniques in biochemistry and molecular biology. Elsevier, Amsterdam.

Kim, S., Wijesekara, I., 2010. Development and biological activities of marine-derived bioactive peptides: A review. Journal of Functional Foods 2 (1), 1–9.

Kinsella, J. E., 1981. Functional properties of proteins: Possible relationships between structure and function in foams. Food Chemistry 7 (4), 273–288.

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

Klompong, V., Benjakul, S., Kentachote, D., Hayes, K., Shahidi, F., 2007b. Comparative study on antioxidant activity of yellow stripe trevally protein hydrolysate produced from alcalase and flavourzyme. International Journal of Food Science and Technology 43, 1019–1026.

Kristinová, V., 2014. Oxidation of marine lipids in liposomes and emulsions mediated by iron and methemoglobin.
URL http://hdl.handle.net/11250/246097

Kristinová, V., Mozuraityte, R., Storrø, I., Rustad, T., 2009. Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. Journal of agricultural and food chemistry 57 (21).

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

Logan, A., Nienaber, U., Pan, X. S., 2013. 1.1 introduction. In: Lipid Oxidation - Challenges in Food Systems. AOCS Press.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., 1951. Protein measurement with the folin phenol reagent. The Journal of biological chemistry 193 (1).

Mazorra-Manzano, M., Ramírez-Suárez, J., Moreno-Hernández, J., Pacheco-Aguilar, R., 2018. 17 - seafood proteins. In: Yada, R. Y. (Ed.), Proteins in Food Processing (Second Edition), second edition Edition. Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 445 – 475.

Meldstad, F., 2015. Hydrolysis of marine cod (gadus morhua) head - utilization of rest raw material from cod for production of ingredients for human consumption.
URL http://hdl.handle.net/11250/2351655

Mendis, E., Rajapakse, N., Kim, S., 2005. Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. Journal of agricultural and food chemistry 53 (3), 581–587.

Minotti, G., Aust, S., 1992. Redox cycling of iron and lipid peroxidation. Lipids 27 (3), 219–226.

Monslaup, M., 2017. Hydrolysates from cod (gadus morhua) heads: In vitro antioxidant activity and structural components. Unpublished.

Mozuraityte, R., Rustad, T., StorrâŠ~, I., November 2006a. Oxidation of cod phospholipids in liposomes: Effects of salts, ph and zeta potential. European Journal of Lipid Science and Technology 108 (11), 944–950.

Mozuraityte, R., Rustad, T., Storrø¸, I., 2006b. Pro-oxidant activity of fe 2+ in oxidation of cod phospholipids in liposomes. European Journal of Lipid Science and Technology 108 (3), 218–226.

Muzolf, M., Szymusiak, H., Gliszczynska-Swiglo, A., Rietjens, I., Tyrakowska, B., 2008. ph-dependent radical scavenging capacity of green tea catechins. Journal of Agricultural and Food Chemistry 56 (3), 816–823.

Mæhre, H. K., Dalheim, L., Edvinsen, G. K., Elvevoll, E. O., Jensen, I.-J., January 2018. Protein determination-method matters. Foods (Basel, Switzerland) 7 (1).

Najafian, L., Babji, A., 2012. A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. Peptides 33 (1), 178 – 185.

Najafian, L., Jafarzade, M., Said, M., Babji, A. S., 2013. Biochemical properties and antioxidant activity of myofibrillar protein hydrolysates obtained from patin (p angasius sutchi. International Journal of Food Science & Technology 48 (10), 2014–2022.

Nenadis, N., Lazaridou, O., Tsimidou, M., 2007. Use of reference compounds in antioxidant activity assessment. Journal of agricultural and food chemistry 55 (14).

Nenadis, N., Wang, L., Tsimidou, M., Zhang, H., 2004. Estimation of scavenging activity of phenolic compounds using the abts•+ assay. Journal of Agricultural and Food Chemistry 52 (15), 4669–4674.

Oliveira, C. C. D., Calado, V. M. D. A., Ares, G., Granato, D., 2015. Statistical approaches to assess the association between phenolic compounds and the in vitro antioxidant activity of camellia sinensis and ilex paraguariensis teas. Critical Reviews in Food Science and Nutrition 55 (10), 1456–1473, pMID: 24918265.

Osnes, K., Mohr, V., 1985. Peptide hydrolases of antarctic krill, euphausia superba. Comparative biochemistry and physiology. Comparative biochemistry 82 (4), 599–606.

Paiva, L., Lima, E., Neto, A., Baptista, J., 2017. Angiotensin i-converting enzyme (ace) inhibitory activity, antioxidant properties, phenolic content and amino acid profiles of fucus spiralis l. protein hydrolysate fractions. Marine Drugs 15 (10).

Picot, L., Ravallec, R., Martine, F.-P., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., Guérard, F., Chabeaud, A., Legal, Y., Alvarez, O., Bergé, J.-P., Piot, J.-M., Batista, I., Pires, C., Thorkelsson, G., Delannoy, C., Jakobsen, G., Johanssonm, I., Bourseau, P., 2010. Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. Journal of the Science of Food and Agriculture 90 (11), 1819–1826.

Qian, Z., Jung, W., Kim, S., 2008. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, rana catesbeiana shaw. Bioresource Technology 99 (6), 1690–1698.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved abts radical cation decolorization assay. Free Radical Biology and Medicine 26 (9), 1231–1237.

Remme, J. F., 2017. Faktaark: Heads up kvalitetsproteiner fra torskehoder. Reviewed 24.03.2018.
URL http://www.fhf.no/prosjektdetaljer/?projectNumber=901308

Remme, J. F., Carvajal, A. K., Indergård, E., Toldnes, B., Slizyte, R., Grimsmo, L., Austens, A., 2018. Sluttrapport - heads up i. Tech. rep., SINTEF Fiskeri og Havbruk AS.

Richardsen, R., Nystøyl, R., Strandheim, G., Marthinussen, A., 2017. Analyse marint restråstoff, 2016. Tech. rep., SINTEF Fiskeri og Havbruk AS, Kontali Analyse AS.

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

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

Sarmadi, B., Ismail, A., 2010. Antioxidative peptides from food proteins: A review. Peptides 31 (10), 1949–1956.

Shahidi, F., 2015. Handbook of Antioxidants for Food Preservation. Elsevier Inc.

Singleton, V. L., Orthofer, R., Lamuela-Raventos, R., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol 299, 152–178.

SINTEF, F., Aquaculture, 2015. New value added products from rest raw material. protein hydrolysates and lipids.

Stoknes, I., Økland, H., 2002. Potensiale for ilandføring og utnyttelse av torskehoder. Tech. rep., Møreforskining.

Stookey, L., 1970. Ferrozine—a new spectrophotometric reagent for iron. Analytical Chemistry 42 (7), 779–781.

Tan, J., Lim, Y., 2015. Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts. Food Chemistry 172, 814–822.

Taylor, W. H., 1957. Formol titration: an evaluation of its various modifications. The Analyst 82 (976), 488–498.

Turner, R., McLean, C. H., Silvers, K. M., 2006. Are the health benefits of fish oils limited by products of oxidation? Nutrition Research Reviews 19 (1), 53–62.

Viollier, E., Inglett, P., Hunter, K., A.N., R., Van Cappellen, P., 2000. The ferrozine method revisited: Fe(ii)/fe(iii) determination in natural waters. Applied Geochemistry 15 (6), 785 – 790.

Zayas, J. F., 1997. Solubility of Proteins. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 6–75.

Zhang, X., Xiong, Y. L., Chen, J., Zhou, L., 2013. Synergistic inhibition of lipid oxidation by pea protein hydrolysate coupled with licorice extract in a liposomal model system. Journal of Agricultural and Food Chemistry 61 (35), 8452–8461.

Zheng, L., Zhao, M., Xiao, C., Zhao, Q., Su, G., 2016. Practical problems when using abts assay to assess the radical-scavenging activity of peptides: Importance of controlling reaction ph and time. Food Chemistry 192, 288 – 294.

Zhong, Y., 2015. 12 - methods for the assessment of antioxidant activity in foods. In: Shahidi, F. (Ed.), Handbook of Antioxidants for Food Preservation. Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 287 – 333.

Šližytė, R., Daukšas, E., Falch, E., Storrø, I., Rustad, T., 2005a. Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (gadus morhua) by-products. Process Biochemistry 40 (3), 1415–1424.

Šližytė, R., Mozuraitytė, R. Martínez-Alvarez, O., Falch, E., Fouchereau-Peron, M., Rustad, T., 2009. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (gadus morhua) backbones. Process Biochemistry 44 (6), 668 – 677.

Šližytė, R., Nguyen, J., Rustad, T., Storro, I., 2004a. Hydrolysis of cod (gadus morhua) by-products: influence of initial heat inactivation, concentration and separation conditions. Journal of aquatic food product technology 13 (2), 31–48.

Šližytė, R., Rommi, K., Mozuraityte, R., Eck, P., Five, K., Rustad, T., 2016. Bioactivities of fish protein hydrolysates from defatted salmon backbones. Biotechnology Reports 11, 99–109.

Šližytė, R., Rustad, T., Storrø, I., 2005b. Enzymatic hydrolysis of cod (gadus morhua) by-products: Optimization of yield and properties of lipid and protein fractions. Process Biochemistry 40 (12), 3680 – 3692.

Šližytė, R., Van Nguyen, J., Rustad, T., Storro, I., 2004b. Hydrolysis of cod (gadus morhua) by-products: Influence of initial heat inactivation, concentration and separation conditions. Journal of Aquatic Food Product Technology 13 (2), 31–48.

Appendix A

Raw materials

Physical descriptions of CPH samples are listed bellow. The dry CPH samples are shown in figures A.1-A.2. The samples dissolved in water are shown in figure A.3. The pH of the samples at a concentration of 10 mg CPG/mL are listed in table A.1.

- **T1**: Fine, even powder, little or no lumps, light sandy-brown colour.
- **T5**: Fine, even powder, some lumps, light sandy-brown colour.
- **T9**: Coarse and grainy, lumpy, brown-yellow colour. Difficult to dissolve in water.
- **T13**: Very fine, even powder, no lumps, lightest colour of the samples. Dissolved well in water.

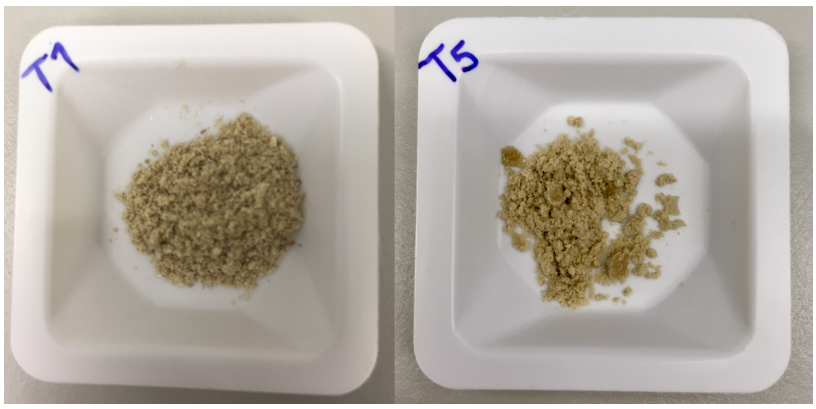

Figure A.1: Sample of T1 from hydrolysis 04.02.2017, sample of T5 from hydrolysis 11.08.2017

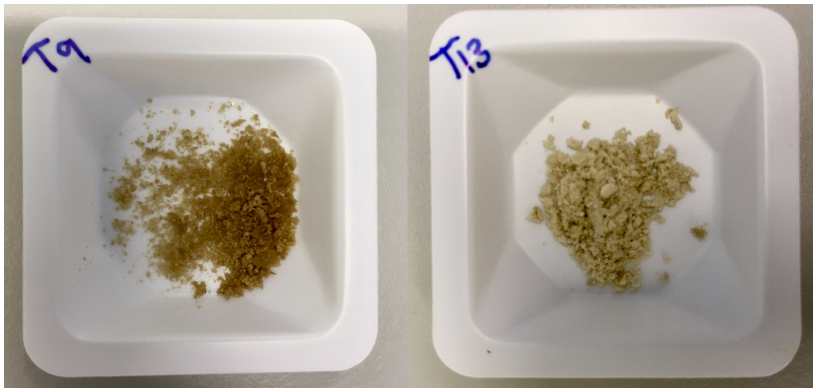

Figure A.2: Sample T9 from hydrolysis 31.08.2017, T13 from hydrolysis 25.08.2017.

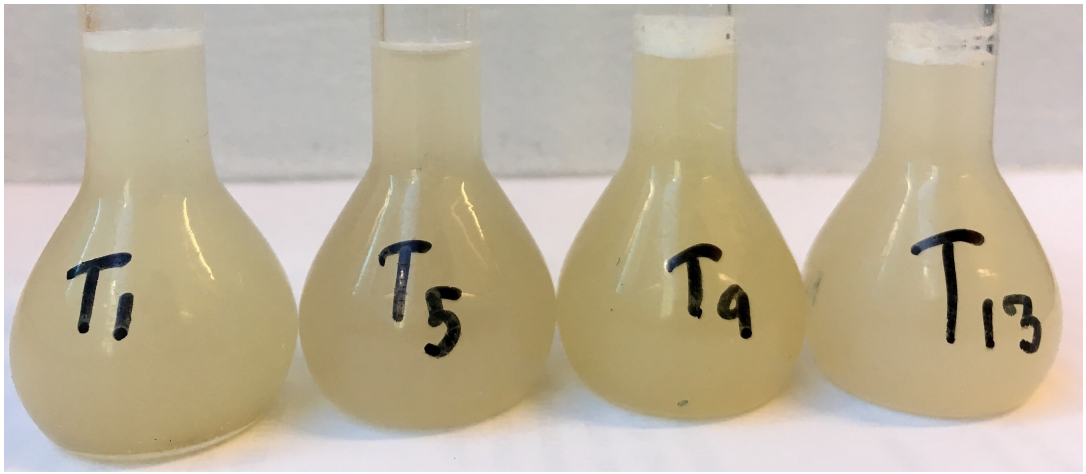

Figure A.3: Samples T1, T5, T9, T13 at a concentration of 10 mg/mL in distilled water.

Table A.1: pH of samples at a concentration of 10 mg/mL

Sample	pH
T1	6.75
T5	6.73
T9	5.94
T13	4.74

Appendix B

Lowry Method - Soluble Protein

Protein determination as described by Lowry et al. (1951) was performed, in collaboration with Margrethe Fossheim Ohnstad, on fractions following ultrafiltration to determine the soluble protein content in the permeate (< 4kDa) and retentate (> 4kDa). The protein content of crude samples was also determined, in which the original concentration was 20 mg CPH powder/mL.

Seven standard solutions of BSA (12,5 - 300 μg/mL) were prepared. Blank (0.5 mL H_2O) was added to a glass tube. Standard solution or suitably diluted sample were each added (0.5 mL) to three parallels of glass tubes. Alkaline copper reagent (2,5 mL) was added to each glass tube and left at room temperature for 10 minutes. Folin-Ciocalteu reagent (0.25 mL) was added and mixed immediately. After 30 minutes of incubation, absorbance at 750 nm was read. Absorbance at 750 nm was plotted against BSA concentration. By linear regression an equation describing the linear relationship between protein concentration and absorbance was calculated and used to calculate the unknown protein concentration in the fractionated samples.

In figure B.1, BSA concentrations are plotted against absorbance.

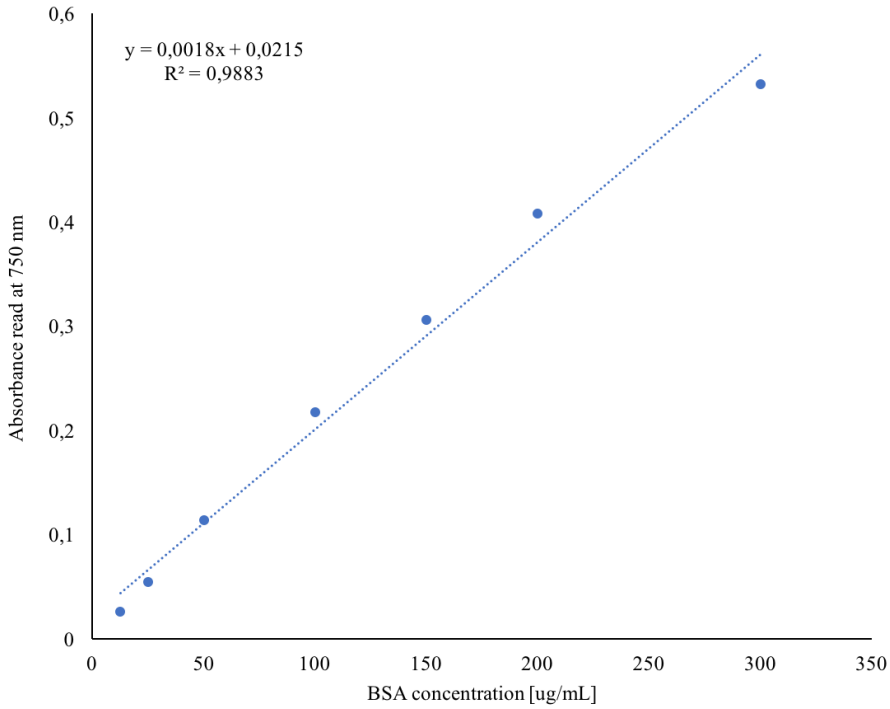

Figure B.1: BSA concentration plotted against absorbance read at 750 nm. By linear regression the equation y = 0.0018x + 0.0215 was found. R^2 = 0.9883.

Table B.1: Protein concentrations determined by the Lowry method.

	Average protein conc. [mg/mL]	SD	protein/CPH[wt.%]
CPH (n = 3)			
T1	10.67	0.82	53.4
T5	8,30	0.35	41.5
T9	6,37	0.53	31.9
T13	10.36	0.60	51.8
Permeate (n = 5)			
T1-R	1,77	0.14	
T5-R	1,77	0.15	
T9-R	2,23	0.98	
T13-R	2,11	0.13	
Retentate (n= 3)			
T1-P	11,35	0.44	
T5-P	11,02	0.72	
T9-P	8,31	0.24	
T13-P	9,63	0.61	

Appendix C

HPLC - TAA and FAA

C.1 TAA

The data processing of HPLC results are shown, using sample T1 as an example. 100 mg sample was weighed in. Figure C.1 displays the data obtained from HPLC to determine the composition of total amino acids in the hydrolysates. The last column, "amount μmol/l" is equivalent to nmol/ml and is used to calculate the amount of each amino acid as mg protein per gram sample.

Table C.1: HPLC raw data for CPH sample T1.

No. Emission	Peak Name Emission	Retention Time min Emission	Area mV*min Emission	Height mV Emission	Relative Area % Emission	Relative Height % Emission	Amount umol/l Emission
1	Asp	1,653	10,626	122,323	12,12	20,23	2,9169
2	Glu	2,718	11,523	61,007	13,15	10,09	3,8217
3	Asn	3,543	0,085	0,620	0,10	0,10	0,0254
4	His	4,408	0,020	0,139	0,02	0,02	0,0096
5	Ser	4,817	4,029	25,855	4,60	4,28	2,0638
6	Gln	5,087	0,028	0,000	0,03	0,00	0,0090
7	Gly/Arg	8,093	22,215	93,106	25,35	15,40	4,4589
8	Thr	8,735	3,595	15,381	4,10	2,54	1,5004
9	Ala	13,207	9,111	50,368	10,40	8,33	3,7123
10	Tyr	14,553	0,025	0,175	0,03	0,03	0,0085
11	Aba	16,707	0,184	0,000	0,21	0,00	0,0532
12	Met	18,183	1,138	9,558	1,30	1,58	0,3446
13	Val	18,477	5,722	51,141	6,53	8,46	1,5451
14	Phe	18,963	2,561	21,057	2,92	3,48	0,8242
15	Ile	19,957	3,945	36,284	4,50	6,00	1,0892
16	Leu	20,298	7,265	64,421	8,29	10,65	2,2037
17	Lys	21,960	5,567	53,234	6,35	8,80	2,2121
Total:			**87,641**	**604,671**	**100,00**	**100,00**	

By multiplying the molecular weight of each amino acid with the concentration found (μmol/l) the amount of amino acid (ug/mL) is found. Taking into account the dilution (D),

mass (M), and volume (V) used, the mg protein/g sample for each amino acid is found as shown in equation C.1.

$$Amino\ acid\ (mg/g) = \frac{C \times V \times D}{1000 \times M} \tag{C.1}$$

The total amount of amino acid was 569 mg/g for T1, as seen in Figure C.2. The weight percentage was calculated from mass by dividing the mass of each amino acid with the mass of total amino acid.

Table C.2: Calculated total amino acid composition and content for CPH sample T1.

Amino acid from HPLC	Mw bound in protein g/mol	Weighed in Dil. 1:1000 nmol/ml	0,1 g ug/ml	mg/g	weight %
Asp	115	2,9169	0,34	67,09	11,79
Glu	129	3,8217	0,49	98,60	17,33
Asn	114	0,0254	0,00	0,58	0,10
His	137	0,0096	0,00	0,26	0,05
Ser	87	2,0638	0,18	35,91	6,31
Gln	128	0,0090	0,00	0,23	0,04
Gly/Arg	98	4,4589	0,44	87,39	15,36
Thr	101	1,5004	0,15	30,31	5,33
Ala	71	3,7123	0,26	52,71	9,26
Tyr	163	0,0085	0,00	0,12	0,02
Aba	85	0,0532	0,00	0,90	0,16
Met	131	0,3446	0,05	9,03	1,59
Val	99	1,5451	0,15	30,59	5,38
Phe	147	0,8242	0,12	24,23	4,26
Ile	113	1,0892	0,12	24,62	4,33
Leu	113	2,2037	0,25	49,80	8,75
Lys	128	2,2121	0,28	56,63	9,95
Total		**24,59**	**2,85**	**569,02**	**100**

The calculated fractions for each parallel for each CPH sample are shown in figures C.3-C.6. The mass of total amino acid calculated for all parallels of each sample is shown in figure C.7.

Table C.3: Fractions (%), average and SD of TAA in sample T1. n=7

T1	2	3	4	5	6	7	8	Average	SD
Asp	12,41	12,82	12,28	12,44	12,23	12,23	12,19	12,4	0,22
Glu	18,24	18,46	17,75	15,58	15,72	15,74	15,55	16,7	1,36
Asn	0,11	0,12	0,12	0,10	0,09	0,06	0,09	0,1	0,02
His	0,05	0,05	0,04	0,10	0,11	0,10	0,12	0,1	0,03
Ser	6,64	6,81	6,48	5,85	5,90	5,90	5,91	6,2	0,41
Gln	0,04	0,02	0,01	0,13	0,12	0,09	0,10	0,1	0,05
Gly/Arg	16,16	16,21	15,60	16,27	16,44	16,44	16,40	16,2	0,29
Thr	5,61	5,61	5,25	5,42	5,22	5,24	5,33	5,4	0,17
Ala	9,75	9,86	9,39	9,13	9,21	9,16	9,15	9,4	0,30
Tyr	0,02	0,03	0,03	0,07	0,06	0,06	0,07	0,0	0,02
Aba	0,17	0,13	0,13	0,18	0,18	0,15	0,20	0,2	0,03
Met	1,67	3,29	3,12	2,09	2,26	2,21	2,29	2,4	0,58
Val	5,66	5,75	5,46	5,97	5,92	5,99	5,96	5,8	0,20
Phe	4,48	4,63	4,28	3,73	3,74	3,74	3,71	4,0	0,40
Ile	4,55	4,54	4,34	4,45	4,44	4,45	4,43	4,5	0,07
Leu	9,21	10,35	9,86	9,00	9,02	9,03	9,00	9,4	0,54
Lys	5,24	5,01	5,85	9,48	9,35	9,41	9,48	7,7	2,19

Table C.4: Fractions (%), average and SD of TAA in sample T5. n=7

T5	1	2	3	4	5	6	7	Average	SD
Asp	9,5	11,2	10,5	10,3	9,9	10,0	10,0	10,2	0,55
Glu	14,0	0,4	14,8	15,0	14,2	14,4	14,3	12,4	5,33
Asn	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,01
His	2,1	2,7	2,3	2,3	2,4	2,3	2,3	2,3	0,18
Ser	6,0	7,9	6,8	6,7	6,9	6,8	6,8	6,9	0,57
Gln	0,0	0,2	0,2	0,2	0,1	0,0	0,0	0,1	0,08
Gly/Arg	25,7	18,0	15,4	15,3	14,9	15,2	15,0	17,1	3,97
Thr	4,4	6,1	5,2	5,2	5,3	5,2	5,2	5,2	0,50
Ala	6,9	9,4	8,0	7,9	8,5	7,9	8,6	8,2	0,78
Tyr	2,3	3,9	3,3	3,2	3,4	3,5	3,5	3,3	0,49
Aba	0,2	0,1	0,1	0,1	0,2	0,2	0,2	0,1	0,06
Met	3,0	4,0	3,3	3,4	3,5	3,5	3,5	3,4	0,30
Val	4,2	5,9	4,9	5,0	5,0	5,0	5,0	5,0	0,48
Phe	3,6	5,0	4,1	4,2	4,3	4,3	4,3	4,3	0,39
Ile	3,4	4,7	4,0	4,0	4,1	4,2	4,1	4,1	0,39
Leu	7,0	9,4	7,8	7,9	8,2	8,3	8,3	8,1	0,72
Lys	7,6	11,0	9,3	9,3	9,1	9,1	9,0	9,2	1,02

Table C.5: Fractions (%), average and SD of TAA in sample T9. n=7

T9	1	2	3	4	5	6	7	Average	SD
Asp	9,4	12,55	12,20	12,40	11,72	6,34	12,28	11,0	2,3
Glu	13,9	16,12	15,46	16,17	14,17	14,95	13,85	14,9	1,0
Asn	0,0	0,11	0,12	0,10	0,09	0,10	0,09	0,1	0,0
His	2,0	0,00	0,18	0,06	0,62	0,61	0,68	0,6	0,7
Ser	6,3	6,32	7,48	6,24	6,76	7,22	7,24	6,8	0,5
Gln	0,0	0,01	0,03	0,00	0,01	0,01	0,03	0,0	0,0
Gly/Arg	26,9	12,72	12,72	12,72	13,21	13,72	12,90	15,0	5,3
Thr	4,5	5,91	5,99	5,81	5,84	5,56	5,13	5,5	0,5
Ala	0,8	8,98	9,05	8,99	8,94	9,56	9,02	7,9	3,1
Tyr	7,1	0,19	0,29	0,26	0,40	0,42	0,39	1,3	2,6
Aba	0,2	0,09	0,11	0,14	0,76	1,03	1,15	0,5	0,5
Met	3,0	2,55	2,65	2,75	2,92	3,07	2,93	2,8	0,2
Val	4,3	6,01	6,02	6,07	5,88	6,27	5,82	5,8	0,7
Phe	3,7	4,59	4,52	4,58	5,14	5,52	5,35	4,8	0,6
Ile	3,4	4,79	4,69	4,77	4,82	5,18	4,66	4,6	0,6
Leu	7,0	9,50	9,21	9,46	9,06	9,96	8,85	9,0	1,0
Lys	7,5	9,56	9,29	9,47	9,64	10,49	9,64	9,4	0,9

Table C.6: Fractions (%), average and SD of TAA in sample T13. n=7

T13	1	2	3	4	5	6	7	Average	SD
Asp	9,4	9,6	9,1	9,6	10,9	10,8	10,8	10,0	0,8
Glu	13,9	14,2	13,7	14,2	14,1	14,2	14,3	14,1	0,2
Asn	0,0	0,1	0,0	0,1	0,1	0,1	0,1	0,1	0,0
His	2,0	1,0	2,0	1,0	0,1	0,1	0,1	0,9	0,9
Ser	6,3	6,3	5,9	6,3	7,6	7,1	7,0	6,7	0,6
Gln	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Gly/Arg	26,9	26,9	26,8	26,9	15,6	15,8	15,7	22,1	6,0
Thr	4,5	4,8	4,4	4,8	5,9	5,8	5,8	5,1	0,7
Ala	0,8	7,5	7,0	7,5	10,1	9,9	9,9	7,5	3,3
Tyr	7,1	0,2	1,7	0,2	0,2	0,1	0,1	1,4	2,6
Aba	0,2	0,1	0,2	0,1	0,1	0,1	0,1	0,1	0,0
Met	3,0	2,7	3,0	2,7	2,6	2,6	2,6	2,7	0,2
Val	4,3	4,4	4,2	4,4	5,6	5,7	5,7	4,9	0,7
Phe	3,7	3,6	3,7	3,6	4,3	4,2	4,2	3,9	0,3
Ile	3,4	3,6	3,4	3,6	4,6	4,7	4,7	4,0	0,6
Leu	7,0	7,2	7,0	7,2	8,8	9,1	9,1	7,9	1,0
Lys	7,5	7,9	8,0	7,9	9,3	9,6	9,6	8,5	0,9

Table C.7: TAA (mg/g CPG) in samples T1, T5, T9, and T13. n=7

mg/g CPH	1	2	3	4	5	6	7	Average	SD
T1	569	556	546	530	545	531	567	549	16
T5	402	663	759	852	979	946	945	792	206
T9	445	690	736	703	358	309	290	504	199
T13	458	735	558	738	1171	1117	1081	837	286

C.2 FAA

The FAA was calculated as shown in C.1.

Table C.8: Free amino acid composition expressed as the weight percentage of total free amino acids of CPH samples T1, T5, T9, and T13 $\pm SD$, n=3. For sample T13, n=1.

Weight percentages [%]				
Amino Acid	T1	T5	T9	T13
Asp	3.7 ± 0.13	3.4 ± 0.01	3.5 ± 0.01	4.1
Glu	7.9 ± 0.18	6.50.14	7.2 ± 0.12	7.2
Asn	0.1 ± 0.06	0.1 ± 0.03	0.1 ± 003	0.1
His	2.5 ± 0.05	2.9 ± 0.02	2.7 ± 0.15	1.8
Ser	9.0 ± 0.54	6.4 ± 0.10	7.7 ± 0.6	6.0
Gln	2.9 ± 0.15	3.2 ± 0.1	3.0 ± 0.3	2.1
Gly/Arg	11.0 ± 0.13	0.4 ± 0.2	10.7 ± 0.3	11.7
Thr	5.2 ± 0.01	5.7 ± 0.12	5.5 ± 0.17	4.8
Ala	11.9 ± 0.2	11.1 ± 0.3	11.5 ± 0.6	11.2
Tyr	3.8 ± 0.06	4.2 ± 0.01	$4.0 \pm .09$	4.5
Aba	0.6 ± 0.7	0.5 ± 0.3	0.6 ± 0.2	0.4
Met	6.7 ± 0.26	7.3 ± 0.2	7.0 ± 0.4	5.5
Val	4.8 ± 0.07	6.0 ± 0.3	5.4 ± 0.2	5.6
Phe	4.8 ± 0.8	6.3 ± 0.3	5.6 ± 0.65	6.5
Ile	3.0 ± 0.15	3.6 ± 0.03	3.3 ± 0.02	3.4
Leu	11.9 ± 0.15	13.5 ± 0.1	12.7 ± 0.1	14.7
Lys	10.2 ± 0.2	8.9 ± 0.1	9.6 ± 0.16	10.5
Total	100	100	100	100

Table C.9: FAA (mg/g CPH) of crude samples.

FAA mg/g CPH	I	II	II	Average	SD
T1	10,49	12,12	11,78	11,46	0,86
T5	21,21	20,23	22,44	21,29	1,10
T9	13,38	13,40	13,16	13,32	0,13
T13	25,44			25,44	n.d.

Table C.10: FAA compositions (%) of <4 kDa fractions.

<4 kDa				
FAA	T1	T5	T9	T13
Asp	2,26	14,22	39,18	29,94
Glu	2,30	1,86	2,37	2,10
Asn	0,06	0,07	0,80	0,47
His	0,85	1,08	1,31	0,65
Ser	2,03	5,47	6,85	7,53
Gln	20,09	40,59	15,11	29,11
Gly/Arg	6,90	3,84	4,63	3,06
Thr	2,66	1,59	1,09	1,08
Ala	4,93	1,73	1,11	0,63
Tyr	4,65	2,74	2,67	2,53
Aba	1,37	0,84	2,11	0,97
Met	7,05	1,52	0,64	1,26
Val	4,68	2,14	2,03	3,22
Phe	7,09	4,41	6,04	3,34
Ile	3,07	2,38	2,26	2,34
Leu	15,09	7,65	6,49	5,52
Lys	14,90	7,86	5,31	6,27
Total	100,00	100,00	100,00	100,00

Table C.11: FAA (mg/g soluble protein) of <4 kDa samples.

mg FAA/ g. sol protein	I	II	III	Average	SD
T1	13,20	12,58	13,54	13,11	0,49
T5	2,32	2,47	2,74	2,51	0,21
T9	1,27	1,27	1,60	1,38	0,19
T13	1,70	1,71	1,81	1,74	0,06

Table C.12: FAA compositions (%) of >4 kDa fractions.

>4 kDa				
FAA	T1	T5	T9	T13
Asp	0,17	0,25	0,41	0,74
Glu	0,47	0,07	0,04	2,19
Asn	0,04	0,11	0,15	0,05
His	4,04	3,19	2,79	2,63
Ser	0,35	0,22	0,15	1,63
Gln	18,22	5,28	6,03	4,64
Gly/Arg	5,07	5,54	5,19	5,99
Thr	0,05	0,28	0,35	3,55
Ala	2,17	3,36	1,76	9,12
Tyr	2,72	6,12	5,49	6,04
Aba	0,57	1,21	1,07	1,16
Met	11,32	10,90	10,79	9,00
Val	11,34	13,64	14,72	7,91
Phe	6,51	9,73	10,17	8,01
Ile	7,89	8,87	9,29	3,71
Leu	19,49	21,47	22,15	18,00
Lys	9,58	9,76	9,45	15,63
Total	100,00	100,00	100,00	100,00

Table C.13: FAA compositions (mg/g soluble protein) of >4 kDa fractions.

FAA mg/g.sol.protein	I	II	III	Average	SD
T1	2,74	2,77	2,96	2,82	0,12
T5	8,71	8,95	8,70	8,79	0,14
T9	3,96	3,96	4,02	3,98	0,04
T13	11,24	11,45	11,37	11,36	0,11

Appendix D

Chromatograms of Standard Compounds

Table D.1: Molecular weights, and elution volume peaks of standards used in gel filtration.

Standard	Molecular weight [kDa]	Peak [mL]
Pepsin	34	10.3
Cytochrome C	12.4	15.1
Aprotinin	6.5	17.9
Vitamin B12	1.3	18.5

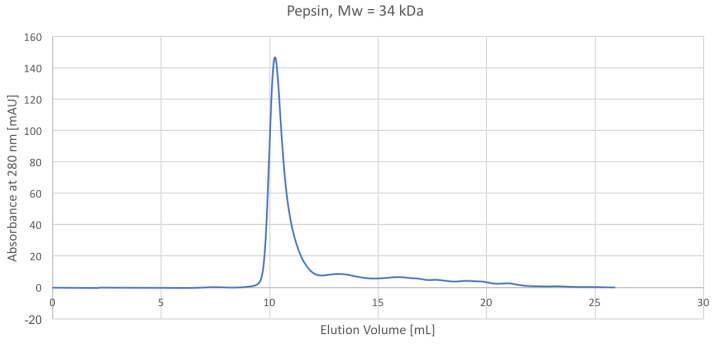

Figure D.1: Chromatogram of pepsin, Mw = 34 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU).

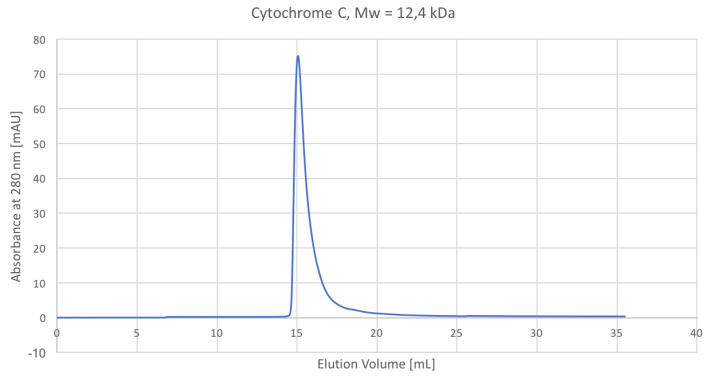

Figure D.2: Chromatogram of cytochrome C, Mw = 12.3 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU).

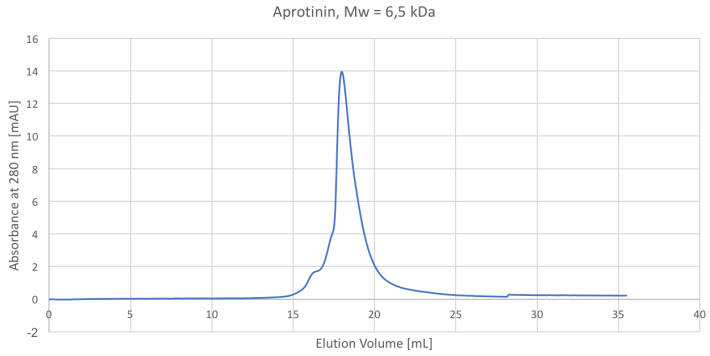

Figure D.3: Chromatogram of aprotinin, Mw = 6.5 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU).

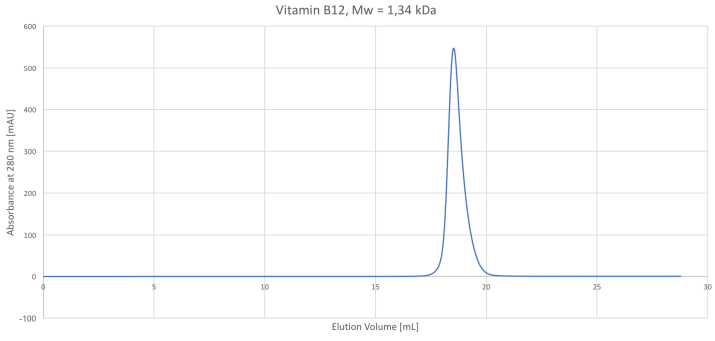

Figure D.4: Chromatogram of aprotinin, Mw = 1.34 kDa, used as standard in FPLC. Retention volume (mL) is plotted against absorbance (mAU).

Appendix E

Antioxidant Assays - Raw Data and Calculations

E.1 ABTS radical scavenging activity

Table E.1 shows the absorbance read at 734 nm for standard PG concentrations (10-50 μM), and UF fractions, measured in three parallels. Eight experiments were conducted for each CPH sample and UF fraction. Based on the measured absorbance of the standard concentrations a standard curve was plotted, shown in figure E.1. Absorbance read at 734 nm was plotted against concentration (μM) of propyl gallate used. By linear regression the following equation was found:

$$y = -0.0074x + 0.5359 \tag{E.1}$$

The absorbance is given by y, the concentration of PG is given by x as micromolars. An example of how E.1 was applied is shown for sample T1-P in equation E.2.

$$x = \frac{0.112 - 0.5359}{-0.0074} = 57.24 \tag{E.2}$$

Based on the concentration calculated in equation E.2 and the protein content measured by the Lowry method (table B.1) the ABTS radical scavenging activity was calculated as equivalent concentrations of the standard compound PG (μ mol/g). The equation used is shown bellow:

$$\mu mol/mL = \frac{\mu M}{1000} \tag{E.3}$$

$$\frac{\mu mol}{g\ protein} = \frac{\mu mol/mL}{g\ protein/mL} \tag{E.4}$$

An example calculation based on T1-P is shown bellow.

Table E.1: Absorbance read at 734 nm and the averages of three parallels of PG standard and hydrolysate samples measured in ABTS assay. Blue T1-T13 is the absorbance read for permeate, green is absorbance read for retentates.

Sample	Conc. [µM]	Parallell 1	Parallell 2	Parallell 3	Average
Blank	0	0,639	0,639	0,639	0,639
Standard 1	10	0,44	0,439	0,437	0,439
Standard 2	20	0,41	0,409	0,407	0,409
Standard 3	30	0,32	0,317	0,315	0,317
Standard 4	40	0,247	0,244	0,241	0,244
Standard 5	50	0,151	0,145	0,143	0,146
T1		0,117	0,118	0,102	0,112
T5		0,115	0,143	0,136	0,131
T9		0,191	0,179	0,171	0,180
T13		0,35	0,333	0,323	0,335
T1		0,232	0,224	0,219	0,225
T5		0,234	0,226	0,22	0,227
T9		0,283	0,274	0,267	0,275
T13		0,409	0,401	0,394	0,401

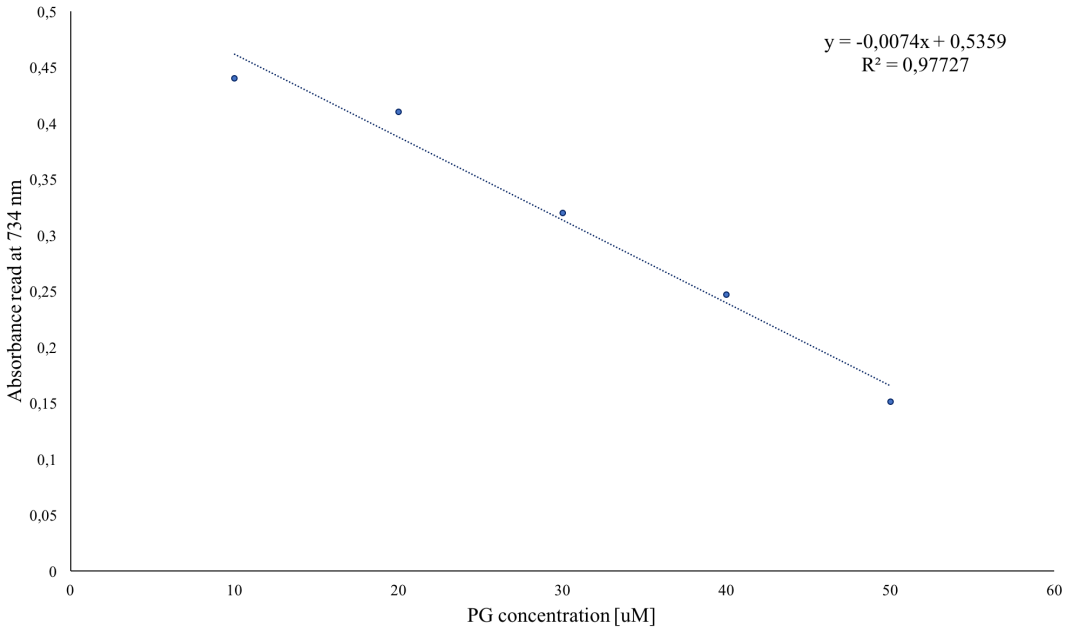

Figure E.1: Standard curve of propyl gallate used in the ABTS assay to determine radical scavenging activity. Absorbance read at 734 nm was plotted against concentration (μM) of propyl gallate used. By linear regression the equation y = -0.0074x + 0.5359 was found.R^2 = 0.97727.

$$\frac{\mu mol}{g\ protein} = \frac{0.05724}{0.00177} = 32.34 \tag{E.5}$$

In table E.2, calculated concentrations (μ M) and equivalent PG concentrations (μmol / g) is for all fractionated samples are shown.

Table E.2: Absrobance measured, and concentration and equivalent ABTS radical scavenging activity calculated for > 4 kDa fractions (P) and < 4 kDa fractions (R).

Sample	Absorbance	Conc. [uM]	Eq. activity [umol/g]
T1-P	0,112	57,24	32,34
T5-P	0,131	54,67	30,89
T9-P	0,180	48,05	21,55
T13-P	0,335	27,10	12,85
T1-R	0,162	72,58	41,01
T5-R	0,178	48,41	27,35
T9-R	0,243	39,63	17,77
T13-R	0,467	9,36	4,43

Results from eight ABTS experiments conducted are found in table E.3.

Table E.3: Results from ABTS assay of permeates and retentates of samples T1, T5, T9, and T13. n = 8.

umol/g	1	2	3	4	5	6	7	8	Average	SD
T1-P	24,76	25,40	27,26	39,73	30,88	42,57	32,34	41,01	32,99	7,22
T5-P	25,19	20,63	27,70	27,40	29,98	29,44	30,89	27,35	27,32	3,24
T9-P	20,45	14,83	19,92	18,19	21,25	19,58	21,55	17,77	19,19	2,21
T13-P	6,95	4,48	9,30	6,28	13,19	6,91	12,85	4,43	8,05	3,43
T1-R	4,04	6,40	5,05	6,40	4,68	6,20	4,05	3,96	5,10	1,02
T5-R	4,26	4,39	4,96	4,39	4,92	4,40	4,25	3,31	4,36	0,47
T9-R	5,10	4,77	5,78	4,77	5,97	4,88	4,76	3,98	5,00	0,59
T13-R	0,24	0,97	2,81	0,97	2,29	1,38	1,02	0,98	1,33	0,77

E.2 Folin-Ciocalteu reducing capacity

The FC reducing capacity of hydrolysate smaples and UF fractions were calculated in a similar way to ABTS radical scavenging activity. The absorbances, measured in three parallels, of standard, permeates and retentates are shown in table E.4. The standard curve is found in figure E.2.

Table E.4: Absorbances read at 725 nm and their averages of standard concentrations of PG and hydrolysate samples measured in FC assay.

	Conc. [mM]	Abs I	II	III	Average
B		0	0,02	0	0
1	0,5	0,034	0,031	0,023	0,029
2	1	0,083	0,109	0,062	0,085
3	1,5	0,147	0,146	0,145	0,146
4	2	0,203	0,23	0,224	0,219
T1-P		0,104	0,07	0,059	0,078
T5-P		0,047	0,085	0,085	0,072
T9-P		0,078	0,06	0,06	0,066
T13-P		0,086	0,056	0,085	0,076
T1-R		0,116	0,122	0,121	0,120
T5-R		0,024	0,025	0,025	0,025
T9-R		0,134	0,135	0,135	0,135
T13-R		0,105	0,112	0,112	0,110

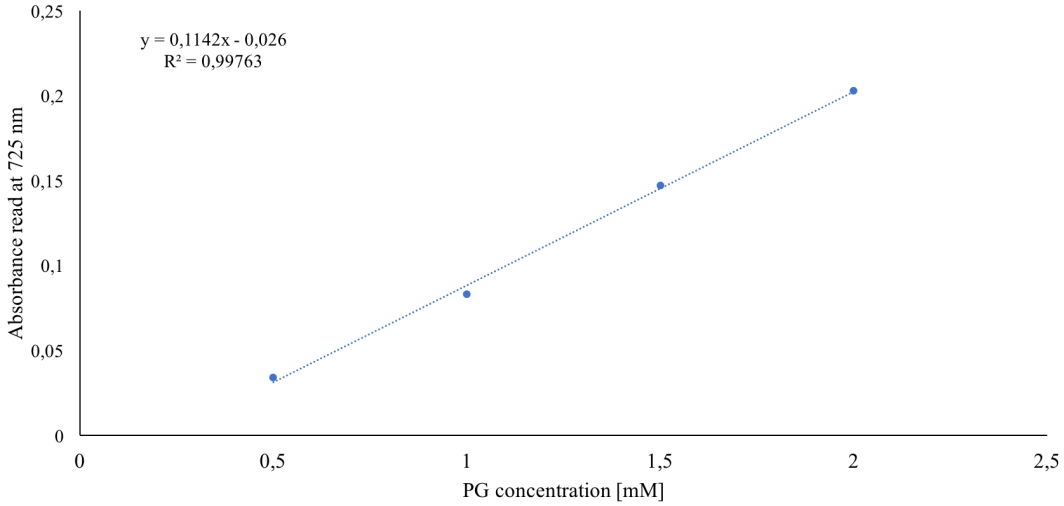

Figure E.2: Standard curve of propyl gallate used in the Folin-Ciocalteu assay to determine the total phenolic concentration. Absorbance read at 725 nm was plotted against concentration (mM) of propyl gallate used. By linear regression the equation y = 0.1261x - 0.0378 was found. R^2 = 0.99601.

From the standard curve shown in Figure E.2, the following equation was found using

linear regression:

$$y = 0.1261x - 0.0378 \tag{E.6}$$

The y represents the absorbance, and the x concentration in millimolars. Using equation E.6, the absorbances of the samples were used to calculate the equivalent concentrations of PG. An example calculation for T13-R is shown bellow.

$$x = \frac{0.110 - (-0.0378)}{0.1261} = 1.17 \tag{E.7}$$

Because the hydrolysate fractions were of different protein concentrations after UF the FC total phenol content was expressed as equivalents of mmol PG per gram of protein. The protein concentrations are based on the values found from the Lowry method as shown in table B.1. Equation E.4 was used to calculate mmol PG/g protein. An example calculation based on sample T13-R is shown in equation E.8.

$$\frac{mmol}{g\ protein} = \frac{0.00117}{0.00963} = 1.21 \tag{E.8}$$

Table E.5: Absorbances measured, concentrations, and equivalent PG concentrations calculated in FC assay.

	Absorbance	Conc. [mM]	Eq. Conc. PG [mmol/g
T1-P	0,078	0,92	5,17
T5-P	0,072	0,87	4,93
T9-P	0,066	0,82	3,69
T13-P	0,076	0,90	4,26
T1-R	0,120	1,25	1,10
T5-R	0,025	0,50	0,45
T9-R	0,135	1,37	1,65
T13-R	0,110	1,17	1,21

The results from the FC assay of permeates and retentates are shown in table E.6.

Table E.6: Results obtained in the FC assay of permeates and retentates of samples T1, T5, T9, and T13 based on eight experiments.

mmol PG/g	1	2	3	4	5	6	7	8	Average	SD
T1-P	4,94	3,54	1,66	3,39	1,62	1,79	1,50	1,97	2,55	1,18
T5-P	4,70	3,66	1,91	4,07	1,81	2,97	2,27	1,90	2,91	1,04
T9-P	3,49	2,85	1,37	2,69	2,39	1,88	2,56	1,53	2,34	0,67
T13-P	4,07	4,15	2,12	3,83	2,26	2,08	1,56	1,69	2,72	1,03
T1-R	1,08	1,18	0,76	1,29	0,86	0,77	0,69	0,77	0,92	0,21
T5-R	0,40	1,15	0,78	0,53	0,98	0,86	0,96	0,77	0,80	0,23
T9-R	1,62	1,32	0,89	0,88	0,98	1,13	0,82	0,78	1,05	0,27
T13-R	1,18	1,17	0,77	1,15	0,92	0,82	0,76	0,80	0,95	0,18

E.3 Iron chelating activity

Absorbance measured for control, crude CPH samples, and the absorbance of protein in samples are shown in table E.7.

Table E.7: Absorbance read at 562 nm of control, sample, and protein, calculated as an average of three measurements (denoted as parallels).

Control	Absorbance Sample				Absorbance Protein			
0,809	I	II	III	Average	I	II	III	Average
T1	0,748	0,736	0,735	0,740	0,129	0,127	0,127	0,128
T5	0,818	0,815	0,814	0,816	0,177	0,176	0,176	0,176
T9	0,8	0,772	0,772	0,781	0,061	0,061	0,061	0,061
T13	0,761	0,761	0,761	0,761	0,056	0,057	0,056	0,056

The chelating activity was calculated as a percentage as shown in figure E.9.

$$Chelating\ Activity(\%) = (1 - \frac{A_{563}sample - A_{562}protein}{A_{562}control}) \times 100 \tag{E.9}$$

An example calculation using sample T1 is shown in equation E.9.

$$Chelating\ Activity(\%) = (1 - \frac{0.740 - 0.128}{0.809}) \times 100 = 75.65\% \tag{E.10}$$

A summary of measured iron chelating activities from eight experiments for crude CPH samples T1, T5, T9, and T13 is found in table E.8.

Table E.8: Iron chelating activity results of samples T1, T5, T9, and T13 given as percentages. n = 8.

% Activity	1	2	3	4	5	6	7	8	Average	SD
T1	75,65	74,43	94,22	79,85	69,17	74,60	68,58	79,20	76,96	8,1
T5	79,03	77,86	78,52	79,71	77,23	81,77	75,56	94,51	80,52	5,9
T9	89,04	77,24	86,61	88,16	82,01	91,13	81,05	84,24	84,93	4,7
T13	87,10	92,61	84,97	99,10	95,78	98,12	79,29	81,81	89,85	7,6

Appendix F

Oxygen Uptake in Liposomes

In figure F.1 the dissolved oxygen is plotted as a function of time. The oxygen concentration in four oxygen electrodes with liposomes are shown in the plot. In addition to iron, either water, T1, T5, and casein was added to the different cells.

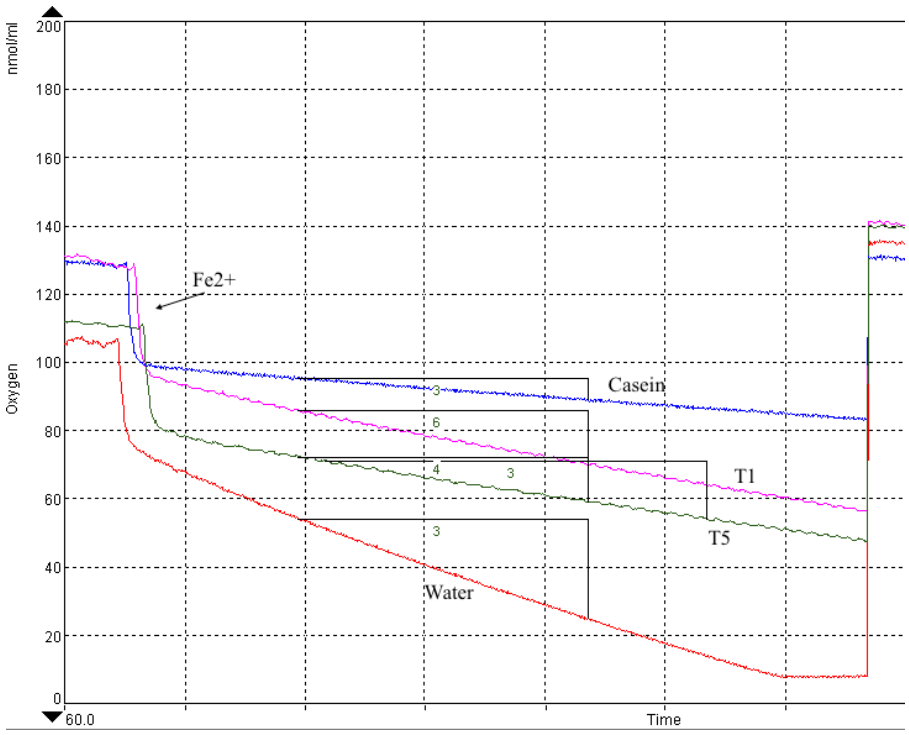

Figure F.1: Dissolved oxygen as a function of time.

Changes in dissolved oxygen in liposomes after addition of Fe^{2+} and either water, T5, T9, or T13 are shown in figure F.2. In table F.1 the OUR reduction rates following addition of Fe^{2+} and different protein samples is shown.

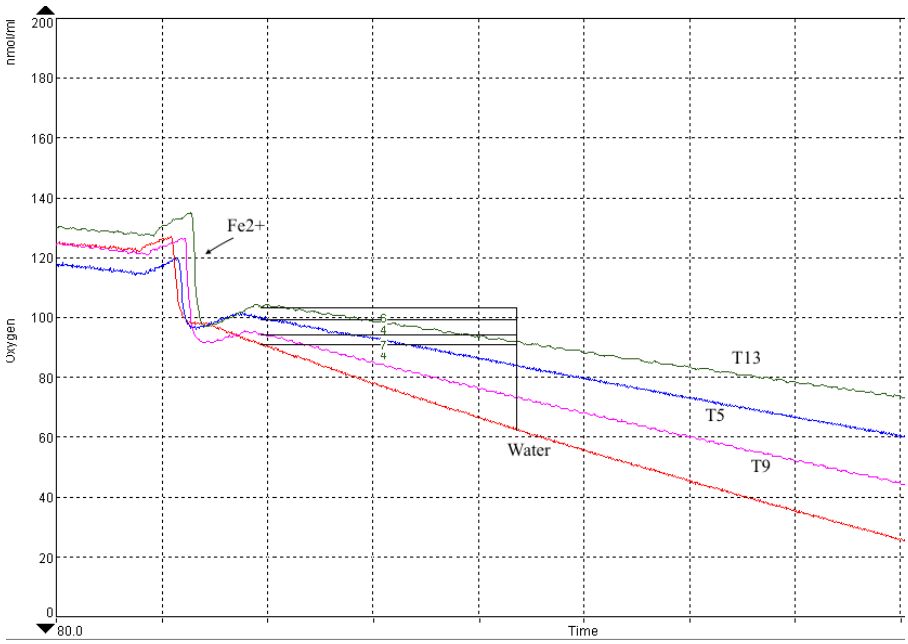

Figure F.2: Dissolved oxygen as a function of time.

Table F.1: Measured OUR reduction rates following Fe^{2+} addition in liposomes.

% inhibition	I	II	III	IV	Average	SD
T1	46,96		45,61	41,12	44,56	3,05743
T5	55,54	44,67	51,02	45,92	49,29	4,99191
T9		25,07			25,07	-
T13		55,83	64,76	59,47	60,02	4,48821
Casein	78,41				78,41	-

Appendix G

ACE-inhibition Activity

Margrethe Fossheim Ohnstad determined the ACE-inhibiting activity of hydrolysates from the pilot project in her thesis project. Inhibition is determined as IC_{50} (half maximal inhibitory concentration), which is the effectiveness of the substance in inhibiting ACE in the conversion of angiotensin I to angiotensin II. A lower IC_{50} value indicates higher effectiveness. In figure G.1, the measured IC_{50} values of CPH samples and permeate (<4 kDa) is shown.

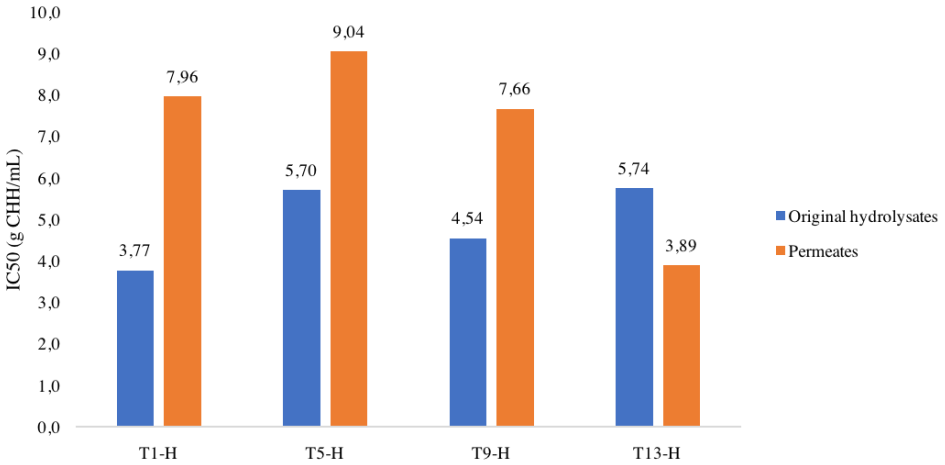

Figure G.1: IC_{50} measured in hydrolysates and permeates.

The data was applied to principal component analysis. Because a low value indicates high ACE-inhibition the sign of the IC_{50} values were reversed so that any positive correlation with ACE-inhibition is associated with high effectiveness.

Appendix H

Principal Component Analysis

The average (x_{avg}) and standard deviation (σ) is calculated for each variable. x_i is the value of each data point.

$$X_{norm} = \frac{x_i - x_{avg}}{\sigma} \tag{H.1}$$

Boxplots of the normalized data are found in figures H.1-H.2.

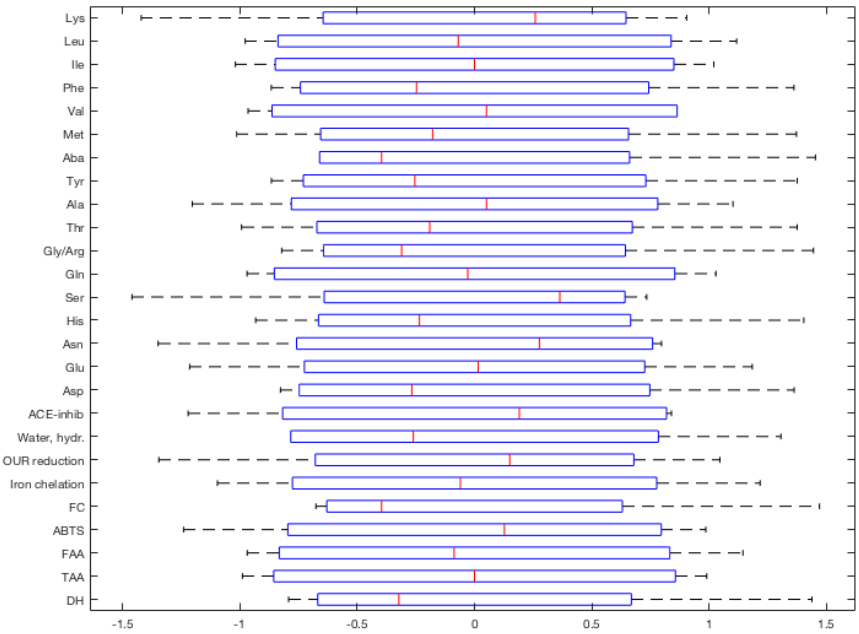

Figure H.1: Boxplot of normalized data variables in CPH samples.

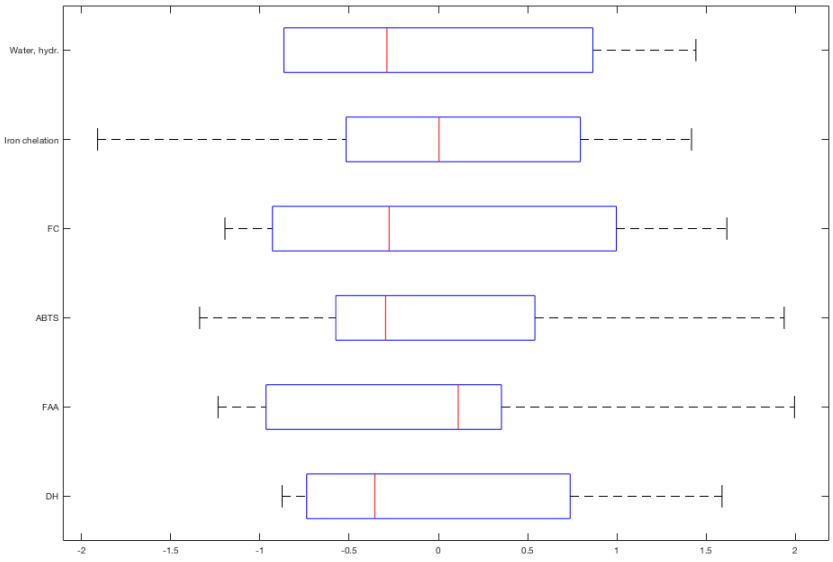

Figure H.2: Boxplot of normalized data from CPH samples and fractions.