Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Aleksandra Stensen

# Investigation of bioactive properties of fish protein hydrolysates: Antioxidant activity and ACE inhibitory activity

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Turid Rustad
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# Abstract

The waste of and less than optimal use of fish by-products is one of the main problems for the fish industry today. Fish resources are limited, and underutilised by-products can have a great ecological impact and affect the economics of the fishing and aquaculture sector. It is therefore of great interest to utilise by-products as optimally as possible. Even though the situation in Norway is relatively good, the white fish area has the potential for improvement. Utilisation can be optimised by obtaining bioactive compounds from by-products. Due to their properties, these compounds can be used in the production of drugs or functional foods.

The main aim of the work in this thesis was to determine the bioactive properties of protein hydrolysates, improve the analytical methods and apply membrane filtration on several hydrolysates. Correlations between structural and bioactive properties of the hydrolysates were also analysed. Cod protein hydrolysates produced by SINTEF Ocean in the pilot project HEADS UP and a commercial salmon protein hydrolysate have been analysed for the presence of antioxidant activity and blood pressure lowering effect (ACE inhibitory activity). In addition, structural properties such as total and free amino acid content, molecular weight distribution, protein content and amount of acid soluble peptides were determined. Ultrafiltration was applied on two hydrolysates to find out if molecular weight influenced bioactivities. However, the separation was not as sharp as desired. Degree of separation was improved slightly by filtrating the hydrolysate twice.

Antioxidant activity was measured by two different methods: ABTS and FC assays. All hydrolysates were shown to have antioxidant activity. Results from the ABTS assay showed that the permeate ($<$ 4 kDa) had significantly higher antioxidant activity compared to crude hydrolysate and retentate ($>$ 4 kDa). Cod protein hydrolysate prepared with citric acid showed the lowest antioxidant activity by the ABTS assay, indicating that scavenging could be dependent on pH. Antioxidant activity increased with increasing content of free tyrosine and free aromatic amino acids. Results from the FC assay did not correlate with results from the ABTS assay. However, these methods differ in what they are measuring, so correlation should be interpreted carefully.

The ACE inhibitory activity of hydrolysates was also determined by two methods, spectrophotometric by Cushman and Cheung (1971) and fluorescence by Sentandreu and Toldrá (2006). After a thorough investigation, a well functioning protocol for Cushman and Cheung was implemented. For Sentandreu and Toldrá's method, some adjustments were needed in the fluorimeter. ACE inhibitory activity found by Sentandreu and Toldrá's method was very low compared to the previous studies. This method did not confirm the need for membrane filtration since UF fractions had lower ACE inhibitory activity. Cushman and Cheung's method showed significantly higher inhibition, but only three hydrolysates were analysed.

Storage time of hydrolysates could have influenced the properties of hydrolysates. Species, part of fish used for analysis, hydrolysis conditions influence the bioactivities. It was concluded that fish protein hydrolysates could have great potential to be used in foods and nutraceuticals, but more studies are needed for evaluation of biofunction availability in protein hydrolysates during storage and after digestion.

# Sammendrag

Utilstrekkelig utnyttelse av fiskerestråstoff er et av hovedproblemene for fiskeindustrien i dag. Ressursene i havet er begrensede, og restråstoff som ikke er godt nok utnyttet kan skape miljøproblemer og samtidig påvirke økonomien i fiskeri og akvakultursektoren. Det er derfor av stor interesse å utnytte biprodukter så optimalt som mulig. Selv om situasjonen i Norge er relativt god, har hvitfisksektor et potensial for forbedring. Utnyttelse kan optimaliseres ved å utvinne bioaktive forbindelser fra restråstoff. På grunn av egenskapene til disse forbindelsene kan de brukes som matingredienser eller til produksjon av legemidler.

Målet med denne oppgaven var å undersøke bioaktive egenskaper til proteinhydrolysater, forbedre analytiske metoder og utføre membranfiltrering på noen hydrolysater. Sammenheng mellom strukturelle og bioaktive egenskaper av hydrolysater ble også undersøkt. Proteinhydrolysater fra torskehoder produsert av SINTEF Ocean i pilotprosjektet HEADS UP og kommersielt produsert laskeproteinhydrolysat ble analysert for antioksidantaktivitet og blodtrykkssenkende effekt (ACE inhiberende aktivitet). I tillegg ble strukturelle egenskaper som totalt innhold og fri innhold av aminosyrer, molekylvektfordeling, proteininnhold, og mengde syreløselige peptider bestemt. To hydrolysater ble membranfiltrert for å finne ut om molekylvekt påvirket bioaktivitetene, men separasjonen var ikke så skarp som ønsket. Ett torskehydrolysat ble filtrert to ganger for å forsøke å forbedre separasjonsgraden. Dette forbedret separasjonen til en viss grad.

Antioksidantaktivitet ble målt med to forskjellige metoder: ABTS metoden og redusering av FC-reagens. Resultatene viste at permeat ($< 4$ kDa) hadde den høyeste inhiberingen av ABTS radikaler, sammenlignet med ufiltrerte hydrolysater og retentat ($> 4$ kDa). Torskeproteinhydrolysat som ble laget med sitronsyre viste den laveste inhiberingen av ABTS radikaler, noe som tyder på at antioksidant aktivitet kan være avhengig av pH. Bidrag fra fri tyrosin og frie aromatiske aminosyrer ble også observert. Det var ingen korrelasjon mellom resultatene fra FC metoden og ABTS metoden. De to metodene måler forskjellige ting, og korrelasjonen må tolkes med forsiktighet.

ACE inhiberende aktivitet ble også bestemt ved to metoder: spektrofotometrisk ved Cushman og Cheungs metode (1971) og fluorescens ved Sentandreu og Toldrás metode (2006). Etter grundig etterforskning ble en velfungerende prosedyre implementert for Cushman og Cheungs metode. For Sentandreu og Toldrás methode ble instillinger i fluorimeteret justert. ACE inihiberende aktivitet funnet med Sentandreu og Toldrás metoden, var svært lav sammenlignet med tidligere studier. Denne metoden bekreftet heller ikke behov for membranfiltrering, siden filtrerte fraksjoner har vist enda lavere ACE inhiberende aktivitet. Cushman og Cheungs metode viste bedre inhibering, men bare tre hydrolysater ble analysert.

Lagringstiden og betingelser kan ha påvirket egenskaper til fiskeproteinhydrolysater. Fiskeart, restråstoff-fraksjoner og hydrolysebetingelser påvirker bioaktiviteter. Det ble konkludert med at fiskeproteinhydrolysater har stort potensial for bruk i matvarer og legemidler, men flere studier er nødvendig for evaluering av bioaktiviteter i proteinhydrolysater under lagring og etter fordøyelsen.

# Preface

This thesis was carried out spring 2019 as a part of the master's degree program in Industrial Chemistry and Biotechnology. The work took place at the Department of Biotechnology and Food Chemistry at the Norwegian University of Science and Technology.

I would like to thank my supervisor, Professor Turid Rustad, for guidance, patience, and support during this whole process. I also wish to thank Oskar Speilberg for his help with experiments, Siri Stavrum and Janna Cropotova for their help in the laboratory. I would like to thank Veronica Hjellnes for all her help with membrane filtration at Kalvskinnet. Days in the laboratory would not be the same without the students who were working there.

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Trondheim, June 2019

Aleksandra Stensen

# Table of Contents

**Abstract** i

**Sammendrag** i

**Preface** ii

**Table of Contents** v

**List of Tables** viii

**List of Figures** x

**Abbreviations** xi

**1 Introduction** 1
- 1.1 Background and motivation . . . 1
- 1.2 Fish by-products or fish rest raw material . . . 2
- 1.3 Fish protein hydrolysate (FPH) . . . 3
- 1.4 Enzymatic hydrolysis . . . 4
- 1.5 Properties of fish protein hydrolysates . . . 5
  - 1.5.1 Antioxidant activity . . . 6
  - 1.5.2 ACE inhibitory activity . . . 11
  - 1.5.3 Ultrafiltration . . . 15
  - 1.5.4 Challenges in the applications of FPH . . . 16
- 1.6 Aims of the thesis . . . 17

**2 Materials and methods** 19
- 2.1 Overview . . . 19
- 2.2 Analysis of the fish protein hydrolysates . . . 19
  - 2.2.1 Determination of degree of hydrolysis . . . 19
  - 2.2.2 Determination of soluble protein concentration . . . 20
  - 2.2.3 Fractionation of hydrolysates by ultrafiltration (UF) . . . 21
  - 2.2.4 Determination of molecular weight distribution . . . 21
  - 2.2.5 Total amino acid and free amino acid amount and composition . . . 22
  - 2.2.6 Acid soluble peptides . . . 23

2.2.7 ACE inhibitory activity . . . 23
2.2.8 Antioxidant activity assays . . . 25
2.3 Statistical Analysis . . . 26

**3 Results and discussion** **29**
3.1 Degree of hydrolysis . . . 29
3.2 Protein content . . . 30
3.3 Amino acid content and composition . . . 32
3.3.1 Total amino acids (TAA) . . . 32
3.3.2 Free amino acids (FAA) . . . 33
3.4 Molecular weight distribution . . . 35
3.5 Ultrafiltration . . . 39
3.6 Acid soluble peptides . . . 41
3.7 Antioxidant activity . . . 43
3.7.1 ABTS radical scavenging activity . . . 44
3.7.2 Folin-Ciocalteu reducing capacity . . . 45
3.8 ACE inhibitory activity . . . 48
3.8.1 Method by Sentandreu and Toldrá . . . 49
3.8.2 Method by Cushman and Cheung . . . 51
3.9 Principal Component Analysis . . . 54

**4 Conclusion** **57**

**5 Further work** **59**

**Bibliography** **60**

**Appendices** **71**

**A Raw materials** **71**

**B Degree of hydrolysis** **73**

**C Protein concentration - the Lowry method** **75**

**D Amino acid content and composition** **77**
D.1 Total amino acid content and composition . . . 77
D.2 Free amino acid content and composition . . . 80

**E Molecular weight distribution** **83**
E.1 Chromatograms of standard proteins . . . 83
E.2 Determination of the peptide size . . . 85

**F Ultrafiltration** **87**

**G Acid soluble peptides** **89**

**H Antioxidant activity assays** **91**
H.1 ABTS radical scavenging activity . . . . . 91
H.2 Folin-Ciocalteu reducing capacity . . . . . 94

**I ACE inhibitory activity** **97**
I.1 Method by Sentandreu and Toldrá . . . . . 97
I.2 Method by Cushman and Cheung . . . . . 99
I.2.1 Hippuric acid extraction . . . . . 99
I.2.2 Determination of $IC_{50}$ . . . . . 99

**J Pearson correlation coefficient** **101**

# List of Tables


1.1 Antioxidant activity of fish protein hydrolysates made from by-products. . . . . 10
1.2 ACE inhibitory activity of fish protein hydrolysates made from by-products. . . 14

3.1 Protein concentrations and percent for all hydrolysates and their fractions. . . . 31
3.2 Amount of free amino acids found in the crude protein hydrolysates. . . . . 34
3.3 Ultrafiltration volumes and dry matter content for crude hydrolysates and their fractions. . . . . 40
3.4 Dry matter and soluble protein content in fractions of T1H and SPH. . . . . 40
3.5 Results for two antioxidant activity assay for all hydrolysates. . . . . 43
3.6 Comparison of FC assay. . . . . 46
3.7 $IC_{50}$ values for UF fractions of T1H and SPH. . . . . 50
3.8 $IC_{50}$ values by Cushman and Cheung's method. . . . . 53

A.1 Hydrolysis conditions and harvest time for cod protein hydrolysates. . . . . 72

C.1 Absorbance measured at 750 nm for different concentrations of BSA (Protein content). . . . . 75
C.2 Protein content in cod protein hydrolysates by SINTEF Ocean. . . . . 76

D.1 Total amino acid composition (T1H, T2H, T3H, T4H). . . . . 78
D.2 Total amino acid composition (T5H, T6H, T13H, SPH). . . . . 79
D.3 Free amino acid amount and composition (T1H, T2H, T3H, T4H). . . . . 80
D.4 Free amino acid amount and composition (T5H, T6H, T13H, SPH). . . . . 81
D.5 Free amino acid amount and composition of T1H and its fractions. . . . . 81
D.6 Free amino acid amount and composition of SPH and its fractions. . . . . 82

E.1 Standards of proteins used for determination of peptide molecular weight. . . . 85
E.2 Approximate molecular weights of peptides presented in hydrolysates. . . . . 85

G.1 Absorbance measured at 750 nm for different concentrations of BSA (ASP). . . 89
G.2 Amount of acid soluble peptides present in crude protein hydrolysates and their fractions. . . . . 90

H.1 Absorbance values for standard used in ABTS assay. . . . . 92
H.2 Absorbance at 734 nm for crude protein hydrolysates and UF fractions. . . . . 93
H.3 Absorbance values for standard used in FC assay. . . . . 94

H.4 Absorbance at 725 nm for crude protein hydrolysates. . . . . 95

I.1 Equations for each concentration of inhibitor (T4H). . . . . 98
I.2 Absorbance values of hippuric acid. Mixing time 60 s. . . . . 99
I.3 Absorbance values of hippuric acid. Mixing time unknown. . . . . 99
I.4 Absorbance values and ACEI for T6H. . . . . 100

J.1 Correlation matrix of the studied parameters for crude protein hydrolysates. . . 101

# List of Figures


1.1 Methods for recovery of proteins and peptides from rest raw material. . . . . 3
1.2 Lipid oxidation reactions. . . . . 7
1.3 Chemical structures of common natural antioxidants. . . . . 8
1.4 Chemical structures of common synthetic antioxidants. . . . . 8
1.5 ACE inhibition mechanism. . . . . 12
1.6 HHL hydrolysis by angiotensin-converting enzyme. . . . . 14
1.7 Example of UF membrane particle separation. . . . . 16

2.1 Workflow of the experiments. . . . . 20
2.2 Theoretical chromatogram from gel filtration. . . . . 22
2.3 Protocol for determination of ACE inhibition activity. . . . . 24

3.1 Values for the degree of hydrolysis for crude protein hydrolysates. . . . . 29
3.2 The total amount of amino acids for crude protein hydrolysates. . . . . 33
3.3 Amount of free amino acids present in the crude protein hydrolysates and their UF fractions. . . . . 35
3.4 Gel filtration chromatogram of T1H, T2H, T3H and T4H. . . . . 36
3.5 Gel filtration chromatogram of T5H, T6H, T13H and SPH. . . . . 36
3.6 Gel filtration chromatogram for crude T1H and its UF fractions. . . . . 37
3.7 Gel filtration chromatogram of permeate of T1H. . . . . 38
3.8 Gel filtration chromatogram of crude SPH and its fractions. . . . . 39
3.9 Acid soluble peptides in the crude protein hydrolysates. . . . . 41
3.10 Acid soluble peptides and soluble protein in crude protein hydrolysates and their UF fractions. . . . . 42
3.11 ABTS radical scavening activity for crude protein hydrolysates and their UF fractions. . . . . 45
3.12 $IC_{50}$ values for crude protein hydrolysates. . . . . 49
3.13 Hippuric acid concentrations as function of absorbance. Vortex time 60 s. . . . 52
3.14 Hippuric acid concentrations as function of absorbance. Vortex time unknown. 53
3.15 Correlations found by PCA. . . . . 55

A.1 Samples of fish protein hydrolysates analysed in this thesis. . . . . 71

C.1 Standard curve of BSA used to determine protein concentrations. . . . . 76

D.1 Raw data for SPH obtained from HPLC analysis. . . . . 78

E.1 Chromatogram of Aprotinin. . . . . 83
E.2 Chromatogram of B12. . . . . 84
E.3 Chromatogram of Cytochrome C. . . . . 84
E.4 Calibration curve for estimation of peptide molecular weight. . . . . 86

G.1 Standard curve of BSA (ASP). . . . . 90

H.1 Standard curve of propyl gallate (ABTS). . . . . 92
H.2 Standard curve of propyl gallate (FC). . . . . 94

I.1 Fluorescence plotted against time for different concentrations of T4H. . . . . 97
I.2 Correlation between ACEI and concentrations of T4H. . . . . 98
I.3 ACEI plotted against concentrations of T6H for determining of $IC_{50}$ value. . . . 100

# Abbreviations

| | | |
|---|---|---|
| ABTS | = | 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| ACE | = | Angiotensin-I converting enzyme |
| ACEI | = | Angiotensin-I converting enzyme inhibition |
| BSA | = | Bovine Serum Albumin |
| CPH | = | Cod protein hydrolysate |
| Da | = | Dalton |
| DH | = | Degree of hydrolysis |
| EAA | = | Essential amino acids |
| ET | = | Electron transfer |
| FAA | = | Free amino acids |
| FC | = | Folin-Ciocalteu |
| FPH | = | Fish protein hydrolysate |
| FPLC | = | Fast protein liquid chromatography |
| HAT | = | Hydrogen atom transfer |
| HPLC | = | High-pressure liquid chromatography |
| Mw | = | Molecular weight |
| MWCO | = | Molecular weight cut-off |
| P | = | Permeate |
| PCA | = | Principal component analysis |
| PG | = | Propyl gallate |
| R | = | Retentate |
| Rcf | = | Relative centrifugal force |
| ROS | = | Reactive oxygen species |
| RP-HPLC | = | Reversed phase high-pressure liquid chromatography |
| RPM | = | Revolutions per minute |
| SD | = | Standard deviation |
| SPH | = | Salmon protein hydrolysate |
| TAA | = | Total amino acids |
| UF | = | Ultrafiltration |

Chapter 1

# Introduction

## 1.1 Background and motivation

The fish industry plays an important role in many countries worldwide. Annual production of fish is approximately 140 million tons, where 80% is intended for human consumption (Benhabiles et al., 2012). However, since fish has a significant requirement for processing, the actual amount of fish consumed by people is not so high (Arvanitoyannis and Kassaveti, 2008). Fisheries and fish processing generate a high amount of fish by-products that are currently underutilized. The amount of fish by-products that are discarded without any intention of recovery is highly variable. It depends on species and fishing areas and can vary from 10% to 50% (Caruso, 2016; Zamora-Sillero et al., 2018).

These discards can have a great environmental impact and significant economic effect (Morales-Medina et al., 2016; Zamora-Sillero et al., 2018). The release of fish waste can affect aquatic ecosystems by changing the community structure and biodiversity of benthic assemblages (Arvanitoyannis and Kassaveti, 2008). Underutilization of fish by-products results in economic loss, whereas these by-products could be a potential source of high value-added products instead of being used for fish meal or discarded as waste (Rustad et al., 2011).

The world population is increasing, and the annual consumption of seafood has been doubled during the last three decades (FAO, 2000). Unfortunately, fish resources are limited, and proper management of fish by-products is a topic that is receiving increased interest. Now, by-products are mostly used for the production of silage, fish meal, feed for fur animals and fish oil, and only around 10% are used for direct and indirect human consumption. The main part of the by-products are used for low value products and will not give any high profit for the fish industry (Rustad et al., 2011). However, processes for the production of these low value products are less technically complicated and less expensive. For example, the silage process does not require any additional cost for the enzymes, because they are already present in the raw material. The only expenses are for acid, and the product will have good nutritional properties (Mohr, 1978).

Fish by-products can be a good source of valuable nutrients, especially heads, frames, belly flaps and parts of the viscera like liver and roe. They contain lipids with long-chain omega-2 fatty acids and high quality proteins. Fish by-products contain up to 10-20% (w/w) of the total fish protein. In addition, by-products are often rich in minerals like iron, zinc, iodine and

selenium, and micronutrients like vitamins A, D, riboflavin, and niacin (Olsen et al., 2014). A large number of studies have been published on the possibilities of making high value products like gelatine, collagen, enzymes and specific proteins, and bioactive peptides from by-products (Arvanitoyannis and Kassaveti, 2008; Rustad et al., 2011). Bioactive peptides and the essential amino acids found in fish by-products can have a great potential to be used in the production of drugs and functional foods (Zamora-Sillero et al., 2018). Peptides obtained from the fish by-products were reported to have different bioactivities such as antioxidant, antihypertensive, antibacterial, cholecystokinin release activity and antiproliferative effect (Ahn, Je and Cho, 2012; Chi et al., 2015; Intarasirisawat et al., 2013).

During the last years a large number of studies have been done on different properties of fish by-products, however, very few products have actually reached the market and been sold in larger quantities. There are several reasons for that. Some of them are an overestimation of market possibilities, too high cost of isolation of specific compounds or challenges with providing the necessary documentation if the product is intended for pharmaceutical use (Olsen et al., 2014).

## 1.2 Fish by-products or fish rest raw material

Fish are usually processed to different degrees before reaching the market or being consumed. Depending on the markets or countries, some species are cut to fillets or parts of fillets, while others are not processed at all. Fillets are considered as more convenient products, and in addition it saves time for the consumer, and skills to eviscerate are not necessary. This has created a higher demand for processed fish products, making larger amounts of by-products available (Olsen et al., 2014).

Processing of fish can include bleeding, gutting, beheading, filleting, skinning and trimming (Rustad et al., 2011). Fish by-products or rest raw material is defined as material left after the primary processing of fish manufacturing process and includes head, skin, trimmings, fins, frames, viscera, and roe (He et al., 2007).

In general, most of the rest raw material from fish and aquaculture industry in Norway is utilized compared to many other countries. In 2016, 76% of the seafood rest raw material was utilized, but it was mostly used for the low value products (Richardsen et al., 2016). However, the area that needs some improvement in utilization is the white fish sector. The largest volumes of non-utilized rest raw material are coming from heads, viscera, and liver from white fish. The reasons for the inadequate management of white fish rest raw material are the lack of good technological solutions and economical motivation. A large part of the rest raw material is generated at sea, and the boats are not equipped to process this material, or just do not have enough space on board. The market is requiring products of high quality with good taste, high nutritional value, adequate shelf life, and competitive price.

White fish heads are representing the highest amount of non-utilized rest raw material with around 65 000 tons in 2016 in Norway (Richardsen et al., 2016). However, cod heads can represent a very attractive source of getting high value products. Earlier they were exported to Nigeria and some countries in Asia, though in the last years both price and export have decreased

significantly. It is therefore of interest to find other ways to utilize this material. Cod heads make up almost 20% of the fish weight and contain around 15% of protein (Remme et al., 2018). Protein hydrolysis is one of the solutions to recover and make use of proteins that can further be used for human consumption.

## 1.3 Fish protein hydrolysate (FPH)

Protein hydrolysis can be defined as a process to chemically or enzymatically break down proteins into peptides of varying sizes (Kristinsson and Rasco, 2000). Several methods exist for the recovery of proteins and peptides from fish rest raw material, such as acid or alkaline hydrolysis, autolysis and enzymatic hydrolysis (**Figure 1.1**). Hydrolysis makes it easier to access the functional peptide sequences and makes peptides physiologically active (bioactive) (Ghaly et al., 2013; Shahidi and Zhong, 2008). Enzymatic hydrolysis is in focus in this work.

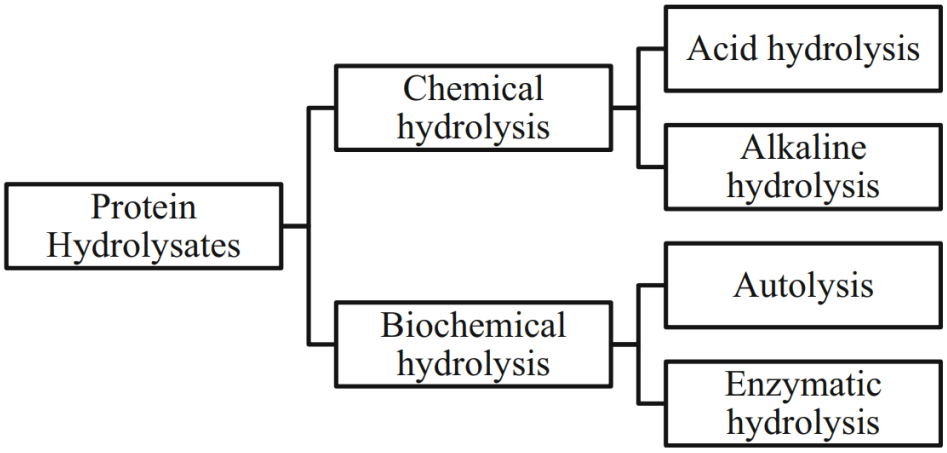



**Figure 1.1:** Methods for recovery of proteins and peptides from rest raw material (Zamora-Sillero et al., 2018).

Chemical hydrolysis (alkaline or acid) is a relatively inexpensive and easy method. However, it is difficult to control, it gives a heterogeneous yield of peptides and reduces the nutritional properties of products (Celus et al., 2007). This process can destroy the natural form of amino acids (L-form), which is essential for the nutritional and physiological activities of peptides. It can also contribute to the production of toxic by-products and D-amino acids (Shahidi and Zhong, 2008).

Enzymatic hydrolysis represents the biochemical method for the production of protein hydrolysates. Autolysis is enzymatic hydrolysis based on the endogenous proteolytic enzymes that are already present in the raw material. It is a simple and cheap operation (Kristinsson and Rasco, 2000). However, this process also has some drawbacks. Endogenous enzymes are a complex mixture, and the different enzymes have different activity requirements. The amount and presence of certain digestive enzymes can vary with season, gender, age, species and part of the species used. It can be difficult to control endogenous enzymes, even when hydrolysis is performed under the same reaction conditions (Kristinsson and Rasco, 2000). Autolysis is not so

reproducible compared to enzymatic hydrolysis based on exogenous enzymes, and properties, quality and stability of protein hydrolysates may vary significantly (Liaset et al., 2000).

## 1.4 Enzymatic hydrolysis

Enzymatic hydrolysis with added enzymes is a process that has some advantages over the previously mentioned processes. For example, it is carried out under milder conditions than chemical hydrolysis. Chemical hydrolysis is often performed at extreme temperatures and pH, and some of the nutritional value of source protein is lost. In contrast, enzymatic hydrolysis allows to control the degree of hydrolysis and retain the nutritional value (Zamora-Sillero et al., 2018). This process allows to control the degree of cleavage of the protein in the substrate. It is possible to obtain hydrolysates with desirable properties by using suitable enzymes and conditions (Kristinsson and Rasco, 2000). Increase in charge density is occurring with a decrease in the molecular size, and this leads to increased solubility.

Protein hydrolysates are made by breaking down proteins into peptides by adding different proteolytic enzymes to the mixture. Usually, the size of the peptides is between 2 and 20 amino acids (Zamora-Sillero et al., 2018). Some of the most commonly used enzymes are Alcalase, papain, pepsin, trypsin, Flavourzyme, and Protamex (Yathisha et al., 2018). Enzyme specificity strongly influences the molecular size and hydrophobicity of hydrolysates (Kristinsson and Rasco, 2000).

Before starting the enzymatic hydrolysis, the raw material is prepared (e.g. by mincing), and if necessary, endogenous enzyme activity is terminated. Termination is usually done by heating the mixture to about 85-95°C. After that, an exogenous enzyme is added at specific conditions required for the raw material used. When the desired degree of hydrolysis is achieved, the enzymatic reaction is stopped by heating or adding strong acids. The remaining suspension is separated into different fractions by centrifugation, sieving, or ultrafiltration. The aqueous phase containing hydrolysates is collected and dehydrated into a soluble powder, which can be included in food formulations (Kristinsson and Rasco, 2000).

Many studies on enzymatic hydrolysis of fish rest raw material have been reported in the literature. Kristinsson and Rasco (2000) have written an extensive review and summarized studies done on this topic. Some of the results were successful, some not, but Kristinsson and Rasco have concluded that there was a potential for the production of fish protein hydrolysates from the rest raw material and hydrolysates could have good functional properties. However, standardized procedures were needed to evaluate the functional properties of fish protein hydrolysates and more studies on the endogenous enzymes should be performed. Slizyte has done a lot of research on fish protein hydrolysates prepared by enzymatic hydrolysis during her PhD thesis (Šližytė, 2004). Both cod and salmon were successfully utilised for the hydrolysis process, and their functional and bioactive properties were studied. It was concluded that properties of FPH were dependent on the hydrolysis time and conditions, what species were utilised and what part of fish was used. The state of the raw material was also important. Melstad (2015) studied the effect of freezing and thawing of the rest raw material (cod heads) on yield and properties of hydrolysates. Protein recovery was slightly higher in the hydrolysates prepared from the fresh cod heads. However, the degree of hydrolysis was lower in the hydrolysates from fresh cod

heads.

## 1.5 Properties of fish protein hydrolysates

The properties of fish protein hydrolysates can be divided into four categories: physiochemical, nutritional, sensory and bioactive. They depend on several factors. Some of the factors are the initial composition of the by-product, type and nature of enzymes used in hydrolysis, conditions of the hydrolysis, such as pH, temperature and time, amount of water added, molecular weight and size of peptides (Sarmadi and Ismail, 2010; Zamora-Sillero et al., 2018). These factors can influence the peptide structure and amino acid sequence. For this reason, the degree of hydrolysis (DH) is a fundamental parameter for the characterization and production of fish protein hydrolysates. It defines peptide bonds that are broken in relation to the original protein (Zamora-Sillero et al., 2018). A high DH indicates more broken peptide bonds and more small peptides, something that will increase the solubility and other properties of the protein.

Physiochemical properties include solubility, foaming, emulsification, water-holding capacity, and the ability to retain and absorb oil (Šližytė, Rustad and Storrø, 2005). These properties are especially important if fish protein hydrolysates are intended for use in food products (Kristinsson and Rasco, 2000). FPHs have been shown to have a good solubility at different ionic strengths and pH levels. This is due to the smaller size of molecules compared to the intact protein and increase in hydrophilicity caused by newly exposed ionizable amino and carboxyl groups of amino acids (Kristinsson and Rasco, 2000). Ionic interactions promote protein-water interactions and lead to an increase in solubility, whereas hydrophobic interactions lead to increased protein-protein interaction and hence decreased solubility. FPH can tolerate strong heat without precipitation. They have good foaming and emulsifying properties and can increase the water holding capacity of food formulations (Halim et al., 2016). Although desirable functional properties are obtained during enzymatic hydrolysis, some undesirable sensory properties such as bitterness can be present in the protein hydrolysates. The composition of the starting material or the hydrolysis process itself can be possible sources for the bitterness (Rustad et al., 2011). A major factor for the bitter taste is hydrophobic amino acids. The bitterness appears because of the particular arrangements of certain chemical groups in peptides. For example, a pair of hydrophobic groups or one hydrophobic group with one basic group together can produce bitterness (Tamura et al., 1990). Extensive hydrolysis to free amino acids can reduce the bitterness. However, free amino acids are not desirable from a functional view. Choosing the most appropriate enzyme for hydrolysis can also help to decrease bitterness. Alcalase is an enzyme with a high preference for hydrophobic amino acids and often yields products of low bitterness (Kristinsson and Rasco, 2000).

The nutritional properties are determined by the content of essential amino acids, which cannot be synthesized by the organism and must be supplied through the diet. Sabeena Farvin et al. (2016) has reported high content of essential amino acids in cod protein hydrolysates. Good digestibility is another advantage of hydrolysates. In addition to the nutritional values, FPHs may also have bioactive properties due to bioactive peptides. These bioactivities can take part in the promotion of human health and may help to prevent chronic diseases (Kim and Wijesekara, 2010). The majority of studies on bioactivities of hydrolysates have been focused on antioxidant activity and ACE inhibitory activity, however other bioactivities such as antibacterial activity,

cholecystokinin release activity and antiproliferative activity have been reported (Zamora-Sillero et al., 2018). Antioxidant activity and ACE inhibitory activity are also the focus in this thesis.

### 1.5.1 Antioxidant activity

#### Lipid oxidation

Before discussing antioxidants, it is important to explain what lipid oxidation is. Lipid oxidation mainly involves the reaction of oxygen with unsaturated fatty acids. It is identified as a free radical chain reaction that occurs in three stages: initiation, propagation, and termination (Schaich et al., 2013).

1. Initiation ($LH \rightarrow L^{\bullet}$)

Since oxygen is normally in the triplet spin state and double bonds are in singlet spin states, the reaction between atmospheric oxygen and lipid double bonds cannot occur directly. An initiator or a catalyst is required to remove an electron from either oxygen or lipid, or to change the electron spin of oxygen. As a result, initial lipid alkyl radicals are formed that can react with oxygen to start lipid oxidation.

2. Propagation

Propagation is the main stage of the whole process. Oxygen reacts with relatively unreactive lipid alkyl radicals, $L^{\bullet}$, transforming them to highly reactive peroxyl radicals, $LOO^{\bullet}$. Further, peroxyl radicals can remove hydrogen from adjacent lipid molecules to form hydroperoxides, LOOH, and a new free radical, $L^{\bullet}$. These new free radicals can again react with oxygen and the previous process is repeated to produce a chain reaction. The chain reaction can continue for a long time as long as oxygen is available and chain is not intercepted. Branching is another step occurring during propagation, where the radical chain reaction expands.

3. Termination

Termination is not about stopping the overall reaction but converting individual lipid radicals to stable non-radical products. Four major mechanisms exist for the formation of these products: racidal recombinations, radical scissions, co-oxidation of non-lipid molecules such as proteins and group eliminations or dismutation. A summary of the whole process is presented in **Figure 1.2**.

Lipid oxidation is an important problem in the storage stability of foods. Once lipid oxidation has started, it will self-propagate and self-accelerate. It is autocatalytic. More than one lipid molecule is oxidized and more than one LOOH is formed per initiation. Very small amounts of pro-oxidants and antioxidants are needed to cause large rate changes. Multiple intermediates and products are produced that change with reaction conditions and time (Schaich et al., 2013).

Precursors of lipid oxidation are always present in foods, and reactions rates, pathways and products can change over time. So this process is dynamic and constantly changing (Schaich et al., 2013). This is one of the most challenging aspects of lipid oxidation. Lipid oxidation is

**Initiation**

$$L_1H \rightarrow L_1^{\bullet}$$

**Propagation**

*Free radical chain reaction established*

$$L_1^{\bullet} + O_2 \rightarrow L_1OO^{\bullet}$$
$$L_1OO^{\bullet} + L_2H \rightarrow L_1OOH + L_2^{\bullet}$$
$$L_2OO^{\bullet} + L_3H \rightarrow L_2OOH + L_3^{\bullet} \text{ etc.} \rightarrow L_nOOH$$

*Free radical chain branching (initiation of new chains)*

$$2L_nOOH \rightarrow L_nO^{\bullet} + L_nOO^{\bullet} + H_2O$$

**Termination**

$$L^{\bullet}, LO^{\bullet}, LOO^{\bullet} \rightarrow \text{non-radical stable products}$$

**Figure 1.2:** The reactions of lipid oxidation: initiation, propagation and termination (adapted from Schaich et al. (2013); Coultate (2009))

not only affecting sensory properties, but it can influence many other aspects of chemistry and quality. Loss of essential amino acids, fat-soluble vitamins, and other bioactive molecules are some of the negative effects of lipid oxidation (Zhong and Shahidi, 2015).

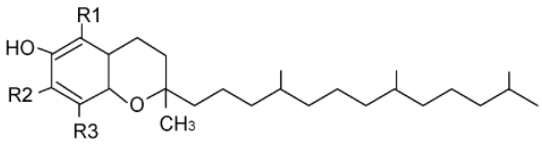
### Antioxidants

There are several ways of reducing or slowing down lipid oxidation, but there are no ways to control it completely. Excluding the initiator or promoter elements, adding antioxidants or controlling the temperature are some of the ways to improve the oxidative stability of lipids. The use of antioxidants is one of the most convenient and effective methods.

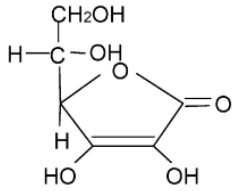
When a compound present in low concentrations significantly delays or inhibit oxidation of a compound that is present in higher concentrations, it can be defined as an antioxidant (Apak et al., 2007). Antioxidants can be chelators, quenchers, oxygen scavengers, free radical scavengers, inhibitors of pro-oxidative enzymes, or antioxidant regenerators (Kendler, 2002). Based on their mode of action, antioxidants can be primary or secondary. Primary antioxidants break the chain reaction of oxidation by scavenging free radical intermediates. They are able to donate a hydrogen atom or an electron to a radical. This prevents the further free radical chain propagation process. Secondary antioxidants can prevent or retard oxidation by suppressing oxidation initiator or accelerators or by regenerating primary antioxidants (Shahidi and Zhong, 2010). Oxidation initiators can be metal ions, singlet oxygen or pro-oxidative enzymes. Chelators are known for binding with metals, and by that preventing the radical formation. Examples are citric acid, phosphoric acid and ethylenediaminetrtraacetic acid (EDTA). Quenchers such as carotenoids deactivate singlet oxygen or other high energy species and redirect it into the less harmful paths.

Compounds that remove or react with oxygen are known as oxygen scavengers. Antioxidant regenerators reduce the radicals formed under the action of primary antioxidants (Berdahl et al., 2010). Ascorbic acid, for example, can regenerate primary antioxidants, tocopherols, from their radicals and then act together in oxidation inhibition (Shahidi and Zhong, 2010).

Antioxidants can be natural or synthetic. Some examples are illustrated in **Figure 1.3** and **Figure 1.4**. Higher plants and their constituents present a good source of natural antioxidants, such as tocopherols and phenols/polyphenols (Zhong and Shahidi, 2015). Fruits and vegetables contain also natural antioxidants, such as ascorbic acid (vitamin C) and carotenoids. Several synthetic antioxidants, such as butylated hydroxy-anisole (BHA), propyl gallate (PG) and butylated hydroxytoluene (BHT), exist and show higher antioxidant activities compared to natural antioxidants. They all act as free radical scavengers. PG inhibits lipid oxidation by trapping peroxy radicals. Synthetic antioxidants are cheap, relatively stable and have been in use for quite a long time. However, due to their toxicity and potential health hazards, use of synthetic antioxidants are becoming limited (Zamora-Sillero et al., 2018; Sabeena Farvin et al., 2016). In addition, consumers desire all natural ingredients. Fish by-products have shown good antioxidant activities and could be an alternative to synthetic antioxidants.

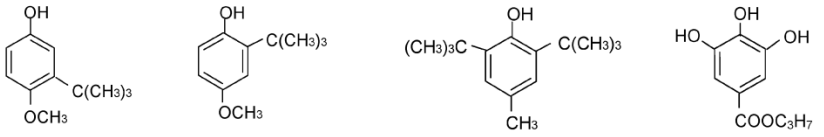



| R1 | R2 | R3 | |
|---|---|---|---|
| $CH_3$ | $CH_3$ | $CH_3$ | α-tocopherol |
| $CH_3$ | H | $CH_3$ | β-tocopherol |
| H | $CH_3$ | $CH_3$ | γ-tocopherol |
| H | H | $CH_3$ | δ-tocopherol |




Ascorbic acid

**Figure 1.3:** Chemical structures of common natural antioxidants; tocopherol and ascorbic acid (Shahidi and Zhong, 2010).




BHA BHT PG

**Figure 1.4:** Chemical structures of common synthetic antioxidants; BHA, BHT and PG (Shahidi and Zhong, 2010).

The effectiveness of antioxidants is usually influenced by their structural features, concentration, temperature, type of oxidation substrate and physical state of the system and also the presence of prooxidants and synergists (Shahidi and Zhong, 2010). Reactivity towards free radicals and other

ROS are determined by the chemical structure of the antioxidant. Correct concentration is also important because at high concentrations antioxidants can exhibit pro-oxidant effects (Zhong and Shahidi, 2015). Efficiency depends on the system environment because the behaviour of antioxidants in bulk oils is not the same as in the oil-in-water emulsions (Shahidi and Zhong, 2010).

To have an antioxidant effect proteins derived from fish by-products should have the two following properties. The first property is the presence of donor electrons and/or an aromatic ring that has several stable configurations. Electrons are transferred to free radicals to neutralize them, and the aromatic ring ensures that peptides donating electrons will not become free radicals themselves. The second property is having hydrophobic character, which is important if the antioxidants are going to be used in e.g. muscle foods. This property helps antioxidants go through the bilipid cell membrane structure into the cell where free radicals are created. Peptides can also act as antioxidants by chelating transition metals and having ferric-reducing power (Shahidi and Zhong, 2008). Marine bioactive peptides have been reported to be effective in scavenging free radicals and reactive oxygen species and in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Kim and Wijesekara, 2010).

Antioxidant activity of proteins is associated with peptide size and molecular weight, composition, sequence, hydrophobicity of free amino acids and peptides (Lassoued et al., 2015; Mazorra-Manzano et al., 2018; Li et al., 2004). Peptides with a molecular weight between 0.5 and 1.5 kDa and chains of 5-16 amino acids have shown to exhibit strong antioxidant effect (Li et al., 2004). Several authors have reported that the presence of hydrophobic amino acids and at least one residue of histidine, phenylalanine, tryptophan or tyrosine in the peptide structure could affect the antioxidant activity of protein hydrolysates (Moayedi et al., 2017; Zamora-Sillero et al., 2018). The imidazole group of histidine residue can participate in hydrogen atom transfer and single electron transfer reactions in order to bind metal ions or neutralize free radicals. Phenylalanine can form more stable hydroxylated derivatives by scavenging hydroxyl radicals. In addition, hydrophobic amino acids make it easier to access the hydrophobic polyunsaturated chain of fatty acids within cell membranes. Unsaturated fatty acids are highly susceptible to oxidative damage (Aluko, 2012). Acidic amino acids, such as glutamic acid and aspartic acid can be chelators of metal ions due to carboxyl and amino groups on the side chains (Zamora-Sillero et al., 2018).

It was observed that contribution to the antioxidant activity of individual free amino acids is slightly lower than the additive effect of peptide amino acids. However, the effect of individual free amino acids can depend on the nature of the free radical and the reaction medium (Aluko, 2012). It was also suggested that the antioxidant capacity of the protein hydrolysates was dependent on the degree of hydrolysis (DH). A higher degree of hydrolysis showed higher antioxidant capacity. It increased the level of carboxyl groups, which lead to the availability of more free electrons (Jamdar et al., 2010; Aluko, 2012).

**Table 1.1** summarizes some of the findings done on the antioxidant activity activity of fish by-products in the recent years. Ahn, Je and Cho (2012) isolated octapeptide Phe-Leu-Asn-Glu-Phe-Leu-His-Val from salmon by-product protein hydrolysate which showed DPPH and ABTS radical scavenging activity and strong ferric reducing activity (Ahn, Je and Cho, 2012). Chi et al. (2015) has hydrolyzed skin from Bluefin leatherjacket, and hydrolysate produced

**Table 1.1:** Antioxidant activity of fish protein hydrolysates made from by-products (Zamora-Sillero et al., 2018).

| Source | Enzyme | Purified sequences | References |
|---|---|---|---|
| Salmon (Scientific name not specified), Pectoral fin | Alcalase, Flavourzyme, Neutrase, papain, Alcalase, pepsin | FLNEFLHV | Ahn et al. (2014) |
| Bluefin leatherjacket (*Navodon septentrionalis*), Heads | Papain | WEGPK, GPP, GVPLT | Chi et al. (2015) |
| Skate (*Taja porosa*), Cartilage | Trypsin and Alcalase | FIMGPY, GPAGDY, IVAGPQ | Pan et al. (2016) |
| Pacific cod (*Gadus macrocephalus*), Skin gelatin | - | LLMLDNDLPP | Himaya et al. (2012) |

with Alcalase showed the highest antioxidant activity against DPPH$^{\bullet}$, HO$^{\bullet}$ and $O_2^{\bullet -}$ radicals. They assumed that the presence of hydrophobic and/or aromatic amino acids had influenced the bioactive property. Sequences of purified peptides from this study are presented in **Table 1.1**. In the other study by Pan et al. (2016) three bioactive hexapeptides were isolated (Phe-Ile-Met-Gly-Pro-Tyr, Gly-Pro-Ala-Gly-Asp-Tyr and Ile-Val-Ala-Gly-Pro-Gln) from *Raja porosa* cartilage. They demonstrated good scavenging activities against DPPH$^{\bullet}$, HO$^{\bullet}$, $O_2^{\bullet}$- and ABTS$^{\bullet +}$ because of their small molecular structure and presence of hydrophobic amino acid residues.

### Measurement of antioxidant activity

Several chemical assays, food and biological model systems exist to measure antioxidant activity. These methods are different in terms of substrate, oxidation initiator, antioxidant mechanism, expression of a result, and ease of operation. One of the mechanisms is measuring scavenging activity against certain types of free radicals or reactive oxygen species (ROS) (Zhong and Shahidi, 2015). Further, the evaluation of radical scavenging can be divided into two types of assays: hydrogen atom transfer (HAT) reaction-based assay and single electron transfer (ET) reaction-based assay. Measurement of antioxidant capacity in these assays can be performed using spectrophotometric, fluorometric, chemiluminescent, NMR (nuclear magnetic resonance) methods, among others. HAT assays are measuring the ability of an antioxidant to quench free radicals by hydrogen donation (Sun et al., 2017). Examples of HAT assays are oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), and inhibition of auto-oxidation of induced low-density lipoprotein (LDL).

ET-based assays measure the ability of an antioxidant to transfer electron to reduce an oxidant. This is reflected in the color change of oxidant. There is a correlation between the degree of colour change and the concentration of the antioxidant in the sample. The following equation represents the electron transfer reaction:

$$\begin{aligned} &\textit{Probe(oxidant)} + \textit{e (from antioxidant)} \rightarrow \\ &\textit{reduced probe} + \textit{oxidised antioxidant} \end{aligned} \tag{1.1}$$

Absorbance can decrease or increase depending on the method used. For example, ABTS and DPPH are decolorization assays, where the absorbance is decreasing with increased concentration of antioxidant. Folin-Ciocalteu (FC) assay and FRAP assay show an increase in absorbance as the antioxidant reacts with the chomogenic reagent (Apak et al., 2007).

The ABTS assay is one the most commonly used methods to measure radical scavenging activity due to its simplicity, solubility in both organic and aqueous media and stability over a wide pH range (Nenadis et al., 2007). The FC assay is another simple and reproducible method. It determines total phenolic content (TPC), which is also an important parameter of total antioxidant capacity (Zhong and Shahidi, 2015). This method is based on the reaction of phenolic compounds with Folin-Ciocalteu reagent under alkaline conditions.

Antioxidant activity assays are easy and effective methods, but there are some drawbacks. Formation and stability of coloured radicals are not always easily achievable, especially in ABTS and DPPH assays (Arnao, 2000; Bondet et al., 1997). Both ABTS and DPPH assays are widely used methods, but DPPH is a free radical that is acquired directly without preparation, while for ABTS chemical or enzymatic reactions should be performed before it can be acquired. $ABTS^{\bullet+}$ generation should be performed, and it can be done by reaction with potassium persulfate, for example. These two radicals also differ in solubility; ABTS is dissolved in both organic and aqueous solvents as it was mentioned before, while DPPH is only dissolved in an organic solvent. This can influence the evaluation of hydrophilic antioxidants (Arnao, 2000).

When using the DPPH assay one should be careful, because when DPPH radical has reacted with an antioxidant, absorbance can be decreased by light, oxygen, pH, and type of solvent (Ozcelik et al., 2003). It is also important to mention that DPPH has a narrow linear range of absorbance versus concentration, and smaller molecules are more likely to reach the radical than the larger molecules (Prior et al., 2005).

The mechanism and redox potential of the Folin reagent are unknown, and oxidation of compounds that are not antioxidants can occur, for example, reducing sugars (Huang et al., 2005). It has also been reported that reaction conditions should be selected carefully in order to get consistent and reliable results.

### 1.5.2 ACE inhibitory activity

Another area where marine-derived bioactive peptides have been shown to have a good effect is in reducing high blood pressure. Hypertension is an important risk factor for the development of cardiovascular diseases. More than 1 billion people worldwide were affected in 2015 (Fan

et al., 2019). Angiotensin-converting enzyme is important in the treatment of hypertension because of its participation in several blood pressure-related systems, i.e., the renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS). High activity of ACE leads to increased vasoconstriction and hypertension (Norris et al., 2012).

ACE catalyses the conversion of inactive angiotensin I to potent vasoconstrictor angiotensin II by the removal of the C-terminal dipeptide. This vasoconstrictor stimulates the secretion of aldosterone, which leads to the retention of sodium and water. These actions result in an increase of artery pressure. In addition, ACE is inactivating vasodilator bradykinin by sequentially removing two C-terminal dipeptides (Hernández-Ledesma et al., 2003). Therefore, by inhibiting the action of ACE, the level of angiotensin II is reduced, while bradykinin is present in a higher amount, leading to a lowering of blood pressure. **Figure 1.5** illustrates the mechanism. Two isoforms of ACE exist: testicular ACE (tACE) and somatic ACE (sACE). sACE can be found in various cells and extracellular fluids, while tACE is only present in germinal cells in male testis (Fan et al., 2019).

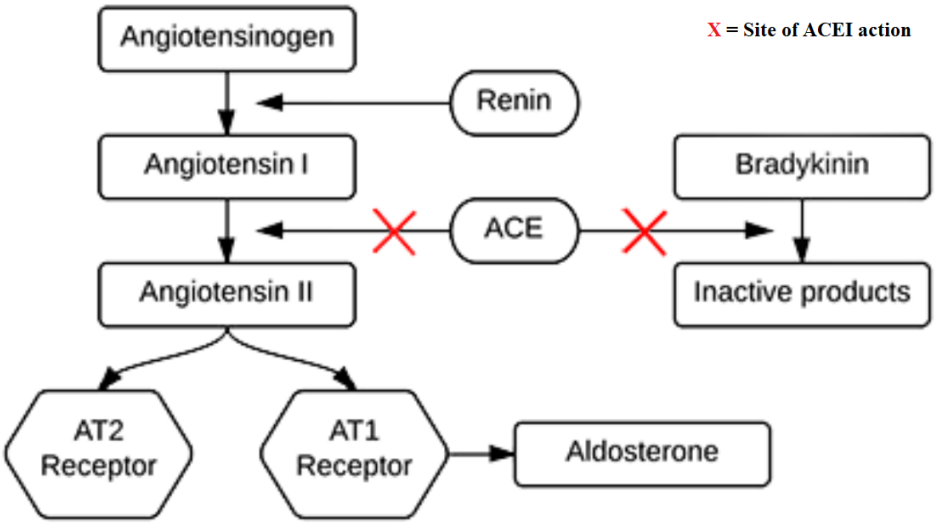



**Figure 1.5:** ACE inhibition mechanism (Barrett et al., 2009).

The most effective treatment of hypertension and heart failure today are synthetic ACE inhibitors, such as Captopril, Enalapril, Alcacepril and Lisinopril. These drugs are effective, but they have certain side effects. Therefore, it is of great interest to find alternatives to these. One of the advantages of using bioactive peptides is reduced potential for toxicity or negative side effects. Another advantage is reduced pressure on kidneys because peptides are metabolized and absorbed into the muscles or used for the synthesis of proteins in the cells (Aluko, 2012). Peptides acting as ACE inhibitors are better tolerated by the human body, however, the dosage required to give any effect is usually higher than for synthetic drugs (Shahidi and Zhong, 2008). To determine the potency of ACE inhibitors $IC_{50}$ values are used. This value is the concentration which gives a 50% inhibition of ACE activity. Protein hydrolysate or peptide is more potent when this value is low (Kim and Wijesekara, 2010; Aluko, 2012). $IC_{50}$ values of drugs are usually in the nanomolar (nM) range, while proteins from food are in micromolar ($\mu$M) or millimolar (mM) range.

Several studies have shown that peptides with low molecular weight and shorter chain length obtained from different fish species exhibited higher ACE inhibitory activity (Yathisha et al., 2018; Lee et al., 2010). The binding channel in the ACE active site is too narrow to accommodate large peptides (Fan et al., 2019). Peptides consisting of 2-4 amino acids were reported to have higher inhibitory potency than peptides of 5-12 amino acids. Free amino acids tyrosine and arginine have shown impressive ACE inhibitory activity (Yathisha et al., 2018). VPP (Val-Pro-Pro) and IPP (Ile-Pro-Pro) are two well-known tripeptides from milk that are easily entering the binding channel and accessing the active site. Therefore, fractionated peptides are assumed to give lower $IC_{50}$ values compared to crude protein hydrolysates. However, bigger peptides also exhibit noticeable ACE inhibitory activity (Sun et al., 2017). It can also be assumed that the interaction between different peptide components contributes to blood pressure reduction (Shahidi and Zhong, 2008).

Salampessy et al. (2015) reported that in the presence of tyrosine and valine in the peptides higher inhibitory activity was observed. They suggested that there was a stronger affinity of inhibitory peptides with these amino acids as C-terminal residues towards ACE. However, these peptides were obtained from the species of fish named trevally, and it could be that amino acid sequence and composition would be different for peptides obtained from other fish species, such as Atlantic cod or salmon. Other studies have also reported that peptides with aromatic and hydrophobic residues, like leucine, valine, alanine, tyrosine, phenylalanine, or tryptophan, at their C-terminus could be potent (Norris et al., 2012). Hydrophilic peptides are less accessible to the active site of ACE and will result in weak or no inhibitory activity (Li et al., 2004). Positively charged amino acids are also good inhibitors (Shahidi and Zhong, 2008). The last four amino acid residues at the C-terminal of long-chain peptides have been shown to influence ACE inhibitory activity. Tyrosine and cysteine, or histidine, tryptophan, and methionine at the C-terminal contribute to ACE inhibition Aluko (2012).

Some peptides from fish by-products are summarized in **Table 1.2**. Intarasirisawat et al. (2013) have reported $IC_{50}$ of 2.49 mg/mL for skipjack roe protein hydrolysate. After ultrafiltration the $IC_{50}$ value for permeate was even lower, 0.76 mg/mL. They have identified several ACE inhibitory peptides, and the most potent was hexapeptide Met-Leu-Val-Phe-Ala-Val (MLVFAV). Ahn, Jeon, Kim and Je (2012) have evaluated ACE inhibitory capacity of protein hydrolysates from salmon pectoral fin and determined lowest $IC_{50}$ (0.36 mg/ml) for hydrolysate prepared with Alcalase. The authors identified three peptides (Val-Trp-Asp-Pro-Pro-Lys-Phe-Asp, Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe and Phe-Asn-Val-Pro-Leu-Tyr-Glu). Another study by Gu et al. (2011) hydrolyzed Atlantic salmon skin protein and found two dipeptides (Ala-Pro and Val-Arg) to be the major contributors to the ACE inhibitory capacity peptides.

### Methods for determination of ACE inhibition activity

Different methods for measuring ACE inhibition are described in the literature. The most common are methods using HHL, AGPP or FAPGG as substrates. One of the first methods described in the literature is by Cushman and Cheung (1971), where HHL is used as a substrate. It is based on the action of ACE enzyme on hippuryl-L-histidyl-L-leucine (HHL) which results in the formation of hippuric acid. This reaction is stopped by adding hydrochloric acid. Addition of ethyl acetate extracts the released hippuric acid. Ethyl acetate is evaporated and the hippuric acid is redissolved in deionised water. The amount of formed hippuric acid is determined by

**Table 1.2:** ACE inhibitory activity of fish protein hydrolysates made from by-products (Zamora-Sillero et al., 2018).

| Source | Enzyme | Purified sequences | References |
|---|---|---|---|
| Skipjack (*Katsuwana pelamis*), Roe | Alcalase | DWMKGQ, MLVFAV, MCYPAST, FVSACSVAG, LADGVAAPA, YVNDAATLLPR, DLDLRKDLYAN | Intarasirisawat et al. (2013) |
| Salmon, Pectoral fin | Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, trypsin | VWDPPKFD, FEDYVPLSCF, FNVPLYE | Ahn et al. (2012) |
| Pacific cod (*Gadus macrocephalus*), Skin gelatin | Pepsin + trypsin + $\alpha$-chymo-trypsin | LLMLDNDLPP | Himaya et al. (2012) |
| Antarctic krill (*Euphausia superba*), Tail | Themoase PC10F | VW, LKY | Hatanaka et al. (2009) |

measuring absorbance at 228 nm. The mechanism for this method is shown in **Figure 1.6**. Hippuric acid and histidylleucine are formed.

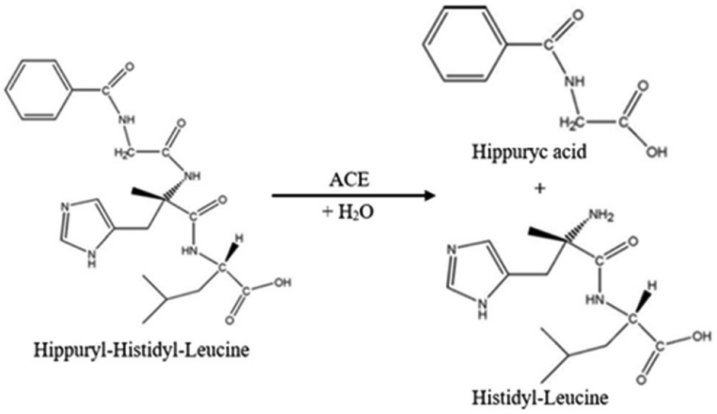



**Figure 1.6:** HHL hydrolysis by angiotensin-converting enzyme (Ahmad et al., 2017).

This method is still frequently used for measuring of ACE inhibitory activity. However, other authors have done several modifications, which affect the buffer composition, the enzyme/substrate ratio, and the reaction time (Hernández-Ledesma et al., 2003). The most used buffer for this method is sodium borate buffer, but potassium phosphate buffer and phosphate buffered-saline

(PBS) are also mentioned in other studies. From the study of Cushman and Cheung (1971) it was observed that activity of ACE was optimal in the presence of 300 mM NaCl. They have studied different concentrations of NaCl, from 0 to 600 mM (Cushman and Cheung, 1971).

Another method described in the literature is using N-(3-[2-furylacryloyl]-Phe-Gly-Gly (FAPGG) as a substrate. It was first introduced by Holmquist et al. in 1979. This method is easier to perform since the step with extraction of the product from the reaction mixture with an organic solvent is not required. The additional source of error from the extraction step is therefore avoided. In this second method ACE is hydrolysing FAPGG to furylacryol-L-phenylalanine (FAP) and glycylglycine (GG). This leads to decrease in absorbance which can be measured at 340 nm. This method has experienced different modifications with fixed time conditions. In a study by Lahogue et al. (2010) these two methods were compared, and it was concluded that FAPGG was more stable than HHL, and less chemicals per sample were used to perform experiment with FAPGG as a substrate.

Studying ACE inhibition with AGPP as a substrate has a lot of advantages over other used methods, because it is easy to carry out, it allows analysis of a high number of samples in a relatively short time and it has only one-step reagent. In addition, this method is accurate and sensitive (Sentandreu and Toldrá, 2006b). The method by Sentandreu and Toldrá is based on using the intramolecularly quenched fluorescent tripeptode o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe($NO_2$)-Pro) as substrate. Hydrolysis of this molecule by ACE leads to formation of fluorescent product o-aminobenzoylglycine (Abz-Gly), which can be measured by using appropriate excitation and emission wavelengths.

All laboratory experiments, regardless of the type of substrate, need large amount of costly chemicals and labor. Recently, computational methods are becoming widely used to predict and understand relationship between peptide structure, bioactivity and their formation during proteolysis. *In silico* (computer-predicted) proteolysis and quantitative structure-activity relationship (QSAR) modelling are used complementary to experimentally work (Pripp, 2007). Several studies have been done for screening of inhibitory peptides of angiotensin I-converting enzyme (Wu et al., 2014; Pripp, 2007). Molecular docking is one of the tools used in virtual screening by *in silico* experiment. The protein is held in a rigid conformation and ligand is "docked" into the active site. Interaction between them is "scored" to determine potential bioactivity of candidate compounds (Pripp, 2007). Wu et al. (2014) have tested the Libdock docking method for screening of ACE inhibitory peptides. They have compared the estimated $\log(1/IC_{50})$ and measured $\log(1/IC_{50})$ of five ACE inhibitory peptides, and the results confirmed the validity for Libdock module. Computation simulation technology can provide the possibility to select promising candidates before going to large-scale experimental screening. This technology will not replace the need for *in vitro* and *in vivo* testing, but can contribute to a molecular understanding of bioactivity (Wu et al., 2014).

### 1.5.3 Ultrafiltration

It was documented that peptides with small molecular weight exhibit stronger antioxidant and ACE inhibitory activities (Lee et al., 2010; Intarasirisawat et al., 2013; Raghavan and Kristinsson, 2009). To obtain the desired molecular weight distributions ultrafiltration (UF) membrane system can be used. The major advantage of this system is the possibility to control molecular weight

distribution by adopting an appropriate UF membrane (Jeon et al., 1999).

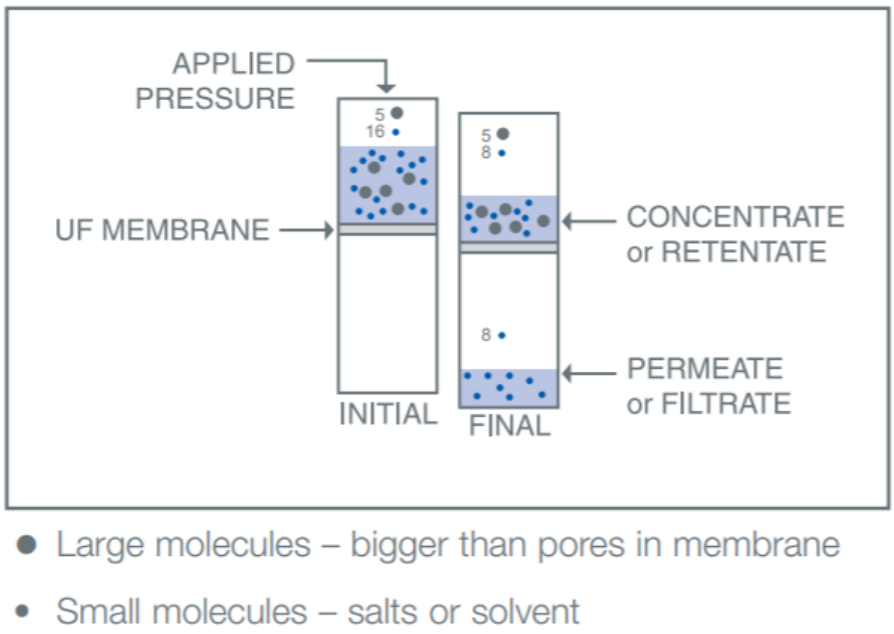



**Figure 1.7:** Example of how UF membrane separate particles according to the MWCO of the membrane (Schwartz, 2003).

Ultrafiltration is a type of membrane filtration where pressure or concentration gradients are used to force water through a semipermeable membrane. This filtration is used for purifying and separating macromolecular solutions of size $10^3$-$10^6$ Da and often applied for filtrating of protein solutions. MW cutoff value is usually expressed in kilodaltons and is the most important characteristic of the ultrafiltration tube (Vas et al., 2008). Water and particles having molecular weight smaller than the cutoff value are going through membrane to the permeate, while high molecular weight components are retained in the retentate (Zydney, 2011). The driving force that pushes peptides through the membrane is usually nitrogen gas (Aluko, 2012). More than one membrane can also be used to obtain several molecular weight fractions (Kristinsson and Rasco, 2000).

### 1.5.4 Challenges in the applications of FPH

Benefits from bioactive peptides found in fish protein hydrolysates have been confirmed in many studies. However, there are not so many studies on the stability of FPHs during storage, processing and consumption (Korczek et al., 2018). Several factors can limit their application in food and pharmaceutical products. Taste and shelf life are the two biggest challenges for the use of protein hydrolysates in foods. It is usual with bitterness and fish odour and taste. Fat content is also desired to be minimal to avoid lipid oxidation, which can greatly influence the taste (Remme et al., 2018).

The high proportion of hydrophobic amino acids is decreasing the water solubility of bioactive peptides. Amino acids can be destroyed by fluctuations in temperature and pH. Cys, Ser, and Thr can be destroyed by alkaline pH, while Asp and Glu are not favoring the acidic environment.

Bioavailability of Lys can be reduced due to the increase in the frequency of the Maillard reaction, caused by the variations in temperature and high pH (Korczek et al., 2018). Freezing and low temperatures can influence the physical and structural properties of proteins/peptides. Oxidation of FPHs is another problem that limits their production. This happens because of the high content of unsaturated fatty acids, which is easily oxidized. Gastrointestinal degradation is also a problem limiting the use of protein hydrolysates. Peptides can lose their bioactivities after oral administration *in vivo*. Often, they do not even reach the target sites before they are degraded by enzymes or the acidic environment in the gastrointestinal tract.

## 1.6 Aims of the thesis

This master's thesis is a continuation of the specialization project performed during autumn 2018, where three cod protein hydrolysates produced by Sintef Ocean as a part of HEADS UP project were analysed for bioactive properties. Antioxidant and ACE inhibition activity was tested. The aim of this thesis was to study more protein hydrolysates, improve the analytical methods and apply membrane filtration on several hydrolysates. In addition, the aim was to analyse correlations between structural and bioactive properties of the hydrolysates. Finally, the influence of storage time and conditions have been discussed.

To learn about structural properties of the hydrolysates the total and free amount and composition of amino acids, molecular weight distribution, protein concentration, degree of hydrolysis and amount of acid soluble peptides were determined. One salmon and one cod protein hydrolysate were ultrafiltrated to separate peptides in two fractions. Molecular weight cut-off of the membrane was 4 kDa.

Antioxidant activity and ACE inhibitory activity was measured on both crude protein hydrolysates and their UF fractions. Each of the bioactivities was measured by two different methods to compare them against each other. For antioxidant activity, the ABTS assay and Folin-Ciocalteu assay were chosen. ACE inhibitory activity was measured by fluorescence method developed by Sentandreu and Toldrá and spectrophotometric method developed by Cushman and Cheung. Analysis of variance (ANOVA) was applied for statistical evaluation. The correlation was evaluated by principal component analysis and Pearson correlation coefficient.

Chapter 2

# Materials and methods

## 2.1 Overview

**Figure 2.1** gives an overview of the work performed during this thesis. The raw material, cod protein hydrolysates (CPH) and salmon protein hydrolysate (SPH), were analysed by different methods. Cod protein hydrolysates were prepared from cod heads by SINTEF Ocean during winter and spring of 2017. Their names start with T and end with H with the number in the middle. Amount of water added varied, with 400 kg for T1H, 100 kg for T6H, 200 kg for T5H and 300 kg for T2H, T3H, T4H and T13H. Enzyme type and concentration were the same for all hydrolysates, except for T3H. Protamex was used for T3H, while a combination of Papain and Bromelain was applied for other hydrolysates. The concentration of Protamex was half the concentration of Papain and Bromelain. The hydrolysate named T13H was added citric acid together with proteolytic enzymes in the hydrolysis process.

The salmon protein hydrolysate was prepared by Hofseth Biocare AS. They make commercial products for human consumption. The hydrolysate is produced according to a standardized process from the fillet of Norwegian salmon. SPH was produced in October 2018 and stored in a cold room at $4^oC$ before arriving at NTNU.

T1H and SPH were filtrated using ultrafiltration to separate proteins in two different fractions, $> 4$ kDa and $< 4$ kDa. Structural properties and bioactivities were analysed using different analytical methods, presented in **Figure 2.1**. All samples were stored in plastic bags in a freezer (-$20^oC$). Protein hydrolysates dissolved in water were stored in the cold room ($4^oC$) for up to two weeks.

## 2.2 Analysis of the fish protein hydrolysates

### 2.2.1 Determination of degree of hydrolysis

The degree of hydrolysis was determined using formol titration as described by Taylor (1957). All hydrolysates, except salmon protein hydrolysate, were analysed by SINTEF Ocean, spring 2017. Measurements for all hydrolysates were performed in triplicates.

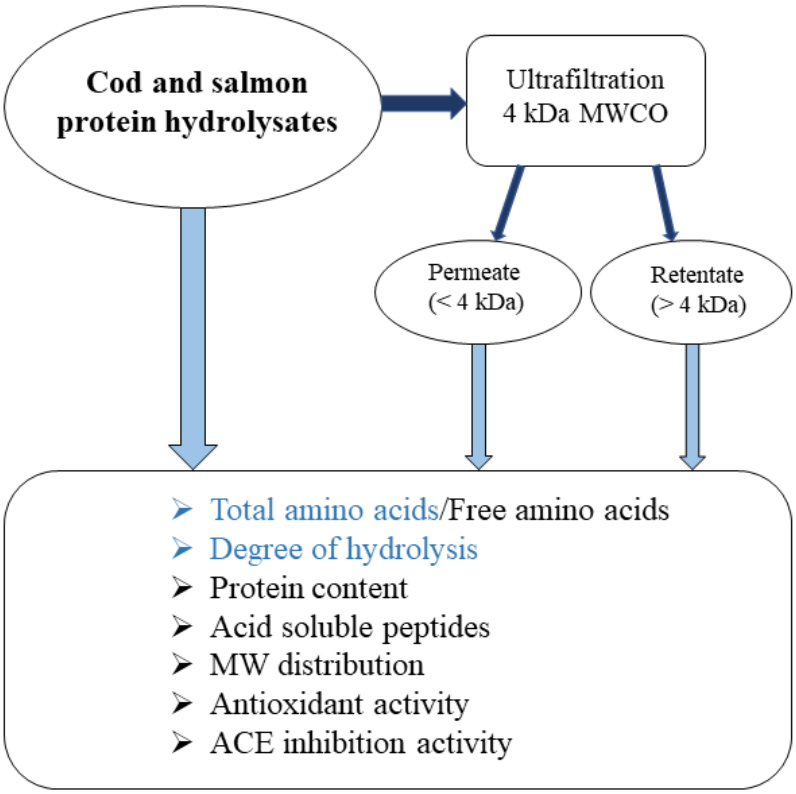



**Figure 2.1:** Workflow of the experiments performed during this master's thesis. Determination of total amino acid content and degree of hydrolysis (highlighted in blue) were performed only for crude protein hydrolysates.

A reaction between formaldehyde and an amino group of an amino acid at neutral or alkaline pH is occurring in this method. This releases a proton that contributes to lowering of pKa of the amino acid-formaldehyde complex. The procedure was started with weighing out 1.5 g of sample and adding distilled water to 50 g. pH was adjusted to 7 by adding 0.1 M NaOH. After that, 10 mL formaldehyde was added, and solution was left for 5 minutes. It was then titrated to a pH of 8.5 with 0.1 M NaOH. The amount of NaOH used for titration was noted down. To calculate DH, the percentage of free amino groups was divided by the total nitrogen content. Detailed calculations are presented in Appendix B.

### 2.2.2 Determination of soluble protein concentration

The protein concentration of fractioned and unfractionated (crude) hydrolysates was determined using the Lowry Method (Lowry et al., 1951). Crude and fractionated protein hydrolysates were prepared by dissolving 100 mg in 10 mL distilled water. Dilution for cod protein hydrolysates was 1:25, but for salmon protein hydrolysate 1:50 was needed.

In the Lowry assay, copper (II) ion reacts with peptide bonds under alkaline conditions to produce a complex, where copper is becoming a monovalent ion. This forms a complex with the aromatic protein residues (tyrosine, tryptophan, and cysteine) and reduces Folin-Ciocalteu reagent that is transformed into an intense blue molecule, heteropolymolybdenum blue (Lowry

et al., 1951). This color change of the sample solution depends on the protein concentration present and can be measured by absorbance at 750 nm.

The procedure for determination of protein concentration consisted of several steps. The first step was to mix 0.5 ml blank/BSA solution/diluted samples with alkaline copper reagent. All tubes were left for 10 minutes. After that, Folin-Ciocalteu reagent (0.25 ml) was added, solutions were mixed and left for 30 minutes. When the time was up, absorbance at 750 nm was measured. A detailed description of the calculations can be found in Appendix C.

### 2.2.3 Fractionation of hydrolysates by ultrafiltration (UF)

T1H (from cod head) and SPH (from salmon fillet) were used for fractionation. To prepare hydrolysates for membrane filtration, 1 g of powder was dissolved in 100 mL distilled water. In total, 300-350 mL of each type were made. The samples were centrifuged at 4500 rcf for 10 minutes and filtered through a 0.45 $\mu$m filter. Prior to UF, they were stored in a freezer at $-20°$C.

UF was performed at NTNU Kalvkinnet in collaboration with PhD Candidate Veronica Hammer Hjellnes and Lab Engineer Oskar Speilberg. MMS AG Membrane system was used for fractionation. 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). There were three membrane cells of 28 cm$^2$ in this system. **Figure 1.7** in Chapter 1 illustrates the principle of ultrafiltration.

The prepared solutions of hydrolysates were poured into the feed tank and filtered through. At least 50 mL should be left in the tank to avoid drying out the pumps. The volume left in the tank after the stop of filtration was referred to as a dead volume or retentate. The filtrate was referred to as the permeate. All volumes were written down to calculate the loss of solution.

### 2.2.4 Determination of molecular weight distribution

Molecular weight distribution was determined using gel filtration on a Fast Protein Liquid Chromatography (FPLC) system. This system separates peptides depending on their size. AktaPurifier FPLC system (GE Healthcare Life Sciences, Uppsala, Sweden) was used. Large peptides are leaving the column first and therefore have a short retention time, while smaller peptides stays in the column for a longer period. A theoretical chromatogram is presented in **Figure 2.2**.

Sodium acetate buffer (0.05 M) at pH 5 was prepared and filtered through a 0.22 $\mu$m filter using suction. Hydrolysates were prepared by dissolving 0.1 g powder in sodium acetate buffer (4 mL) and filtering with 0.22 $\mu$m filter. Samples from ultrafiltration were used directly (without dilution) for analysis because of the low concentration of hydrolysate. Both UF fractions and crude protein hydrolysates were analysed.

A Superdex Peptide 10/300 GL column was used for separation. It has an MW-range of 100-7000 Da. The experiment was performed at a flow rate of 0.5 mL/min and absorbance of 280 nm. The measurement was started after the insertion of a syringe with the sample solution. The 100 $\mu$l of the sample solution are going through the column, but to be safe 300-400 $\mu$l were inserted. To estimate the molecular weight of the detected peptide fractions, three standards with

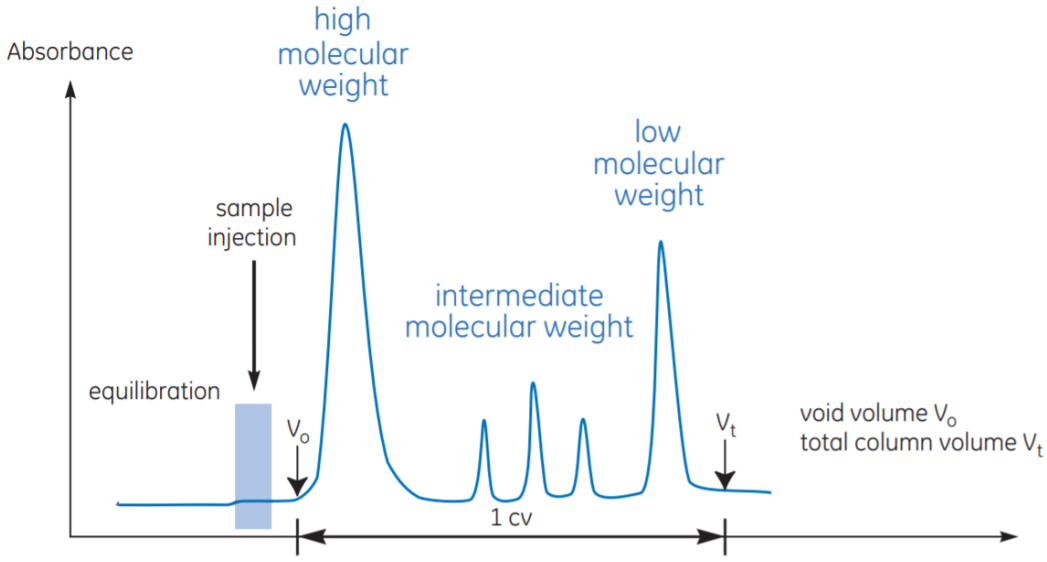



**Figure 2.2:** Theoretical chromatogram from gel filtration (GE Healthcare, 2010).

known molecular weights were used (Aprotinin - 6.5 kDa, B12 - 1.4 kDa and Cytochrome C - 12.4 kDa). Calculations and chromatograms can be found in Appendix E.

### 2.2.5 Total amino acid and free amino acid amount and composition

High-pressure liquid chromatography (HPLC) was used for the determination of the amount of total amino acids (TAA) and free amino acids (FAA). This method allows to separate different amino acids and establish the concentration of each of these amino acids. A Nova-Pak Column Reversed-Phase 4 $\mu$m Spherical Silica was used for HPLC.

#### Total amino acid amount and composition

Results from the total amount of amino acids for cod head protein hydrolysates were taken from the master's thesis of Ayat Asfour. TAA in salmon protein hydrolysate (SPH) was determined as described by Blackburn et al. (1968). First, samples (50 mg) were hydrolyzed in 6 M HCl (2 mL) for 22 hours at 105°C. After cooling, they were transferred to glass beakers using distilled water. To reach a pH of 7, NaOH was added. After that samples were filtrated through Whatman glass microfibre filter GF/C using suction and transferred to measuring flasks of 10 mL. Samples were diluted 1:500, filtered again through 0.22 $\mu$m and transferred to HPLC vials (0.205 mL). The HPLC analysis was conducted by NTNU employee Siri Stavrum.

#### Free amino acid amount and composition

The amount of free amino acids was determined by the protocol of Osnes and Mohr (1985). 1 mL of each hydrolysate (2 mg/mL) was mixed with 0.25 mL of 10% sulphosalicylic acid. Solutions were left in a cold room for 30 minutes. After the time was up, they were centrifuged for 15 minutes at 4500 rcf. The supernatant was mixed again with sulphosalicylic acid and centrifuged to check if all proteins had precipitated. When no more precipitation was observed, samples

were diluted 1:25 with distilled water and filtered using a 0.22 $\mu$m filter. After filtration samples were transferred into HPLC glass vials and delivered to Siri Stavrum for further analysis.

### 2.2.6 Acid soluble peptides

The amount of acid soluble peptides was found using the method described by Hoyle and Merritt (1994). Trichloroacetic acid (TCA) was used to precipitate proteins. Amino acids and small peptides can be dissolved in TCA, but larger peptides and proteins are precipitated.

2 ml of protein solution were mixed with 2 ml of 20% TCA and left at room temperature for 30 minutes. All samples were filtered, crude protein hydrolysates and retentate of T1H were diluted 1:50, while permeates were diluted 1:25, and the retentate of SPH was diluted 1:50. After that, they were analysed using the Lowry method (Lowry et al., 1951).

### 2.2.7 ACE inhibitory activity

Angiotensin-converting enzyme (ACE) inhibitory activity was measured by two different methods, the spectrophotometrical method by Cushman and Cheung (1971) and the fluorescence method by Sentandreu and Toldrá (2006a).

#### Method by Cushman and Cheung (1971)

Three hydrolysates (T5H, T6H, and SPH) were analysed by this method. The principle behind this method is to use a spectrophotometer to measure the amount of hippuric acid produced from the reaction between the substrate, N-hippuryl-histidyl-leucine (HHL), and angiotensin-converting enzyme (ACE). From this, ACE inhibition and $IC_{50}$ value can be calculated. The protocol is based mainly on Cushman and Cheung (1971), but also on two other articles, Dragnes et al. (2009) and Cao et al. (2010). The flowchart of the final protocol is presented in **Figure 2.3**.

HHL (Sigma-Aldrich, cat. no. H1635) with a concentration of 5 mM was used as a substrate. It was prepared by dissolving the required amount in sodium borate buffer, 100 mM, pH 8.3, with 300 mM NaCl. Enzyme, ACE, 5 mU, was dissolved in the same buffer. Both solutions were kept in the freezer until the day of experiment.

ACE-inhibition percentage was calculated in accordance with **Equation 2.1**:

$$ACEI(\%) = \frac{B - A}{B - C} \times 100 \tag{2.1}$$

where A represents absorbance in the presence of ACE, inhibitor and substrate, B absorbance of the substrate and ACE and C absorbance in the presence of only substrate. $IC_{50}$ can be found by plotting ACEI(%) against inhibitor concentrations. Graphs and calculations are presented in Appendix I.

#### Method by Sentandreu and Toldrá (2006a)

In this assay, the substrate, o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe($NO_2$)-Pro) is hydrolysed by ACE, resulting in formation of two products, fluorescent

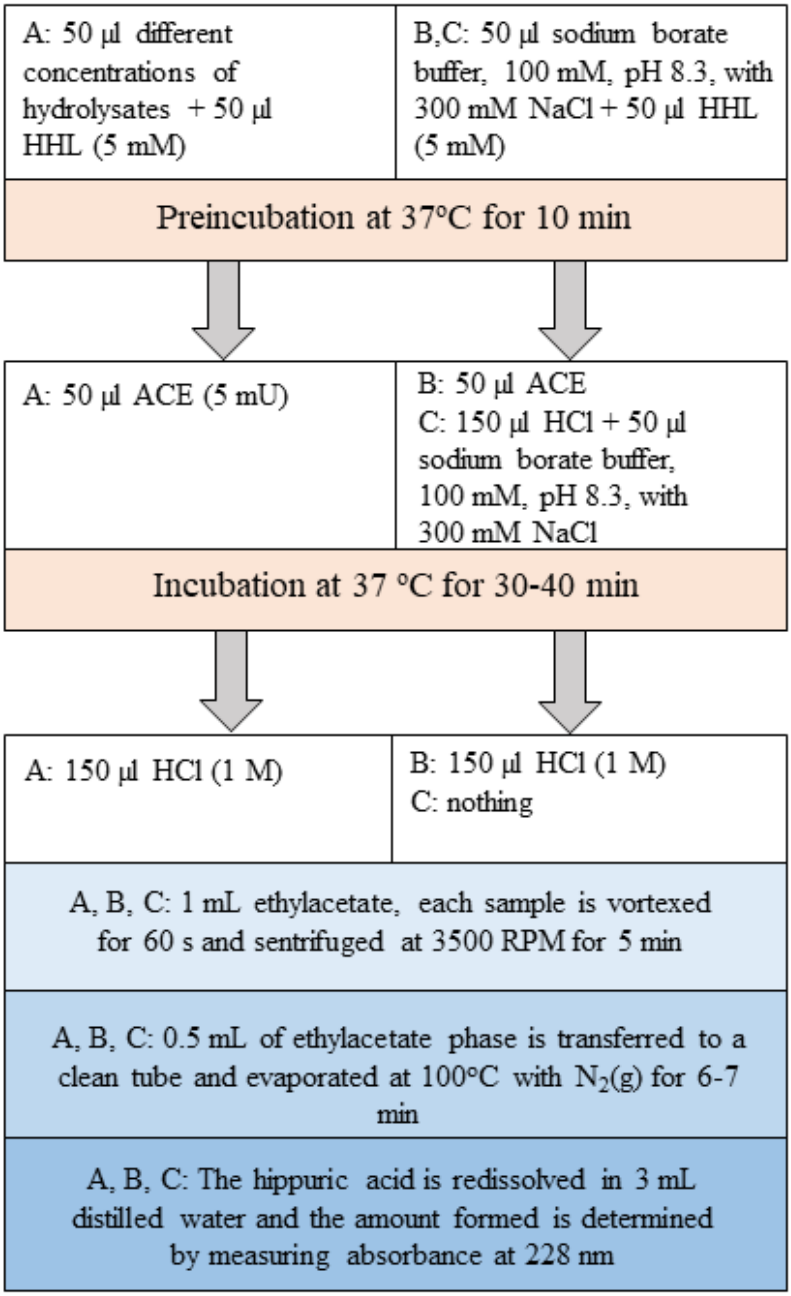



**Figure 2.3:** Flowchart of protocol for spectrophotometric method for determination of ACE inhibition activity (Cushman and Cheung, 1971; Dragnes et al., 2009; Cao et al., 2010).

Abz-Gly and Phe($NO_2$)-Pro. If ACE is inhibited, no fluorescence is detected. By using appropriate wavelengths for excitation (355-375 nm) and emission (400-430 nm), the amount of fluorescent product can be measured by a microplate reader.

Abz-Gly-Phe($NO_2$)-Pro (Bachem, cat. no. M-1100) was dissolved in 150 mM Tris-Base buffer containing 1.125 M NaCl to a concentration of 0.45 mM. Exposure to light should be avoided until use. The enzyme solution was prepared by first dissolving ACE from rabbit lung (Sigma-Aldrich, cat. no. A6778) in 300 mM Tris-base buffer (pH 8.3) with 2 $\mu$M $ZnCl_2$ and

glycerol (1:1). In this state, ACE can be stored in the freezer for more than one year. On the day of the experiment, the enzyme solution was thawed and diluted in 150 mM Tris-base buffer (pH 8.3) to a concentration of 7.5 $\mu$g/mL (enzyme activity of approximately 3 mU/mL). UF fractions were freeze-dried before the experiment. Freeze-drying was performed by NTNU employees Oskar Speilberg and Siri Stavrum. Inhibitor solutions were prepared by dissolving crude powder/UF fraction in 10 mL of 150 mM Tris-Base buffer. Different concentrations were used to evaluate degree of ACE inhibition (0.5, 1, 5, 10, 20, 30 mg/mL for crude hydrolysates and 1, 2, 4, 6, 8, 10 mg/mL for UF fractions).

To determine ACE inhibitory activity, 50 $\mu$l of inhibitor solution and 50 $\mu$l of ACE solution were added to a black microplate and shaken carefully for a few seconds. After that, microplate with inhibitor and enzyme was pre-incubated at 37$^o$C for 10 minutes. The substrate solution was pre-incubated simultaneously in the same conditions. The enzyme reaction was started by adding 200 $\mu$l of substrate solution to the microplate with inhibitor and enzyme. The microplate was gently shaken and fluorescence was measured every 5 minutes for 45 minutes. All measurements were compared to negative control, a sample without inhibitor. In the sample without inhibitor, the amount of fluorescence was expected to increase, while in the samples with inhibitor it was expected to decrease.

TECAN Spark® multimode microplate reader was used as a fluorometer. It was important to choose the correct settings in the software. The number of flashes was set to 15, settle time was 1 ms, and a manual gain of 60 was chosen. Fluorescence was plotted against time for all the concentrations, including negative control (blank). The degree of ACE inhibition (in percent) was calculated using the following **Equation 2.2**:

$$ACEI(\%) = (1 - \frac{slope\ inhibitor}{slope\ blank}) \times 100 \tag{2.2}$$

where “slope inhibitor” and “slope blank” were found from curve of degradation of APGG during a given time interval. To find the slopes, linear regression was applied. In the end, ACEI (%) was plotted against concentrations and inhibitor concentration ($IC_{50}$) was determined.

### 2.2.8 Antioxidant activity assays

Two different spectrophotometric assays were used for measuring antioxidant activity: ABTS and Folin-Ciocalteu. Propyl gallate was used as a standard for both methods. Crude protein hydrolysates, permeate and retentate were analysed.

#### Preparation of hydrolysate solutions

Crude protein hydrolysates (200 mg) were dissolved in distilled water to a total volume of 10 mL. After centrifugation at 4500 rcf for 15 minutes, all samples were filtered. The supernatants were further diluted with methanol (80%) suitably for the antioxidant assay used. Samples were stored in a cold room at $4 - 5$°C for a week. Hydrolysates used for ultrafiltration were prepared by dissolving 1 g in 100 mL distilled water.

### ABTS radical scavenging activity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay is the method often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids (Nenadis et al., 2004). The radical is produced chemically by oxidation with $K_2S_2O_8$. It is reduced in the presence of the antioxidant molecule, giving rise to a weak green colour.

The $ABTS^{\bullet+}$ solution was prepared by mixing 25 ml of 7 mM aqueous ABTS solution and 440 $\mu$l of 140 mM potassium persulfate ($K_2S_2O_8$) solution. This solution was covered with aluminium foil and left at room temperature overnight to react. On the next day, it was diluted with methanol (80%) until an initial absorbance value of $0.75 \pm 0.05$ at 734 nm was reached. 10 mM propyl gallate in 80% methanol was used as a stock solution for making a standard curve. A series of dilutions was made: 10, 20, 30, 40 and 50 $\mu$M.

To start the reaction 2 ml $ABTS^{\bullet+}$ was mixed with 200 $\mu$l extract/standard solutions/blank (80% methanol). After 6 minutes absorbance was measured with water as a reference. The following dilutions of hydrolysates were used for this assay: 1:10 for crude protein hydrolysates and retentates, and 1:5 for permeates.

### Folin-Ciocalteu assay

Folin-Ciocalteu assay is the simplest method that is available for the measurement of phenolic content in products (Agbor et al., 2014). The basis for this method is the oxidation of the phenolic compound in a carbonate solution using the reagent, $3H_2O \times P_2O_5 \times 13WO_3 \times 5MoO_3 \times 10H_2$.

Folin-Ciocalteu phenol reagent consists of heteropoly acids, phosphomolybdic and phosphotungstic acids. Molybdenum and tungsten are in the $6^+$ oxidation state. When these metals are reduced, the colour of the solution becomes blue and the mean oxidation state is between 5 and 6.

This assay was performed by combining the protocols by Singleton et al. (1999) and Nenadis et al. (2007). Propyl gallate in four different concentrations was used as a standard (0.5, 1, 1.5 and 2 mM). 5 ml distilled water, 0.5 ml Folin-Ciocalteu phenol reagent (FCR) and 0.1 ml hydrolysate/standard/blank (80% methanol) were mixed together and left for 3 minutes before adding 1.5 ml 20% $Na_2CO_3$. Solutions were mixed again, and distilled water was added to get a total volume of 10 ml. After one-hour incubation at room temperature, absorbance at 725 nm was measured with water as a reference. Hydrolysates were diluted 1:5 with 80% methanol.

## 2.3 Statistical Analysis

All measurements were performed at least in triplicate, and the results were presented as means $\pm$ standard deviations (SD). One-way analysis (ANOVA) was applied on antioxidant and ACE inhibition assays to assess for any significant differences between the means of crude and fractionated hydrolysates. Differences between the means were considered statistically significant at $p < 0.05$. Pearson correlation coefficient (r) was used for evaluation of the correlation between the properties for crude protein hydrolysates.

Principal Component Analysis, PCA, was performed on the crude protein hydrolysates. PCA is a multivariate technique that reduces the dimensionality of large data sets but retains most of the variation (Ringnér, 2008). It is performed by identifying directions, called principal components. The variation along these directions is maximal. Each sample can be represented by relatively few numbers instead of thousands of variables. It is possible to plot the samples, visually assess differences and similarities between them and determine if they can be grouped (Ringnér, 2008).

Chapter 3

# Results and discussion

## 3.1 Degree of hydrolysis

Degree of hydrolysis for protein hydrolysates varied from 8.36% to 15.81%, with SPH having the lowest DH, and T6H having the highest. Measurements for cod head protein hydrolysates were performed by SINTEF Ocean during HEADS UP project. Processing parameters for the hydrolysis of CPH were quite similar. T1H, T2H, T4H, T5H, T6H, and T13H were treated with two enzymes, Papain and Bromelain under 50$^{o}$C. T3H was treated with Protamex, and enzyme concentration was half of compared to hydrolysates treated with Papain and Bromelain. To T13H, citric acid was added in addition to these two enzymes.

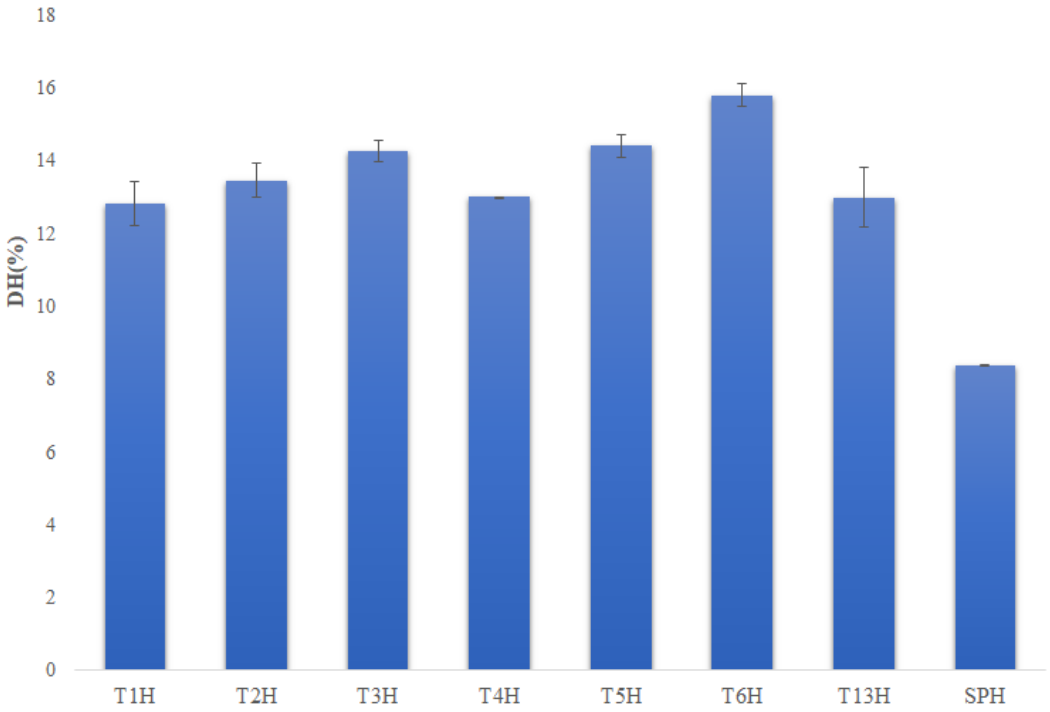



**Figure 3.1:** Values for the degree of hydrolysis for crude protein hydrolysates (mean ± SD, n = 3.

**Figure 3.1** shows the degree of hydrolysis for all hydrolysates. DH of cod head hydrolysates

measured by the master student, Ayat Asfour differ slightly from the results obtained by SINTEF Ocean, however, the same method was used. The main reason for these discrepancies is most likely that experiments were done in different laboratories. In another work by Melstad (2015) cod heads were hydrolysed with Protamex and the degree of hydrolysis was determined to be between 13.63% and 20.60%. Since all hydrolysates were prepared with the same conditions, the autolysis of raw material was assumed to be the reason for the differences in DH. The highest DH was observed for frozen/thawed samples. The hydrolysates from the HEADS UP project were prepared from fresh cod heads. This could probably explain the slightly lower DH values compared to the results of Melstad (2015).

The amount of water used for hydrolysis could have influenced the amount of peptide bonds that were broken. CPH that had a different amount of water added during hydrolysis was T1H, T5H and T6H, with 400, 200, and 100 kg water used, respectively. Since T6H had the highest DH, while T1H had the lowest, it is reasonable to conclude that water influenced the DH. In the final report by Remme et al. (2018) it was also mentioned that a clear correlation between the amount of water and the degree of hydrolysis was observed. DH (%) was decreasing with an increasing amount of water. However, in the study by Šližytė et al. (2004) on the backbones and liver from farmed cod, the amount of water added during the hydrolysis did not influence DH significantly. They have found that the dry yield of FPH decreased and emulsion yield increased with the reduction of the amount of water. SPH was a commercially prepared hydrolysate and conditions for hydrolysis were not specified. Therefore, it is difficult to compare it with the CPHs.

Remme et al. (2018) have also found that enzyme type played an important role on DH. Protamex gave higher DH compared to Papain and Bromelain. From the study by Šližytė, Daukšas, Falch, Storrø and Rustad (2005) on different cod by-products (viscera, backbones), DH only slightly depended on the enzyme type used.

Finally, it is important to mention that the method used for the determination of the degree of hydrolysis can also influence the results (Rutherfurd, 2010). In et al. (2002) compared three different methods: formol titration, pH-stat and SN-TCA. Little correlation was observed among these three methods. The values for DH determined by formol titration were half of those found by the pH-stat method across all enzyme concentrations. SN-TCA gave even larger differences with formol titration.

Since the degree of hydrolysis affects the size and hence the amino acid composition of the peptides, it is logical to speculate that it can also influence the bioactive properties of hydrolysates. Several studies have indicated that DH can be correlated with antioxidant activity and ACE inhibitory activity (Geirsdottir et al., 2011; Jamdar et al., 2010; Chen et al., 2013). This is further discussed in sections 3.7 and 3.8.

## 3.2 Protein content

The soluble protein concentration was determined using the Lowry method (Lowry et al., 1951). For T1H and SPH protein concentration was determined for both crude hydrolysates and UF fractions. Results are presented in **Table 3.1**. Initial concentration for crude protein hydrolysates was 10 mg/mL.

**Table 3.1:** Average protein concentrations in mg/mL and percent for all hydrolysates and their fractions. Initial concentration of FPH was 10 mg/mL.

| | **Average protein concentration (mg/mL)** | **Protein concentration (%)** |
|---|---|---|
| **Crude protein hydrolysates** | | |
| T1H | $6.61 \pm 0.03$ | 66.13 |
| T2H | $7.19 \pm 0.11$ | 71.87 |
| T3H | $6.62 \pm 0.21$ | 66.21 |
| T4H | $7.42 \pm 0.37$ | 74.17 |
| T5H | $7.03 \pm 0.11$ | 70.33 |
| T6H | $6.46 \pm 0.17$ | 64.60 |
| T13H | $6.32 \pm 0.18$ | 63.21 |
| SPH | $10.38 \pm 0.39$ | 103.89 |
| **UF fractions** | | |
| T1H P | $1.98 \pm 0.13$ | |
| T1H R1 | $15.66 \pm 1.06$ | |
| T1H R2 | $3.43 \pm 0.04$ | |
| SPH P | $2.17 \pm 0.07$ | |
| SPH R | $31.42 \pm 2.74$ | |

The soluble protein concentration varied from 6.32 mg/mL to 10.38 mg/mL (63.21% to 103.89% of dry weight), with the lowest concentration observed for T13H, while the highest was for SPH. Cod head protein hydrolysates showed similar concentrations for all samples. Remme et al. (2018) has reported protein content around 80% for the same cod head hydrolysates. It was higher than the obtained results, however, the method for determination was not specified. Most likely, the Kjeldahl method was used to determine nitrogen content and then to convert it to protein content by multiplying with 6.25. In this method, it is assumed the same nitrogen content for all peptides and proteins, while amino acid composition and the number of nitrogen atoms usually vary (Mæhre et al., 2018). It is therefore highly probable that numbers obtained by this method can be overestimated.

Values in this master's thesis were obtained by the Lowry method (Lowry et al., 1951), which is based on the light absorption by the functional groups or regions within the protein. This method is simple, available and is measuring the amount of soluble proteins, which could be another reason for disagreement with Remme et al. (2018), where, presumably, the total protein content was measured. Overestimation of protein content is also possible using the Lowry method. It is based on the reaction of aromatic amino acids with Folin-Ciocalteu reagent, but a wide range of other compounds can react with this reagent. Other disadvantages that could influence results are the slow reaction rate and instability of some reagents (Peterson, 1979). Hence, results from the Lowry method should also be interpreted carefully.

In general, all FPHs showed good solubility. If proteins are intended for use in foods, they should have high solubility. Enzymatic hydrolysis can improve solubility by increasing the number of polar groups. It also converts some hydrophobic groups to hydrophilic groups. Release of small soluble peptides and new carboxylic and amine groups from amino acids lead to an increase in

solubility after hydrolysis (Kristinsson and Rasco, 2000).

The report by Remme et al. (2018) concluded that the amount of water during the hydrolysis influenced protein concentration. Šližytė et al. (2004) has reported that the dry yield of FPH decreased and emulsion yield increased when the amount of water was reduced. From **Table 3.1** no correlation was observed. Type of enzyme has not influenced the protein concentration either, and this was also the conclusion from Remme et al. (2018).

Protein solubility of cod head hydrolysates obtained by Monslaup (2018) was lower than the results presented in **Table 3.1**. The same method for determination was used, and differences were unexpected. Šližytė, Rustad and Storrø (2005) have reported total protein content between 68.0% and 83.5% for cod protein hydrolysates, using the Kjeldahl's method.

All permeates showed a low concentration of proteins in the solutions, while retentates had high concentrations of protein. This is an expected result since most of the water will pass through the membrane and end up in the permeate.

## 3.3 Amino acid content and composition

Several studies have reported that amino acid composition, peptide structure and amount of free amino acids can influence bioactive properties of fish protein hydrolysates (Morales-Medina et al., 2016; Halim et al., 2016; Šližytė, Rustad and Storrø, 2005; Zamora-Sillero et al., 2018). Consequently, analysis of total amino acids and free amino acids was performed.

### 3.3.1 Total amino acids (TAA)

Total amino acid content and composition of cod protein hydrolysates were determined by Asfour (2018). Salmon protein hydrolysate was analysed during the work on this thesis. Results for crude protein hydrolysates can be found in **Figure 3.2**. The standard deviation for cod protein hydrolysates was not specified. Appendix D.1 shows calculations of results presented in **Figure 3.2** and the amount of different amino acids present in these hydrolysates. Total amino acid content and composition were not measured for UF fractions due to the limited amount of solutions. However, it would be of great interest to compare the differences between crude protein hydrolysate and its fractions.

The highest total amino acid content was found to be 703.11 mg/g FPH in T4H. T3H showed the lowest amount of amino acids, 358.02 mg/g FPH. The most abundant amino acids found in all three hydrolysates were glycine/arginine (Gly/Arg), glutamic acid (Glu), aspartic acid (Asp), serine (Ser), leucine (Leu) and lysine (Lys). The same results were observed in other studies (Sabeena Farvin et al., 2016; Jensen et al., 2013). T2H and SPH had the highest amount of tyrosine and the lowest amount of alanine compared to other hydrolysates. A high amount of glutamine was found in T5H, T6H, and T13H. T4H contained almost twice as high amount of glutamic acid compared to the rest of hydrolysates. This hydrolysate contained the highest amount of glycine and arginine (111.32 mg/g). The amount of total amino acids for most hydrolysates was slightly low. This is due to the presence of other components in FPHs. Hydrolysates contained ash, and Asfour (2018) has reported between 7 - 10% of ash in the CPHs.

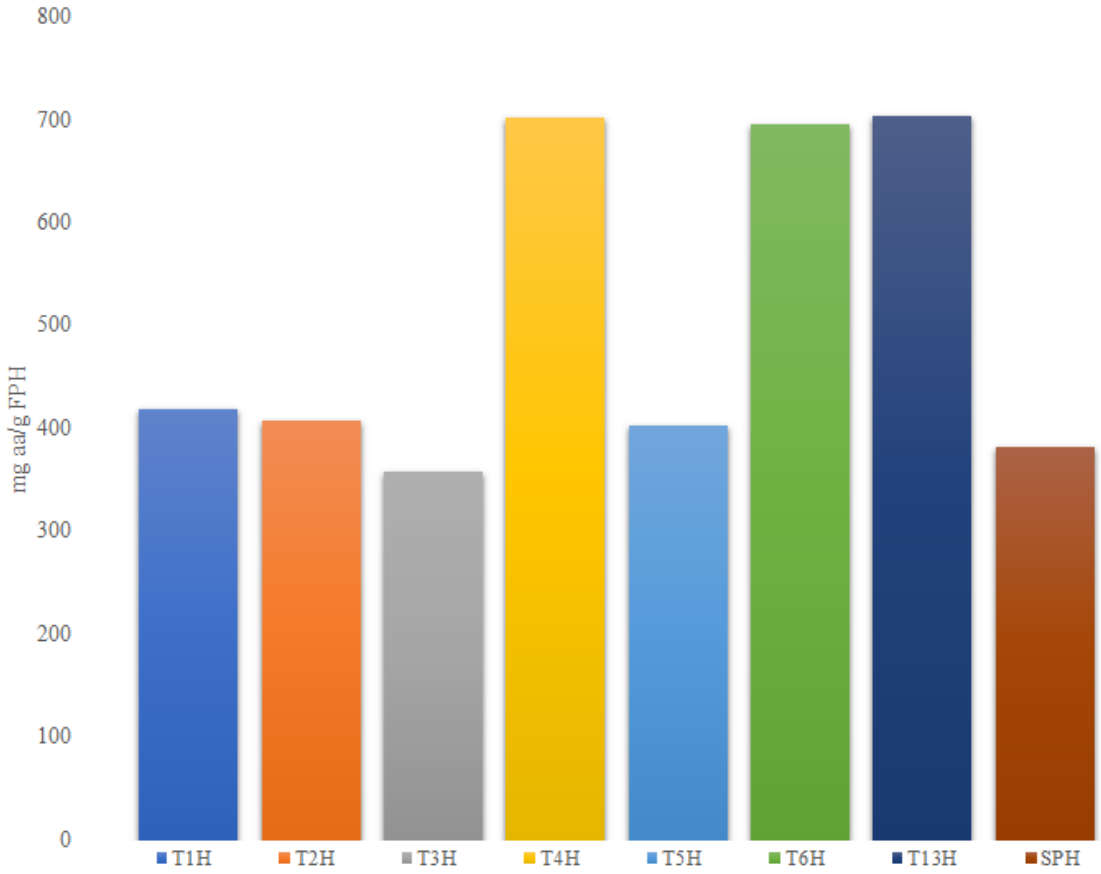



**Figure 3.2:** The total amount of amino acids for crude protein hydrolysates presented in mg amino acid per g crude protein hydrolysate. Values are given as means (n=3). Standard deviation was not specified.

Not all amino acids can be determined by HPLC analysis because of poor recovery during acid hydrolysis. One of the amino acids that can be destroyed during acid hydrolysis is tryptophan. This could be another reason for low values.

### 3.3.2 Free amino acids (FAA)

The amount of free amino acids was measured for both crude protein hydrolysates and UF fractions of T1H and SPH. Results for crude protein hydrolysates can be found in **Table 3.2** and Appendix D.2. The hydrolysate named T3H had the highest content of free amino acids (37.13 mg/g FPH powder). T3H was the only CPH treated with a different enzyme (Protamex). The lowest content of free amino acids was observed in SPH with the value of 6.37 mg/g FPH powder. The reason for such a variation between the free amino acid amount in the CPHs is unclear, since most the hydrolysates were treated with the same enzyme, except for the T3H, and T13H was added citric acid. Compared to the results of the study by Šližytė, Rustad and Storrø (2005) on viscera from cod, the results presented in **Table 3.2** were low. Melstad (2015) has studied cod heads, and her results for free amino acids were also slightly higher. Amount of free amino acids ranged between 48.37 mg/g and 54.25 mg/g after 60 min hydrolysis.

Free amino acids amount was found to be positively correlated with the degree of hydrolysis in the study by Šližytė et al. (2016) on hydrolysates from defatted salmon backbones. This

correlation was also found between the FAA amount of CPHs and SPH and DH (r = 0.81, $p < 0.05$).

**Table 3.2:** Amount of free amino acids found in the crude protein hydrolysates. Values are expressed as means.

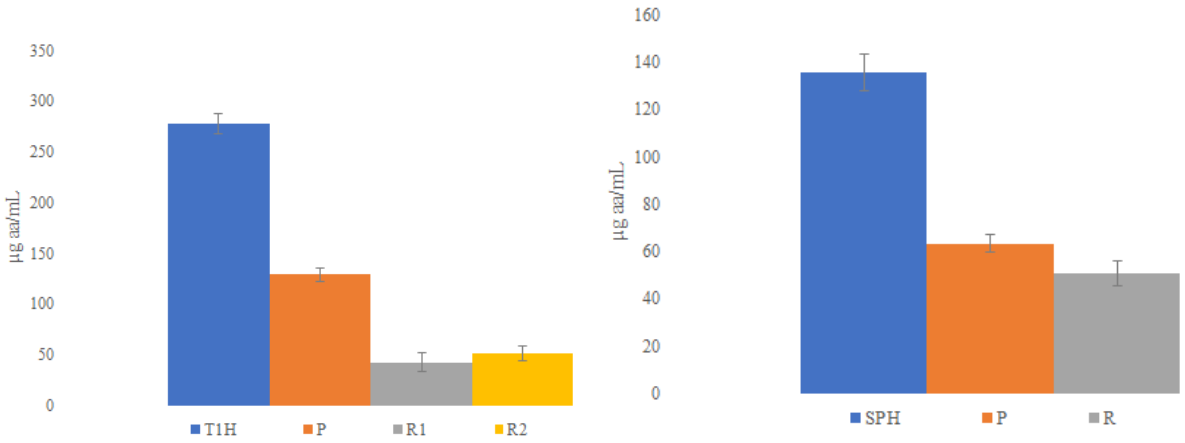
| | **FAA** | |
|---|---|---|
| | $\frac{mg\ aa}{g\ FPH}$ | $\frac{FAA}{TAA}$ (%) |
| **T1H** | 13.62 ± 0.95 | 3.25 |
| **T2H** | 20.48 ± 3.72 | 5.02 |
| **T3H** | 37.13 ± 4.52 | 10.37 |
| **T4H** | 20.31 ± 3.60 | 2.89 |
| **T5H** | 27.28 ± 3.66 | 6.77 |
| **T6H** | 28.01 ± 0.87 | 4.03 |
| **T13H** | 25.88 ± 6.10 | 3.67 |
| **SPH** | 6.37 ± 1.22 | 1.67 |

The predominant free amino acids in all hydrolysates were serine, alanine, glutamic acid, methionine, leucine, and lysine. All the amino acids were also found in high concentrations in the analysis of total amino acid, except for methionine. A low amount of methionine in the total amino acid analysis can be explained by possibly poor recovery during acid hydrolysis and hence inaccurate quantitation of the original content (Keutmann and Potts Jr, 1969). Cod protein hydrolysates contained more glycine/arginine, threonine, and valine than salmon protein hydrolysates.

T1H and SPH and their UF fractions are presented in **Figure 3.3a** and **Figure 3.3b** in mg amino acid per mL. As it is seen from these figures, permeates contain a higher amount of free amino acids, compared to the retentates. Taking into account the measured volumes for each fraction, permeates contain a significantly higher amount of free amino acids compared to the retentates. It is expected since most of the free amino acids were supposed to go through the membrane and end up in the permeate. However, some free amino acids will always stay in the retentate. Some loss of the free amino acids was also observed, but it was expected and loss for protein and dry matter is explained in the section about ultrafiltration.

Detailed information about free amino acid composition is presented in Appendix D.2. Most of the free amino acids were present in a lower amount in the retentate, however, some were present in higher amounts compared to the permeates. For example, in the retentate fraction of SPH, the amount of glutamine, phenylalanine, isoleucine, and lysine was higher than in the permeate. For T1H fractions the permeate contained a higher amount of all free amino acids.

The dominating free amino acids of SPH and its fractions were alanine, phenylalanine, and lysine. The study by Liaset et al. (2003) on hydrolysates from salmon frame also reported a high amount of free alanine, but phenylalanine and lysine were not present in high amounts. The high amount of glutamate and leucine was found instead. T1H and its fractions had most of serine, glutamine, glycine/arginine, alanine, valine, isoleucine and leucine. Sabeena Farvin et al. (2016) found the same amino acids to be dominating in the commercial cod protein hydrolysate.




**(a)** T1H and its UF fractions.

**(b)** SPH and its UF fractions.

**Figure 3.3:** Amount of free amino acids present in the crude protein hydrolysates and their UF fractions. Values are given as means ± SD (n = 4 for (a) and n = 3 for (b)).

## 3.4 Molecular weight distribution

The molecular weight distribution of protein hydrolysates was determined using a FPLC system. Both UF fractions and crude protein hydrolysates were analysed. For UF fractions, the degree of separation was studied. To estimate the approximate molecular size of peptides present in hydrolysates, standards with known molecular weight were used, Aprotinin (6.5 kDa), Cytochrome C (12.4 kDa) and Vitamin B12 (1.3 kDa). Chromatograms of these standards and calculations are presented in Appendix E.

**Figure 3.4** and **Figure 3.5** show molecular weight distribution of crude protein hydrolysates. Values for absorbance at 280 nm (mAU) are plotted against elution volume (mL). In addition, vertical lines in these figures represent elution volumes for standards.

First of all, chromatograms of all hydrolysates show a certain number of peaks, indicating that they contain a wide range of molecular sizes. Most of the cod protein hydrolysates have their first peaks between 12.4 kDa and 6.5 kDa, but closer to 6.5 kDa. Five cod hydrolysates (T1H, T3H, T5H, T6H, and T13H) have small peaks at an elution volume of around 7 mL, indicating the possible presence of molecules larger than 12.4 kDa. However, since these peaks are quite far from peaks of the used standards, it is difficult to give any accurate value for the molecular weight. The same applies to peaks appearing after elution volume of 30 mL. All other peaks are relatively similar for cod head hydrolysates.

The enzyme type did not influence molecular weight distribution significantly, however, T3H treated with Protamex had a more distinct peak at an elution volume of 16.6 mL ($\sim$ 7 kDa) than other hydrolysates treated with Papain and Bromelain.

From the report on the cod head hydrolysates, it was observed that most of the hydrolysates contained peptides between 1-10 kDa (Remme et al., 2018). This is not in total agreement with the results in **Figure 3.4** and **Figure 3.5**, where peptide sizes smaller than 1 kDa and bigger than 10 kDa were detected. However, the highest absorbance is observed in this range, indicating that

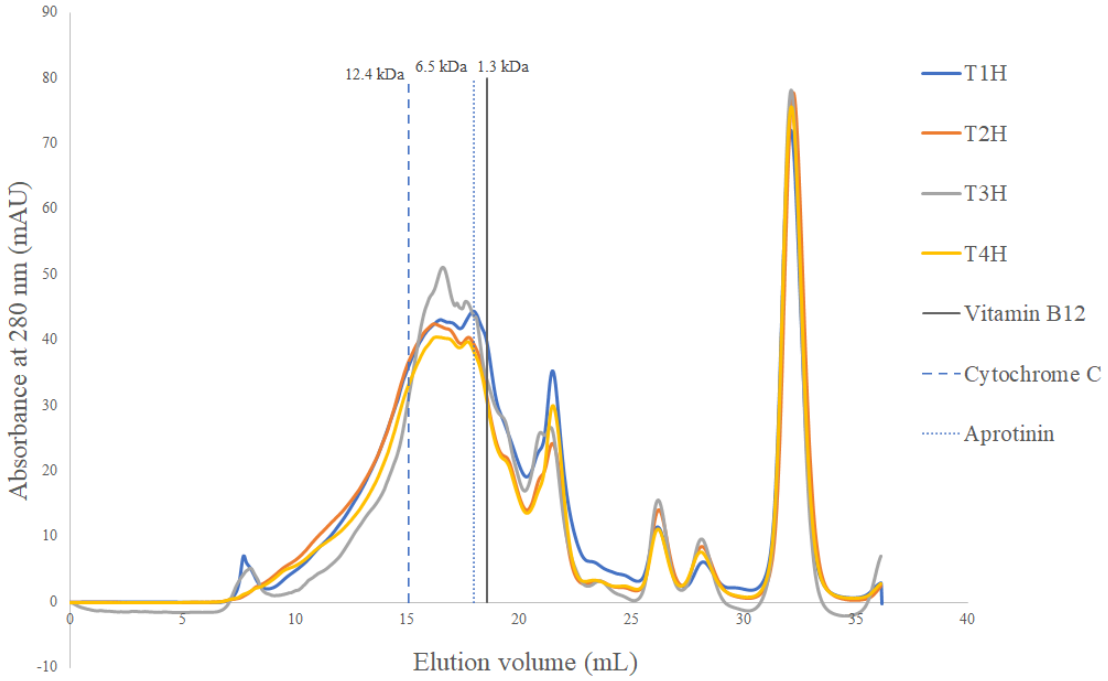



**Figure 3.4:** Gel filtration chromatogram presenting molecular weight distributions of T1H, T2H, T3H, T4H and peaks of known standards (B12, Aprotinin and Cytochrome C).

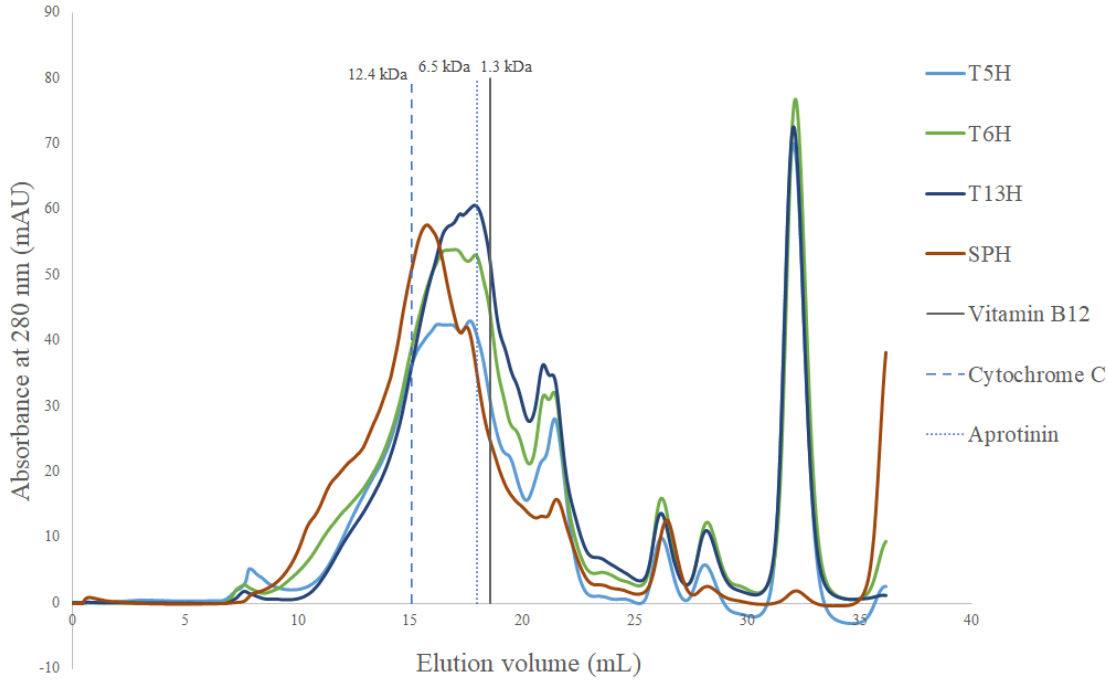



**Figure 3.5:** Gel filtration chromatogram presenting molecular weight distributions of T5H, T6H, T13H, SPH and peaks of known standards (B12, Aprotinin and Cytochrome C).

most of the peptides had a size between 1-10 kDa.

Molecular weight distribution for salmon protein hydrolysate differed from the rest of the

hydrolysates to some degree. The first distinct peak was observed close to the peak of Cytochrome C, 12.4 kDa. This can be explained by the degree of hydrolysis. SPH had the lowest DH, and therefore fewer peptide bonds were broken resulting in larger molecular sizes of the peptides compared to other hydrolysates with higher DH. Other peaks appeared around the same elution volumes as for the other hydrolysates. Significantly high absorbance and the sharp-cut peak is observed for all the hydrolysates, except SPH, at around 33 mL. Low molecular weight compounds were possibly responsible for this peak, or it was due to disturbances.

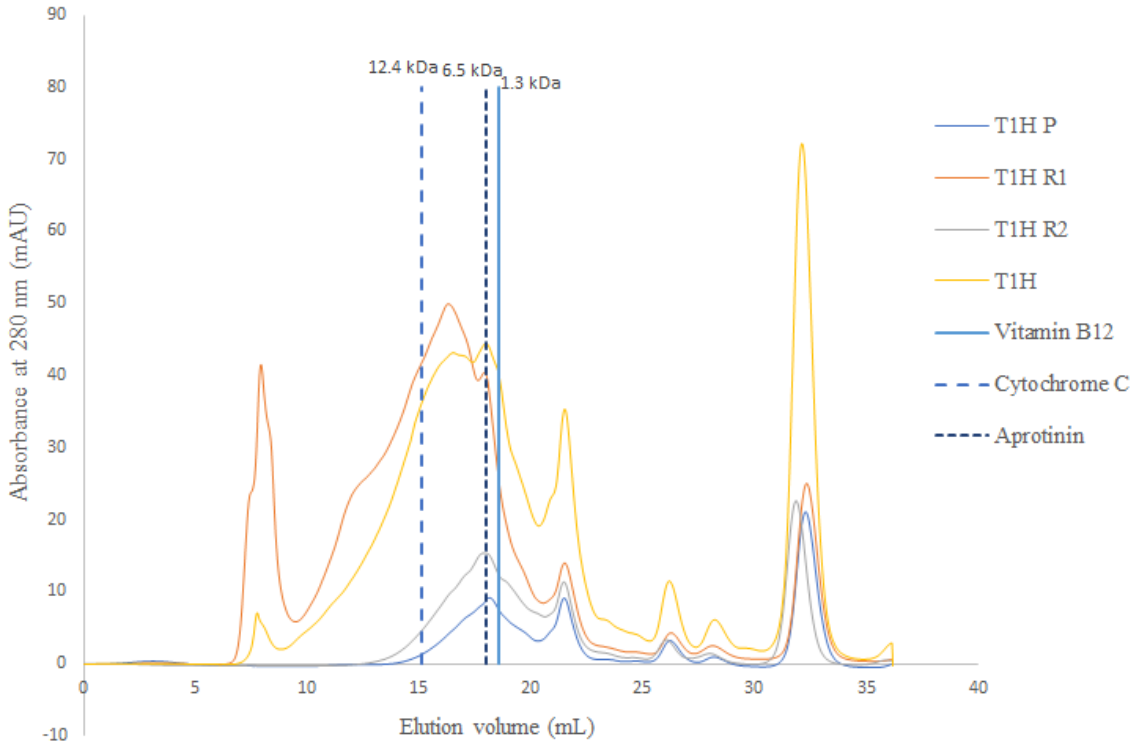



**Figure 3.6:** Gel filtration chromatogram of molecular weight distribution for crude T1H and its UF fractions. Peaks of known standards (B12, Aprotinin and Cytochrome C) is presented for comparison.

**Figure 3.6** shows molecular weight distribution of crude T1H and its fractions. Elution volumes of standards are also plotted in the same figure. T1H was ultrafiltrated in the specialization project, but the results did not give the desired separation. The permeate still contained molecules larger than the molecular weight cut-off of the membrane. It was therefore decided to filtrate this hydrolysate twice, i.e. filtrate the obtained permeate one more time. This gave one permeate (P) and two retentates (R1 and R2), which are presented in **Figure 3.6**. From this figure, it can be observed, that both retentates contained a high amount of small peptides. From the earlier studies by Bourseau et al. (2009) and Picot et al. (2010), it was observed that separations were not so sharp as it was desired, and peptides of the same size could be found in both fractions. It is nearly impossible to remove all small peptides and amino acids from the retentate using ultrafiltration. Part of peptides of smaller sizes will always be retained in the retentate, though only peptides smaller than MWCO of the membrane are expected to be found in the permeate. In addition, it is important to remember about the concentration polarization phenomenon. It occurs when molecules bigger than molecular weight cut-off are accumulated near the membrane wall because they are totally or partially rejected by the membrane. Thus, the concentration at the

membrane surface increases and becomes saturated, leading to a decreasing of permeate flux. A gel layer of dissolved proteins on the membrane surface can be formed and by that slow down or even stop the transport of other molecules through the membrane (Grandison, 1996). Bourseau et al. (2009) have studied the impact of UF on two industrial FPHs. They concluded that a decrease in retention and permeation flux was caused by an increase in polarization. Therefore, it is probably more correct to refer to the retentate as a fraction that contains a high amount of peptides bigger than 4 kDa, not just the fraction consisting exclusively of peptides bigger than 4 kDa. If it is desired to remove small molecules from the retentate, diafiltration can be applied. This process helps to reduce the ionic strength of the retentate by adding water to the system (Schwartz, 2003).

The first distinct peak for the permeate is on elution volume of 18.12 mL (∼4 kDa), while for the second retentate it lies at 17.96 mL (∼7 kDa). This proves that the second ultrafiltration has changed the molecular weight distribution of the permeate to some extent. However, since the calibration curve for the standards was not perfect (Appendix E), it could lead to inaccurate estimation of the peptide sizes. This should be taken into account. Comparing to the results from the specialization project, double filtration did not improve the sharpness of separation. **Figure 3.7** illustrates chromatograms of the permeate of T1H analysed during the specialization project (T1H P 2018) and during this thesis (T1H P 2019). The only difference between these filtrations is in the first peak, which appeared first for T1H P 2019, but absorbance started to increase for T1H P 2018 first. Everything else was almost identical.

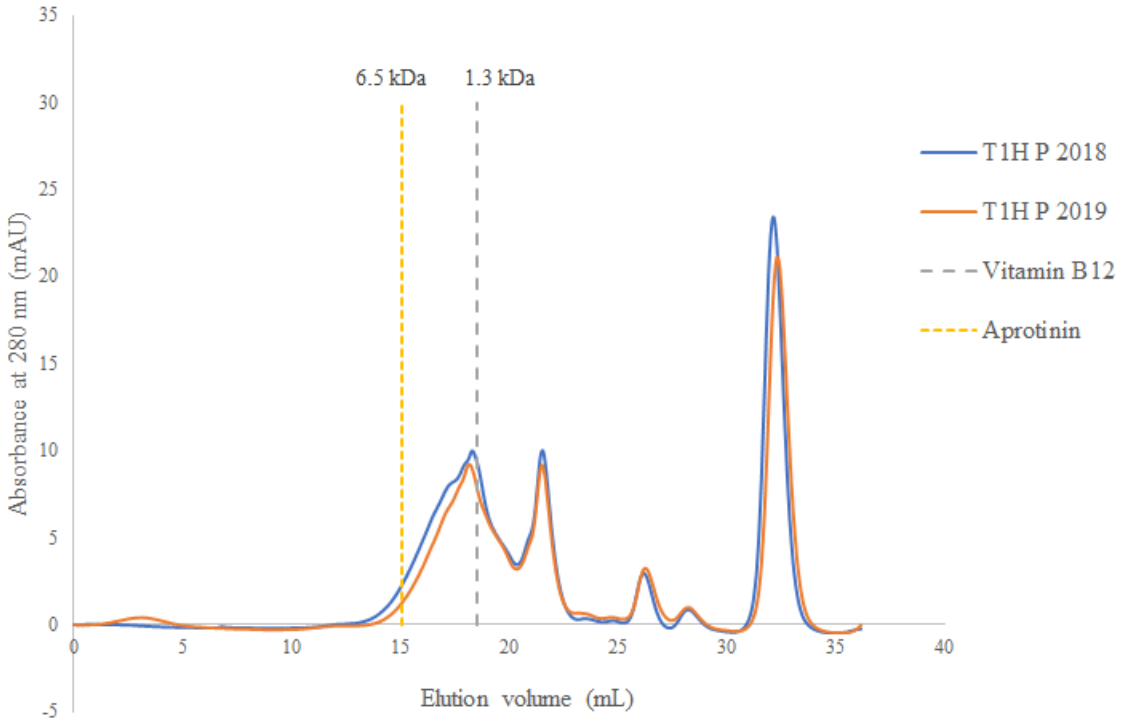



**Figure 3.7:** Gel filtration chromatogram of molecular weight distribution for permeate of T1H analysed during specialization project (filtrated once) and during this thesis (filtrated twice).

**Figure 3.8** shows a chromatogram of salmon protein hydrolysate and its fractions. Ultrafiltration

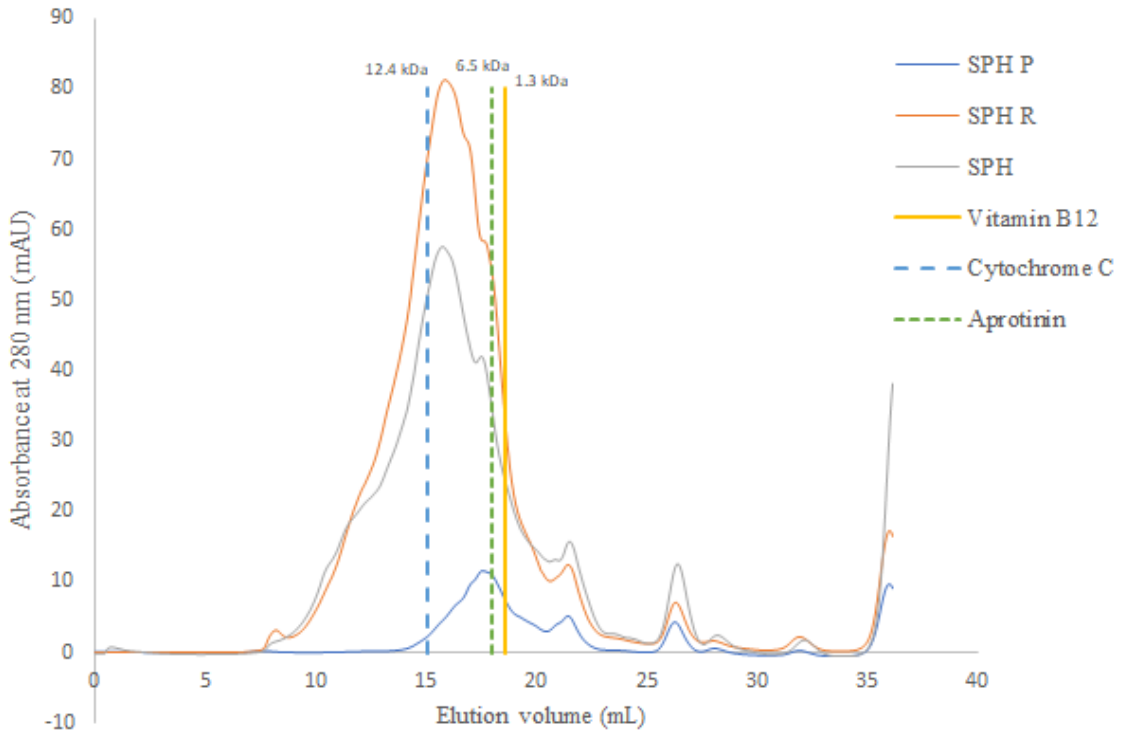



**Figure 3.8:** Gel filtration chromatogram of molecular weight distribution for crude SPH and its UF fractions. Peaks of known standards (B12, Aprotinin and Cytochrome C) is presented for comparison.

was only applied once on this hydrolysate. The largest peptides were removed and appeared only in the retentate, but the degree of separation was not as sharp and accurate as preferable for the permeate. Probably, this occurred because of polarization, or because the membranes were old and had imperfections in the pores, something that could result in slight contamination. To achieve better separation, new filtration of the permeate could be recommended.

Ultrafiltration has been found to increase bioactive properties of fish protein hydrolysates (Jeon et al., 1999; Sun et al., 2017; Yathisha et al., 2018). Consequently, it was of interest to study the differences between the crude hydrolysate and UF fractions. The influence of ultrafiltration on bioactivities is discussed in later sections.

Ideally, the gel filtration chromatogram should contain several distinct peaks corresponding to different sizes. All hydrolysates had some distinct peaks, but also one or two broad peaks covering a wide molecular weight range. This can probably indicate that there were a lot of molecules with a molecular size close to each other, something that led to overlapping peaks. Successful gel filtration depends primarily on choosing the right conditions for the sample. Flow rate, sample volume, viscosity, bed height, column packing quality, and gel filtration medium are some of the factors that can influence resolution.

## 3.5 Ultrafiltration

Ultrafiltration was performed on one cod protein hydrolysate and one salmon protein hydrolysate. The degree of separation was discussed in the previous section about molecular weight distri-

bution. To calculate protein recovery from membrane filtration mass balance was carried out. The loss was calculated based on protein content and dry matter content. Volumes and DM(%) for T1H and SPH are presented in **Table 3.3**, and a detailed explanation of calculations can be found in Appendix F.

**Table 3.3:** Volumes from ultrafiltration and dry matter content for crude hydrolysates and their fractions.

| | **Volume (mL)** | **DM (%)** |
|---|---|---|
| **T1H** | | |
| Crude hydrolysate | 350 | 0.78 |
| Permeate | 205 | 0.27 |
| Retentate 1 | 85 | 1.45 |
| Retentate 2 | 60 | 0.40 |
| **SPH** | | |
| Crude hydrolysate | 300 | 1.10 |
| Permeate | 231 | 0.18 |
| Retentate | 69 | 3.30 |

**Table 3.4:** Dry matter content and soluble protein content in fractions of T1H and SPH and loss during ultrafiltration.

| | **DM (mg)** | **Protein (mg)** |
|---|---|---|
| **T1H** | | |
| Crude hydrolysate | 2716 | 2314 |
| Permeate | 549 | 406 |
| Retentate 1 | 1234 | 1331 |
| Retentate 2 | 240 | 206 |
| Loss | 693 | 371 |
| **SPH** | | |
| Crude hydrolysate | 3300 | 3000 |
| Permeate | 416 | 501 |
| Retentate | 2277 | 2168 |
| Loss | 607 | 331 |

The loss for SPH was lower than for CPH for both dry matter and protein content, 18.4% and 11.0% for SPH and 25.5% and 16.0% for T1H. Ultrafiltration was performed once on SPH, while permeate from CPH was filtrated again. This resulted in a higher loss. Loss, in general, happened because of the retention of some particles or peptides on the membranes or pipes of the instrument. Protein content was slightly higher than DM in Retentate 1 of T1H and Permeate of SPH. Probably, overestimation of the protein content by the Lowry method has occurred.

The protein loss was also calculated for T1H during the specialization project. The result was 45%, while the protein loss in this thesis was 25%. The main reason for the difference was inaccurate volumes. They were not measured properly during the specialization project. During this thesis, accurate volumes were used for calculation, and results are more reliable and accurate.

Ohnstad (2018) reported very low protein loss for T1H (less than 1%), but volumes were not measured exactly, and protein content for unfiltered hydrolysate was not determined properly.

## 3.6 Acid soluble peptides

Precipitation of proteins by using trichloroacetic acid (TCA) was performed on crude protein hydrolysates and UF fractions. **Figure 3.9** represents the values obtained for crude protein hydrolysates in percent, and **Figure 3.10** represents the amount of acid soluble peptides and soluble protein content for crude protein hydrolysates and their UF fractions in mg/mL. The amount of acid soluble peptides in % dry weight was lying between 55.28% and 84.82%. All calculations are explained and illustrated in Appendix G.

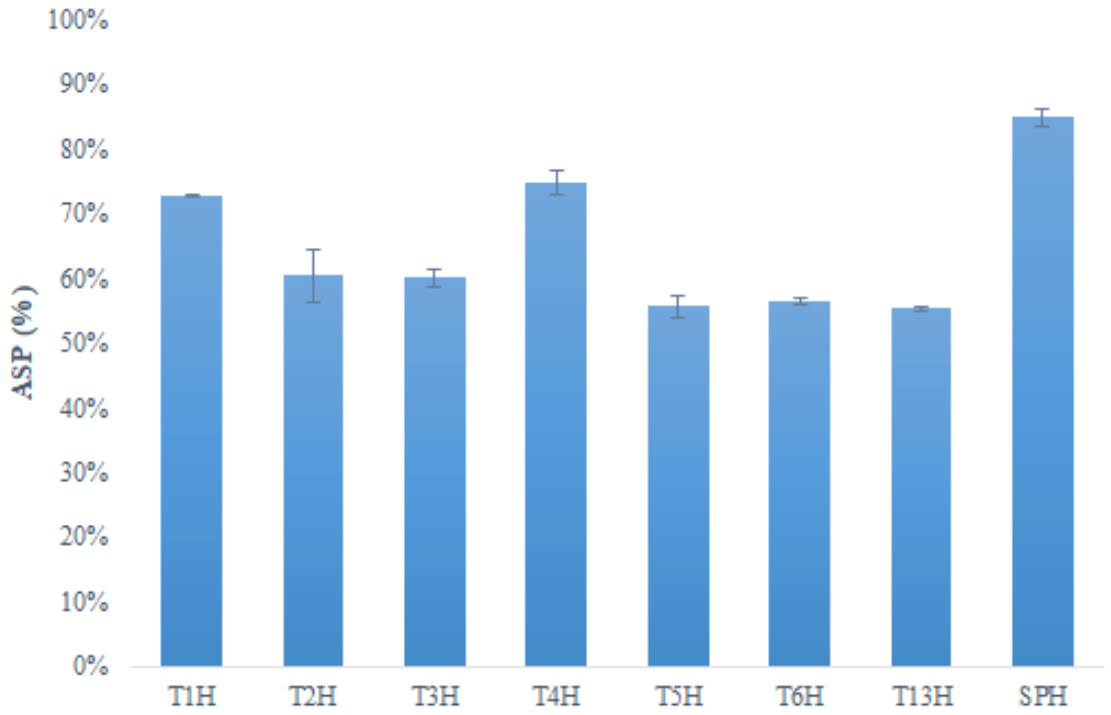



**Figure 3.9:** The amount of acid soluble peptides (% dry weight) in the crude protein hydrolysates. Values are given as means ± SD (n = 3).

The amount of acid soluble peptides is expected to be somewhat lower than the total amount of soluble proteins presented in the hydrolysates. Obtained values were slightly lower, except for T1H where ASP (%) was higher than soluble protein content (%). The reason could be the underestimation of the results for soluble protein content due to the possible interferences present in the CPHs. Mixing CPH with TCA could have removed these interferences (Peterson, 1979).

SPH showed the largest difference between the amount acid soluble peptides and soluble protein content. The size of peptides soluble in TCA can vary, Greenberg and Shipe (1979) have reported their size to be around 3-4 amino acids in 10% TCA. Yvon et al. (1989) showed that all the peptides containing less than seven amino acid residues were soluble in 12% TCA. Therefore, results are indicating that even though SPH contains more soluble protein, it contains

more peptides with high molecular weight. This was also confirmed by the chromatogram from molecular weight distribution. On the other hand, so high acid soluble peptide content of CPHs indicates that most of the soluble peptides were small enough to be acid soluble. Free amino acids could also have influenced the amount of acid soluble peptides. The Folin phenol reagent reacts with free amino acids, even though the color intensity is less pronounced than for peptides (Peterson, 1979; Lowry et al., 1951). Free amino acids could also contribute to the color formation by cross reacting with the formed copper complexes (Overrein, 2010). CPHs contained a higher amount of free amino acids compared with the SPH. Hydrophobicity of the peptides has also been found to be important for the solubility in TCA as reported by Yvon et al. (1989).

Asfour (2018) has analysed the same CPHs and reported the amount of acid soluble peptides to vary from 44.79% to 65.11%. Slight differences in results were possibly due to performance of the experiment by different people or due to the storage time. Meldstad (2015) has reported very high acid soluble peptide content of cod head hydrolysates, almost 100%. She has also investigated the relationship between DH and TCA soluble peptides, but no correlation was found. The correlation between DH and ASP in this thesis was difficult to evaluate since values for DH of CPHs were not so different.

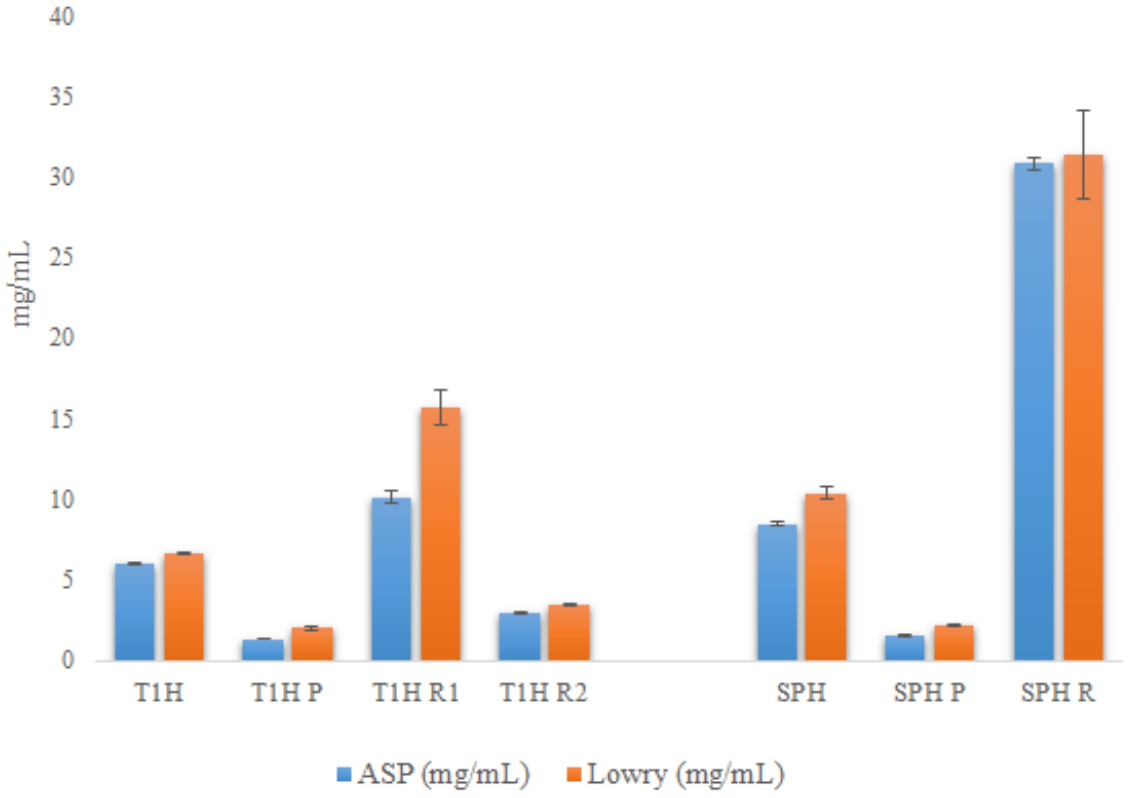



**Figure 3.10:** The amount of acid soluble peptides (mg/mL) and soluble protein content (mg/mL) in the crude protein hydrolysates and their UF fractions. Values are given as means ± SD (n = 3).

**Figure 3.10** is illustrating the amount of acid soluble peptides and soluble protein content in two hydrolysates and their fractions. The concentration of acid soluble peptides was highest for retentates. According to the principle of ultrafiltration, retentates are the most concentrated

and measured volumes for the retentates were low, so it is logical that the concentration was high. Difference between soluble protein and acid soluble peptides in the retentate of T1H can be explained by the fact that retentates are enriched in bigger peptides, that are not soluble in TCA. The amount of acid soluble peptides and soluble protein content in SPH were almost the same, something that was not expected. The overestimation of ASP could have occurred. The amount of ASP in permeates is the lowest, but the volume of the permeates was high, something that led to quite diluted solutions.

## 3.7 Antioxidant activity

Antioxidant activity was measured by two spectrophotometric assays, ABTS and FC. During the specialization project, a third method was also applied, DPPH radical scavenging activity was measured. This assay has been frequently used for the analysis of fish protein hydrolysates (Klompong et al., 2012; Šližytė et al., 2016; Sabeena Farvin et al., 2016). Different concentrations of CPHs were tested, but none of them gave absorbance values within the standard curve. Due to these difficulties, it was decided to exclude the results of this assay in the master's thesis.

During the ABTS assay reduction in colour/decreasing absorbance is associated with increasing concentration of the standard, while for the FC assay, absorbance is increasing with increasing concentrations. Results are presented in **Table 3.5**. Antioxidant activity for UF fractions was determined only by the ABTS assay. Detailed information about calculations and standard curves are presented in Appendix H.

**Table 3.5:** Results for two antioxidant activity assay for all hydrolysates (mean ± SD, n = 3).

| | **Antioxidant assay** | |
|---|---|---|
| | ABTS [$\mu$mol/ g protein] | FC [mmol/g protein] |
| **Crude protein hydrolysates** | | |
| T1H | 27.9 ± 2.3 | 0.15 ± 0.01 |
| T2H | 33.7 ± 1.8 | 0.14 ± 0.01 |
| T3H | 27.1 ± 0.7 | 0.13 ± 0.02 |
| T4H | 30.9 ± 0.6 | 0.14 ± 0.01 |
| T5H | 30.0 ± 1.3 | 0.15 ± 0.02 |
| T6H | 22.3 ± 7.9 | 0.18 ± 0.01 |
| T13H | 18.9 ± 9.8 | 0.17 ± 0.03 |
| SPH | 25.2 ± 0.9 | 0.10 ± 0.00 |
| **UF fractions** | | |
| T1H P | 107.0 ± 5.5 | |
| T1H R1 | 24.9 ± 6.9 | |
| T1H R2 | 62.1 ± 0.7 | |
| SPH P | 99.2 ± 6.4 | |
| SPH R | 6.7 ± 1.8 | |

### 3.7.1 ABTS radical scavenging activity

Radical scavenging activity of crude protein hydrolysates and their UF fractions measured by the ABTS assay was calculated as equivalent concentrations of propyl gallate per gram of soluble protein. This method was easy to perform and did not require much time per sample.

The antioxidant activity of T13H was significantly lower ($p < 0.05$) than for the other hydrolysates. T4H had the highest antioxidant activity. The same result was obtained by Monslaup (2018) for T13H. The only difference between T13H and other hydrolysates is that citric acid was added. It is usually added to decrease pH and by that inhibit bacterial growth. Citric acid is also a good chelating agent and can reduce oxidation. It forms complexes with metals and makes them soluble (Majeti and Freitas, 2003). Measuring iron chelating activity would probably give different results for the antioxidant activity of T13H. In the ABTS assay, citric acid could influence the solubility of hydrolysate or influence the reaction between radical and antioxidant. It was reported that scavenging measured with ABTS assay was dependent on pH (Zheng et al., 2016), and pH of T13H was lower than for other hydrolysates. The reducing capacity may be suppressed under the acidic conditions (MacDonald-Wicks et al., 2006).

Garcia et al. (2012) reported that the antioxidant activity of fish protein hydrolysates increased with increasing DH. Other studies have also confirmed this finding (Thiansilakul et al., 2007a; Jamdar et al., 2010). However, ABTS and DH presented no correlation for cod protein hydrolysates with an r of only 0.03. However, DH values for CPHs were not so different, hence correlation should be evaluated carefully. Another study by You et al. (2009) has concluded that limited hydrolysis can lead to better antioxidant activity. ABTS antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysate has increased greatly with increasing DH and reached a maximum value at DH of 23%. However, a further increase of DH decreased antioxidant activity. Correlation between salmon protein hydrolysate and DH was not possible to evaluate since it was only one hydrolysate.

The amount of free amino acids may give some information about antioxidant activity (Sabeena Farvin et al., 2016). The hydrolysate with the highest amount of free amino acids was T3H, but the highest ABTS scavenging activity was observed for T2H. Correlation was not observed ($r = 0.19$). The same study has reported that amino acids as Lys and Tyr can exert antioxidant effects. T2H had a very high amount of total Tyr (17.88%), but SPH contained even more Tyr (20.18%). The amount of total Lys in T2H was not significantly different from other hydrolysates. Amount of free Tyr was the highest in T2H, indicating that this probably contributed to the antioxidant activity.

Cheison et al. (2007) and Zamora-Sillero et al. (2018) have reported a strong correlation between the antioxidant activity and hydrophobic and aromatic amino acids. This trend was not observed between ABTS and the total amount of hydrophobic amino acids and ABTS and total amount of aromatic amino acids analysed here. The amount of free aromatic amino acids and ABTS antioxidant activity presented a significant correlation with an r of 0.83 ($p < 0.05$). Possibly being part of peptides and proteins has prevented amino acids from exerting antioxidant activity.

The results for filtrated hydrolysates are presented in **Figure 3.11**. Antioxidant activity for crude protein hydrolysates is compared to their UF fractions. A highly significant difference

is observed between crude protein hydrolysates and permeates ($p < 0.05$). Ultrafiltration has improved the antioxidant activity for the fraction containing smaller peptides (< 4 kDa). Retentates showed lower antioxidant activity compared to both crude hydrolysate and permeate. The second retentate from T1H showed higher antioxidant activity compared to crude hydrolysate and its first retentate, though lower than for the permeate. It is understandable because even if the second retentate does not contain exclusively small peptides, it does not contain large peptides either. It is also proving that smaller peptides exhibit stronger antioxidant activity. This finding confirms results from the previous studies where increased antioxidant activity was observed for fractionated hydrolysates (Picot et al., 2010; Sabeena Farvin et al., 2016; Jeon et al., 1999). However, Shahidi and Zhong (2008) stated that lower antioxidant activity was observed in permeates and retentates of food protein hydrolysates, because of the loss of additive effect of peptides of different sizes. Monslaup (2018) has also reported higher antioxidant activity for crude protein hydrolysates compared to UF fractions.

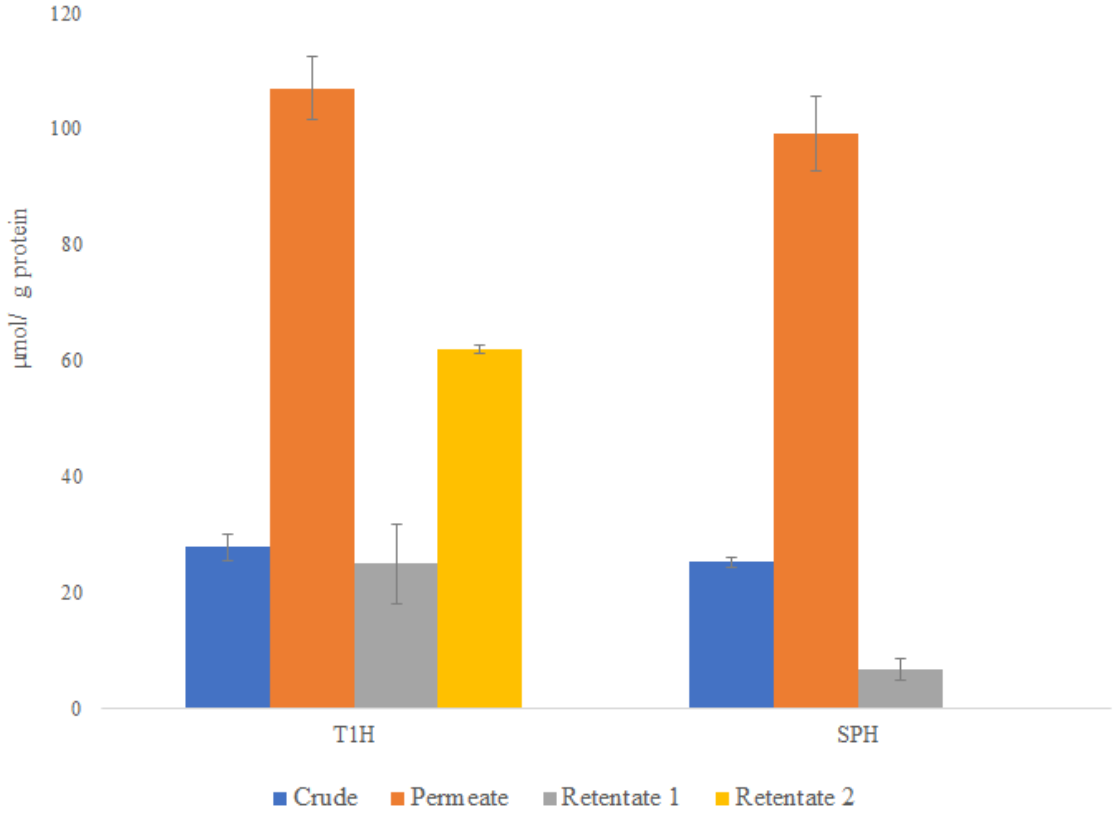



**Figure 3.11:** ABTS radical scavening activity for crude protein hydrolysates and their UF fractions (T1H and SPH).

### 3.7.2 Folin-Ciocalteu reducing capacity

Folin-Cioalteu assay measured the total phenolic content (TPC). The phenolic ring can donate hydrogen and act as a direct radical scavenger. This method for measuring antioxidant activity differed from the previous one by increasing in absorbance with increasing concentration of the

standard compound. Results are expressed as mmol of propyl gallate equivalent per g soluble protein and presented in **Table 3.5**. Standard curve and calculations can be found in Appendix H.2.

The FC assay needed to be modified to be used on the FPHs. During the specialization project absorbance values for the standard curve were greater than 1 for the highest concentrations using the protocol by Nenadis et al. (2007) and it was desired to avoid so high absorbance values. Instead, a combination of protocols by Nenadis et al. (2007) and by Singleton et al. (1999) was used which gave lower absorbance values. The difference in these two protocols was in the amount/proportions of antioxidant and $Na_2CO_3$ solution added. A comparison of these two protocols is presented in **Table. 3.6**.

**Table 3.6:** Comparison of FC assay by Nenadis et al. (2007) vs. Singleton et al. (1999).

| Materials | Nenadis et al. | Singleton et al. |
|---|---|---|
| **Methanolic solutions** | 0.5 - 2.0 mM | 0.5 - 2.0 mM |
| **Distilled water** | 8 mL | 7.9 mL |
| **Antioxidant solution** | 0.5 mL | 0.1 mL |
| **Folin-Ciocalteu reagent** | 0.5 mL | 0.5 mL |
| **$Na_2CO_3$** | 1 mL (20%) | 1.5 mL (Saturated) |
| **Total volume** | 10 mL | 10 mL |
| **Absorbance** | 725 nm | 760 nm |

Concentrations of standard solution (PG) were the same and the amount of Folin-Ciocalteu reagent was 0.5 mL for both protocols. The original procedure by Singleton et al. (1999) proposed to measure absorbance at 760 nm after 2 hours, while Nenadis et al. (2007) proposed 725 nm after 1 hour. It was decided to use 725 nm for absorbance measurements and 1-hour waiting, because it gave reliable results. However, other incubation times and wavelengths were not tested.

The absorbance values for Nenadis et al. (2007) were between 0.308 and 1.397 nm, while the protocol of Singleton et al. (1999) gave values from 0.061 to 0.318 nm. Singleton values were also more stable and had standard deviation 0.005 versus 0.019 from protocol by Nenadis et al. (2007). It was also considerably easier to find appropriate dilutions for the samples. Further measurements were based on the combination of these two protocols, with volumes of antioxidant and $Na_2CO_3$ solution proposed by Singleton et al. (1999) and wavelength proposed by Nenadis et al. (2007).

Values for cod protein hydrolysates found by FC assay did not show large variation, but the differences were statistically significant ($p < 0.05$). Salmon protein hydrolysate had the lowest activity ($0.10 \pm 0.00$ mmol/g protein). For CPHs, T3H showed the lowest antioxidant activity ($0.13 \pm 0.02$ mmol/g protein). Both T3H and SPH had the lowest amount of total amino acids, probably this resulted in low FC reducing capacity. FC assay is based on the reaction between oxidant and tyrosine/tryptophan (Sánchez-Rangel et al., 2013). However, under conditions used for determination of total amino acids content tryptophan is unstable (Çevikkalp et al., 2016). Therefore, amount of tryptophan in hydrolysates is unknown. Amount of tyrosine is known, but

hydrolysates with the highest amount of this amino acids did not show the highest FC reducing capacity. T6H and T13H had the highest antioxidant activity, but the amount of tyrosine was highest in SPH and T2H.

Singleton et al. (1999) mentioned that the FC assay should be interpreted carefully because of interferences and the ability of FC reagent to oxidize nonphenolic reducing agents, such as certain amino acids. Interferences can be enhancing, additive or inhibitory, so results for antioxidative activity can be influenced by that.

A very weak but not significant ($p > 0.05$) negative correlation was found between ABTS and FC assays (r = -0.33). Although these two assays are both based on electron transfer, which measures the reductive capacity of an antioxidant, different chemicals are involved and end results can therefore differ. Rate constants are also different for different antioxidants and oxidants, and therefore the overall antioxidant capacity will vary (MacDonald-Wicks et al., 2006). Because of the mentioned differences, these methods should be compared carefully. ABTS and FC antioxidant activities were calculated based on the amount of soluble protein measured by the Lowry method. This could influence the results because the Lowry method has some drawbacks which were discussed in section 3.2.

Despite the fact that no correlation between ABTS and DH was observed, FC and DH have showed moderate significant correlation (r = 0.78), by that supporting studies where DH has influenced antioxidant activity.

In the study by MacDonald-Wicks et al. (2006), it was stated the antioxidant capacity was more related to the ability of hydrogen atom donation than to the redox potential of the compound. They have therefore concluded, that electron transfer was not as relevant to antioxidant capacity *in vivo* and it was more difficult to study novel antioxidants using ET-based assays.

All antioxidant assays can be used with different concentrations of standards and different standards, so it is difficult to compare results with other studies. Also, different solvents are used, and optimal incubation time can therefore vary. For example, in the study by Katsube et al. (2004), FC assay was used to study antioxidant activity of edible plant products. They have used epigallocathechin 3-gallate (EGCG) as a standard and 70% (v/v) ethanol as a solvent. Values from the FC assay ranged from 188.5 to 1.0 $\mu$mol of EGCG equivalent per g sample, depending on the plant. Values in this thesis was expressed per g soluble protein, therefore, values from Katsube et al. (2004) would probably differ somewhat. Compared to this study, antioxidant activity of FPHs was quite high, even though it should be interpreted carefully since overestimation is possible. Most of the studies with FC assay are performed on the plant extracts or wines, for fish protein hydrolysates DPPH and ABTS assays are used more often.

The current situation is that there is no single antioxidant assay that can be applied for all the compounds (Apak et al., 2007). Food antioxidants are diverse, and reactions involved in the measurement of antioxidant activity are complex. Consequently, it is recommended to use methods based on different reaction mechanisms and with varying conditions to get appropriate results. Lastly, *in vitro* results should be supported with *in vivo* results to truly describe antioxidant activity, if tested compounds are desired to be used in food. Food model systems can be used for this purpose. It is usual to perform most antioxidant activity assessments in oil

(Zhong and Shahidi, 2015). In the work by Medina et al. (2012) activity of an antioxidant, caffeic acid, was reviewed in different fish lipid systems, such as bulk fish oils, liposomes from cod roe phospholipids, fish oil-in-water emulsions, washed cod mince and crude horse mackerel mince. The obtained results were interesting; the antioxidant activity of caffeic acid was different in the studied systems.

All protein hydrolysates have been stored in the freezer (-20$^o$C) for at least a year. It could influence stability, functional and bioactive properties of these hydrolysates. Hydrolysate T1H was found to be slightly sticky, and solubility was not as high as for other hydrolysates. This hydrolysate was prepared in February 2017, while other cod protein hydrolysates were prepared in March and May 2017. Decrease in solubility might be due to the aggregation of the peptides. As the storage time increased, destruction of antioxidative compounds could occur. In a study by Thiansilakul et al. (2007b), stability of protein hydrolysates from round scad (*Decapterus maruadsi*) was studied at two different storage temperatures (4$^o$C and 25$^o$C) for 6 weeks. It was observed a slight decrease in DPPH radical scavenging activity for both temperatures. Cod protein hydrolysates were stored for much longer time. Temperature fluctuations can reduce the bioavailability of Lys because of increased frequency of the Maillard reaction (Klompong et al., 2012).

## 3.8 ACE inhibitory activity

Angiotensin-converting enzyme inhibitory activity was measured by two different methods, by Sentandreu and Toldrá and by Cushman and Cheung. The first method is quite recent (2006) and has not been reported so frequently in the literature, but in the published articles it has shown good results. The Cushman and Cheung method (1971) has quite a long history and has been tested on different products. However, different modifications have been done. For example, different buffers have been used, and the extraction/quantification of released hippuric acid was performed in different ways. Bougatef et al. (2008) have quantified the released hippuric acid by RP-HPLC, while Yu et al. (2013) have used the extraction with ethyl acetate and measurement of absorbance as it was in the original protocol by Cushman and Cheung (1971).

The biggest limitation with the Cushman and Cheung's method is the extraction step. This step is the additional source of error that can be avoided using other methods. The extraction step is also significantly reducing the amount of samples that could be analysed during the day. Sentandreu and Toldrá presented a rapid and simple method without the extraction step that can allow analysis of a high number of samples. Using the fluorescence method it was possible to analyse up to 4 samples during 3 hours, while Cushman and Cheung's method required 3 hours for only one sample. Taking several samples at the same time would be difficult since it would lead to different incubation time for the samples and could affect the results.

Another big difference in these methods is the substrate. Cushman and Cheung are using HHL, while Sentandreu and Toldrá are using AGPP. The first one is cheaper and easier to purchase in bigger amounts. The price for 25 mg of HHL was 253 NOK, while AGPP cost 4390 NOK for 25 mg at Sigma Aldrich. To analyse one hydrolysate (6 different concentrations + blank) it will cost around 33 NOK with Cushman and Cheung's method, whereas the price with Sentandreu and Toldrá's protocol will be 191 NOK per hydrolysate. This is a noticeable

difference, hence all advantages and disadvantages should be evaluated before choosing the method.

### 3.8.1 Method by Sentandreu and Toldrá

The ease of the performance is a definite advantage of this method. However, during the specialization project results obtained by this method were not reliable. It was possible to calculate $IC_{50}$ values for just two out of five samples, and most of the concentrations gave negative ACE inhibition. The highest negative ACE inhibition was 200%. Fluorescence was expected to increase linearly during at least 30 minutes from the paper by Sentandreu and Toldrá (2006b). During the specialization project, linearity was observed for only 15 minutes. Calculations were checked thoroughly several times, all solutions were prepared again with fresh enzyme and substrate, but it did not improve the results. Another fluorimeter at the department was also tested, but this changed nothing. The problem was finally solved by adjusting settings in the software of TECAN Spark® multimode microplate reader. This software was also newly installed. It turned out standard settings for gain, flashes and settle time did not work for this method. Changing these settings gave considerably better results with linearity for the whole period of measurement and improved values for inhibition. It made it possible to estimate $IC_{50}$ values, though some of the samples still showed quite a low degree of inhibition. Results are presented in **Figure 3.12**. T2H had significantly lower ACE inhibitory activity compared to other hydrolysates ($p < 0.05$), while SPH had the highest ACE inhibitory activity.

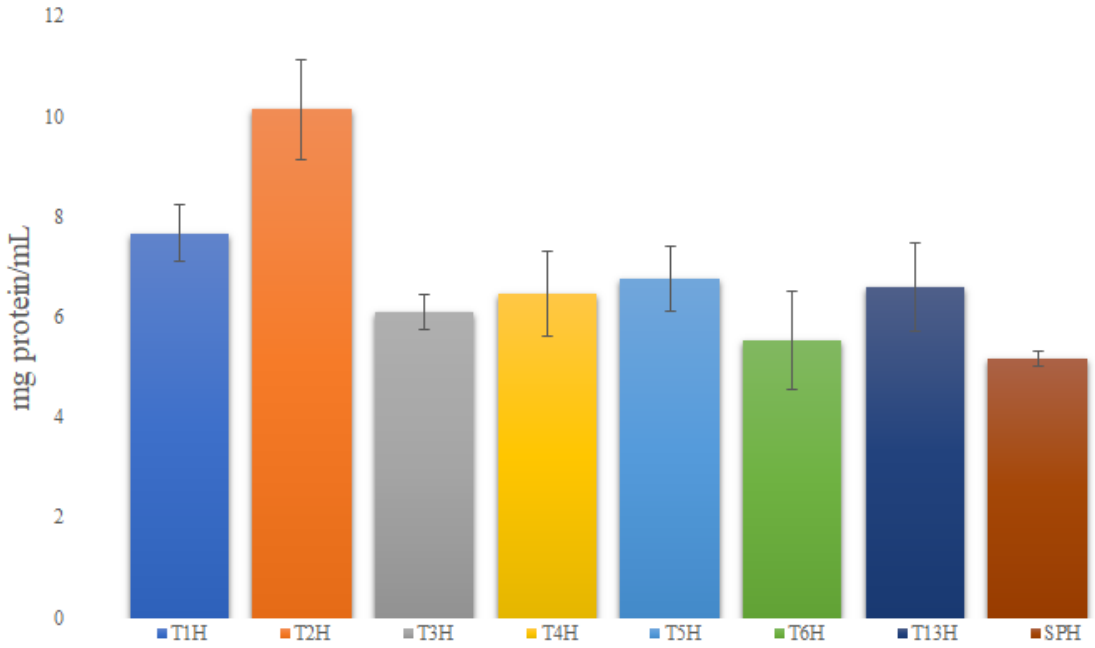



**Figure 3.12:** $IC_{50}$ values for crude protein hydrolysates, presented as mg protein per mL and expressed as the mean ± SD (n = 3).

The obtained results differed from what was achieved by Ohnstad (2018). She was analysing the same cod hydrolysates. She obtained lower $IC_{50}$ values. One of the reasons for the discrepancy could be that she used different settings in the fluorimeter, the software was different, or ACE

inhibitory activity of hydrolysates has decreased during the storage time. However, results from permeate were in agreement with Ohnstad (2018), the fraction with small peptides and free amino acids did not increase ACE inhibitory activity. **Table 3.7** is representing $IC_{50}$ values for permeates of T1H and SPH and the second retentate of T1H. Even though different concentrations of retentates were investigated, only a very low degree of inhibition was observed and it was difficult to calculate any reliable $IC_{50}$ values. It can, therefore, be assumed that fractionation of hydrolysates was not necessary, even though several studies indicated the opposite (Sun et al., 2017; Yathisha et al., 2018). Peptide interactions can contribute to ACE inhibitory activity, and ultrafiltration can remove these interactions (Raghavan and Kristinsson, 2009). It can also be suggested that another membrane size could be used. It could be that the peptide size in the permeate was too small, and small peptides did not have ability to inhibit the ACE enzyme. From **Table 3.7** value for the second retentate is lower than for permeate, indicating that this assumption could be true.

**Table 3.7:** $IC_{50}$ values for UF fractions of T1H and SPH, where P is permeate and R2 is the second retentate of T1H expressed as the mean $\pm$ SD (n = 3).

| | **T1H P** | **T1H R2** | **SPH P** |
|---|---|---|---|
| **$IC_{50}$ (mg/mL)** | 22.23 $\pm$ 2.04 | 15.37 $\pm$ 1.25 | 8.03 $\pm$ 0.78 |

Salmon protein hydrolysate showed more potent ACE inhibitory activity, despite the fact that it consisted of bigger peptides than the cod protein hydrolysates as it was observed from molecular weight distribution plots. The reason for the higher inhibitory activity in salmon protein hydrolysate could also be because of what parts of the fish that were utilised for hydrolysis. Cod protein hydrolysates were prepared from the head, but salmon protein hydrolysates were prepared from the fillet. The result from the study by Dragnes et al. (2009) reported the highest ACE inhbibitory activity in the fillet of cod and salmon compared to other fish parts.

For orientation, peptides from fermented soybean treated with chymotrypsin and pepsin had $IC_{50}$ = 0.019 mg/mL, peptides from pea protein hydrolysate had $IC_{50}$ = 0.07 mg/mL and squid gelatin hydrolysate had $IC_{50}$ value of 0.340 mg/mL (Aluko, 2012). Captopril had $IC_{50}$ value of 0.0013 $\mu$g/mL (Fitzgerald and Meisel, 2000). Other studies reported similar values (Chen et al., 2013; Intarasirisawat et al., 2013). $IC_{50}$ value of sardinelle protein hydrolysate varied between 1.24 and 7.4 mg/mL (Bougatef et al., 2008). From Ohnstad (2018) $IC_{50}$ values for crude cod protein hydrolysates were between 2 to 4 mg/mL approximately. Results from **Figure 3.12** represent higher values. The reasons for these large discrepancies could be changes in the properties of the hydrolysates. They were stored in the freezer for almost two years. It could influence the properties. Another reason could be different substrates used for the measurement of ACE inhibitory activity for the mentioned hydrolysates. Ohnstad (2018) was the only one who measured ACE inhibition with the same method and substrate as in this thesis.

Hydrophobic and aromatic amino acids were reported to influence ACE inhibitory activity (Yu et al., 2013). Peptides with phenylalanine, tyrosine, or proline at the C-terminus had higher potency of inhibitory activity according to this study. The hydrophobicity at the N-terminus may also contribute to the inhibition activity. No correlation was observed between the amount of hydrophobic and aromatic amino acids and inhibitory activity. The amount of free amino acids did not influence the inhibitory activity either. As for the antioxidant activity, DH was also

reported to influence the ACE inhibitory activity (Raghavan and Kristinsson, 2009). However, no correlation was observed. The absence of correlation between the factors that were reported to have an impact can indicate that more studies should be done to verify the results of this method.

A moderate correlation was found between ACE inhibitory activity and ABTS scavenging activity (r = 0.62), but it had a p-value of less than 0.05. From this, it can be assumed that the same bioactive peptides could be responsible for both ACE inhbition and antioxidant activity, but more hydrolysates should be analysed to get a significant correlation. Several studies analysing these two bioactivities found positive correlation (Šližytė et al., 2016; Paiva et al., 2017).

### 3.8.2 Method by Cushman and Cheung

When this method was first used, it gave very unstable results, so different approaches have been tested to find what the reason for this was. First of all, Cushman and Cheung's method contains more steps in the procedure compared to the method by Sentandreu and Toldrá. Step with extraction is the key step because it determines how much hippuric acid would end up in the final solutions. If this step is not performed properly, the amount of extracted hippuric acid can be significantly lower than the actual amount of hippuric acid formed during the reaction between substrate and enzyme. The direct kinetic spectrophotometric assay is not possible due to the insufficient spectral difference between HHL and hippuric acid. Hence, the extraction step is required for this method.

Values for absorbance after extraction are supposed to give a straight decreasing line for increasing concentrations of the sample. If any mistakes are made during this extraction step, final results will be greatly influenced. Therefore, it is particularly important to perform this step properly. In order to check if this step was the reason for unstable results, different concentrations of hippuric acid were prepared in the borate buffer and extracted directly with ethyl acetate. Observations have shown that the results were better when each sample was vortexed for exactly 60 seconds after addition of ethyl acetate. When the time was varying, results for the same parallel differed significantly. **Figure 3.13** and **Figure 3.14** represent results obtained by using different concentrations of hippuric acid when mixing time was exactly 60 seconds and when the time was not taken. **Figure 3.14** does not give any sense, absorbance is decreasing with increasing concentration of hippuric acid.

In the paper by Cushman and Cheung (1971) the influence of different conditions was described, and some of the conditions were therefore tested in this thesis. While concentrations and activity of substrate and enzyme were kept the same, different buffer and reaction conditions were tested. In this thesis sodium borate buffer, 0.01 M, pH 8.3 was used. To exclude that buffer could influence the results, another buffer was also tested, sodium phosphate buffer, 0.02 M, pH 8.3. This buffer gave even less consistent results with absorbance lower than zero for most of the samples, and still highly variable absorbance values. Further measurements were performed in sodium borate buffer. These findings did not confirm previous results from Hernández-Ledesma et al. (2003), where 0.2 M phosphate buffer provided a faster reaction rate of the enzyme on the substrate compared to a borate buffer.

The very first experiment was performed with a borate buffer without any NaCl added. Differences between results obtained with buffer containing chloride ions and buffer lacking these

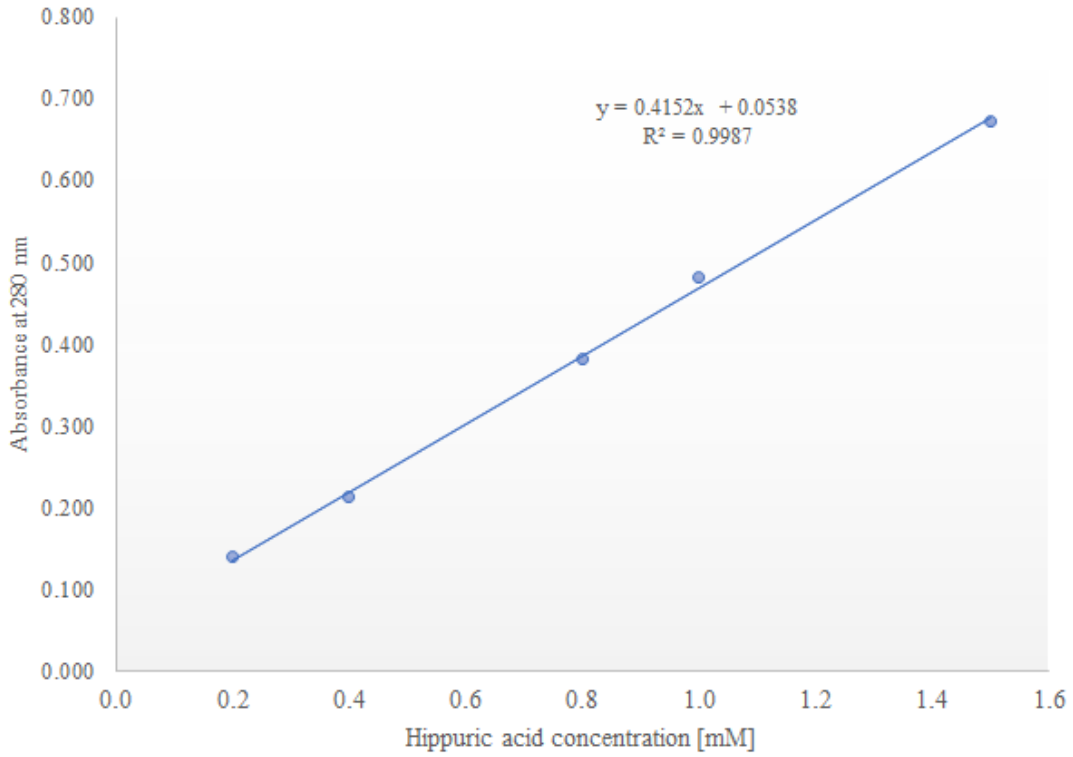



**Figure 3.13:** Different concentrations of hippuric acid extracted with ethyl acetate plotted against absorbance values at 228 nm. Vortexed for 60 seconds.

were highly significant. There was almost no enzyme activity observed in the first experiment. This result was in agreement with the study by Cushman and Cheung (1970), where it was found that the rate of angiotensin-converting enzyme prepared in buffer containing NaCl was 7.5 times greater than the rate in the assays lacking chloride ion.

The incubation time was another factor that could influence the experiment. Most of the measurements were performed with 30 minutes incubation, but for two of the experiments, the incubation time was extended to 45 minutes to observe whether the sensitivity of the method improved. No significant differences were observed between these two incubation times, indicating that such a small increase in the incubation time was not necessary and was therefore not used in further measurements. However, from the other study, it was observed that the increase of the incubation time to 80 min leads to an increase in absorbance (Hernández-Ledesma et al., 2003). Due to the limited time, longer incubation times were not investigated.

Another problem that has occurred during the measurements was that the highest concentrations of inhibitor solutions (hydrolysates) gave very unstable and inaccurate values for absorbance. The reason for that could be the presence of compounds that were extracted by ethyl acetate and absorb at 228 nm in addition to hippuric acid in the samples (Hernández-Ledesma et al., 2003). These compounds, therefore, could be responsible for interferences in the measurements. Hence, it was decided to exclude the two highest concentrations (20 mg/mL and 30 mg/mL).

Three different hydrolysates were analysed using this method. $IC_{50}$ values were calculated and presented in the **Table 3.8**. A detailed explanation of calculations can be found in Appendix E.2.

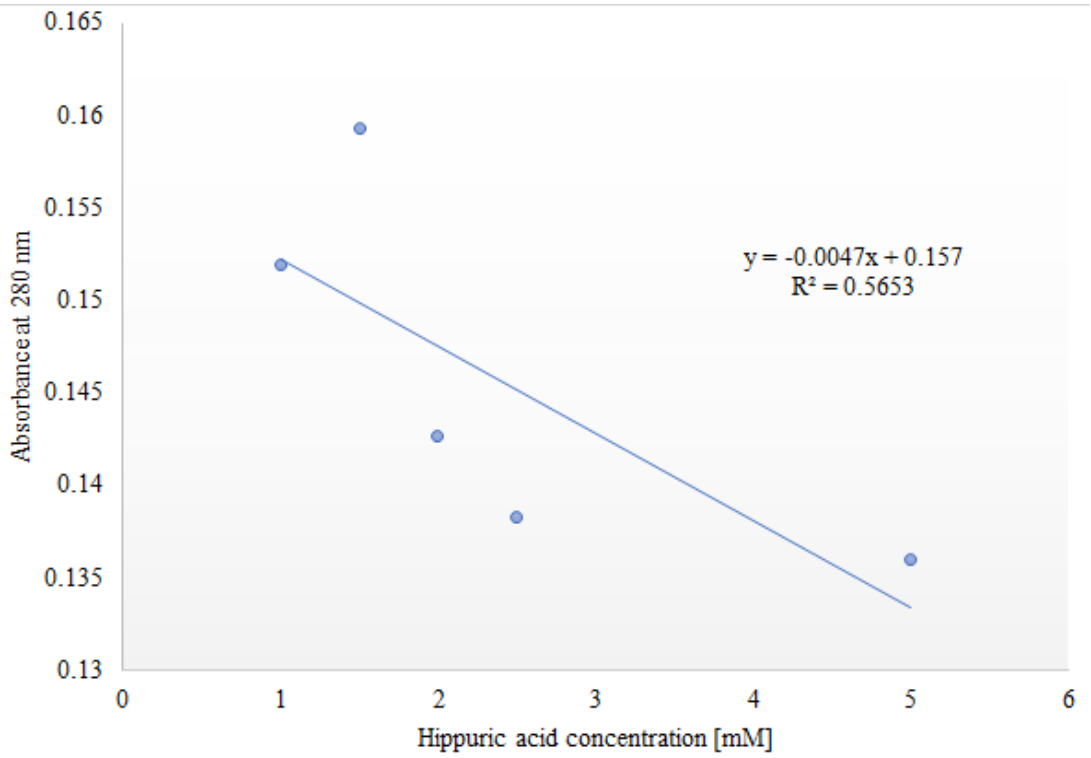



**Figure 3.14:** Different concentrations of hippuric acid extracted with ethyl acetate plotted against absorbance values at 228 nm. Time for vortexing was not taken.

Using fresh enzyme and substrate solutions as well as mixing for exactly 60 seconds during

**Table 3.8:** Concentrations of hydrolysates needed for 50% inhibition of ACE. Values are given as the mean ± SD (n = 3).

| | **T5H** | **T6H** | **SPH** |
|---|---|---|---|
| **$IC_{50}$ (mg/mL)** | 1.16 ± 0.18 | 1.24 ± 0.29 | 1.70 ± 0.27 |

the extraction step improved results significantly. Increasing concentration of inhibitor gave an increasing degree of ACE inhibition, and $IC_{50}$ value was easy to calculate from the curves obtained by plotting ACE inhibition against inhibitor concentrations, which can be found in Appendix E.2. The highest ACE inhibitory activity was found for T5H, T6H was on the second place and SPH showed the lowest activity. The amino acid composition of T5H and T6H was more or less the same, while SPH had a significantly lower amount of glutamine and alanine, but a much higher amount of tyrosine. Findings by Salampessy et al. (2015), where the presence of tyrosine and valine improved ACE inhibitory activity, were not confirmed. T5H and T6H had a higher percentage of hydrophobic amino acids (49.24% and 50.91%) compared to SPH (37.46%), indicating that it can influence ACE inhibition. The same result was reported in a comprehensive review on functional and bioactive properties of fish protein hydrolysates by Halim et al. (2016).

**Comparison of the two methods**

Both methods showed that hydrolysates exhibited ACE inhibition, but the $IC_{50}$ values obtained by Cushman and Cheung's method were significantly lower than values obtained by the fluorescence method. Results obtained by Sentandreu and Toldrá's method were high. It is not so profitable/valuable to research on these hydrolysates if the amount of hydrolysate that is needed to be ingested is too high. Cushman and Cheung's method gave results that can actually be investigated further and tested in food model or biological model systems. Commercially produced fermented sour milk analysed by the Cushman and Cheung's method had $IC_{50}$ of 0.38 mg/mL, which is not so far away from cod and salmon hydrolysates (Fahmi et al., 2004). Such a large difference in the results from these two methods is the challenge.

Methods by Sentandreu and Toldrá and by Cushman and Cheung were negatively correlated with the correlation coefficient (r) of 0.76. This was not expected, the positive correlation would be more reasonable. However, only three hydrolysates were analysed by Cushman and Cheung method. To establish and evaluate the accurate relationship between these methods, analysis of more hydrolysates should be considered. Sentandreu and Toldrá (2006b) have reported a good positive correlation between these two methods, however, they did not indicate the $IC_{50}$ values.

It is possible to assume that the hydrolysates contained some compounds that interfered with bioactive peptides preventing them to exert inhibition of ACE. Hence, to obtain the highest possible ACE inhibitory activity, it is recommended to purify bioactive peptides. Fahmi et al. (2004) has reported that isolated peptides exerted higher activity (lower $IC_{50}$ values) than the original hydrolysate.

From the study of Jeon et al. (1999), fractionation of cod frame hydrolysates improved the ACE inhibitory effect. Fractions of $< 3$ kDa had $IC_{50}$ values of 0.08 mg/mL, while the original hydrolysate had around 0.32 mg/mL. Another study by Raghavan and Kristinsson (2009) has reported an absence of increased ACE inhibitory activity in the tilapia fractionated hydrolysates. This study even showed decreased activity compared to crude hydrolysates. It was assumed that the loss of synergetic action amongst all the peptides led to lower activity. Lee et al. (2010) came also to the same conclusion analysing tuna frame fractions. A fraction of 1-5 kDa exerted highest ACE inhibitory activity, with a fraction of 5-10 kDa on the second place, and fractions with molecular weight less than 1 kDa exhibited the lowest activity.

During this thesis, it was concluded that measurement of ACE inhibition on fish protein hydrolysates with Cushman and Cheung's method should be performed with borate buffer and NaCl. After the addition of ethyl acetate, the solution should be mixed exactly one minute, otherwise, results will be unreliable and unstable. For Sentandreu and Toldrá's method, it is particularly important to use the right settings in the fluorimeter, because it can influence the results for ACE inhibition significantly.

## 3.9 Principal Component Analysis

**Figure 3.15** illustrates the results of the PCA performed on the crude protein hydrolysates. Principal component 1 (F1) explained 48.17% of the total variance, while principal component 2 (F2) explained 27.43%. The following variables were included in the analysis: degree of

hydrolysis, total and free amino acid content, ACE inhibition activity (by Sentandreu and Toldrá (2006a)), ABTS radical scavenging activity, FC reducing capacity, protein solubility (by the Lowry method) and amount of acid soluble peptides. The closer variables are to each other, the more positively correlated they are. Information obtained from the F1 is more important than the information from the F2. PCA was not applied for the UF fractions since there were too few samples. PCA required at least five samples to give better information about the correlations (Oliveira et al., 2015).

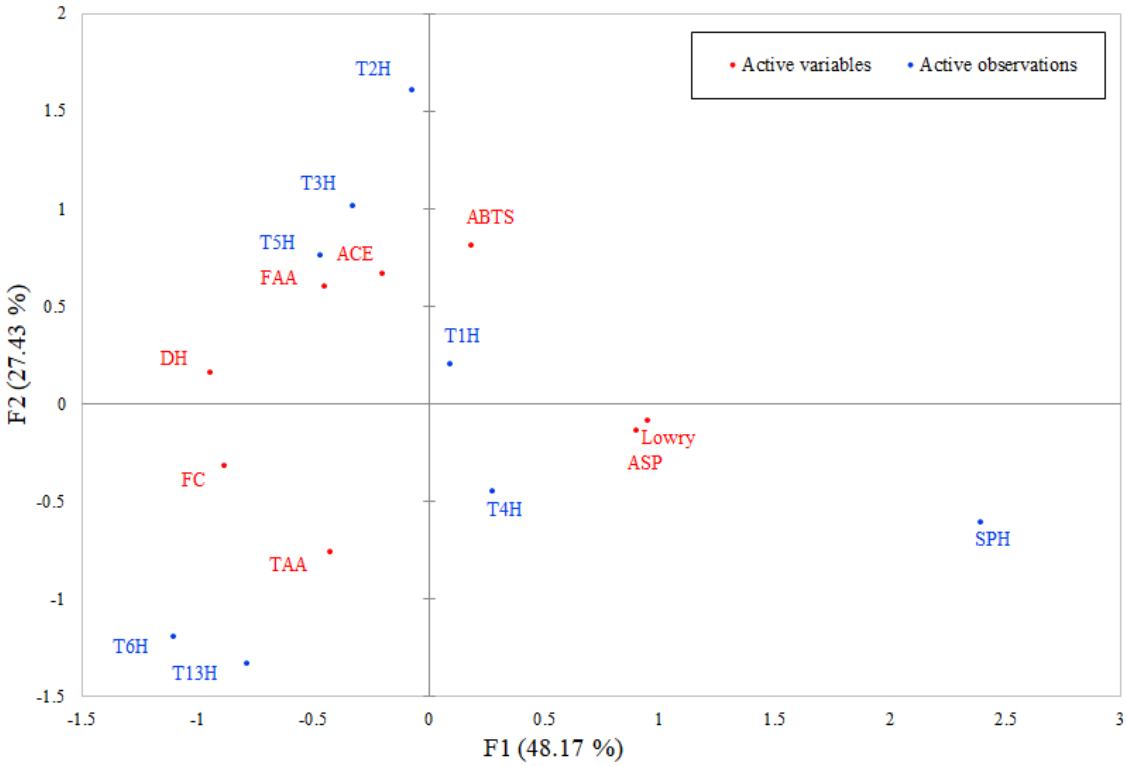



**Figure 3.15:** Biplot showing correlations in the crude protein hydrolysate found by PCA.

Soluble protein concentration was positively correlated with the amount of acid soluble peptides. High solubility indicated a high amount of acid soluble peptides. This was already discussed in section 3.6. ACE inhibitory activity showed some correlation with the amount of free amino acids and ABTS radical scavenging activity. As was mentioned before, several studies have found a positive correlation between antioxidant and ACE inhibiting activities of fish protein hydrolysates (Šližytė et al., 2016; Paiva et al., 2017). The FC assay was slightly correlated with the total amino acid content and DH. An increase in the degree of hydrolysis has been associated with an increase in antioxidant activity in several studies (Garcia et al., 2012; Thiansilakul et al., 2007a; Jamdar et al., 2010). PCA confirmed also that mechanisms in the ABTS assay and the FC assay may be unrelated and therefore no significant correlation was observed from **Figure 3.15**.

Salmon protein hydrolysate was situated far away from all other samples and variables, indicating that it differed significantly from cod protein hydrolysates. It is logical since structural and bioactive properties are usually dependent on the species, part of fish used for hydrolysis and hydrolysis conditions. Among the cod protein hydrolysates, T6H and T13H were grouped,

and T5H and T3H were grouped. It means that the PCA showed similarities in structural and bioactive properties within the group, but differences between the groups. T5H and T3H had a similar degree of hydrolysis, while T6H and T13H had similar protein content and FC reducing capacity. However, similar hydrolysis conditions were applied for all cod protein hydrolysates. The mixture of Papain and Bromelain was used for hydrolysis of all samples, except T3H. The amount of water differed, but grouped hydrolysates did not have the same amount of water. The harvested period was also different, but T5H and T6H were placed in different groups even though they were harvested during the same month.

Chapter 4

# Conclusion

Two bioactive properties of fish protein hydrolysates and their relationship with structural properties were studied in this master's thesis. The structure and composition of fish protein hydrolysates were analysed by determining degree of hydrolysis, total and free amino acid content, protein concentration, amount of acid soluble peptides and molecular weight distribution. Two hydrolysates were fractionated by ultrafiltration with MWCO of 4 kDa. The degree of separation was not as sharp as desired. However, the second filtration improved separation to some degree, but it also led to a bigger loss.

Fish protein hydrolysates consisted of peptides of different sizes with a low amount of free amino acids. Salmon protein hydrolysate differed from cod protein hydrolysates by the presence of bigger peptides and a lower degree of hydrolysis. The degree of hydrolysis was correlated with the amount of water used during hydrolysis.

Each bioactivity was analysed by two different methods. Antioxidant activity was determined by measuring ABTS radical scavenging activity and FC reducing capacity. The FC assay was slightly modified to get lower absorbance values. The ABTS assay showed that UF fraction with peptides $<$ 4 kDa improved antioxidant activity compared to a crude hydrolysate and fraction with peptides $>$ 4 kDa. Cod protein hydrolysate prepared with citric acid showed the lowest antioxidant activity by the ABTS assay, indicating that scavenging was dependent on pH. The amount of free tyrosine and the total amount of free aromatic amino acids influenced antioxidant activity. Results from the FC assay did not correlate with results from the ABTS assay.

ACE inhibition activity was measured by spectrophotometric and fluorescence methods. Some problems occurred with the performance of Cushman and Cheung's method, but after a thorough investigation, a well functioning protocol was implemented. Some adjustments in the fluorimeter were needed for Sentandreu and Toldrá's method. $IC_{50}$ values by Sentandreu and Toldrá method were quite high, while Cushman and Cheung's method gave significantly lower values. Values from Sentandreu and Toldrá method did not show any correlation with the amount and content of amino acids or degree of hydrolysis. Ultrafiltration did not show to affect ACE inhibitory activity, therefore it can be considered unnecessary.

Fish protein hydrolysates are a good source of compounds with antioxidant and antihypertensive effects, but these effects depend on species, parts of fish used for hydrolysis and possibly

storage time and conditions. It is therefore recommended that correlations between these factors and bioactivities are investigated further.

Chapter 5

# Further work

For further investigation of the bioactive properties of fish protein hydrolysates, new analysis methods can be applied. More hydrolysates should be evaluated in order to give more trustworthy results. For ACE inhibitory activity and antioxidative activity, membrane filtration can be applied with different cut-off values. Bioactive peptides can be purified from the hydrolysates to determine the sequences. More hydrolysates should be analysed by Cushman and Cheung's method to compare it with Sentandreu and Toldrá's method. Other antioxidant assays based on different mechanisms can be tested to get more understanding. Model food systems can be used for the evaluation of an antioxidant activity.

More studies could be conducted on the effect of storage time and conditions on properties of FPH. Bioactive properties as well as functional and sensory can be studied by incorporation hydrolysates in the food products. *In vivo* studies are needed to understand the possibilities of using hydrolysates in the foods and nutraceuticals.

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Appendix A

# Raw materials

Cod protein hydrolysates were produced by SINTEF Ocean in the pilot project HEADS UP. Cod heads were used as a raw material. **Table A.1** provides description of hydrolysis conditions and harvest time of cod protein hydrolysates. **Figure A.1** illustrates samples of all fish protein hydrolysates analysed in this thesis.

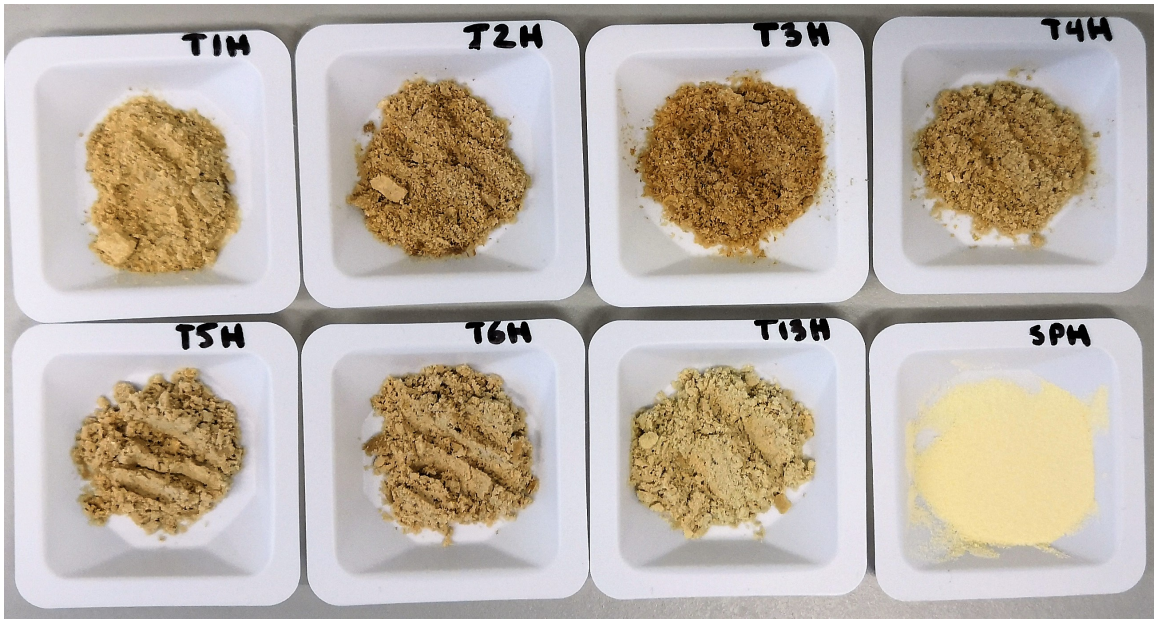



**Figure A.1:** Samples of fish protein hydrolysates analysed in this thesis.

**Table A.1:** Information about hydrolysis conditions and harvest time for cod protein hydrolysates. Temperature of hydrolysis was 50$^{o}$C for all hydrolysates and 400 kg raw material were used per experiment.

| CPH | Month of harvest (2017) | Water (kg) | Enzyme | Enzyme concentration (% w/w raw material) |
|---|---|---|---|---|
| T1H | February | 400 | Papain + Bromelain | 0.1 |
| T2H | February | 300 | Papain + Bromelain | 0.1 |
| T3H | February | 300 | Protamex | 0.05 |
| T4H | March | 300 | Papain + Bromelain | 0.1 |
| T5H | March | 200 | Papain + Bromelain | 0.1 |
| T6H | March | 100 | Papain + Bromelain | 0.1 |
| T13H | May | 300 | Papain + Bromelain (+ citric acid) | 0.1 |

Appendix B

# Degree of hydrolysis

First, % free amino groups should be determined by applying the following equation:

$$\frac{A \times B \times 14.007 \times 100}{C \times 1000} = \%\ free\ amino\ groups\ (D) \tag{B.1}$$

where A is amount of NaOH used, B - concentration of the solution used for titration (0.1 M NaOH) and C is amount of the sample (g).

After that, degree of hydrolysis (%) is calculated by utilizing **Equation B.2**.

$$\frac{D \times 100}{E} = DH\ (\%) \tag{B.2}$$

where D is % free amino groups and E is % N (% protein divided by 6.25). Values from the Lowry method were used for E.

For example, for SPH, 14.398 mL NaOH were used for the first parallel with mass of 1.5047 g. It was assumed that SPH consisted of 100% protein. % free amino groups (D) was calculated to be:

$$\frac{14.398 \times 0.1 \times 14.007 \times 100}{1.5047 \times 1000} = 1.34 \tag{B.3}$$

Degree of hydrolysis (%) was then:

$$\frac{1.34 \times 100}{\frac{100}{6.25}} = 8.38 \tag{B.4}$$

Appendix C

# Protein concentration - the Lowry method

Protein concentration in crude protein hydrolysates and UF fractions was determined by using the Lowry method (Lowry et al., 1951). 100 mg crude protein hydrolysate was dissolved in 10 mL distilled water. Permeate (<4 kDa) and retentates (>4 kDa) had initial concentration of 10 mg/ml.

To establish the relationship between protein concentration and absorbance, a standard curve using bovin serum albumin (BSA) was obtained. BSA stock solutions were prepared in different concentrations (12.5, 25, 50, 100, 150, 200 and 300 $\mu$g BSA/ml). Results for absorbance for BSA are presented in **Table C.1**.

**Table C.1:** Absorbance measured at 750 nm, average and standard deviation for different concentrations of standard protein (BSA) are presented.

| BSA [$\mu$g/mL] | OD750 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| 12.5 | 0.021 | 0.021 | 0.028 | 0.024 | 0.004 |
| 25 | 0.043 | 0.043 | 0.046 | 0.044 | 0.002 |
| 50 | 0.085 | 0.081 | 0.084 | 0.083 | 0.002 |
| 100 | 0.159 | 0.165 | 0.174 | 0.166 | 0.007 |
| 150 | 0.239 | 0.235 | 0.231 | 0.235 | 0.004 |
| 200 | 0.290 | 0.293 | 0.291 | 0.292 | 0.002 |
| 300 | 0.398 | 0.435 | 0.400 | 0.411 | 0.021 |

Concentrations of bovin serum albumin was plotted against absorbances to obtain a standard curve. Results are shown in **Figure C.1** and the equation for standard curve is:

$$y = 0.0014x + 0.0168, \tag{C.1}$$

where y is absorbance, and x is concentration of BSA.

Protein content of hydrolysates was calculated in $\mu$g/mL using equation for standard curve. Final answers were corrected for dilution and converted to mg/mL.

Examples of calculation for T2H is shown in the following equations:

$$x = \frac{0.212 - 0.0168}{0.0014} = 139.43 \tag{C.2}$$

To get the final answer value from equation F is multiplied with dilution factor (DF) and divided by 1000 to get the units mg/mL:

$$\frac{139.43 \times 50}{1000} = 6.97 \tag{C.3}$$

**Table C.2** represents protein content (%) of fish protein hydrolysates determined by SINTEF

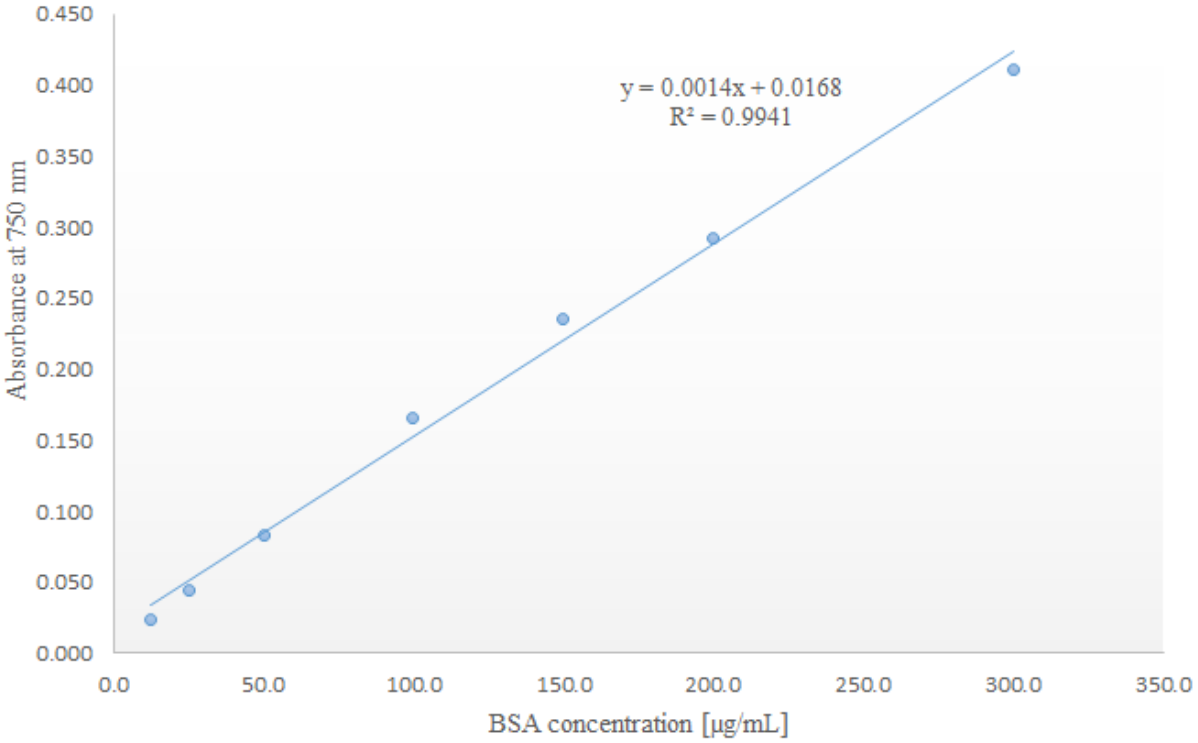



**Figure C.1:** Standard curve of BSA used to determine protein concentrations in crude protein hydrolysates and UF fractions. BSA concentration in $\mu$g/mL is plotted against absorbance at 750 nm. Equation for this curve is y = 0.0014x + 0.0168.

Ocean.

**Table C.2:** Protein content in cod protein hydrolysates determined by SINTEF Ocean (Remme et al., 2018).

| Hydrolysate | Protein content (%) |
|---|---|
| T1H | 81.57 |
| T2H | 81.40 |
| T3H | 80.20 |
| T4H | 85.00 |
| T5H | 83.75 |
| T6H | 83.75 |
| T13H | 75.63 |

Appendix D

# Amino acid content and composition

## D.1 Total amino acid content and composition

The total amino acid composition was determined by HPLC analysis. To convert results from HPLC to mg amino acids per gram, following equation was used:

$$\frac{mg\ aa}{g} = \frac{\frac{nmol\ aa}{mL} \times M_{w,aa}(\frac{g}{mol}) \times V_{FPH}(mL) \times DF}{1 \times 1000 \times 1000 \times m_{FPH}(g)} \tag{D.1}$$

where nmol aa/mL is obtained from HPLC analysis (**Figure D.1**, last column), values for $M_{w,aa}$ are defined as molecular weight of amino acids when bound to protein, DF is dilution factor, $V_{FPH}$ and $m_{FPH}$ are volume and mass of fish protein hydrolysate. Values for CPHs were obtained by Asfour (2018), SPH was analysed during this thesis. Results are presented in **Table D.1** and **Table D.2**.

| 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 | 1.520 | 12.129 | 108.736 | 9.52 | 15.79 | 4.5121 |
| 2 | Glu | 2.297 | 15.808 | 71.047 | 12.41 | 10.32 | 5.3109 |
| 3 | Asn | 3.243 | 0.009 | 0.055 | 0.01 | 0.01 | 0.0047 |
| 4 | His | 4.067 | 1.441 | 8.168 | 1.13 | 1.19 | 0.7452 |
| 5 | Ser | 4.422 | 6.717 | 37.688 | 5.27 | 5.47 | 3.0356 |
| 6 | Gln | 4.622 | 0.394 | 4.070 | 0.31 | 0.59 | 0.2205 |
| 7 | Gly/Arg | 7.495 | 46.772 | 171.279 | 36.71 | 24.87 | 9.8998 |
| 8 | Thr | 8.123 | 4.442 | 17.452 | 3.49 | 2.53 | 2.1424 |
| 9 | Ala | 12.677 | 12.772 | 59.180 | 10.03 | 8.59 | 6.2546 |
| 10 | Tyr | 14.328 | 1.116 | 7.279 | 0.88 | 1.06 | 0.4870 |
| 11 | Aba | 16.133 | 0.062 | 0.405 | 0.05 | 0.06 | 0.0252 |
| 12 | Met | 18.000 | 2.625 | 20.700 | 2.06 | 3.01 | 1.0026 |
| 13 | Val | 18.302 | 4.835 | 38.373 | 3.80 | 5.57 | 1.7078 |
| 14 | Phe | 18.827 | 2.399 | 19.228 | 1.88 | 2.79 | 1.0042 |
| 15 | Ile | 19.802 | 3.435 | 27.469 | 2.70 | 3.99 | 1.2410 |
| 16 | Leu | 20.153 | 6.209 | 44.942 | 4.87 | 6.53 | 2.4675 |
| 17 | Lys | 21.907 | 6.230 | 52.650 | 4.89 | 7.64 | 2.8495 |
| **Total:** | | | **127.397** | **688.719** | **100.00** | **100.00** | |

**Figure D.1:** Raw data for SPH obtained from HPLC analysis.

**Table D.1:** The average total amino acid composition presented in mg/g FPH. All values except T2H and SPH were determined by Asfour (2018). Standard deviation was not specified.

| | **T1H** | **T2H** | **T3H** | **T4H** |
|---|---|---|---|---|
| **Asp** | 44.93 | 38.42 | 36.12 | 76.81 |
| **Glu** | 66.59 | 53.42 | 56.77 | 100.08 |
| **Asn** | 0.08 | 0.29 | 0.21 | 0.19 |
| **His** | 9.47 | 8.94 | 7.83 | 8.62 |
| **Ser** | 27.40 | 26.49 | 24.68 | 46.77 |
| **Gln** | 0.00 | 2.04 | 0.00 | 0.73 |
| **Gly/Arg** | 63.94 | 59.53 | 58.42 | 111.32 |
| **Thr** | 20.67 | 19.31 | 17.51 | 38.13 |
| **Ala** | 10.82 | 72.88 | 8.42 | 8.34 |
| **Tyr** | 35.29 | 4.17 | 32.13 | 65.56 |
| **Aba** | 0.78 | 0.62 | 0.76 | 0.92 |
| **Met** | 13.98 | 11.81 | 11.63 | 22.83 |
| **Val** | 20.65 | 18.85 | 16.44 | 36.48 |
| **Phe** | 17.25 | 15.89 | 14.03 | 30.09 |
| **Ile** | 16.64 | 14.17 | 13.14 | 29.29 |
| **Leu** | 33.62 | 27.15 | 27.69 | 60.70 |
| **Lys** | 37.30 | 33.73 | 32.26 | 66.22 |
| **Total** | 419.41 | 407.70 | 358.04 | 703.08 |

**Table D.2:** Continuation of Table D.1. The average total amino acid composition presented in mg/g FPH. All values except T2H and SPH were determined by Asfour (2018). Standard deviation was not specified.

| | **T5H** | **T6H** | **T13H** | **SPH** |
|---|---|---|---|---|
| **Asp** | 11.87 | 21.23 | 26.54 | 39.50 |
| **Glu** | 25.38 | 42.08 | 50.09 | 52.49 |
| **Asn** | 0.37 | 0.64 | 1.36 | 0.05 |
| **His** | 13.43 | 21.69 | 15.85 | 7.64 |
| **Ser** | 19.55 | 33.18 | 31.01 | 19.71 |
| **Gln** | 31.14 | 45.39 | 42.64 | 2.26 |
| **Gly/Arg** | 43.71 | 80.33 | 86.91 | 73.06 |
| **Thr** | 19.39 | 31.17 | 26.56 | 16.11 |
| **Ala** | 16.41 | 34.38 | 36.78 | 77.06 |
| **Tyr** | 41.34 | 74.72 | 75.52 | 2.42 |
| **Aba** | 4.45 | 6.70 | 8.15 | 0.16 |
| **Met** | 28.06 | 47.79 | 37.98 | 9.54 |
| **Val** | 23.26 | 40.53 | 35.52 | 12.81 |
| **Phe** | 22.98 | 38.69 | 41.73 | 10.89 |
| **Ile** | 13.57 | 22.71 | 21.64 | 10.49 |
| **Leu** | 52.71 | 95.48 | 95.24 | 19.84 |
| **Lys** | 35.20 | 59.23 | 71.27 | 27.80 |
| **Total** | 402.82 | 695.94 | 704.79 | 381.82 |

## D.2 Free amino acid content and composition

The amount of free amino acids was also determined by HPLC analysis. In this analysis, crude protein hydrolysates and UF fractions of T1H and SPH were analysed. **Equation D.2** was used to find the amount of free amino acids in mg free amino acids/g:

$$\frac{mg\ free\ aa}{g} = \frac{\frac{nmol\ aa}{mL} \times M_{w,aa}(\frac{g}{mol}) \times 1.25 \times V_{FPH}(mL) \times DF}{1 \times 1000 \times 1000 \times m_{FPH}(g)} \tag{D.2}$$

where nmol aa/mL is obtained from HPLC analysis, values for $M_{w,aa}$ are defined as molecular weight of amino acids when bound to protein, DF is dilution factor, $V_{FPH}$ and $m_{FPH}$ are volume and mass of fish protein hydrolysate. **Table D.3** and **Table D.4** are representing the average free amino acid amount and composition in mg/g protein hydrolysate.

**Table D.3:** The average free amino acid amount and composition of T1H, T2H, T3H and T4H in mg free amino acid per g protein hydrolysate (n = 3, ± SD).

| | **T1H** | **T2H** | **T3H** | **T4H** |
|---|---|---|---|---|
| **Asp** | 0.46 ± 0.09 | 0.41 ± 0.12 | 1.03 ± 0.18 | 0.29 ± 0.09 |
| **Glu** | 0.97 ± 0.14 | 0.99 ± 0.16 | 1.33 ± 0.11 | 1.12 ± 0.13 |
| **Asn** | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.00 | 0.04 ± 0.02 |
| **His** | 0.34 ± 0.01 | 0.46 ± 0.08 | 0.84 ± 0.10 | 0.56 ± 0.17 |
| **Ser** | 1.67 ± 0.01 | 0.77 ± 0.12 | 1.48 ± 0.09 | 0.70 ± 0.17 |
| **Gln** | 0.32 ± 0.09 | 2.41 ± 0.49 | 3.28 ± 0.55 | 1.82 ± 0.37 |
| **Gly/Arg** | 1.34 ± 0.06 | 1.92 ± 0.36 | 2.04 ± 0.26 | 1.65 ± 0.32 |
| **Thr** | 0.65 ± 0.04 | 0.98 ± 0.22 | 1.76 ± 0.26 | 0.87 ± 0.33 |
| **Ala** | 1.78 ± 0.11 | 4.91 ± 0.87 | 7.63 ± 0.71 | 4.95 ± 0.69 |
| **Tyr** | 0.45 ± 0.09 | 0.33 ± 0.05 | 0.59 ± 0.08 | 0.34 ± 0.05 |
| **Aba** | 0.02 ± 0.01 | 0.33 ± 0.04 | 0.47 ± 0.04 | 0.42 ± 0.04 |
| **Met** | 1.54 ± 0.02 | 1.17 ± 0.20 | 2.01 ± 0.24 | 1.29 ± 0.19 |
| **Val** | 0.56 ± 0.02 | 0.89 ± 0.15 | 1.90 ± 0.23 | 0.84 ± 0.10 |
| **Phe** | 0.64 ± 0.03 | 0.88 ± 0.12 | 2.51 ± 0.32 | 0.92 ± 0.15 |
| **Ile** | 0.25 ± 0.02 | 0.52 ± 0.13 | 2.27 ± 0.34 | 0.61 ± 0.18 |
| **Leu** | 1.34 ± 0.20 | 2.04 ± 0.42 | 5.61 ± 0.64 | 2.29 ± 0.51 |
| **Lys** | 1.26 ± 0.11 | 1.44 ± 0.22 | 2.37 ± 0.47 | 1.58 ± 0.33 |
| **Total** | 13.62 ± 0.47 | 20.48 ± 3.72 | 37.13 ± 4.52 | 20.31 ± 3.60 |

**Table D.5** and **Table D.6** are showing the average free amino acid amount and composition of T1H, SPH and their UF fractions given as $\mu$g amino acid per mL.

**Table D.4:** Contiuation of Table D.3. The average free amino acid amount and composition of T5H, T6H, T13H and SPH in mg free amino acid per g protein hydrolysate (n = 3, ± SD).

| | T5H | T6H | T13H | SPH |
|---|---|---|---|---|
| **Asp** | 0.65 ± 0.12 | 0.98 ± 0.15 | 1.14 ± 0.19 | 0.08 ± 0.02 |
| **Glu** | 1.40 ± 0.18 | 1.18 ± 0.32 | 1.73 ± 0.41 | 0.29 ± 0.07 |
| **Asn** | 0.03 ± 0.00 | 0.00 ± 0.00 | 0.03 ± 0.01 | 0.02 ± 0.00 |
| **His** | 0.75 ± 0.09 | 0.15 ± 0.02 | 0.24 ± 0.30 | 0.26 ± 0.06 |
| **Ser** | 1.16 ± 0.14 | 0.50 ± 0.30 | 0.65 ± 0.35 | 0.18 ± 0.04 |
| **Gln** | 2.16 ± 0.34 | 1.23 ± 0.21 | 1.06 ± 0.33 | 0.37 ± 0.06 |
| **Gly/Arg** | 2.51 ± 0.34 | 3.71 ± 0.12 | 3.43 ± 0.76 | 0.34 ± 0.05 |
| **Thr** | 1.41 ± 0.25 | 1.75 ± 0.06 | 1.16 ± 0.10 | 0.22 ± 0.04 |
| **Ala** | 6.27 ± 0.85 | 1.24 ± 0.05 | 1.06 ± 0.35 | 1.13 ± 0.18 |
| **Tyr** | 0.44 ± 0.09 | 4.05 ± 0.38 | 3.48 ± 0.87 | 0.15 ± 0.02 |
| **Aba** | 0.38 ± 0.02 | 0.02 ± 0.03 | 0.01 ± 0.01 | 0.06 ± 0.01 |
| **Met** | 1.66 ± 0.19 | 1.58 ± 0.19 | 1.29 ± 0.34 | 0.39 ± 0.04 |
| **Val** | 1.34 ± 0.18 | 1.64 ± 0.19 | 1.40 ± 0.33 | 0.35 ± 0.06 |
| **Phe** | 1.29 ± 0.16 | 1.44 ± 0.24 | 1.39 ± 0.46 | 0.58 ± 0.33 |
| **Ile** | 0.85 ± 0.04 | 1.07 ± 0.03 | 0.89 ± 0.20 | 0.37 ± 0.04 |
| **Leu** | 2.97 ± 0.46 | 4.49 ± 0.16 | 3.98 ± 0.89 | 0.92 ± 0.12 |
| **Lys** | 2.01 ± 0.24 | 2.97 ± 0.13 | 2.92 ± 0.62 | 0.66 ± 0.12 |
| **Total** | 27.28 ± 3.66 | 28.01 ± 0.87 | 25.88 ± 6.10 | 6.37 ± 1.22 |

**Table D.5:** The average free amino acid amount and composition of T1H and its UF fractions presented as $\mu$g aa/mL (n = 3, ± SD).

| | T1H | P | R1 | R2 |
|---|---|---|---|---|
| **Asp** | 7.72 ± 0.54 | 1.67 ± 1.09 | 0.21 ± 0.21 | 0.18 ± 0.08 |
| **Glu** | 11.73 ± 1.12 | 4.98 ± 1.04 | 0.09 ± 0.13 | 0.03 ± 0.03 |
| **Asn** | 0.21 ± 0.05 | 0.12 ± 0.08 | 0.01 ± 0.00 | 0.02 ± 0.00 |
| **His** | 13.89 ± 1.34 | 1.88 ± 0.22 | 0.43 ± 0.19 | 1.06 ± 0.36 |
| **Ser** | 14.32 ± 2.34 | 4.41 ± 0.70 | 0.16 ± 0.11 | 0.19 ± 0.07 |
| **Gln** | 14.15 ± 1.54 | 13.27 ± 0.48 | 8.93 ± 1.45 | 6.96 ± 0.64 |
| **Gly/Arg** | 16.87± 2.45 | 11.96 ± 0.58 | 4.82 ± 1.36 | 4.83 ± 0.23 |
| **Thr** | 9.92 ± 0.89 | 3.94 ± 1.30 | 0.21 ± 0.08 | 0.05 ± 0.01 |
| **Ala** | 51.84 ± 4.23 | 35.48 ± 3.10 | 5.87 ± 1.67 | 9.74 ± 0.73 |
| **Tyr** | 15.68 ± 2.43 | 2.46 ± 0.14 | 0.78 ± 0.21 | 1.21 ± 0.13 |
| **Aba** | 9.30 ± 1.02 | 1.88 ± 0.31 | 0.51 ± 0.06 | 1.14 ± 0.49 |
| **Met** | 10.18 ± 1.30 | 6.29 ± 1.54 | 5.75 ± 1.18 | 5.37 ± 1.09 |
| **Val** | 17.24 ± 2.95 | 5.65 ± 0.58 | 2.73 ± 0.67 | 3.43 ± 0.74 |
| **Phe** | 6.76 ± 0.43 | 7.53 ± 0.46 | 1.96 ± 0.48 | 3.06 ± 0.60 |
| **Ile** | 43.61 ± 3.05 | 3.45 ± 0.27 | 0.45 ± 0.19 | 1.04 ± 0.17 |
| **Leu** | 18.07 ± 1.46 | 13.75 ± 1.08 | 6.88 ± 1.79 | 7.76 ± 1.69 |
| **Lys** | 15.78 ± 0.98 | 10.35 ± 0.61 | 2.41 ± 0.44 | 5.38 ± 1.39 |
| **Total** | 277.29 ± 10.94 | 129.07 ± 6.64 | 42.21 ± 9.03 | 51.44 ± 7.67 |

**Table D.6:** The average free amino acid amount and composition of SPH and its UF fractions presented as $\mu$g aa/mL (n = 3, $\pm$ SD).

| | **SPH** | **P** | **R** |
|---|---|---|---|
| **Asp** | 1.85 $\pm$ 0.31 | 1.12 $\pm$ 0.35 | 0.17 $\pm$ 0.26 |
| **Glu** | 6.52 $\pm$ 0.07 | 3.38 $\pm$ 0.55 | 0.06 $\pm$ 0.06 |
| **Asn** | 0.52 $\pm$ 0.06 | 0.10 $\pm$ 0.11 | 0.01 $\pm$ 0.00 |
| **His** | 5.92 $\pm$ 0.24 | 1.86 $\pm$ 0.38 | 0.27 $\pm$ 0.19 |
| **Ser** | 4.06 $\pm$ 0.21 | 2.00 $\pm$ 0.33 | 0.11 $\pm$ 0.08 |
| **Gln** | 7.97 $\pm$ 0.89 | 2.88 $\pm$ 0.19 | 3.59 $\pm$ 0.42 |
| **Gly/Arg** | 7.37 $\pm$ 0.02 | 3.14 $\pm$ 0.33 | 1.16 $\pm$ 0.02 |
| **Thr** | 4.78 $\pm$ 0.05 | 2.89 $\pm$ 0.10 | 1.36 $\pm$ 1.32 |
| **Ala** | 24.64 $\pm$ 0.29 | 15.36 $\pm$ 1.43 | 3.09 $\pm$ 0.37 |
| **Tyr** | 3.21 $\pm$ 0.32 | 1.92 $\pm$ 0.13 | 0.92 $\pm$ 0.07 |
| **Aba** | 1.34 $\pm$ 0.36 | 0.25 $\pm$ 0.16 | 0.07 $\pm$ 0.06 |
| **Met** | 8.14 $\pm$ 0.13 | 3.93 $\pm$ 0.27 | 2.66 $\pm$ 0.36 |
| **Val** | 7.78 $\pm$ 0.33 | 3.83 $\pm$ 0.13 | 2.80 $\pm$ 0.21 |
| **Phe** | 14.91 $\pm$ 4.18 | 5.28 $\pm$ 1.37 | 9.30 $\pm$ 0.89 |
| **Ile** | 7.93 $\pm$ 0.27 | 3.07 $\pm$ 0.47 | 6.14 $\pm$ 1.04 |
| **Leu** | 19.70 $\pm$ 0.60 | 9.80 $\pm$ 1.69 | 7.76 $\pm$ 1.26 |
| **Lys** | 14.52 $\pm$ 0.89 | 4.81 $\pm$ 0.48 | 11.38 $\pm$ 1.98 |
| **Total** | 141.15 $\pm$ 7.67 | 65.62 $\pm$ 3.95 | 50.87 $\pm$ 5.21 |

Appendix E

# Molecular weight distribution

## E.1 Chromatograms of standard proteins

Molecular weight distribution of crude protein hydrolysates and UF fractions was determined by using FPLC system. Three different standard proteins were used to compare the obtained results: Aprotinin, Vitamin B12 and Cytochrome C. Chromatograms for these proteins are presented in **Figure E.1**, **Figure E.2** and **Figure E.3**.

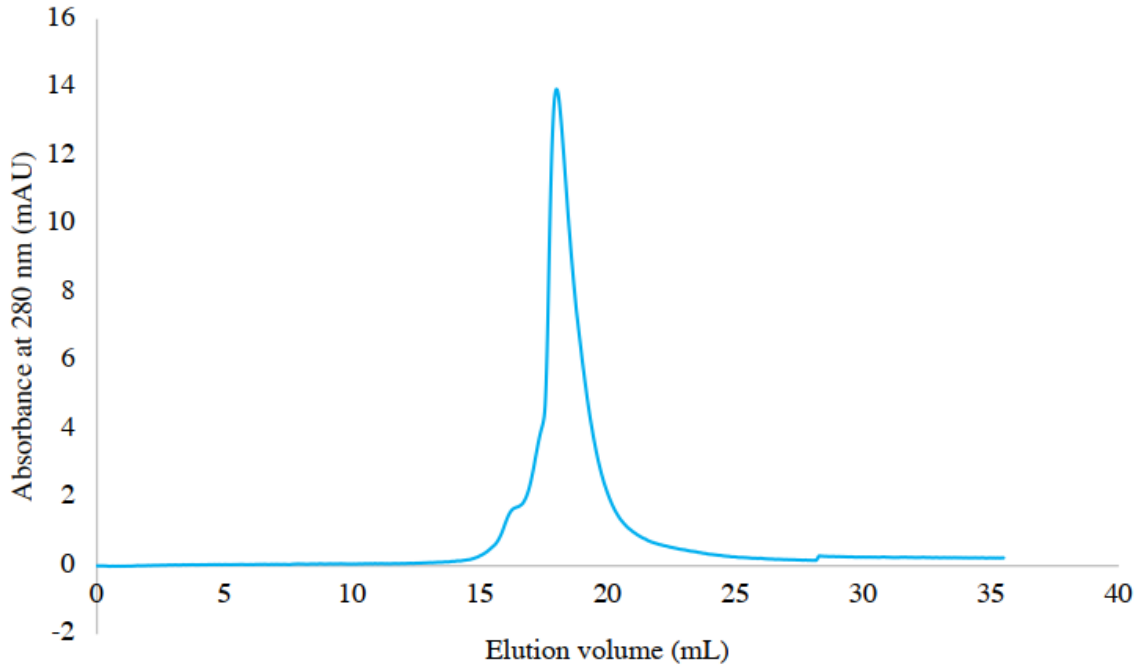



**Figure E.1:** Chromatogram of Aprotinin, Mw = 6.5 kDa, obtained by gel filtration and used as a standard. Absorbance (mAU) is plotted against time (min).

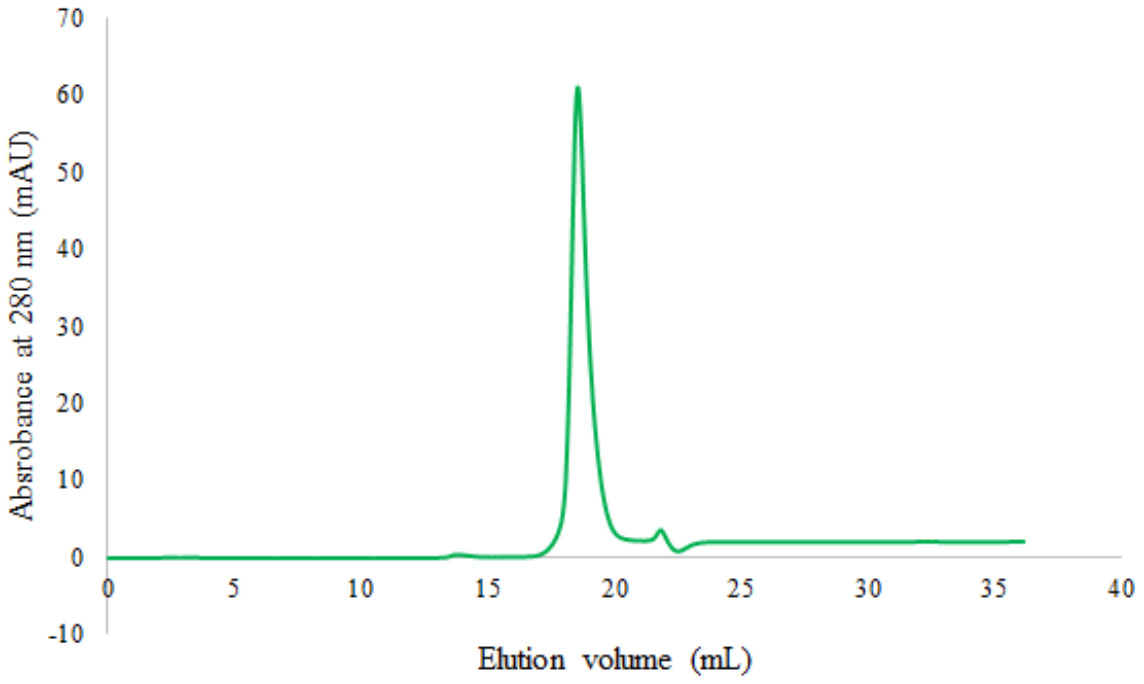



**Figure E.2:** Chromatogram of B12, Mw = 1.34 kDa, obtained by gel filtration and used as a standard. Absorbance (mAU) is plotted against time (min).

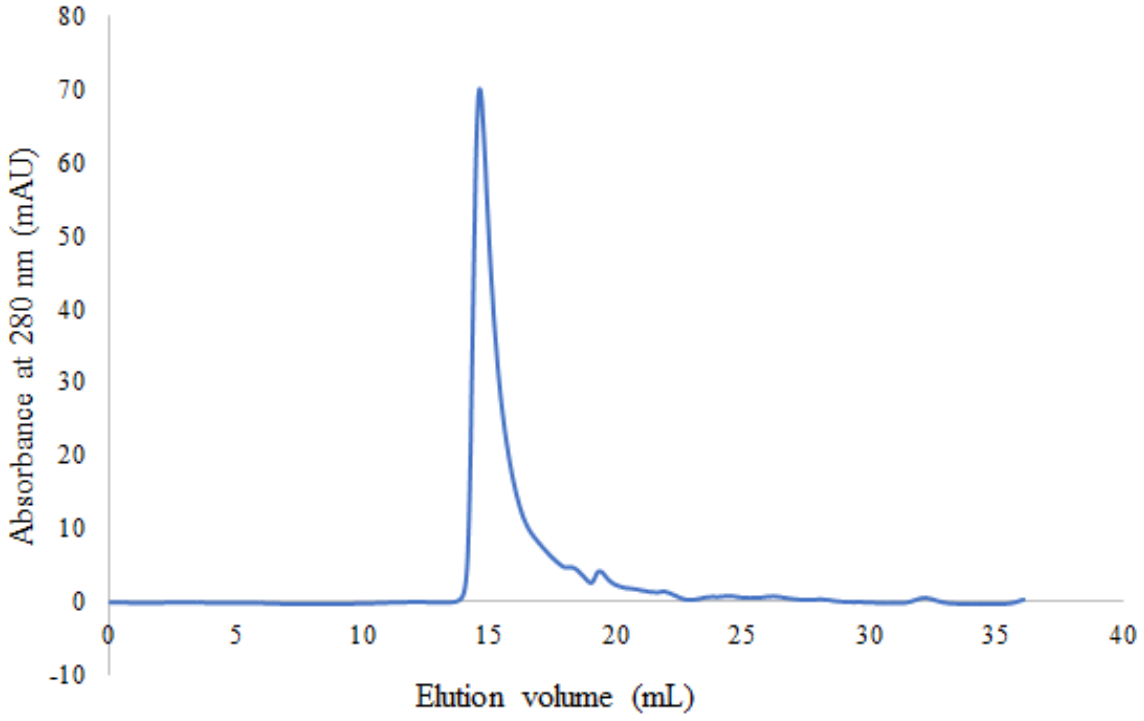



**Figure E.3:** Chromatogram of Cytochrome C, Mw = 12.3 kDa, obtained by gel filtration and used as a standard. Absorbance (mAU) is plotted against time (min).

## E.2 Determination of the peptide size

To estimate approximate molecular weight of peptides present in the hydrolysates, relationship between elution volume parameters and the logarithm of molecular weight of standards was used. First, partition coefficient, $K_{av}$, was calculated using the following equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}, \tag{E.1}$$

where $V_e$ is the elution volume for the protein, $V_0$ is the column void volume (8 mL) and $V_t$ is the total bed volume (24 mL). **Table E.1** represents information about the standards.

**Table E.1:** Standards of proteins used for determination of peptide molecular weight with their molecular weight, $M_w$, elution volume, $V_e$, and logarithmic molecular weight, $\log_10(M_w)$.

| **Standard** | **$M_w$ (kDa)** | **$V_e$ (mL)** | **$K_{av}$** | **$\log_{10}(M_w)$** |
|---|---|---|---|---|
| Cytochrome C | 12.38 | 15.1 | 0.44 | 4.09 |
| Aprotinin | 6.51 | 17.99 | 0.62 | 3.81 |
| Vitamin B12 | 1.36 | 18.56 | 0.66 | 3.13 |

$K_{av}$ values for each protein standard were plotted against the corresponding logarithmic molecular weight and linear regression was applied to find the relationship. Plot is illustrated in **Figure E.4**. After calculating $K_{av}$ for the most distinct peaks of hydrolysates, approximate molecular weights of peptides were estimated using the regression line. Results for T1H, SPH and its fractions can be found in **Table E.2**. Molecular weights for other cod protein hydrolysates were not calculated, since they were similar to T1H and because of the low $R^2$-value leading to very inaccurate results.

**Table E.2:** Approximate molecular weights of peptides presented in hydrolysates (T1H and SPH) and its fractions.

| **Hydrolysate** | **$M_w$ (kDa)** |
|---|---|
| Crude T1H | $\sim$ 7, 6, 0.6, 0.1 |
| T1H Permeate | $\sim$ 4, 0.6, 0.1 |
| T1H Retentate 2 | $\sim$ 5, 0.6, 0.1 |
| Crude SPH | $\sim$ 10, 7, 0.6, 0.05 |
| SPH Permeate | $\sim$ 6, 0.6, 0.05 |

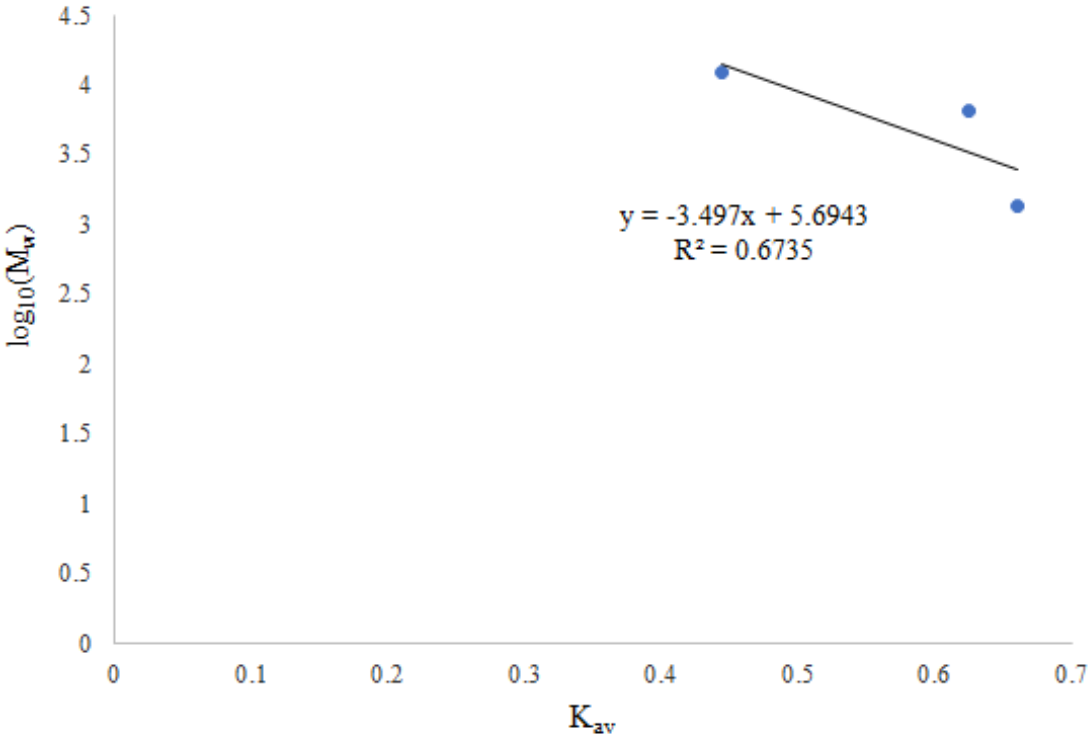



**Figure E.4:** Calibration curve for estimation of peptide molecular weight.

Appendix F

# Ultrafiltration

Recovery from the ultrafiltration was determined based on the protein content and the dry matter content. To calculate based on the protein content, results from the Lowry method and measured volumes from the ultrafiltration were used. Example of protein content in crude SPH is presented in **Equation F.1**.

$$300\,mL \times 10\,\frac{mg}{mL} = 3000\,mg\;protein \tag{F.1}$$

Protein content for other samples was calculated in the same manner.
Protein loss (mg) was calculated using the following equation:

$$Protein\,loss = Protein\,in\,FPH - Protein\,in\,P - Protein\,in\,R \tag{F.2}$$

where Protein in FPH was protein content in crude fish protein hydrolysate, P and R were permeate and retentate, respectively. To find protein loss in %, result from **Equation F.2** was divided by total amount of protein in crude protein hydrolysate (Protein in CPH).

For dry matter content, it was assumed that 1 mL hydrolysate was equal to 1 g hydrolysate. First, mass of each fraction and crude hydrolysate were calculated. It was done by using volumes and DM (%) from **Table 3.3**. For example, for crude SPH:

$$\frac{1.1\%}{100\%} \times 300\,mL = 3.3\,g \tag{F.3}$$

By assuming that 100% of all dry matter content was in crude protein hydrolysate, it was possible to find dry matter loss.

Appendix G

# Acid soluble peptides

Amount of acid soluble peptides was determined by the Lowry method after precipitation of proteins with trichloroacetic acid (TCA) (20%). BSA was used as a standard compound and concentrations used were the same as for the measurement of protein content in Appendix C. Results for absorbance can be found in **Table G.1**. Standard curve was obtained by plotting BSA concentrations against absorbances (**Figure G.1**). Equation for the standard curve is:

$$y = 0.002x + 0.0494 \tag{G.1}$$

Using equation of standard curve, concentrations for hydrolysates were calculated. They are reported in **Table G.2**. These concentrations were corrected for dilution and converted to mg/mL and percent. Dilution factor for crude protein hydrolysates and first retentate of T1H was 50, for permeates and T1H R2 – 25, and for retentate of SPH - 100. Calculations were performed in the same manner as for protein content in Appendix C.

**Table G.1:** Absorbance measured at 750 nm, average and standard deviation for different concentrations of standard protein (BSA) are presented.

| BSA [$\mu$g/mL] | OD750 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| 12.5 | 0.050 | 0.042 | 0.065 | 0.052 | 0.012 |
| 25 | 0.083 | 0.081 | 0.079 | 0.081 | 0.002 |
| 50 | 0.161 | 0.157 | 0.154 | 0.157 | 0.004 |
| 100 | 0.284 | 0.278 | 0.272 | 0.278 | 0.006 |
| 150 | 0.365 | 0.383 | 0.363 | 0.370 | 0.011 |
| 200 | 0.475 | 0.468 | 0.462 | 0.468 | 0.007 |
| 300 | 0.627 | 0.618 | 0.634 | 0.626 | 0.008 |

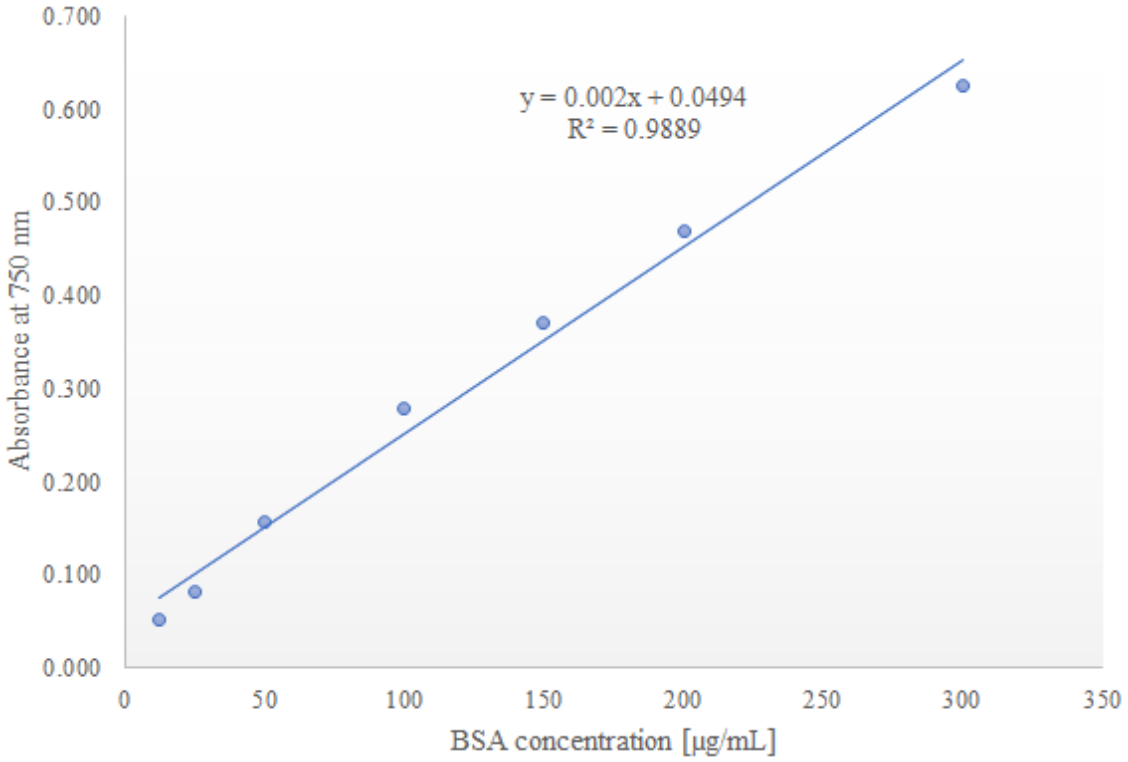



**Figure G.1:** Standard curve of BSA used to determine amount of acid soluble peptides in crude protein hydrolysates and UF fractions. BSA concentration in $\mu$g/mL is plotted against absorbance at 750 nm. Equation for this curve is y = 0.002x + 0.0494.

**Table G.2:** Amount of acid soluble peptides present in the crude protein hydrolysates and their fractions. Initial concentration of hydrolysates were 10 mg/mL. Values are expressed as the mean ± SD (n = 3).

| | **ASP** | |
|---|---|---|
| | **[mg/mL]** | **[%]** |
| **CPH** | | |
| T1H | 6.00 ± 0.03 | 60.04 |
| T2H | 7.49 ± 0.84 | 74.88 |
| T3H | 5.56 ± 0.27 | 55.64 |
| T4H | 5.64 ± 0.39 | 56.35 |
| T5H | 5.53 ± 0.36 | 55.28 |
| T6H | 7.27 ± 0.20 | 72.68 |
| T13H | 6.04 ± 0.11 | 60.37 |
| SPH | 8.48 ± 0.25 | 84.82 |
| **UF fractions** | | |
| T1H P | 1.30 ± 0.00 | |
| T1H R1 | 10.14 ± 0.69 | |
| T1H R2 | 2.94 ± 0.07 | |
| SPH P | 1.58 ± 0.13 | |
| SPH R | 30.82 ± 0.84 | |

Appendix H

# Antioxidant activity assays

## H.1 ABTS radical scavenging activity

**Table H.1** represents absorbance and antioxidant activity measured for the standard compound, propyl gallate (PG). Following concentrations were used: 10, 20, 30, 40 and 50 $\mu$M. Standard curve in **Figure H.1** was plotted based on the measured absorbances. Using linear regression following equation was obtained:

$$y = -0.007x + 0.5345 \tag{H.1}$$

where y is absorbance and x is concentration of propyl gallate in micromolar. This equation was used to calculate equivalent calculations of PG. An example of calculation is shown in **Equation H.2**. Sample of T1H had absorbance 0.277, so using **Equation H.1**, x can be found:

$$x = \frac{0.277 - 0.535}{-0.007} = 36.786 \tag{H.2}$$

To get the final answer in $\mu$mol/ g protein, it was necessary to perform more steps since **Equation H.2** gave concentration in $\mu$M and dilution was not considered. Following calculations were performed to get the desired answer:

$$\frac{\frac{Average\ concentration \times DF}{1000}}{\frac{Protein\ concentration\ (Lowry)}{1000}} = \frac{\frac{36.786 \times 10}{1000}}{\frac{6.610}{1000}} = 55.652 \tag{H.3}$$

DF is dilution factor and protein concentration are the results found in the experiment performed by the Lowry method. Values can be found in Appendix C. **Table H.2** presents results for all the samples.

**Table H.1:** Absorbance values for standard used in ABTS assay.

| Propyl Gallate [$\mu$M] | OD734 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| 10 | 0.464 | 0.462 | 0.464 | 0.463 | 0.001 |
| 20 | 0.409 | 0.404 | 0.401 | 0.405 | 0.004 |
| 30 | 0.319 | 0.310 | 0.308 | 0.312 | 0.006 |
| 40 | 0.256 | 0.253 | 0.261 | 0.257 | 0.004 |
| 50 | 0.195 | 0.179 | 0.191 | 0.188 | 0.008 |

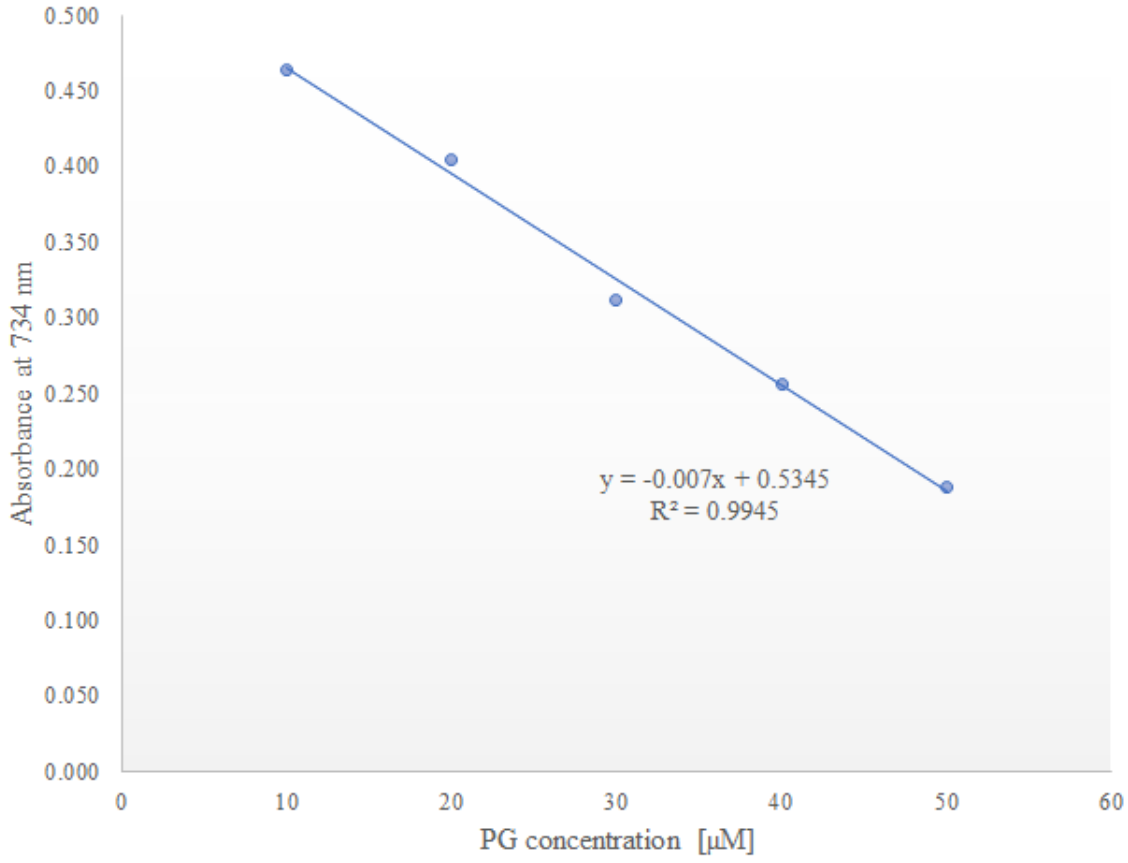



**Figure H.1:** Standard curve of propyl gallate, where concentration in $\mu$M is plotted against absorbance at 734 nm. Equation for it is found by linear regression (y = -0.007x + 0.5345).

**Table H.2:** Absorbance at 734 nm measured for crude protein hydrolysates and UF fractions of T1H and SPH. Concentration and equivalent ABTS radical scavenging activity are calculated from the equation of standard curve. Values are given as the mean (n =3).

| | **Absorbance** | **Concentration [$\mu$M]** | **Eq. concentration PG [$\mu$mol/ g protein]** |
|---|---|---|---|
| **Crude protein hydrolysates** | | | |
| T1H | 0.277 | 36.84 | 27.87 $\pm$ 2.26 |
| T2H | 0.443 | 24.25 | 33.73 $\pm$ 1.76 |
| T3H | 0.482 | 17.95 | 27.12 $\pm$ 0.74 |
| T4H | 0.451 | 22.95 | 30.93 $\pm$ 0.63 |
| T5H | 0.463 | 21.10 | 30.02 $\pm$ 1.32 |
| T6H | 0.417 | 21.61 | 22.30 $\pm$ 2.63 |
| T13H | 0.441 | 17.91 | 18.89 $\pm$ 3.28 |
| SPH | 0.437 | 25.23 | 25.23 $\pm$ 0.93 |
| **UF fractions** | | | |
| T1H P | 0.239 | 42.38 | 107.02 $\pm$ 5.53 |
| T1H R1 | 0.262 | 39.08 | 24.96 $\pm$ 6.86 |
| T1H R2 | 0.237 | 42.57 | 62.05 $\pm$ 0.67 |
| SPH P | 0.234 | 43.05 | 99.19 $\pm$ 6.44 |
| SPH R | 0.336 | 28.43 | 6.66 $\pm$ 1.85 |

## H.2 Folin-Ciocalteu reducing capacity

Calculations for FC assay were performed in the same way as for the ABTS assay. The same standard was used, but concentrations for standard curve were 0.5, 1, 1.5 and 2 mM. Consequently, the final results were in mmol/g protein, not in $\mu$mol/g protein as for the ABTS assay. A standard curve and absorbances for propyl gallate can be found in **Figure H.2** and **Table H.3**. Equation from the standard curve was used to find concentrations for the hydrolysates. The final answer was based on the protein concentrations found by the Lowry method.

**Table H.3:** Absorbance measured at 725 nm, average and standard deviation for different concentrations of propyl gallate.

| Propyl Gallate [mM] | OD725 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| 0.5 | 0.063 | 0.065 | 0.065 | 0.064 | 0.001 |
| 1 | 0.148 | 0.149 | 0.148 | 0.148 | 0.001 |
| 1.5 | 0.225 | 0.214 | 0.221 | 0.220 | 0.006 |
| 2 | 0.270 | 0.293 | 0.281 | 0.281 | 0.012 |

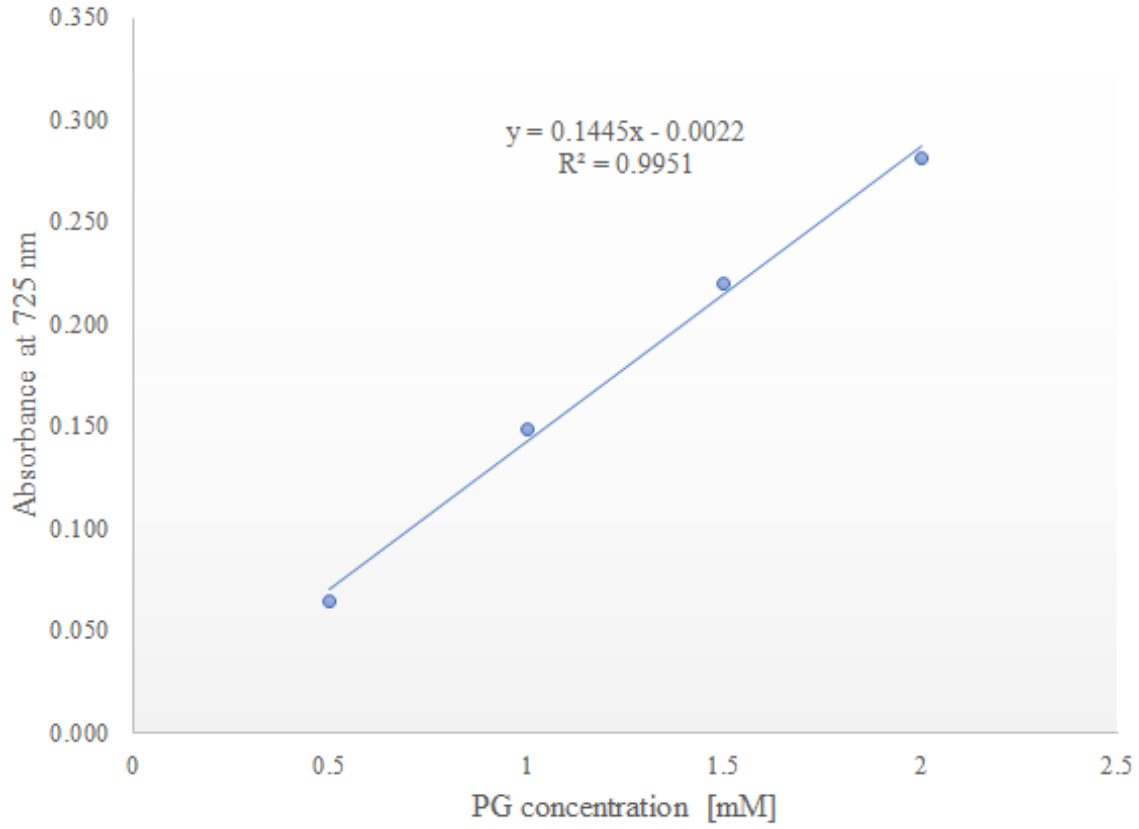



**Figure H.2:** Standard curve of propyl gallate used to determine antioxidant activity in crude protein hydrolysates and their UF fractions. PG concentration in $\mu$g/mL is plotted against absorbance at 725 nm. Equation for this curve is y = 0.1445x - 0.0022.

**Table H.4:** Absorbance at 725 nm measured for crude protein hydrolysates. Concentration and equivalent FC reducing capacity are calculated from the equation of standard curve.

| | **Absorbance** | **Concentration [mM]** | **Eq. concentration PG [mmol/g protein]** |
|---|---|---|---|
| T1H | 0.062 | 0.51 | $0.15 \pm 0.01$ |
| T2H | 0.069 | 0.49 | $0.14 \pm 0.01$ |
| T3H | 0.120 | 0.85 | $0.13 \pm 0.02$ |
| T4H | 0.074 | 0.53 | $0.14 \pm 0.01$ |
| T5H | 0.075 | 0.53 | $0.15 \pm 0.02$ |
| T6H | 0.072 | 0.57 | $0.18 \pm 0.01$ |
| T13H | 0.065 | 0.52 | $0.17 \pm 0.03$ |
| SPH | 0.069 | 0.49 | $0.10 \pm 0.00$ |

Appendix I

# ACE inhibitory activity

## I.1 Method by Sentandreu and Toldrá

Six different concentrations of each hydrolysate were used for the analysis of ACE inhibitory activity. For crude hydrolysates and retentates they were 0.5, 1, 5, 10, 20, 30 mg/mL, and for permeate – 1, 2, 4, 6, 8, 10 mg/mL. Permeate and retentate were freeze-dried before the analysis of ACE inhibition.

An example of the fluorescence measured for different concentrations of T4H is presented in **Figure I.1**. Linear regression was applied for each concentration and by using **Equation 2.2**, ACEI (%) was calculated. Results can be found in **Table I.1**.

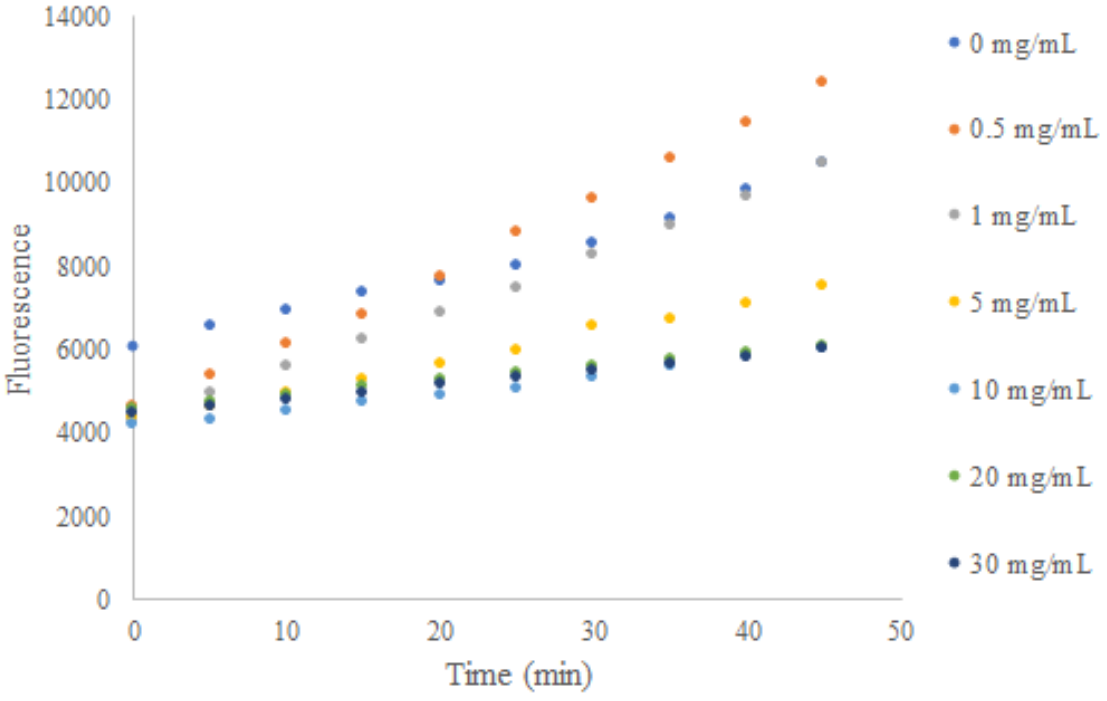



**Figure I.1:** Fluorescence plotted against time (min) for different concentrations of T4H for determining of ACE inhibitory activity.

Calculated ACEI (%) was plotted against the concentration of hydrolysate, and by using logarithmic regression, **Equation I.1** was obtained. To get a more precise equation, two last concentrations were omitted from the graph.

$$y = 0.4589ln(x) - 0.4946 \tag{I.1}$$

**Table I.1:** Equations for each concentration of inhibitor (T4H) obtained by linear regressing, and ACE inhibition (%).

| Concentration of T4H (mg/mL) | Equation | ACEI (%) |
|---|---|---|
| 0 | y = 93.928x + 5940.1 | 0.00 |
| 0.5 | y = 174.06x + 4445.2 | -85.31 |
| 1 | y = 135.24x + 4246.3 | -43.98 |
| 5 | y = 71.732x + 4264.5 | 23.63 |
| 10 | y = 41.784x + 4118.5 | 55.51 |
| 20 | y = 34.126x + 4585.4 | 63.67 |
| 30 | y = 34.721x + 4450.1 | 63.03 |

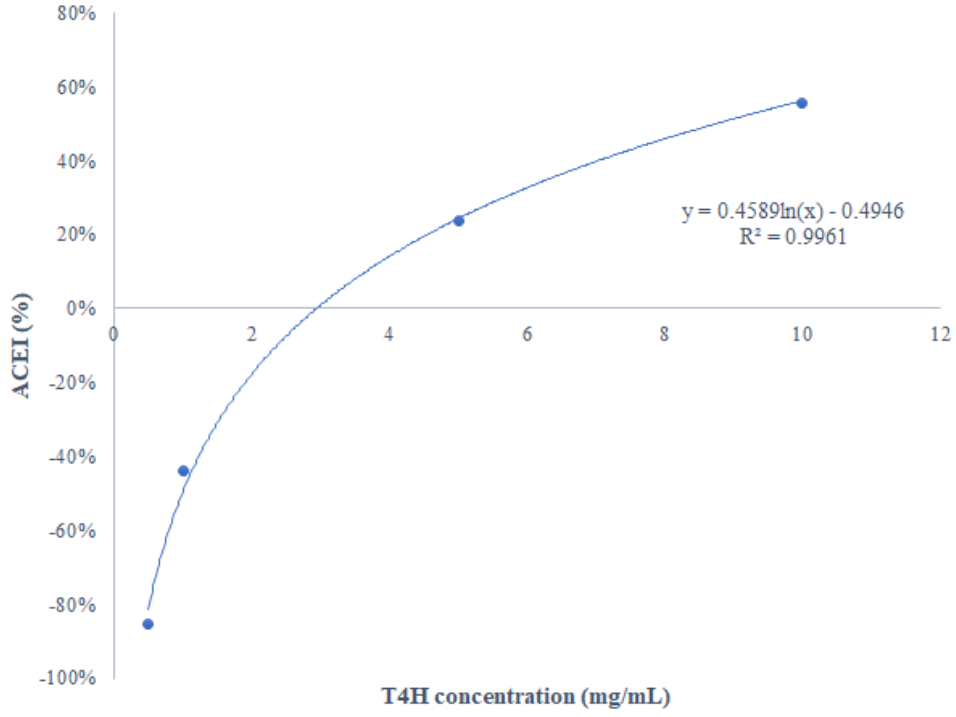



**Figure I.2:** Logarithmic correlation between ACEI (%) and concentrations of hydrolysate T4H (mg/mL).

Since $IC_{50}$ is the concentration of inhibitor needed to inhibit 50% of ACE, **Equation I.1** can be used to calculate this values.

$$IC_{50} = exp(\frac{50 + 0.4946}{0.4589}) = 8.74\, mg\, CPH/mL \tag{I.2}$$

To get units in mg protein/mL, results from the Lowry method are used. For example, there were 74.17% protein found in T6H. $IC_{50}$ value for T4H is therefore 6.48 mg protein/mL.

## I.2 Method by Cushman and Cheung

### I.2.1 Hippuric acid extraction

**Table I.2** and **Table I.3** illustrate absorbance values for extracted hippuric acid, when samples were vortexed for exactly 60 s and when they were not. This was done to control if the extraction step was the reason for unstable results.

**Table I.2:** Absorbance values for different concentrations of hippuric acid extracted with ethyl acetate. Mixing time was 60 s.

| Hippuric acid [mM] | OD228 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| **0** | 0.036 | 0.049 | 0.040 | 0.042 | 0.007 |
| **0.2** | 0.13 | 0.143 | 0.145 | 0.139 | 0.008 |
| **0.4** | 0.214 | 0.214 | 0.215 | 0.214 | 0.001 |
| **0.8** | 0.384 | 0.382 | 0.377 | 0.381 | 0.004 |
| **1.0** | 0.480 | 0.488 | 0.476 | 0.481 | 0.006 |
| **1.5** | 0.669 | 0.681 | 0.666 | 0.672 | 0.008 |

**Table I.3:** Absorbance values of hippuric acid extracted with ethyl acetate. Mixing time was not measured.

| Hippuric acid [mM] | OD228 | | | $\bar{x}$ | SD |
|---|---|---|---|---|---|
| | I | II | III | | |
| **5** | 0.147 | 0.128 | 0.133 | 0.136 | 0.010 |
| **2.5** | 0.130 | 0.140 | 0.145 | 0.138 | 0.008 |
| **2** | 0.139 | 0.134 | 0.155 | 0.143 | 0.011 |
| **1.5** | 0.146 | 0.195 | 0.137 | 0.159 | 0.031 |
| **1** | 0.078 | 0.119 | 0.185 | 0.152 | 0.054 |

### I.2.2 Determination of $IC_{50}$

Five different concentrations of each hydrolysate were used to measure ACE inhibition by this method - 15, 10, 5, 1 and 0.5 mg/mL. ACEI (%) was calculated by **Equation 2.1** using measured absorbance. Further, these values were plotted against concentrations, and by applying logarithmic correlation $IC_{50}$ values were determined. Absorbance values for T6H is presented in **Table I.4**.

Logarithmic correlation gave the following equation (**Figure I.3**):

$$0.3204ln(x) + 0.2911 \tag{I.3}$$

This equation was used to determine $IC_{50}$ value:

$$IC_{50} = exp(\frac{50 - 0.2911}{0.3204}) = 1.92\, mg\, CPH/mL \tag{I.4}$$

To get units in mg protein/mL, results from the Lowry method are used. There was 64.6% protein, $IC_{50}$ value is therefore 1.24 mg protein/mL.

**Table I.4:** Absorbance values and ACEI (%) for T6H.

| | OD228 | | | $\bar{x}$ | SD | ACEI (%) |
|---|---|---|---|---|---|---|
| | I | II | III | | | |
| **B** | 0.113 | 0.127 | 0.150 | 0.130 | 0.019 | |
| **C** | 0.053 | 0.062 | 0.035 | 0.050 | 0.014 | |
| **A (mg/mL)** | | | | | | |
| **15** | 0.038 | 0.037 | 0.047 | 0.041 | 0.006 | 111.67 |
| **10** | 0.045 | 0.049 | 0.044 | 0.046 | 0.003 | 105.00 |
| **5** | 0.057 | 0.059 | 0.071 | 0.062 | 0.008 | 84.58 |
| **1** | 0.132 | 0.104 | 0.112 | 0.108 | 0.014 | 27.50 |
| **0.5** | 0.118 | 0.124 | 0.132 | 0.125 | 0.007 | 6.67 |

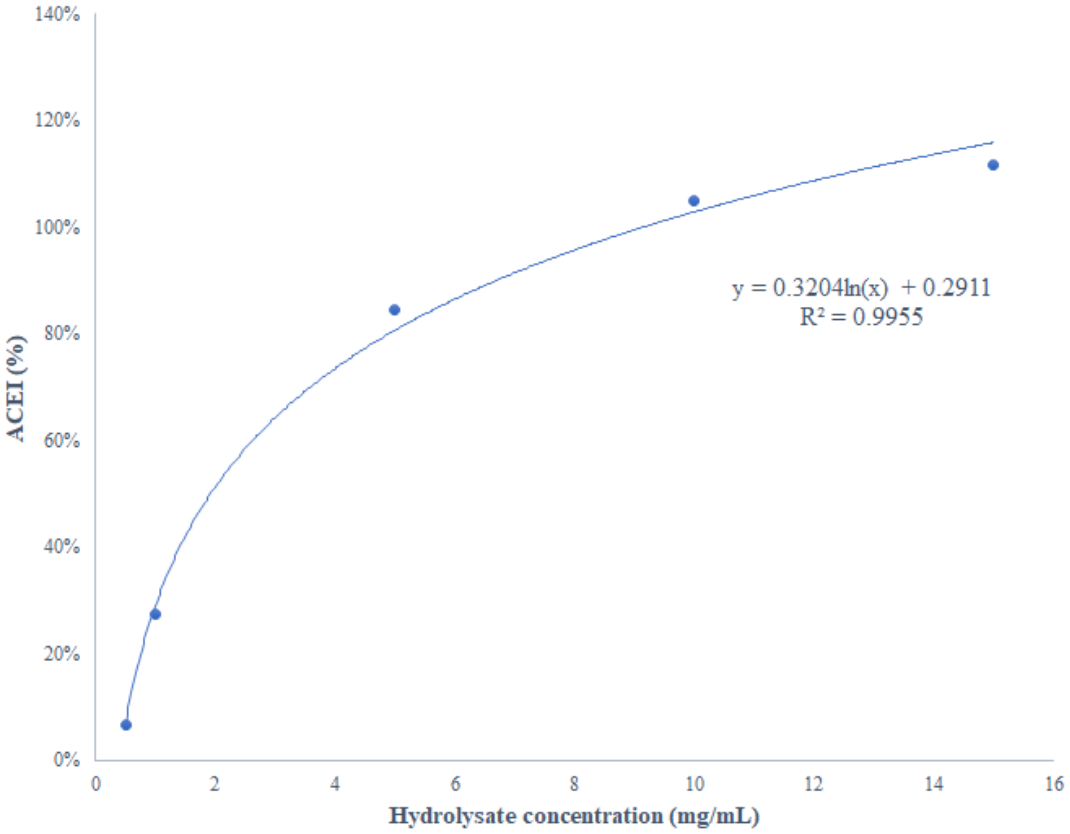



**Figure I.3:** ACEI(%) plotted against concentrations of T6H for determining of $IC_{50}$ value.

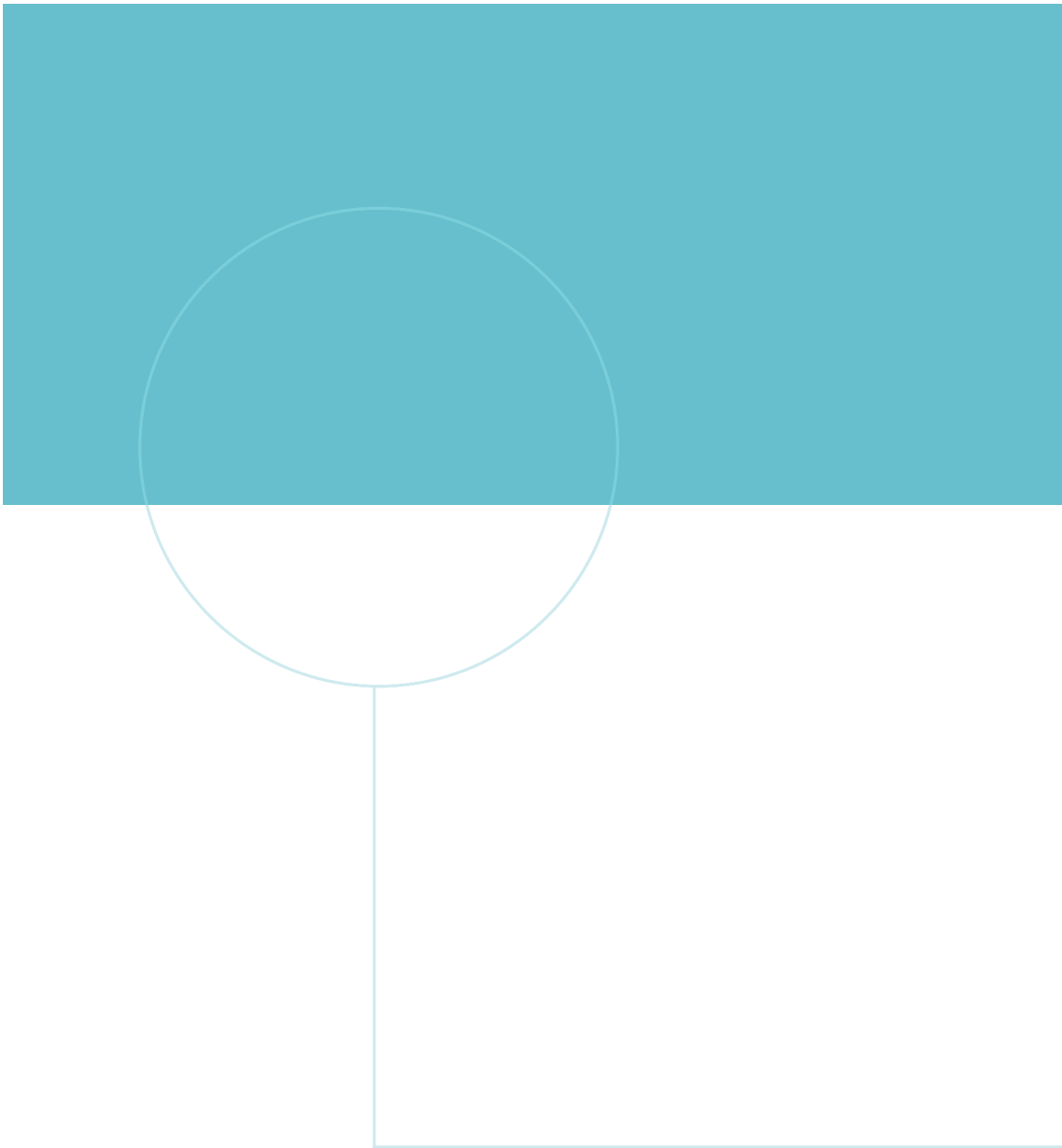
Appendix J

# Pearson correlation coefficient

**Table J.1** illustrates the correlation matrix of the studied parameters for crude protein hydrolysates. A Pearson correlation coefficient can vary from -1 to 1. A value of 0 indicates that there is no correlation, positive values indicate the positive correlation, while negative values indicate the negative correlation.

**Table J.1:** Correlation matrix of the studied parameters for crude protein hydrolysates.

| | **ABTS** | **FC** | **ACEI** | **FAA** | **Lowry** | **TAA** | **DH** | **ASP** |
|---|---|---|---|---|---|---|---|---|
| ABTS | **1** | -0.326 | 0.620 | 0.191 | 0.105 | -0.478 | 0.033 | 0.188 |
| FC | -0.326 | **1** | 0.102 | 0.013 | -0.840 | 0.637 | 0.784 | -0.697 |
| ACEI | 0.620 | 0.102 | **1** | 0.081 | -0.299 | -0.255 | 0.187 | -0.229 |
| FAA | 0.191 | 0.013 | 0.081 | **1** | -0.451 | -0.414 | 0.544 | -0.577 |
| Lowry | 0.105 | -0.840 | -0.299 | -0.451 | **1** | -0.348 | -0.885 | 0.787 |
| TAA | -0.478 | 0.637 | -0.255 | -0.414 | -0.348 | **1** | 0.304 | -0.210 |
| DH | 0.033 | 0.784 | 0.187 | 0.544 | -0.885 | 0.304 | **1** | -0.829 |
| ASP | 0.188 | -0.697 | -0.229 | -0.577 | 0.787 | -0.210 | -0.829 | **1** |



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