Master's thesis

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

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Functional Properties and Effect of Ultrafiltration and *in vitro* Digestion on Antioxidant Properties in Saithe *(Pollachius virens)* Backbone Hydrolysates

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Ida-Johanne Jensen
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Abstract

In Norway, the annual production of seafood was 3.55 million tons in 2019, whereof 0.96 million tons ended up as by-products. These by-products are not regarded as directly saleable but can be recycled into new products for human consumption. By-products from white fish (cod, haddock, ling, tusk, and saithe) are relatively poorly utilized (61 %) compared to aquaculture. One way to improve the utilization of white fish by-products is to produce fish protein hydrolysate (FPH) by enzymatic hydrolysis which is nutritious and has previously shown good functional and bioactive properties. Further, it has been shown that small peptides (1-5 kDa) have higher bioactive activity like antioxidant activity and that they can be concentrated with membrane filtration.

To investigate the potential for utilization of by-products from white fish, saithe protein hydrolysates (SPH) were produced by enzymatic hydrolysis of backbone from saithe. The aim of the thesis was to investigate the functional properties, and antioxidant activity of the SPHs, and explore how the antioxidant activity was affected by membrane filtration and *in vitro* gastrointestinal digestion. The functional properties analysed, were solubility, water holding capacity, and emulsifying properties. The relationship between structural properties and antioxidant activity was analysed by examining the degree of hydrolysis, the molecular weight (MW) distribution and the amino acid composition. The antioxidant activity was analysed with the assays: (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing ability of plasma (FRAP), and oxygen radical absorbance capacity (ORAC)). Further, the SPHs were ultra-filtrated twice (UF1 and UF2) with two different MW cut-offs (MWCO) to evaluate which MW showed the best antioxidant activity. For ultrafiltration (UF) 1, the SPHs were filtrated with a 150 kDa and 4 kDa MWCO membrane. For UF 2, the SPHs were filtrated with a 150 kDa and 2 kDa MWCO membrane.

The SPHs had a degree of hydrolysis of 18 %, a high solubility of 100 %, and a high abundance (300 mg/g) of small peptides (2-1 kDa). The abundance of small peptides resulted in poor water holding capacity and emulsifying properties in the SPHs compared to other FPHs. Hence, production of SPH with a lower DH might favour the functional properties. The MW distribution showed that UF1 was able to separate peptides into fractions based on size. Further, the SPHs and the fractions had a high proportion of essential amino acids and thus, a high nutritional value. The results from the antioxidant activity assays showed that the SPHs can work as radical scavengers by reducing the free ABTS+• radical, have reducing ability of free radicals, and have ORAC. The UFs increased FRAP and ORAC to some extent, where the FRAP was increased for the larger fractions (>150 kDa, 150-2 kDa) and ORAC was increased for the <4 kDa fraction. The ABTS assay showed that the UFs did not improve the free radical scavenging activity and imply that an additive effect of peptides with different sizes might have the best radical scavenging activity. In short, the results in this thesis indicate that UF is probably not necessary for concentrating bioactive peptides to increase antioxidant properties. Lastly, *in vitro* digestion decreased ABTS and FRAP activities, and increased ORAC for some of the SPHs. Thus, the SPHs have shown that white fish by-product hydrolysates have the potential to be used as nutraceuticals. However, white fish by-product hydrolysates have most likely a higher potential to be used as a multi-functional food ingredient.

Sammendrag

I 2019 produserte Norge 3,55 millioner tonn sjømat hvorav 0,96 millioner tonn var biprodukter. Disse biproduktene anses ikke som direkte salgbare, men de kan omgjøres til nye produkter for humant konsum. Biprodukter fra hvit fisk (torsk, hyse, lange, brosme og sei) er relativt dårlig utnyttet (61 % utnyttelse) sammenlignet med biprodukter fra lakseindustrien og har derfor et stort potensial. Et forslag for å øke utnyttelsen av biprodukter fra hvit fisk er å produsere fiskeproteinhydrolysat (FPH) ved enzymatisk hydrolyse. Fiskeproteinhydrolysat er næringsrike og har vist gode funksjonelle og bioaktive egenskaper tidligere og kan derfor ha potensial som funksjonelle matingredienser eller som kosttilskudd for humant konsum. Det er også vist at små peptider (1-5 kDa) ofte har høyere bioaktivitet, som antioksidantaktivitet, og at slike kan bli oppkonsentret ved hjelp av membranfiltrering.

For å undersøke en mulig utnyttelse av biprodukter fra hvit fisk, ble seiproteinhydrolysater (SPH) produsert med enzymatisk hydrolyse av ryggbein fra sei. Målet med denne master-oppgaven var å undersøke funksjonelle- og antioksidantegenskaper i SPH, og videre ble det undersøkt hvordan antioksidantegenskapene ble påvirket av membranfiltrering og *in vitro* fordøyelse. Sammenhengen mellom strukturelle egenskaper og antioksidantaktivitet ble analysert ved å undersøke hydrolysegraden, molvektsfordelingen og aminosyre-sammensetningen. Antioksidantaktiviteten ble undersøkt med analysene 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing ability of plasma (FRAP) og oxygen radical absorbance capacity (ORAC). Seiproteinhydrolysatene ble ultrafiltrert to ganger (UF1 og UF2) med to forskjellige membraner basert på molvekten for å avgjøre hvilke peptidstørrelser som viser høyeste antioksidantaktivitet. Ved UF1 ble det filtrert med en 150 kDa og deretter en 4 kDa membran, mens ved UF2 ble det filtret med en 150 kDa og så en 2 kDa membran.

Seiproteinhydrolysatene hadde en hydrolysegrad på 18 %, en høy proteinløselighet (100 %) og en høy forekomst (300 mg/g) av små peptider (2-1 kDa). På grunn av det høye innholdet av små peptider, hadde SPHene dårlig vannbindingsevne og emulgerende egenskaper sammenlignet med andre FPHer. Hydrolysegraden kan derfor være for høy for ønskede funksjonelle egenskaper. Molvektsfordelingen viste at UF1 separerte peptidene basert på størrelse, der fraksjonen <4 kDa hadde høyere konsentrasjon av mindre peptider. Videre ble det funnet et høyt innhold av essensielle aminosyrer som gjør at SPHene har en høy næringsmiddelverdi. Resultatene fra antioksidantaktivitetanalysene viste at SPH kan hemme ABTS-radikaler, kan redusere frie radikaler og at de har ORAC-aktivitet. Ultrafiltreringen økte FRAP og ORAC til en viss grad, hvor FRAP-verdiene økte i de store fraksjonene ($>$150 kDa, 150-2 kDa) og ORAC-verdiene økte i <4 kDa fraksjonen. Analysen ABTS, viste at ultrafiltreringene ikke økte egenskapen til å hemme ABTS-radikaler og antyder at en synergisk effekt av flere peptidstørrelser er en fordel. Resultatene i denne oppgaven kan indikere at det ikke er nødvendig å ultrafiltrere for å øke konsentrasjonen av antioksidantpeptider for å øke antioksidantaktiviteten. Til slutt, *in vitro* fordøyelse av SPHene reduserte ABTS- og FRAP-aktivitetene, mens ORAC-aktiviteten økte hos noen av SPHene. Utifra analysene, kan det derfor antas at hydrolysater fra biprodukter til hvit fisk kan ha potensiale til å bli brukt som et kosttilskudd, men mest sannsynlig høyere potensiale til å bli brukt som en multifunksjonell matvareingrediens.

Preface

This thesis was conducted spring 2021 as a part of the master's degree program in Chemical Engineering and Biotechnology. The work was carried out at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology. The work was conducted as a part of a PhD thesis under the NTNU funded project OPTiMAT.

I would like to give my special thanks to my supervisor, associate Professor Ida-Johanne Jensen, and my co-supervisors PhD candidate Veronica Hammer Hjellnes and Professor Turid Rustad for all the guidance and support. In addition, I wish to thank laboratory engineer, Siri Stavrum, for all her technical support.

At last, I would like to thank my family, friends, and boyfriend for all encouragement during this process, which was especially needed during the coronavirus pandemic.

Trondheim, June 2021

Stine Marie Pettersen

The Specialization Project

The introduction, the materials and methods, and the appendix sections are based on the specialization project [1] but extended.

In the specialization project, the saithe protein hydrolysates (SPH) were investigated for protein content, lipid content, amino acid composition, and for antioxidant properties. Further, the SPH were ultra-filtrated, where the $<$4 kDa was analysed for antioxidant properties. In this thesis, the $>$150 kDa and 150-4 kDa fractions from the specialization project were further investigated for antioxidant properties and for amino acid composition. Further, a second ultrafiltration was done in this thesis for comparison. In addition, functional properties, and the effect of *in vitro* digestion on antioxidant properties in SPH were investigated.

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Abbreviations

AAA	Aromatic Amino Acid
AAPH	2,2'- azobis-(isobuttersa ureamidin) dihydroclorid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
AUC	Area Under the Curve
BJI	Saithe Backbone from January, hydrolysis I
BJII	Saithe Backbone from January, hydrolysis II
BOI	Saithe Backbone from October, hydrolysis I
BOII	Saithe Backbone from October, hydrolysis II
BSA	Bovine Serum Albumin
Cys	Cysteine
DH	Degree of Hydrolysis
DM	Dry Matter
EAA	Essential Amino Acid
EAI	Emulsion Ability Index
EC	Emulsification Capacity
ES	Emulsification Stability
ET	Electron Transfer
FAA	Free Amino Acids
FL	Fluorescein
FPH	Fish Protein Hydrolysate
FRAP	Ferric Reducing Ability of Plasma
GI	Gastrointestinal
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
HAA	Hydrophobic Amino Acid
His	Histidine
HPLC	High-Pressure Liquid Chromatography
HT	Hydrogen Transfer
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
MW	Molecular weight
MWCO	Molecular weight cut-off
ORAC	Oxygen Radical Absorbance Capacity
PB	Phosphate Buffer
PG	Propyl Gallate
PGE	Propyl Gallate Equivalent
Phe	Phenylalanine

Pro	Proline
RRM	Rest Raw Materials
Ser	Serine
SPH	Saithe Protein Hydrolysate
TAA	Total Amino Acids
TE	Trolox Equivalent
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UF	Ultrafiltration
UF1	Ultrafiltration with 150 kDa and 4 kDa cut-off
UF2	Ultrafiltration with 150 kDa and 2 kDa cut-off
Val	Valine
WHC	Water Holding Capacity

1 Introduction

As much as 8.9 % of the world's population is suffering from starvation [2], meanwhile an estimated one-third of all food produced is wasted [3]. Food ends up as waste because consumers and retailers throw away food, food is spoilt during transportation, and because of poor harvesting practices [3]. In addition, the world's population is expected to increase to 9.7 billion by 2050, which further increases the need for more food [2]. Already, it is estimated that 3 billion people depend on marine food resources [4], and fish and seafood is a limited food resource that is threatened by overfishing, pollution, and ocean acidification [4]. Hence, in a sustainable future, food cannot be wasted, and resources must be utilized in a more sustainable way.

In Norway, the annual production of seafood was 3.55 million tons in 2019, whereof 0.96 million tons ended up as by-products [5]. Consumers prefer fish fillets instead of whole fish [6] and the fish fillets can represent as little as 1/3 of the fish [7]. Hence, the production of the fish fillets for the consumers produces by-products that the fish industry can utilize. The by-products are not regarded as directly saleable but can be recycled to new products for human consumption. The by-products include cut-offs, skin, viscera, heads, bone, by-catch, and fish that are not preferred for human consumption [8]. To emphasize the potential for new products for human consumption, the term "rest raw material" (RRM) is commonly used instead of by-products [8]. Marine RRM consist of valuable nutrients like fat-soluble vitamins, long-chain omega-3 fatty acids, minerals and contains high quality proteins with the essential amino acids (histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val)) [9]. The RRM can e.g., be used to produce fish protein hydrolysate (FPH), gelatine, oil extracts, and collagen [8]. Fish protein hydrolysates are hydrolysed proteins that are nutritious and have shown good functional and bioactive properties [8, 10, 11] (Section 1.2) and may have the potential as a multi-functional food ingredients or nutraceutical [12]. So, as the world's second largest seafood exporter [13], Norway has great potential to reduce waste and create high-value products from seafood RRM for human consumption.

In 2019, Norway utilized 84 % of the produced marine RRM [5]. A major part of the utilized RRM is used for processing of silage (44 %), oil and protein production for aquaculture (20 %), fish meal and oil production (18 %), human seafood consumption (10 %), feed for fur animals (5 %), and human consumption of cod liver oil/extracts (3 %) (Figure 1.1). In short, only 13 % of the utilized marine RRM is used for human consumption. The lowest utilization of marine RRM is the utilization of white fish RRM (61 % of 298 000 tons) and shellfish RRM (51 % of 14 800 tons) [5]. White fish (cod, haddock, ling, tusk, and saithe) are poorly utilized compared to aquaculture and pelagic fish and have big potential for improved utilization.

The harvest of white fish is divided between smaller coastal vessels and deep-sea vessels. The smaller coastal vessel fish daily, while deep-sea trawlers can be at sea for days [13]. For the latter vessels, on-board handling like freezing is required to maintain the high quality of the fish. The quality of RRM depends on the quality of the fish, and it is necessary to have an on-board sorting system combined with freezing to obtain high quality RRM [13]. The advantage of a sorting system is the separation of the less stable

viscera and blood from fractions like backbone and head [8]. Another solution could be on-board processing with biotechnology tools like enzymatic hydrolysis (section 1.1) and silage [13]. In this thesis, saithe (*Pollachius virens*) backbone was used as raw material for enzymatic hydrolysis. Backbone and cut-off represent 18 % of the available RRM from white fish [5].

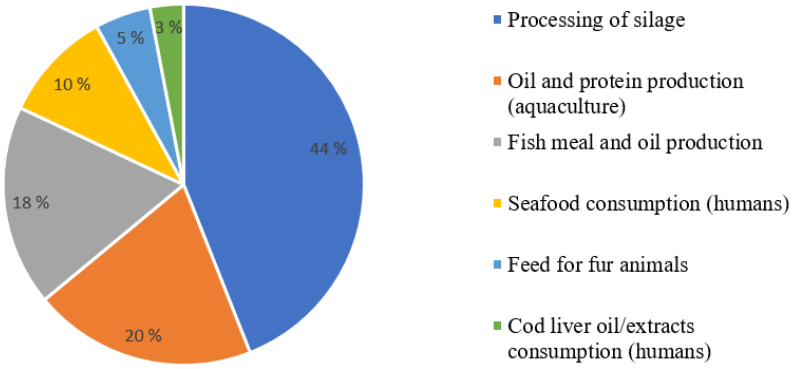

Figure 1.1: The applications of utilized marine rest raw materials in Norway. The figure is adapted from Richardsen et al. (2019) [5].

1.1 Enzymatic hydrolysis

Proteins are made of one or several chains of amino acids linked together by peptide bonds. To form a protein, a condensation reaction occurs between amino acids and water molecules are lost. The process of hydrolysis is the opposite reaction of condensation where water molecules are introduced to the peptide bonds resulting in breaking of the bonds. The main goal of hydrolysis is to increase the solubility by reducing the molecular size of the proteins into peptides [10]. Increased solubility may improve functional and bioactive properties [10] (Section 1.2). The fraction of peptide bonds cleaved can be defined as the degree of hydrolysis (DH) [14]. It is often expressed as a percentage:

$$\%DH = \frac{n}{n_T} \cdot 100, \tag{1.1}$$

where n is the total number of moles of peptide bonds in 1 mole of protein and n_T is the number of moles peptide bonds cleaved per mole of protein. A high DH indicates more peptide bonds cleaved.

The process of hydrolysis can be conducted chemically with strong acids/bases at high temperatures, or biochemically under milder conditions with enzymes as biological catalysts [10]. Chemical hydrolysis is relatively cheap to conduct but results in reduced nutritional qualities of the product [10]. Biochemical hydrolysation can be done by autolysis or enzymatic hydrolysis. Under autolysis, the hydrolysation is effectuated by the endogenous enzymes existing in the raw material, while in enzymatic hydrolysis, it is effectuated by exogenous enzymes that are added. Hence, autolysis is cheaper to perform, but due to

variations in enzyme content within a species, different end products may occur [10]. In enzymatic hydrolysis, the enzyme cost is higher, but the hydrolysis can be more controlled.

The breakage of peptide bonds in proteins is also called proteolysis [14]. Proteases are enzymes that perform proteolysis, and they can be classified into exopeptidases that break peptide bonds at the C- and N-terminus of the protein or endopeptidases that break the peptide bonds within the protein. Endopeptidases results in faster degradation of the proteins in the hydrolysate, because of faster reduction of the peptides molecular weight (MW).

For enzymatic hydrolysis, enzymes can come from plant, microbial or animal sources. Commonly used proteases are papain, Alcalase®, pepsin, bromelain, Flavourzyme®, Protamex®, and trypsin [12]. The choice of enzymes depends on the desired outcome of the hydrolysis, price, their specificity (Table 1.1), and efficacy, and they must be food grade. It has recently been shown that a mixture of bromelain from pineapple and papain from papaya resulted in preferred low bitterness in the applied FPH [15]. The choice of enzymes, therefore, affects the resulting product and must be carefully considered. Further, the hydrolysis process and conditions vary with different substrates [10]. Hence, the unique combination of substrate and enzyme must be investigated to find the proper reaction conditions for wanted properties.

Table 1.1: Commonly used proteases for enzymatic hydrolysis and their specificity [14].

Protease	Specificity
Alcalase®	Nonspecific
Bromelain	Ala–aa; Tyr-aa
Chymotrypsin	Phe-aa; Trp-aa; Tyr-aa
Papain	Lys-aa; Arg-aa; Phe-aa; Gly-aa
Pepsin	Leu-aa; Phe-aa
Trypsin	Lys-aa; Arg-aa

aa: Indicates any of 20 amino acid residues

1.1.1 The enzymatic hydrolysis process

As mentioned, the reaction conditions for enzymatic hydrolysis can vary depending on the substrate, and the activity of the exogenous enzyme, but the general enzymatic hydrolysis process to produce FPH is described in this section (Figure 1.2).

The enzymatic hydrolysis of fish raw material starts with solubilizing the raw material by e.g., mincing, and by adding water. Before adding the exogenous protease to the mixture, the temperature and pH are adjusted to the optimal condition for the enzyme. Thereby the hydrolysis starts. It can also be necessary to terminate endogenous enzyme activity before the exogenous protease is added. The reaction is then terminated by heat inactivation or low pH inactivation of the enzyme after the required reaction time for wanted properties is achieved [10].

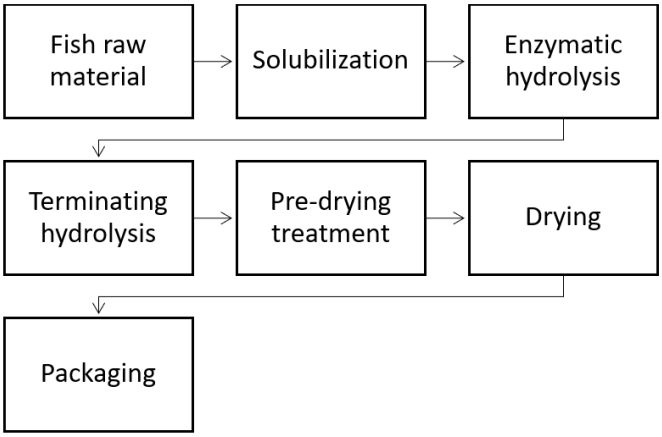

Figure 1.2: Overview of the enzymatic hydrolysis process to produce of fish protein hydrolysates. The figure is adapted from Petrova et al. (2018) [16].

For maximized utilization of the hydrolysate, it must be purified from the insoluble fraction and the fat fractions in the hydrolysate. The fat fraction is mainly removed for unwanted fat oxidation of the hydrolysate. These pre-drying separation treatments can be centrifugation or filtration [16]. If a certain quality/functionality of the hydrolysate is required, smaller peptides can be filtrated from the hydrolysate by microfiltration, ultrafiltration, or nanofiltration. After purification and separation of the proteins, the hydrolysate can be further pasteurized at high temperatures to eliminate possible microorganisms [16]. On the other hand, pasteurising may lead to denaturation of the peptides [17]. At last, the hydrolysate with the wanted quality is dried with spray dryers, vacuum freeze dryers or roller drum dryers [16] and packaged.

1.1.2 Ultrafiltration

Ultrafiltration (UF) is a purification process that can be used to separate smaller molecules from larger molecules (Figure 1.3). Inside the module, a selective barrier with a size of 300-500,000 Da of MW or pore size of 0.001-0.1 μm called a membrane [18] is situated. The membrane separates the feed into two streams which are called retentate and permeate. The retentate stream contains the molecules which were not able to cross the membrane, and the permeate stream contains the molecules that crossed the membrane [18]. The driving force in UF is often pressure by nitrogen gas that operates with a driving force between 2-10 bars.

A challenge with UF is membrane fouling which is an unwanted layer of material that accumulates at the membrane surface or inside the pores. For protein separation with UF, hydrophilic membranes can be used. It is argued that most proteins are hydrophobic at their iso-electric point [19]. As a result, the use of hydrophilic membranes will cause less membrane fouling compared to hydrophobic membranes that can increase protein-membrane interactions. In the early stages of filtration, the fouling is mostly influenced by protein-membrane interactions, but later in the process, it depends more on protein-protein interactions [19]. Protein-membrane and protein-protein interactions can cause a fouling layer which decreases the permeability of the membrane. This layer

depends strongly on pH, but also on the salt concentration [20]. At the iso-electric point, the layer will be more packed and harder to permeate. At low pH, it has been shown that the layer gains a more open structure that is easier to permeate [19]. Also, a high salt concentration can lower protein-protein interactions [20]. Membrane fouling can therefore change the permeability of the membrane and the degree of separation. Hence, UF is influenced by operating conditions like pressure, but also physicochemical conditions. The operating and physicochemical conditions must be optimized to enhance the best selectivity of the membrane [20].

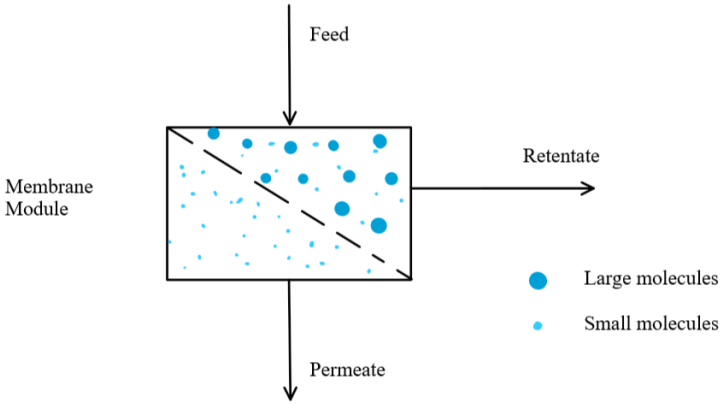

Figure 1.3: A diagram of a basic membrane filtration system. The membrane separates the larger and smaller molecules from each other. The figure is adapted from Chen et al. (2011) [18].

1.2 Properties of FPH

As mentioned, one way of utilizing the nutritious RRM from white fish is to produce FPH by enzymatic hydrolysis. The FPH consists of mainly peptides, where the peptide size depends on DH. The functional and bioactive properties depend on the choice of raw material, hydrolysis conditions, enzymes, and the degree of hydrolysis [10]. For human consumption, FPH of dark fish muscle has limited value because of higher fat content and its potential of oxidation which creates an unwanted flavour and colour [21].

1.2.1 Functional properties

Functional properties of proteins are defined as ”physicochemical characteristics which affect the behaviour of protein in food systems during processing, manufacturing, storage and preparation”[22]. The properties include solubility, water holding capacity (WHC), foaming, emulsifying properties, and gelling. They are affected by the DH, the shape, amino acid composition and sequence, and the distribution of charges of the peptide content [10]. The degree of hydrolysis of FPH, i.e., the reduction in peptide size, which results in increased solubility, is the main reason for improved functional properties [14]. The reduced size increases the amount of ionizable groups, and exposure of hydrophobic groups, thus increasing the solubility [23]. A too high DH, however, can result in a loss of functional properties [10].

Water holding capacity is defined as the ability to bind water and retain it against gravitational force within a protein matrix [24]. Smaller peptides are more hydrophilic, and it has been shown that smaller sized peptides affect WHC greater than larger peptides [25]. However, too small peptides can decrease the WHC due to the lower ability to entrap the water compared to larger peptides [26]. A product with high WHC will often improve the texture of the food product and be juicier. It has been shown that FPH added to fish mince increased the WHC [27, 28].

It has also been shown that FPH can contribute to emulsifying properties [27]. An emulsion is a mixture between two immiscible liquids like oil and water, where one of the liquids is dispersed in the other one as small droplets [29]. If oil is dispersed in water as a continuous phase, the emulsion is called an oil-in-water emulsion, and if opposite, water-in-oil emulsion. An emulsion can be induced by shear forces, cavitation, turbulence, and particle collision by using equipment like high-speed mixers, pressure homogenisers, colloid mills, and ultrasonic homogenisers to disperse one of the liquids into the other one [30]. The homogenised mixture is an unstable system because of the interfacial tension between the polar and nonpolar phases. To lower the interfacial tension an emulsifier is needed [31]. An emulsifier must be amphiphilic to comprise both the hydrophobic and the hydrophilic phases at the oil-water interface [31]. Hence, amphiphilic proteins can work as an emulsifier [32]. These proteins stabilise the system by rearranging the hydrophobic groups in the oil phase and the hydrophilic groups in the aqueous phase [32]. The rearranging creates a viscoelastic film that surrounds the droplet and protects it from coalescence and flocculation through electrostatic repulsion and steric hindrance [29, 32, 33]. It has been shown a positive correlation between the solubility of proteins and emulsifying properties [34, 35]. Therefore, FPH has the potential to be used as emulsifiers. High protein solubility allows a rapid diffusion to an interface and flexibility to unfold, which are two important features for an effective emulsifier [32, 33, 36]. On the other hand, too small peptides with good solubility cannot unfold and reorient at the interface. Generally, the emulsifying properties are improved up to DH < 10 % [14]. The emulsifying properties also depend on the conditions such as pH, temperature, ionic strength, etc. [10]. Two methods to measure the emulsifying properties of FPH are emulsifying capacity and emulsifying stability. Emulsifying capacity is defined as mL emulsified oil per g of protein, while emulsifying stability is defined as the ability to resist changes in the properties of the emulsion over time [10].

1.2.2 Bioactive properties

The definition of a bioactive compound is a compound which could interact with one or more component(s) of the living tissue by presenting a wide range of probable effects [37]. In other words, bioactive peptides have a health benefit beyond the nutritional value. It has been shown that FPH possess bioactive peptides with bioactive properties such as antioxidant activity, antihypertensive, anti-inflammatory, and anticancer activity [27, 38–41]. The peptides with bioactive properties are encrypted in the original protein and need to be released to be functional. These peptides can be released during digestion or processing/hydrolysis of the fish [42]. Non-digested or non-processed fish proteins do not have bioactive properties because of poor accessibility to the bioactive peptide sequence [43]. Thus, the DH affect the bioactive properties. With these bioactive properties, FPH

has a great potential to be used as a nutraceutical and as a functional food for human consumption [43].

1.3 Oxidation

The overall chemical reaction where electrons are transferred between two species is called a redox reaction. This involves both a loss and gain of electrons, where oxidation is the loss of electrons and reduction is the gain of electrons. Atoms that have an unpaired electron, called free radicals, are highly unstable and reactive. They work as oxidizing agents by seeking to gain an electron from other compounds. In return the free radical becomes neutral, but the other compound is turned into a free radical. This is called a chain reaction. Chain reactions caused by free radicals are happening daily in our bodies and in food. To generate energy in the form of ATP (adenosine triphosphate) in the human body, oxygen is essential. The generation of ATP is formed through oxidative phosphorylation where electrons from electron carriers are transferred to molecular oxygen through the electron transportation chain. The oxygen is then reduced to water, and ATP is generated. In addition, it produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) [44]. These compounds consist of free radicals such as superoxide radical ($O_2^{\bullet -}$) and hydroxy radical ($OH^{\bullet}$), and of non-radical compounds such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The non-radical ROS can initiate the production of free radicals, where the Fenton reaction catalysed by iron (II/III) that produce hydroxyl radicals is one example. Further, free radicals can be produced by enzymes, aging, genetics, traditional risk factors (smoking, diabetes, hypertension, etc.), and by environmental risk factors (air pollution, heavy metals, etc.) [45]. Even thou free radicals are a natural part of the human body; a too high concentration will cause oxidative stress. It has been shown that oxidative stress can lead to several serious effects [44, 46, 47] such as chronic diseases, cancer, and heart failure [45]. In addition, they can damage important macromolecules in the body, disturb cell homeostasis and cause cancer [46].

In food, unsaturated fatty acids can be oxidized to hydroperoxides by autoxidation, photosensitized oxidation, and by enzymatic oxidation. The autooxidation is a free radical mechanism that involves oxygen and consist of three phases: initiation phase, propagation phase, and termination phase:

Initiation:
$HO^{\bullet}$ + R-H $\longrightarrow$ $R^{\bullet}$ + H_2O

Propagation:
a) $R^{\bullet}$ + O_2 $\longrightarrow$ $ROO^{\bullet}$
b) $ROO^{\bullet}$ + RH $\longrightarrow$ ROOH + $R^{\bullet}$

Termination:
$R^{\bullet}$ + $R^{\bullet}$ $\longrightarrow$ R-R
$R^{\bullet}$ + $ROO^{\bullet}$ $\longrightarrow$ ROOR
$ROO^{\bullet}$ + $ROO^{\bullet}$ $\longrightarrow$ ROOR + O_2

The initiation phase is started by an initiator, e.g., hydroperoxide decomposition, metals, light, and singlet oxygen [48]. In this phase, a lipid radical ($R^{\bullet}$) is produced. In the propagation phase, lipid peroxyl radicals ($ROO^{\bullet}$) are produced and reacting with unsaturated fatty acids if oxygen and unsaturated fatty acids are available. At last, when it is no longer sufficient oxygen or fatty acids, they start to react with each other to create stable end-products. The hydroperoxides can then create odours, discoloration, and off flavours when they are degraded to ketones, alcohols, and aldehydes [48]. Typical rate effectors are high temperature, UV-light, surface area, water activity, transition metals, pigments, and oxygen [48]. Hence, to inhibit lipid oxidation oxygen must be excluded and the food must be stored in darkness. In addition, antioxidants can be added.

1.4 Antioxidant activity

Antioxidants are defined as molecules that inhibit oxidation and can scavenge free radicals, be metal chelators, inactivate ROS, and inactivate enzymes [46]. Thus, they are important for the inhibition of oxidative stress in the human body and for the inhibition of lipid oxidation in food. Important mechanisms of antioxidants to neutralize free radicals are electron transfer (ET), hydrogen atom transfer (HAT) and to be metal chelators. Also, it is an advantage if the antioxidant has an aromatic ring structure that contributes with resonance delocalization. The resonance delocalization makes the antioxidant, even with an unpaired electron, more stable than the free radical [48]. Further, antioxidants are divided into three levels of action: free radical scavengers (primary antioxidants), preventive antioxidants (secondary antioxidant), and repair and de novo antioxidants [46]. A free radical scavenger transfers an electron/hydrogen to the free radical, preventive antioxidants remove initiators by e.g., metal chelating activity, and repair antioxidant are for example proteolytic enzymes that remove oxidised proteins. Examples of chelating activity are to reduce metals, react with metals to form insoluble complexes, or sterically hinder the metals [49].

Antioxidants are often naturally present in biological tissues, but food processing can impair antioxidants or produce more free radicals [48]. An example is that thermal processing can decompose lipid hydroperoxides that produce free radicals [48]. Therefore, it may be necessary to add antioxidants to processed food. The most common free radical scavengers that are added to food are tocopherols, synthetic phenolics, plant phenolics, and ascorbic acid (vitamin C) [48]. Tocopherols have a hydroxylated ring with a phytol chain and a well-known, commonly used tocopherol is vitamin E and Trolox. Other examples of antioxidants are plant phenolics like rosemary extract, thiols like cysteine and glutathione, and synthetic phenolics like butylated hydroxy-toluene (BHT), butylated hydroxyannisole (BHA), and propyl gallate (PG) [48]. It is now suspected that BHT and BHA can induce DNA damage and show toxic effects [50, 51] as well as instability at high temperatures. Hence, the carcinogenic nature, instability, the strict regulations of the synthetic antioxidants and consumer's demand for natural products have made the manufactures more focused on natural antioxidants [46]. The use of BHT and BHA is decreasing because consumers demand natural products.

Today, it is no standardized method to measure total antioxidant activity [52]. Thus, a combination of several antioxidant activity assays is necessary to measure different

antioxidant mechanisms. Often, the antioxidant activity is measured indirectly with *in vitro* assays or with model systems that simulates natural conditions of oxidation.

1.4.1 Bioactive peptides as antioxidants

Bioactivity in peptides has been thoroughly reviewed and the interest has increased [21, 25, 43], especially antioxidant activity. Several publications have indicated that peptides from fish hydrolysates can have antioxidant properties [53–55], and that FPH can inhibit lipid oxidation, scavenge free radicals and ROS, reduce metals, and chelate [11, 27, 55] (Figure 1.4). The hydrolysate's antioxidant capacity depends on the size, degree of hydrolysis, amino acid composition and sequence of peptides, and the amount of free amino acids (FAA) [53, 56, 57]. For lipid oxidation inhibition, hydrophobic amino acids (glycine (Gly), alanine (Ala), Val, Leu, Met, and Ile) have been shown to be important for lipid-protein interactions [58] and for bioactivity inside cells that are surrounded by a double layer of lipids.

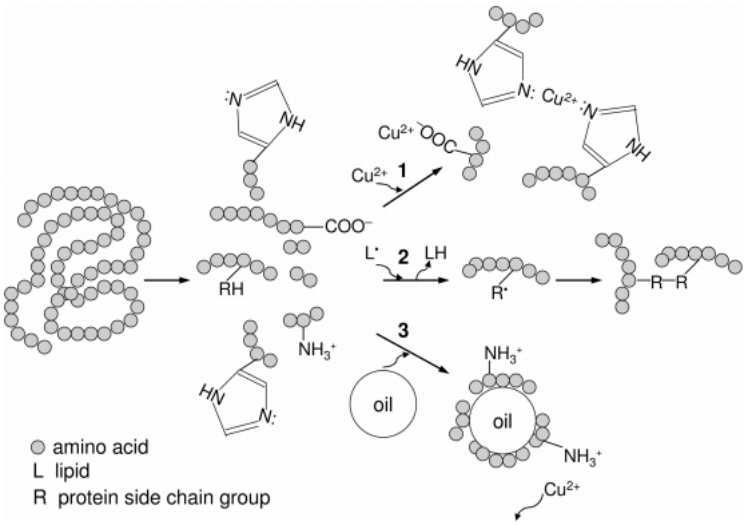

Figure 1.4: The figure presents three examples of how peptides can work as an antioxidant. Reaction 1: metal chelator. Reaction 2: Free radical scavenger. Reaction 3: Physical hindrance. (Figure [59])

In general, peptides with antioxidant activity have a chain length of 2-20 amino acids [43], and it has been shown that low MW (1-5 kDa) peptides have higher antioxidant activity [53, 57, 60, 61]. However, there are some exceptions. Intarasiriawat et al. (2012) showed that defatted skipjack roe hydrolysate fractions with a MW of 5.5 had the strongest ABTS radical scavenging activity and metal chelating activity [54]. Moreover, it has also been shown that Alaska pollack frame protein hydrolysate with peptides with MW below 1 kDa had the highest antioxidant activity [53]. In other words, the ideal size of peptides for antioxidant activity has not been found.

Alemán et al. (2011) [60] showed that ABTS radical scavenging activity in hydrolysed gelatine increased with increasing DH % up to 30 % [60]. Comparable, Intarasiriawat et al. (2012) [54] found that defatted skipjack roe hydrolysate had a reduction of ABTS radical scavenging activity with increased DH. Moreover, they found that the DPHH

radical scavenging and reducing power decreased with increasing DH and that superoxide scavenging activity and metal chelating activity increased with increasing DH up to 40 % DH. At 50 % DH, the metal chelating activity decreased, and one reason can be that smaller peptides cannot form stable complexes with metals.

Amino acids that have been documented to be antioxidants are tyrosine (Tyr), Met, His, Lys, cysteine (Cys), and Trp [62, 63]. In a study on cod protein hydrolysates conducted by Saabena Farvin et al. (2016), they found that peptides with a MW below 0.6 kDa with the dominating content of Tyr and His showed high radical scavenging activity. Also, arginine (Arg)-, Tyr-, and Phe-rich peptides had higher reducing power [62]. The antioxidant activity of the aromatic amino acids Tyr and Phe is because of their phenolic group that serve as good hydrogen donors [21]. The imidazole group of His do also possess proton-donation ability [21]. Other amino acids suspected to have reducing power are negatively charged glutamic (Glu) and aspartic acid (Asp) [64]. Further, the position of the amino acids is important for the antioxidant activity of peptides [56, 65]. Saito et al. (2003) tested libraries consisting of peptides containing either His or Tyr residues, and peptides with proline (Pro)-His-His which have known antioxidant activity. Their work showed antioxidant activity also depends on the positioning of the amino acids and the sequences. Also, FAAs have shown antioxidant activity [66], but in general, peptides show higher antioxidant activity than FAAs.

1.5 Utilization of FPH

As discussed above, FPH may exhibit good functional and bioactive properties as well as containing high quality protein. In addition, enzymatic hydrolysis increases the bioavailability [10]. Thus, FPH can be utilized as a protein supplement in e.g., sports nutrition or in diet-related diseases such as liver failure, food allergy, and Chron's disease [67]. Some adults and children have allergy reactions towards food proteins like egg proteins, cow's milk proteins, and soy proteins [14], but hydrolysis of these proteins can lower their allergenicity. Even though the possibilities of producing high-value products for humans have been thoroughly reviewed [8, 9, 40, 68], only a few products are commercially available on the market. One reason is that it is difficult and costly to provide documentation for health claims since it requires *in vivo* studies in humans [9]. In addition, the properties can behave differently after gastrointestinal digestion. Therefore, more research on *in vitro* digestion of FPH is necessary (Section 1.5.1). Thus, the most realistic use of FPH today is as feed or food ingredient than as functional food or nutraceutical [9, 40]. However, FPH can work as a multipurpose functional ingredient in food where one example is that FPH can be used as both an emulsifier and as an antioxidant that inhibits lipid oxidation. Even so, FPH have two additional challenges.

Lipid oxidation decreases shelf-life, create distaste and discoloration of FPH, and some peptides in FPH are considered bitter which give an undesirable taste. Bitter peptides are related to hydrophobicity, and peptides with more than 1.3 kcal mol^{-1} mean residue hydrophobicity [14] and a MW $<$6 kDa are known to be bitter [69]. Thus, if the DH is kept at 3-5 %, the hydrolysate will have low bitterness [8]. Also, if the hydrolysate is completely hydrolysed to free amino acids, the bitterness will be lowered because the FAAs are less bitter than the hydrophobic peptides [8]. The bitterness of FPH can also

be reduced by several other techniques like using specific peptidases, active carbon, and chromatographic separation. It has for example been shown that a mixture of bromelain and papain or the use of flavourzyme results in preferred low bitterness in the FPH [15].

1.5.1 Gastrointestinal digestion of FPH

The human gastrointestinal (GI) tract consists of the mouth, oesophagus, stomach, and intestines. This system digests the food we consume by degrading the carbohydrates, lipids, and proteins in the food into smaller units, that can be absorbed and utilized in our body. The GI digestion can therefore change the properties of FPH by reducing the size of the peptides. The food is mechanically as well as enzymatically degraded in cooperation with the liver, pancreas, and gallbladder. Digestion of proteins start already in our mouth where they are mechanical degraded by chewing before they enter the stomach. The stomach contains gastric juices with hydrochloride acid and enzymes such as pepsinogen. The low pH (1.5-3.5) activates pepsinogen to the active form pepsin [70] and denatures the protein to reveal the polypeptide chain. The polypeptide chain can now be cleaved by the protease pepsin. Pepsin cleaves peptide bonds in connection to hydrophobic amino acid residues, where it prefers to cleave after Leu and Phe [71]. After the stomach, the smaller polypeptides enter the small intestine which has a pH of 6-7. From the pancreas, trypsin and chymotrypsin are excreted which further degrades the smaller polypeptides. Trypsin prefers to cleave polypeptides at Arg and Lys residues at the carboxy side, and chymotrypsin prefer to cleave peptide bonds at aromatic amino acids residues (Phe, Trp, Tyr, and His) [70].

1.6 Aim of the thesis

The overall aim of this master thesis was to investigate functional properties, and antioxidant activity of saithe protein hydrolysates (SPH) produced by enzymatic hydrolysis of backbone from saithe, and how the antioxidant activity is affected by membrane filtration and *in vitro* GI digestion. The overall aim was divided into 4 sub-goals:

- Analyse the functional properties solubility, WHC, and emulsifying properties.
- Evaluate relationship between structural properties (molecular size and amino acid content) and antioxidant activity.
- Investigate the effect of UF on the degree of separation and antioxidant activity with two different cut-offs.
- Explore the effect of *in vitro* digestion of SPH.

2 Materials and Methods

Saithe protein hydrolysates used for analyses in this thesis were produced by PhD student Veronica Hammer Hjellnes with enzymatic hydrolysis. The work was conducted as a part of a PhD thesis under the NTNU funded project OPTiMAT.

2.1 Raw material

Saithe (*Pollachius virens*) (n=9) was caught in the Trondheimsfjord, Norway, in October (O) 2019 and January (J) 2020. The fish were bled immediately on the fishing vessel, kept on ice for transport and separated into head, backbone, fillet and viscera by hand in the laboratory facility at NTNU Kalvskinnet within a day. The separated parts from the fish were vacuum packed and frozen at -40 °C until analyses. Saithe backbone was used as raw material for the enzymatic hydrolysis.

Upon enzymatic hydrolysis, the fractions were thawed overnight at 4 °C and minced by an industrial grinder (OMAS Meat Mincer Tritacarne TS 22E) with hole size 5 mm and then refrozen at -40 °C.

2.2 Enzymatic hydrolysis

The enzymatic hydrolysis of saithe backbone (B) was performed by PhD student Veronica Hammer Hjellnes. Two enzymatic hydrolysis (I, II) were done respectively on saithe backbone from October (O) 2019 and January (J) 2020 (Figure 2.1).

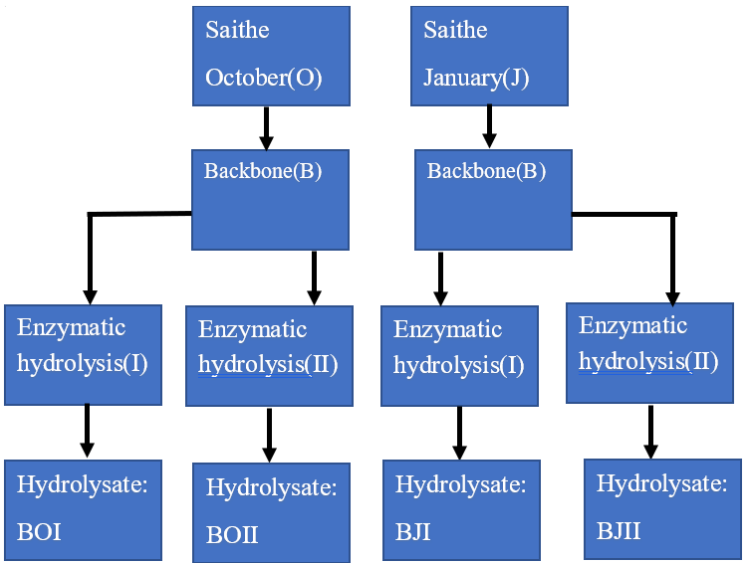

Figure 2.1: Schematic overview of enzymatic hydrolysis of saithe backbone. Two enzymatic hydrolysis (I, II) were done respectively on saithe backbone from October (O) 2019 and January (J) 2020.

Hundred grams of the raw material was thawed overnight at 4 °C and mixed with an equal amount of preheated water (50 °C). The mixture was then transferred to a bioreactor (Syrris Atlas, Model No. 2101000) with thermostats (Huber Ministat 125) and a stirring

speed of 500 rpm. When the mixture had reached 50 °C a 0.1 % 1:1 mixture of papain and bromelain (Merck, Germany) was added. The reaction was terminated after 60 minutes by heat inactivation (> 90 °C, 10 min), before the mixture was centrifuged (900 rpm, 10 min, 20 °C) and frozen at -20 °C. The centrifugation resulted in three fractions: lipids, hydrolysate, and sludge. These fractions were frozen and then separated by using a scalpel. The hydrolysates were filtered, freeze dried (Labconco FreeZone 12) and frozen to -40 °C until analyses (Figure 2.2).

Figure 2.2: Freeze dried saithe hydrolysate from backbone.

2.3 Analyses of saithe protein hydrolysates

Figure 2.3 shows a schematic overview of the analyses of SPHs, and the fractions generated from ultrafiltration (UF). Two UFs were performed on SPHs, one in the specialization project (UF1) and one in the master project (UF2). In the specialization project, the SPHs were UF with a 150 kDa and 4 kDa MW cut-off (MWCO) membrane. In this thesis, the SPHs were UF with 150 kDa and 2 kDa MWCO membranes to compare with UF1. Hydrolysate BJII was only filtrated with 2 kDa MWCO membrane in this thesis because the 150 kDa MWCO membrane broke. The SPHs and the <4 kDa fraction from UF1 were analysed for antioxidant activity in the project and analysed for molecular weight distribution in this thesis. The >150 kDa and 150-4 kDa fraction from UF1 were analysed for antioxidant activity in this thesis. Also, all the fractions from the UFs were analysed for amino acid content in the thesis, if sufficient material.

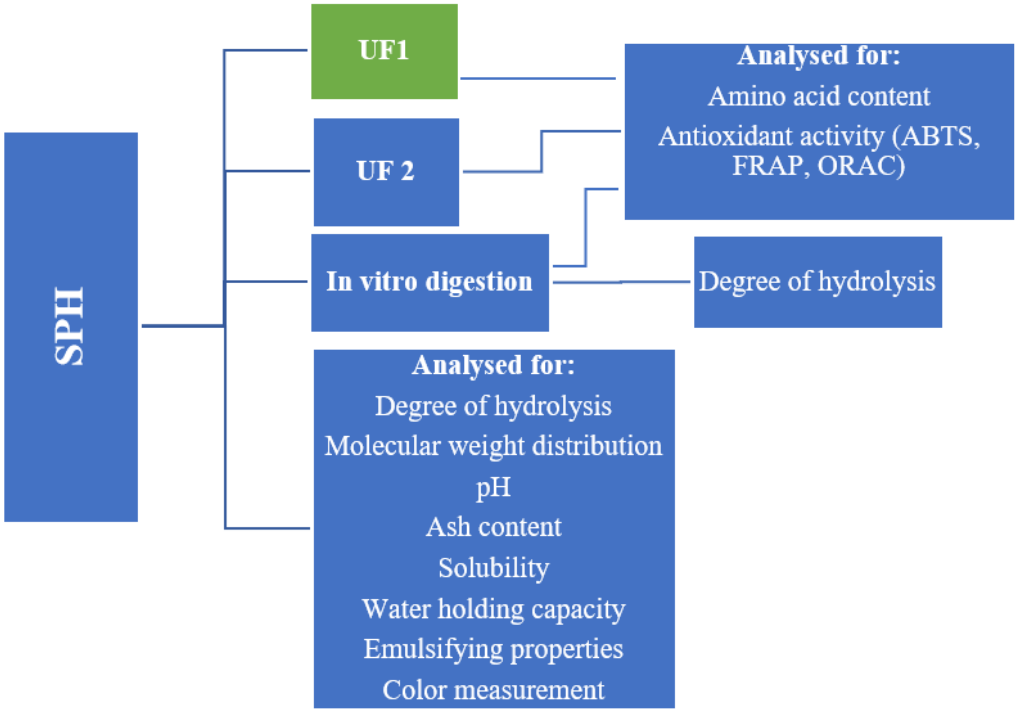

Figure 2.3: An overview of the analyses of saithe protein hydrolysates (SPH) of backbone and fractions performed during the master's project. Two ultrafiltrations (UFs) were performed on SPH, one in the specialization project (UF1) (indicated with green) and one in the master project (UF2).

2.4 Degree of hydrolysis

The degree of hydrolysis of the SPH and digested SPH was determined as described by Taylor (1957) [72], and the analysis on the SPH was performed by Veronica Hammer Hjellnes. Distilled water (50 g) was added to an amount of sample (1-0.5 g) and the pH was adjusted to 7.0 with 0.1 M NaOH before 10 mL of formaldehyde was added. After 5 minutes the solution was titrated to pH 8.5 with 0.1 M NaOH. The degree of hydrolysis was calculated as:

$$\%\text{Free amino groups} = \frac{A * B * 14.007 * 100}{C * 1000}, \tag{2.1}$$

where A= mL NaOH used, B= concentration of NaOH, C= g sample and 14.007 is the molecular weight of nitrogen. The degree of hydrolysis was then calculated as shown in equation 2.2.

$$\%\text{Degree of hydrolysis} = \frac{D * 100}{E}, \tag{2.2}$$

where D= % free amino groups and E= % N in sample.

2.5 Molecular weight distribution

Molecular weight distribution of SPH and the <4 kDa UF fractions was analysed by Innolipid AS with HPLC.

2.6 pH-measurements

The pH of SPH was measured with a pH-meter (Mettler Toledo MP 220) of 1 % concentration of the SPH in distilled water.

2.7 Ash and dry matter content

Dry matter (DM) and ash content were measured gravimetrically. To measure DM, the samples were placed in a heating cabinet at 105 °C for 24 hours. To measure ash content, the samples were placed in a muffle furnace at 550 °C for 24 hours.

The ash content was measured by PhD student Veronica Hammer Hjellnes.

2.8 Functional properties

2.8.1 Solubility

Solubility was measured with the Lowry method as previously described (Lowry et al., 1951) [73]. The alkaline copper reagent was prepared daily by mixing 1 mL of 1 % $CuSO_4 \cdot 5H_2O$, 1 mL 2 % Potassium Sodium Tartrate, and 100 mL Na_2CO_3 in 0.1 M NaOH. The Folin-Ciocalteu reagent was diluted 1:3 with doubly distilled (dd) water. Subsequently, a standard curve was prepared from a series of dilutions (12.5-300 μg/ml) from a stock solution of bovine serum albumin (BSA) (1000 μg/ml). The SPH were dissolved to the concentration of 1 % in distilled water and diluted to 1:100.

Thereafter, 0.5 mL of the sample/standard/blank were mixed immediately with 2.5 mL alkaline copper reagent and incubated for 10 minutes. Then 0.25 mL Folin reagent was added to the tubes, mixed immediately and incubated for 30 minutes. The absorbance was measured spectrophotometrically (Pharmacia Biotech Ultrospec 2000) at 750 nm.

2.8.2 Water holding capacity

Measurement of water holding capacity (WHC) was conducted as described by Eide et al. (1992) [74]. Fresh, filleted, and skinned cod (*Gadus morhua*) (n=2) caught in Barents Sea were bought in the store five days after catch. The fillets were minced three times by a grinder (OMAS Meat Mincer Tritacarne TS 22E) with hole size 5 mm. The mince was then immediately frozen to -20 °C and kept frozen upon analysis.

Two grams of the minced cod muscle were centrifuged in tubes filled with glass beads (Sigma 202 centrifuge) (1500 rpm, 5 min). The WHC was determined as the weight loss after centrifugation (Δr) and with the water content (V1) in the sample:

$$WHC(\%) = \frac{V1 - \Delta r}{V1} * 100\% \tag{2.3}$$

The water content was found by drying samples of the minced cod muscle in a heating cabinet for 24 hours at 105 °C. The same procedure was performed with minced cod muscle mixed with different SPH concentrations (1 %, 2 % and 3 %). Measurements were performed in quadruplicates.

2.8.3 Emulsifying properties

Emulsifying properties of the SPH were determined as described in Šližytė et al. (2009) [27]. Three different solutions of SPH (1 %, 2 % and 3 %) in 4 mL distilled water were mixed with 4 mL of rapeseed oil in a centrifuge tube and homogenized (IKA T18 digital Ultra-Turrax) (12000 rpm, 90 seconds). The mixture was then centrifuged (Sigma 202 centrifuge) (5000 rpm, 3 min) and the volumes of the separated phases were determined. Emulsification capacity was expressed as mL of emulsified oil per g of SPH. Thereafter, emulsion stability was determined by centrifugation (Sigma 202 centrifuge) (5000 rpm, 3 min) of the sample tubes after 24 hours at room temperature. The emulsion stability was expressed as the percentage of emulsion remaining after 24 hours at room temperature. Measurements were performed in duplicates.

2.8.4 Colour measurement

The colour of the SPH was measured with a CR-400-chromameter (Konica Minolta) by PhD student Veronica Hammer Hjellnes. L* indicated lightness, a* indicated redness, and b* indicated yellowness of the SPH. The whiteness (w*) was then calculated from these three values using equation 2.4.

$$W* = 100 - [(a^*)^2 + (b^*)^2 + (L^* - 100)^2]^{1/2} \tag{2.4}$$

2.9 Ultrafiltration

First, the prepared solution of SPH (500 mL, 1 % SPH) was filtered through a ceramic membrane with a 150 kDa CO membrane and an area of 28 cm^2. Then the permeate was filtered through three hydrophilic polyether sulfone flat-sheet membranes with a CO at 2 kDa (Supplier: Trisep) and a total area of 84 cm^2. In the specialization project, hydrophilic polyether sulfone flat-sheet membranes 4 kDa CO membrane (NADIR UH004/UH005 P,MICRODYN-NADIR) was used instead of 2 kDa.

The SPH solution was poured into the feed tank and filtrated through the membrane with a nitrogen gas pressure of 5 bar as a driving force. At the system's dead-volume (50 mL), the pump was stopped to avoid the pump going dry. At last, the permeate and the retentate were freeze dried (Labconco FreeZone 12), and frozen at -20 °C.

2.10 Analysis of free amino acids

Free amino acids (FAA) in SPH fractions were determined as described by Osnes and Mohr (1985) [75]. An amount of 10 mg sample was dissolved in 1 mL of dd water, and then the dissolved sample was vortexed with 0.25 mL 10 % sulphosalicylic acid and cooled down at 4 °C for 30 minutes before centrifugation (eppendorf Centrifuge 5415

R) (10 000 rpm, 10 min). To control if all the protein in the sample had precipitated, 0.125 mL 10 % sulphosalicylic acid was added to 0.5 mL supernatant from the parallels and the procedure above was repeated. If the sample had no precipitated protein, the procedure could be continued with this supernatant. If not, the procedure had to be repeated until no precipitated protein. Thereafter, the samples were diluted 1:100 with dd water and filtered through a 0.20 μm filter (VWR international, 25 mm syringe filter). Finally, 0.205 mL of the samples was used for HPLC that was performed by Siri Stavrum at NTNU with the o-phtalaldehyde (OPA) pre-column derivatization method. The column used was a waters HPLC column (Nova-Pak column Reversed-Phase 4 μm Spherical Silica) with methanol as mobile phase A, $NaCooCH_3$ with oxolane as mobile phase B, Sigma P0532 as precolumn derivatization reagent, and Sigma AAS18 as amino acid standard. The instrument (UltiMate 3000 Dionex) was equipped with a pump, column oven, autosampler and a fluorescence detector (Dionex RF 2000) with 330 nm as excitation wavelength and 438 nm as emission wavelength.

2.11 Analysis of total amino acids

Total amino acid (TAA) content in SPH fractions was determined as described by Blackburn et al. (1978) [76]. An amount of 50 mg sample was added 1 mL 6 M HCl and hydrolysed for 22 hours at 105 °C and then cooled down. The content was flushed with dd water, and the pH was adjusted to pH 6.5-7.5 with NaOH. Subsequently, the samples were filtered through a Whatman glass microfibre filter GF/C with a diameter of 25 mm (GE Healthcare, Great Britain) by using a vacuum pump (Heto MASTER JET) and the volume was adjusted to 10 mL with dd water. The samples were further diluted to 1:500 with dd water, filtered through a 0.20 μm filter (VWR international, 25 mm syringe filter) and transferred to HPLC vials. The HPLC was performed by Siri Stavrum at NTNU with the o-phtalaldehyde (OPA) pre-column derivatization method. The column used was a waters HPLC column (Nova-Pak column Reversed-Phase 4 μm Spherical Silica) with methanol as mobile phase A, $NaCooCH_3$ with oxolane as mobile phase B, Sigma P0532 as precolumn derivatization reagent, and Sigma AAS18 as amino acid standard. The instrument (UltiMate 3000 Dionex) was equipped with a pump, column oven, autosampler and a fluorescence detector (Dionex RF 2000) with 330 nm as excitation wavelength and 438 nm as emission wavelength.

2.12 Antioxidant activity assays

Three different methods were used for measuring antioxidant-activity in SPH and the fractions; ABTS Assay, FRAP assay, and ORAC assay. ABTS and FRAP assay are spectrophotometric methods, while the ORAC assay is a fluorescence method that measures the decay of fluorescence over time. The standard curves used were propyl gallate (PG) for ABTS assay and Trolox for the FRAP and ORAC assays.

2.12.1 ABTS Assay

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay is a decolourisation assay for both lipophilic and hydrophilic antioxidants, and it is stable for a wide pH range

[77, 78]. In the ABTS assay, the ABTS molecule is oxidised with potassium persulfate ($K_2S_2O_8$) to create a stable radical. $ABTS^{+\bullet}$ is a radical mono-cation with a strong blue green colour, and by reduction of an antioxidant, it will lose its colour. Radical scavenging activity was determined in SPHs and SPH fractions from UF with the ABTS assay as described by Re et al. (1999) [78] and further developed by Nenadis et al. (2004) [77].

The ABTS reaction mixture was prepared by mixing 25 mL 7mM ABTS solution and 440 μL 140 mM $K_2S_2O_8$, and subsequently, it was incubated overnight at room temperature in darkness. Thereafter, the mixture was diluted with methanol (80%) until an absorbance of 0.75 $\pm$ 0.05 at 734 nm. From a 10 mM PG stock solution in methanol (80 %) a standard curve was made with dilutions between 10-50 μM of PG in methanol (80%) (Appendix F). To prepare the SPH samples, they were dissolved in distilled water to the concentration of 1 % and then diluted to 1:20 with 80 % methanol.

Thereafter, 200 μL of extract, standard solutions and blank (80 % methanol) were mixed with 2 mL of ABTS solution and vortexed. The samples were incubated for 6 minutes at room temperature in darkness, and the absorbance was measured spectrophotometrically (UV-1800, SHIMADZU UV spectrophotometer) at 734 nm with water as a reference.

2.12.2 FRAP assay

Ferric Reducing Ability of Plasma (FRAP) assay is a measurement of the antioxidant ability to reduce (electron transfer) ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron [79]. The reaction is conducted at low pH (3.6) and the reduction by an antioxidant will result in a deep blue coloured ferrous-tripyridyltriazine (TPTZ) complex [79]. The FRAP was determined in SPHs and SPH fractions from UF as described by Benzie and Strain (1996) [79] with modifications.

The FRAP solution was prepared daily by mixing 5 mL of 19 mM $FeCl_3{\cdot}6H_2O$, 5 mL of 10 mM TPTZ in 40 mM HCl solution, and 50 mL of 300 mM acetate buffer, pH 3.6(1.505 g $C_2H_3NaO_2$ and 8 mL of $C_2H_4O_2$ per 500 mL buffer solution). Subsequently, it was incubated at 37 °C until use. Trolox (97 %) was used as a standard with a series of dilutions in distilled water with concentrations between 31.25-1000 μM (Appendix G). At last, the SPH were dissolved to the concentration of 1% in distilled water.

Thereafter, 10 μL of the sample/standard solutions were mixed with 30 μL distilled water, and 300μL FRAP solution in a 96 well microplate. Then, all the samples were incubated for 30 minutes at 37 °C in darkness, and the absorbance was measured spectrophotometrically (PowerWave XS, BioTek) at 593 nm with water as a reference.

2.12.3 ORAC assay

Oxygen Radical Absorbance Capacity (ORAC) assay is a method based on the oxidation of a fluorescent probe. The probe is oxidised by peroxyl radicals that are produced by the free radical initiator AAPH (2,2'-azobis-(isobuttersa ureamidin)-dihydroclorid) [80]. Oxidation of the probe leads to quenching of the fluorescent probe, and therefore, the fluorescence decreases over time. Antioxidants can delay the oxidation of the probe by reacting with the peroxyl radicals until the antioxidant activity is consumed by hydrogen

transfer. The ORAC was determined in SPHs and SPH fractions from UF as described by Dávalos et al. (2004) [80].

An 88 nM fluorescein sodium salt stock solution in 75 mM phosphate buffer (PB) (Na_2HPO_4, pH 7.4) was prepared and diluted to a final concentration of 55 nM in the well upon analysis. A 153 mM AAPH solution in 75 mM PB was prepared daily and kept on ice during the experiment. Trolox (97 %) was used as standard with concentrations between 6.25-100 μM (Appendix H). At last, the SPH were dissolved to the concentration of 1 % in PB and diluted to 1:200 with PB.

An amount of 125 μL of the diluted FL solution was added to the inner wells of a 96 well black microplate (NUNC) and then 25 μL of the standard, sample or PB for blank was added. Thereafter, the micro plate was incubated at 37 °C for 15 min before 50 μL of APPH was added. The fluorescence was measured kinetically every minute for one hour and 10 min with a TECAN, SPARK 20M at 37 °C. The plate was shaken for three seconds prior to each reading. The excitation wavelength was 485 nm, and the emission wavelength was 535 nm. The antioxidant capacity was calculated as area under the curve (AUC).

2.13 In vitro digestion

In vitro digestion of the SPH was conducted based on the method developed by Jensen et al. (2009) [81]. An amount of 1.25 g of SPH was dissolved in 5 mL dH_2O and then 25 mL dH_2O was added to the solution. Thereafter, the sample was adjusted to pH 2 (exact pH-value noted) and incubated at 37 °C until the solution was 37 °C. To the solution, a 0.5 mL pepsin solution (0.5 g pepsin (Sigma P6887) in 10 ml dH_2O) was added and then it was incubated at 37 °C at 110 rpm for two hours. The exact pH-value was noted before and after the adjusting of the sample to pH 6.5 with NaOH. Then, 1 mL trypsin-chymotrypsin solution (1.25 g trypsin (Sigma 93615) and 0.25 g chymotrypsin (Sigma C4129) in 10 mL dH_2O) was added and incubated at 37 °C at 110 rpm for 2.5 hours. The exact pH was noted after the incubation and then the samples were frozen directly at -80 °C. At last, the samples were freeze dried (Labconco FreeZone 12), frozen at -20 °C, and analysed for free amino acids, the degree of hydrolysis and antioxidant activity.

2.14 Statistical analyses

Results were presented as average $\pm$ standard deviation of 3 parallels unless otherwise stated. For standard calculations, Excel 2016 (Microsoft, USA) was used. For determination of significant difference, IBM SPSS Statistics 26 with a significant level of 5 % was used. The samples were assumed to be independent and normal distributed; the errors were assumed to be independent and random, and the variances were assumed to be equal. Independent t-test was used to analyse significant different for molecular weight distribution and *in vitro* GI digestion results. Tukey's test was applied on DH, protein solubility, EC, ES, FAA, TAA, and on antioxidant assays. Pearson correlation coefficient (r) was used to investigate correlation between amino acid composition and antioxidant properties.

3 Results and discussion

3.1 Degree of hydrolysis

The DH was determined by formol titration and reflects the fraction of peptide bonds cleaved during hydrolysis. The DH for SPH varied from 17.5 % to 18 %, where BOII and BJI had the lowest DH and BJII had the highest (Table 3.1). All SPHs were produced under the same reaction conditions. In comparison, hydrolysates from saithe heads and cod heads produced with the same conditions had a DH of respectively 14.8-18.1 % and 11 % [39, 82]. The variations of DH under the same conditions can be a result of individual difference between the raw materials regarding enzyme, fat, ash, and protein content. A raw material with e.g., lower protein content and higher enzyme content will result in a higher DH than opposite. Thus, it is important with several parallels to even out the individual differences between the raw materials.

Table 3.1: The degree of hydrolysis (DH) of hydrolysates (I and II) of backbone (B) of saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean $\pm$ SD, n=2).

	BOI	**BOII**	**BJI**	**BJII**
DH [%]	17.9 ± 0.1	17.5 ± 0.1	17.5 ± 0.1	18.0 ± 0.3

3.2 Molecular weight distribution

The MW distribution was analysed for characterization of the size of peptides and for investigating the degree of separation for UF1 (Section 3.4). The MW distribution of SPHs and <4 kDa fraction from UF1 were analysed by Innolipid AS with HPLC (Figure 3.1). For all MWs a significant difference ($p \leq 0.05$) in peptides sizes between hydrolysates and <4 kDa fraction was observed. Peptides with MW 2-1 kDa were most abundant in both the SPHs and in the <4 kDa fraction. Further, it is a clear distinction between the SPHs and the <4 kDa, where the SPHs have a higher content of larger peptides, and the <4 kDa fraction have a higher content of smaller peptides. The significant difference in the amount of peptides for all MWs imply that UF1 was able to separate peptides into fractions based on size.

A lot of research has focused on the relationship between peptide sizes and bioactivity. The ideal size of peptides for antioxidant activity, has however, not been found, and the preferred size varies with antioxidant mechanisms and surrounding conditions. However, several studies have reported that low MW (1-5 kDa) have higher antioxidant activity [53, 57, 60, 61]. It was expected a higher concentration of peptides with MW <4 kDa in the permeate compared to the SPHs, but the permeate had a higher concentration of peptides with a MW $\leq$1 kDa. Based on literature, it can therefore be expected that the SPHs will have higher antioxidant activity than the permeate, although higher antioxidant activity has also been reported in fractions with peptide sizes below 1 kDa [53].

The degree of hydrolysis reflects the fraction of peptide bonds cleaved during hydrolysis and thus, affects the MW distribution. It was no significant ($p > 0.05$) difference in the

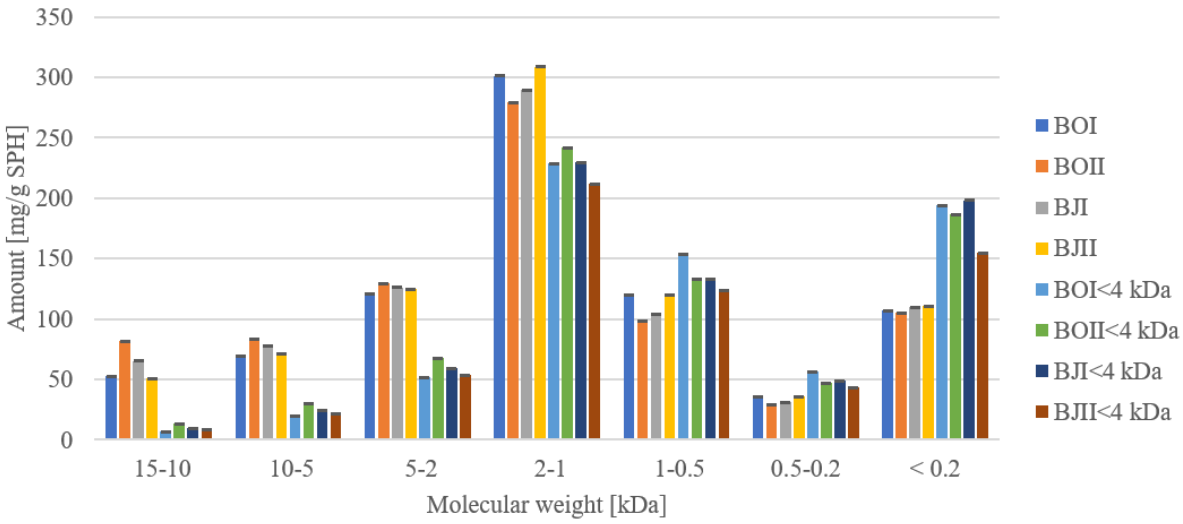

Figure 3.1: The molecular weight distribution (kDa) of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) and in <4 kDa fraction from ultrafiltration 1 (mean ± SD, n=2).

DH between the samples, which is confirmed by the similarities in the MW distribution between the SPHs.

3.3 Functional properties

Functional properties of FPH depends on, amongst other factors, DH, solubility, pH, and ionic strength [10]. For the discussion of protein solubility, water holding capacity, and emulsifying properties, pH and ash content were measured (Table 3.2). The pH of the SPHs was measured to be between 6.6-6.8, and the ash content was measured to be between 1.1-5.4 %. It must, however, be emphasized that variations in the ash content were large and thus, the results are inaccurate. This can be a result of inaccuracies of the method for samples with a low ash content or because of heterogeneous material. In another thesis, it was found an ash content of 1.3-1-9 % in raw, baked, and boiled saithe fillets [83]. Hence, it can be suspected that the ash content is low in the SPHs. The ash content in saithe backbone could, however, be higher because of minerals from the backbone.

Table 3.2: pH-measurements and ash content of dry matter (DM) of hydrolysates (I and II) of backbone (B) of saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean±SD, n=3).

Samples	pH	Ash content [% of DM]
BOI	6.6 ±0.0	1.1 ±1.8
BOII	6.7 ±0.0	-
BJI	6.7 ±0.0	5.3 ±1.8
BJII	6.8 ±0.0	5.4 ±0.3

3.3.1 Solubility

A high protein solubility is an advantage for good functional properties, and the protein solubility of the SPHs was determined using the Lowry method (Table 3.3, Appendix A). The solubility of all the SPHs was around 100 % ($p>0.05$). The reduced peptide sizes, increases the amount of ionizable groups, and the exposure of hydrophobic groups, thus increasing the solubility [23]. The high solubility is therefore consistent with that the SPHs had the most peptides in the size range of 2-1 kDa. Further, the solubility can change with pH because proteins have different ionized state depending on the pH [14]. At their iso-electric point, the proteins possess their lowest solubility. The Lowry method is conducted under basic conditions, and hence, the measured solubility is affected by the pH of the media. Contradictory to this, Pacheco-Aguilar et al. (2008) [84] and Gbogouri et al. (2004) [85] showed respectively that Pacific whiting hydrolysates and salmon by products hydrolysates had almost 100 % solubility over a wide pH range. The solubility of the SPHs was only measured under basic conditions, but the dissolved SPHs had a pH around 6.7. Thus, the solubility can be lower than measured.

Table 3.3: Protein solubility of hydrolysates (I and II) of backbone (B) of saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean $\pm$ SD, n=3 except BOI where n=2).

	BOI	**BOII**	**BJI**	**BJII**
Protein solubility [%]	99.9 ± 0.5	101.2 ± 0.5	99.7 ± 3.4	109.2 ± 1.4

The Lowry method is commonly used for protein solubility determination for FPH, because the hydrolysate fraction represents the water-soluble fraction from the hydrolysis. The higher solubility in BJII (109.2 %) is consistent with the higher protein content measured with the Kjeldahl method (96.3 %). In the specialization project, the protein content was found to be between 94.1-96.3 % [1]. The overestimated protein solubility with the Lowry method is likely to be due to the Folin-Ciocalteu reagent that also reacts with other compounds such as unsaturated fatty acids, inorganic ions, and metal complexes [86]. It is noteworthy to mention that the results from the Kjeldahl method may also be overestimated. The commonly used conversion factor assumes that the nitrogen content in food proteins is 16 % [87], however, other nitrogen sources in fish will also falsely be calculated as protein resulting in a higher estimated protein content. In the specialization project [1], the lipid content was measured to be between 1-5 % (with large variations). Hence, the ash and lipid content can react with Folin-Ciocalteu reagent and contribute to an overestimation of the protein solubility.

3.3.2 Water holding capacity

An increased WHC will contribute with a juicier texture of the food product. The WHC of SPHs added to minced cod muscle was determined by a method based on centrifugation (Table 3.4, Appendix B). Generally, the addition of SPHs to the minced cod muscle resulted in an increased WHC, however, the results were contradictory. While the addition of 1 % SPH to the minced cod muscle resulted in a significantly ($p\leq0.05$) increased WHC (3.1-5.2 %), the addition of 3 % SPH resulted in a significantly ($p\leq0.05$) decreased WHC for minced cod muscle added with BOII (5.8 %). In comparison, Šližytė

et al. (2009) [27] showed that the addition of 1 % fresh cod backbone hydrolysates to minced cod muscle increased the WHC with 16 %. Interestingly, Roland (2021) [88] added 3 % of different chicken protein hydrolysates to minced cod muscle, where some of the hydrolysates resulted in decreased WHC as in this project. Even though the WHC decreased after addition of 3 % SPH, the water loss after centrifugation was the same or less after the addition, except for 3 % BOII (Table B.1 in Appendix B). The experimental analysis is based on the water content of the minced cod muscle with added SPH, and the difference in weight after centrifugation is used for the calculation of WHC. The water content decreased with the increasing addition of the freeze-dried SPHs. Thus, the addition of dry matter can be the main effect for reduced water loss rather than the effect of WHC. This may explain why 1-2 % addition of SPH increased the WHC, while 3 % addition of SPHs decreased the WHC.

Table 3.4: Difference in water holding capacity (ΔWHC) after adding different concentrations (conc.) of hydrolysates (I and II) of backbone of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) to minced muscle (mean ± SD, n=4, except BOI 1 % where n=3).

		ΔWHC [%]			
WHC cod [%]	**SPH conc.**	**BOI**	**BOII**	**BJI**	**BJII**
63.6 ± 0.5	1 %	3.1 ± 0.3*	3.4 ± 0.7*	4.6 ± 0.6*	5.2 ± 0.8*
56.3 ± 0.6	2 %	3.4 ± 0.4	2.4 ± 1.1	1 ± 0.8	1.1± 0.9
61.4 ± 0.8	3 %	-2.0 ± 0.8	-5.8 ± 1.2*	-3.1 ± 0.3	-1.1 ± 1.3

*Denotes a significant ($p \leq 0.05$) difference between minced cod muscle and minced cod muscle with added SPH.

Water holding capacity depends on the molecular size of the peptides and solubility [10, 25]. The MW distribution showed that most of the peptides were 2-1 kDa large. An increased number of small peptides increases the number of free amino- and carboxyl groups that can bind to water and increase WHC compared to the native protein. They will, however, have less capacity to entrap water due to their small size. Šližytė et al. (2009) [27] showed that FPH added to minced cod muscle increased the WHC, but the WHC decreased with increased hydrolysis time where the DH went from 21.4 % to 24.3%. They showed that after a 25- and 45-minutes hydrolyses, the most abundant peptide sizes were 80 kDa to 1 kDa, while in their 60 min hydrolysis, the most abundant peptide sizes were <1 kDa. Also, a high solubility, like in the SPHs, decreases the WHC [25]. This might imply that a less extensively hydrolysed SPH would have greater WHC. The ash content may also affect the WHC, because the ash may contain ions that can interact with the free amino- and carboxyl groups. Consequently, the SPHs could have less interactions with water, and the WHC would be reduced. At last, pH can also affect the WHC of the cod muscle and the SPHs. As mentioned previously, proteins have different ionized state depending on the pH and are electrically neutral at their iso-electric point. The pH-measurements showed that the SPHs had an average pH of 6.7, which is the same pH as cod muscle [89], and therefore, the SPHs did not likely affect the pH of the cod mince. However, peptides are known to increase the protein-protein interaction at their iso-electric point due to higher hydrophobicity and therefore decrease protein-water

interaction. It could therefore be an advantage to adjust the pH of the cod muscle mince and the SPHs.

Small hydrophobic peptides (<6 kDa) can give FPH a bitter taste, and the MW distribution showed that most of the peptides were 2-1 kDa large. The SPHs were produced with an enzyme mixture of bromelain and papain that have shown to reduce bitter peptides [15]. Nevertheless, it is important to consider the bitterness of the SPHs as future work if addition of SPHs to e.g., fish cakes is going to be used. A low concentration of SPHs added to minced cod showed the best increase in WHC, and thus, the low concentration of SPHs can be an advantage for the taste of the food product.

3.3.3 Emulsifying properties

Peptides are amphiphilic molecules and can therefore act as emulsifiers that stabilize emulsions. The emulsifying properties of the SPHs were determined by homogenisation of rapeseed oil, water, and SPHs (Table 3.5). In general, the EC increased with increasing protein concentration (significantly $p \leq 0.05$), while the ES was higher at low protein concentrations. The hydrolysate BJI, exhibited a significantly ($p \leq 0.05$) higher EC (26.7-33-6 mL/g SPH) at all SPHs concentration, and at 2-3 % SPHs concentration, BJII exhibited a significantly ($p \leq 0.05$) lower EC (2.5-4.1 mL/g SPH) compared to the other hydrolysates. The highest, significant ($p \leq 0.05$) stability was shown in 2 % BJII (100 %), and 3 % BOII had the lowest ES (17.5 %) compared to the other hydrolysates. The correlation between protein concentration and EC has been confirmed previously [54, 90]. In a previous study, cod hydrolysate with DH of 21.4-24.3 %, exhibited an average EC of 81 mL/g of FPH and an average ES of 95 % [27]. In comparison the SPHs investigated in this study showed lower EC and ES. This is however, higher compared to the average EC of hen protein hydrolysate (ranging from 0.1 mL/g protein powder to 5.25 mL/g protein powder) [90].

Emulsifying properties depend on the sizes of the peptides and solubility [10]. Most of the peptides were 2-1 kDa large, but the peptides ranged from 15 kDa to below 0.2 kDa. One study showed that protein hydrolysate from defatted skipjack roe had the best emulsion ability index (EAI) at 5 % DH [54] when the DH ranged from 5-50 %. In the same study, it was also shown a weaker EAI as the DH increased. The same correlation between DH and EC has been confirmed in other studies [91, 92]. The small peptides in the SPHs are most likely too hydrophilic, which can also be confirmed from the high solubility of the SPHs. A high solubility is an advantage for rapid diffusion to the interface between the oil and water. However, if the peptides are not amphiphilic, most of the peptides will be in the water phase and thus, the poor EC. An increasing DH has also been confirmed to decrease ES [25, 85, 92]. The smaller peptides cannot unfold and reorient at the interface like the larger peptides [85]. Hence, to improve emulsifying properties of saithe backbone hydrolysates, a less extensive hydrolysis might be required.

Table 3.5: Emulsification capacity (EC) expressed as mL of emulsified oil per g of saithe protein hydrolysate (SPH), and emulsification stability (ES) as percentage of initial emulsion after 24 hours at room temperature and centrifugation (mean ± SD, n=2). Hydrolysates (I and II) were produced from backbone of saithe caught in October (BOI and BOII) and in January (BJI and BJII).

SPH concentration	Sample	EC [mL/g SPH]	ES [%]
1 %	BOI	5.0 ± 2.5^{a1}	66.7 ± 33.3^{a1}
	BOII	3.7 ± 1.3^{a1}	75.0 ± 25.0^{a1}
	BJI	27.3 ± 0.6^{b1}	18.2 ± 0.0^{a1}
	BJII	2.5 ± 0.0^{a1}	75.0 ± 25.0^{a1}
2 %	BOI	10.6 ± 1.8^{a12}	24.3 ± 4.3^{a1}
	BOII	13.8 ± 1.3^{a2}	22.5 ± 2.5^{a1}
	BJI	33.6 ± 0.2^{b2}	22.2 ± 0.0^{a1}
	BJII	2.5 ± 0.0^{c1}	100.0 ± 0.0^{b1}
3 %	BOI	16.5 ± 0.2^{a2}	15.0 ± 0.0^{a1}
	BOII	16.5 ± 0.2^{a2}	17.5 ± 2.5^{a1}
	BJI	26.7 ± 0.5^{b1}	52.5 ± 10.0^{a1}
	BJII	4.1 ± 0.0^{c2}	30.0 ± 10.0^{a1}

Different letters (a-c) within the same column and SPH concentration indicate significant differences ($p \leq 0.05$) between the hydrolysates. Different numbers (1-2) within the same column indicate significant difference ($p \leq 0.05$) between the different concentrations of the same hydrolysate.

The hydrolysates BJI and BJII, showed a large difference in EC. As the pH, protein solubility, and MW distribution is not significantly different between the hydrolysates, the observed difference can be a coincidence. It may also be inaccuracy when conducting the method of measuring the EC. The method is easy to perform, but the samples were hand-homogenized and visually inspected, and no instrument was used for measuring the formed emulsion.

3.3.4 Colour measurement

A white colour on the hydrolysates is more appealing to consumers than a dark colour [10]. Therefore, the colour is important to evaluate if hydrolysates from white fish have the potential to be used as a functional food ingredient or as a nutraceutical. The colour was evaluated by measuring lightness (L*), redness (a*), and yellowness (b*) (Table 3.6), and the whiteness was calculated (equation 2.4). It was no significant difference ($p > 0.05$) in colour between the SPHs. The lightness component ranges from 0 to 100, where 100 is representing the brightest white. As the SPHs have a lightness around 90, the appearance of the SPHs is a bright colour (Figure 2.2). A negative redness component will have an appearance against green, and a positive yellowness will have an appearance against yellow. In total, the SPHs got a high whiteness around 80. Comparable, another study that obtained hydrolysates from cut cod backbones, had a lightness of 85.8, a redness of -0.5, and a yellowness of 19.6 after 60 minutes enzymatic hydrolysis [27].

The whiteness of the hydrolysate from cut cod backbone was calculated to be 75.8, and the appearance was a light-yellow colour. Thus, the hydrolysates investigated in this study had an appealing colour like white fish hydrolysates from other studies.

Table 3.6: Colour measurement of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3). Whiteness is based on calculations (Equation 2.4).

SPH	Lightness (L*)	Redness (a*)	Yellowness (b*)	Whiteness
BOI	91.77 ± 0.12	-0.67 ± 0.03	16.09 ± 0.09	81.91 ± 0.07
BOII	91.74 ± 0.07	-0.90 ± 0.03	16.29 ± 0.10	81.71 ± 0.06
BJI	89.93 ± 0.15	-0.87 ± 0.02	18.26 ± 0.14	79.13 ± 0.14
BJII	89.51 ± 0.05	-1.09 ± 0.13	15.25 ± 0.08	81.46 ± 0.09

3.4 Ultrafiltration

Studies have reported that UF can increase antioxidant activity in FPH by concentrating bioactive peptides [38, 93, 94]. The UFs, namely UF1 and UF2, resulted in five fractions: >150 kDa, 150-4 kDa, 150-2 kDa, <4 kDa, and <2 kDa. The smaller peptides are expected to be concentrated in the permeates (<4 kDa, <2 kDa), and the larger peptides are expected to be concentrated in the retentates (>150 kDa, 150-4 kDa, 150-2 kDa). The SPHs and the fractions were analysed for amino acid composition and antioxidant activity. The SPHs and the <4 KDa fraction were also analysed for MW distribution. The relationship between structural properties and antioxidant activity will be discussed (Section 3.4.2). The dry matter content of the different fractions from UF2 was analysed, but not presented (Appendix C).

Membrane filtration is today widely used because of its simplicity, but UF separation of proteins is a complex process [95]. Besides, the membrane with a given MWCO value is only an indicative value, and poor selectivity is a common problem [95]. The separation of the peptides with UF1 was not as sharp as desired since the permeate contained peptides with MW sizes above 4 kDa. Separation with UF depends on several factors such as pressure, membrane material, and physicochemical conditions. For UF, it is common to use 2-10 bars as pressure, and in UF1/UF2 5 bars was used. A higher pressure will be more efficient due to higher permeate flux, but it can also increase the risk of forcing higher MW peptides through the membrane. Thus, the pressure can be one reason for peptides with higher MW in the permeate than the MWCO value. However, a study of UF of FPH with a 4 kDa MWCO membrane made of modified polyether sulfone showed that the retention of high MW peptides increased with the pressure (10-30 bar) [96]. This could be because the high pressure alters the apparent MWCO to be lower. Hence, it can explain the high concentration of peptides with a MW of 1-0.5 kDa. Further, membrane fouling is a common problem for UF. In the specialization project [1], the loss of SPH was 0.6-1.2 g after UF1, while in UF2 the loss of SPH was 1.1-1.4 g (Table C.1 in Appendix C). The loss of SPH could be due to materials being left in the system, but also due to membrane fouling. In both UFs, a ceramic membrane was used followed by a hydrophilic polyether sulfone membrane with different MWCOs. The hydrophilic membrane for protein separation is often used to decrease the risk of

membrane fouling. Also, the physicochemical conditions affect the protein layer on the surface of the membrane [19]. The SPHs had a pH of 6.6-6.8, and at the iso-electric point, which are often around pH 7, the layer will be more packed and harder to permeate [19]. The MW distribution was not analysed for UF2, but one can expect from the MW distribution of UF1, that it will contain peptides with higher MWs than 2 kDa. A study showed that multilayer UF resulted in a higher selectivity, specifically on small-sized peptides [95]. Therefore, an interesting further work would be to ultra-filtrate SPHs with multilayer UF.

3.4.1 Amino acid content

The TAA and FAA composition in the SPHs and in the UFs fractions were analysed using HPLC. The amino acids Gly and Arg were not possible to separate with the column and are therefore presented as one value. Further, only 17 of 20 amino acids were detected with HPLC. The used standard (Sigma AAS18) for HPLC did not contain Trp, and acid hydrolysis also leads to the destruction of the Trp structure [97]. Therefore, Trp was not detected. Further, Pro was not detected because the used method (o-phtalaldehyde (OPA) pre-column derivatization) for HPLC and the detector cannot detect Pro. Cysteine are unstable during acid hydrolysis, and can be converted to relatives [98], and thus, not detected with HPLC.

The crude SPHs were analysed for TAA and FAA in the specialization project [1]. If sufficient material, the fractions were analysed for TAA and FAA. Calculations and detailed amino acid composition can be found in Appendix D and E.

Free amino acid content

The FAA content in SPHs and the UF fractions was determined with HPLC (Table 3.7). The $<$2 kDa fraction had a significantly ($p \leq 0.05$) higher content of FAA (86.8-157.2 mg FAA/g), and the retentates had a lower FAA content (22.3-36.5 mg FAA/g) compared to the SPHs (47.0-60.1 mg FAA/g). The retentates from BJII had a significantly lower ($p \leq 0.05$) content compared to the crude SPHs. In another study, the FAA content of cod by-products hydrolysates was 48 mg/g FPH [99], which resembles the same level as the SPHs. Stensen (2019) [38], however, found a lower FAA content of 6.37-37.12 mg FAA/g FPH. In contrast to what was observed in this study, Stensen (2019) [38] found that the highest amount of FAA was in crude, and not in the permeates as in this study. Monslaup (2018) [39] reported the same, but most likely they were not representative because of HPLC errors. However, it is expected a higher amount of FAA in the permeates since they can pass through the membrane due to their small size. Sabeena Farvin et al. (2016) [62], who fractionated cod protein hydrolysates, also found a higher content of FAA in the smallest fraction $<$3 kDa.

In general, the most abundant amino acids were Asp, Ala, Leu, Lys, Gly/Arg, and Glu in SPHs and the fractions (Table D.1-D.5 in Appendix D). It was, however, some exceptions like fraction $<$2 kDa had a high amount of serine (Ser) in BOI, glutamine (Gln) in BOII, and Val in BJII. Also, the Glu amount was generally higher in the retentates. The least abundant amino acids in SPHs and the fractions were asparagine (Asn) and His. The high abundance of Leu, Lys, Glu, Ala, Gly/Arg have also been reported by several other

Table 3.7: Free amino acid content (mg/g) in saithe protein hydrolysate (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa) and UF2 (150-2 kDa, <2 kDa) of hydrolysates (I and II) of backbone (B) from saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=2-3).

Fraction	**BOI** [mg FAA/g]	**BOII** [mg FAA/g]	**BJI** [mg FAA/g]	**BJII*** [mg FAA/g]
SPH	51.5 ± 0.8^{a12}	47.0 ± 1.5^{a1}	58.9 ± 3.5^{a2}	60.1 ± 1.0^{a2}
>150 kDa	25.9 ± 0.5^{a1}	26.8 ± 0.7^{a1}	31.2 ± 1.6^{a12}	35.7 ± 1.9^{b2}
150-4 kDa	33.0 ± 3.4^{a12}	22.3 ± 3.6^{a1}	36.5 ± 0.2^{a2}	31.5 ± 1.7^{b12}
150-2 kDa	25.48 ± 0.5^{a1}	25.3 ± 0.3^{a1}	32.8 ± 0.0^{a2}	26.7 ± 0.7^{b1}
<2 kDa	157.2 ± 5.0^{b1}	86.8 ± 12.2^{b1}	130.6 ± 25.2^{b1}	146.4 ± 6.6^{c1}

Different letters (a-c) within the same column indicate significant differences ($p \leq 0.05$) between the fractions. Different numbers (1-2) within the same row indicate significant difference ($p \leq 0.05$) between the hydrolysates. *Only filtrated with a 2 kDa molecular weight cut-off membrane in UF2.

studies of FPH [38, 39, 62]. The amino acids Glu, Asp, and Arg are suspected to have reducing power that can contribute with antioxidant activity [62, 64]. Further, it has been shown that Gly and Lys can contribute with antioxidant activity [62, 66]. Thus, the abundance of these amino acids are advantageous for the antioxidant properties of the hydrolysates.

A positive correlation between DH and FAA content has previously been documented [38, 42]. In this study, the DH and the FAA content was not significantly ($p>0.05$) different between the hydrolysates, except BOI that differed from BJI/BJII, and no correlation between FAA and DH could be observed in this study.

Total amino acid content

The TAA content in SPHs and the UF fractions was determined by acid hydrolysis and then detection with HPLC (Figure 3.2). The >150 kDa and 150-2 kDa had a higher TAA content (755-951 mg/g), and significantly ($p \leq 0.05$) higher content for BOI and BOII, than the crude SPHs (532-680 mg/g) and the <4 kDa fraction (477-613 mg/g). The most abundant amino acids in all fractions were Asp, Glu, Gly/Arg, Ala, Leu and Lys (Table E.1-E.4 in Appendix E). Stensen (2019) [38] that studied cod head protein hydrolysates, found the same abundance of amino acids in addition to Ser. Further, Jensen et al. (2013) [100] also found the most abundant amino acids to be Glu, Asp, Ala, Leu, and Lys in cod. The least abundant amino acids were Asn and Gln in all fractions, but during complete hydrolysis, the amine group in Asn and Gln can be liberated [101]. Thus, Asp can be the sum of Asp and Asn, and Glu can be the sum of Gln and Glu. Further, Trp, Cys and Pro were not detected in this HPLC method, which means that in total 17/20 amino acids were detected. Previously, the protein content was found to be 94.1-96.3 %, by the Kjeldahl method, and the protein solubility was found to be around 100 %. Thus, a high TAA content was expected, but the crude SPHs and the <4 kDa fraction had a

TAA content of 477-680 mg/g SPH. The undetected amino acids and the applied method can be two reasons for underestimated TAA content. The method for measuring TAA content involves several steps where material can be lost during the procedure. Hence, the TAA analysis can underestimated the amino acid content, while Kjeldahl and Lowry can overestimate the protein content as discussed earlier.

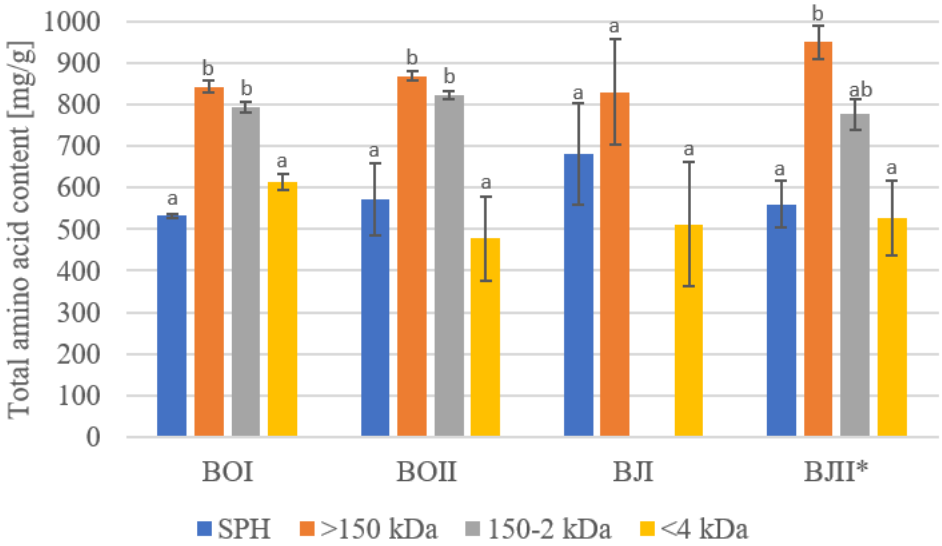

Figure 3.2: Total amino acid content (mg/g) in saithe protein hydrolysate (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, <4 kDa) and UF2 (150-2 kDa) of hydrolysates (I and II) of backbone (B) from saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=2-3). Different letters (a-b) indicate significant ($p \leq 0.05$) difference between the fractions within the hydrolysate. *Only filtrated with a 2 kDa molecular weight cut-off membrane in UF2.

From the TAA-analysis, the average essential (EAA), hydrophobic (HAA), and aromatic (AAA) amino acid proportion in the SPHs and UF's fractions were calculated (Table 3.8). The fraction <4 kDa had a significant ($p \leq 0.05$) higher proportion of HAA (37.8 %) and EAA (44.9 %). Hydrophobic amino acids have been shown to contribute with antioxidant activity [58] and thus, the <4 kDa fraction can contribute with better lipid-protein interaction and higher bioactivity. The 4 kDa membrane was made of hydrophilic polyether sulfone to reduce the risk of membrane fouling. Hence, hydrophobic peptides would permeate the membrane easier than hydrophilic peptides. Thus, the hydrophilic membrane can be one reason for a higher concentration of hydrophobic AA in the <4 kDa fraction. Another reason can be that the smaller peptides had a higher content of HAA.

The nutritional value of proteins depends on the content of EAA [102], and the SPHs and the fractions had a high proportion of EAA (32-45 %), and thus, a high nutritional value. It is also discussed that small MW peptides and HAA content contribute with an increased bioavailability [10, 103, 104]. Hence, the combination of increased bioavailability and a high nutritious value can give food products with added SPHs an increased nutritional value. The SPHs may also have the potential to be utilized as protein supplement in e.g., sports nutrition or in diet-related diseases. However, the potential and the bitterness of

the SPHs must be further investigated.

Table 3.8: Average essential (EAA), hydrophobic (HAA), and aromatic (AAA) amino acid proportion in saithe protein hydrolysate (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, <4 kDa) and UF2 (150-2 kDa) of hydrolysates (I and II) of backbone (B) from saithe caught in October (BOI and BOII) and in January (BJI and BJII).

SPH	EAA [%]	HAA [%]	AAA [%]
BOI	42	32	7
BOII	35	27	4
BJI	32	25	4
BJII	42	32	6
>150 kDa			
BOI	39	31	6
BOII	39	31	6
BJI	40	31	6
BJII	40	31	6
>2 kDa			
BOI	42	31	7
BOII	41	31	6
BJI	-	-	
BJII	41	32	5
<4 kDa			
BOI	44	36	8
BOII	45	38	7
BJI	45	40	6
BJII	45	38	7

EAA:His, Thr, Met, Val, Phe, Ile, Leu, Lys **HAA**:Ala, Tyr, Met, Val, Phe, Ile, Leu **AAA**: His, Tyr, Phe

3.4.2 Antioxidant activity

There is no standardized method to measure total antioxidant activity, and most methods analyse one specific mechanisms of action [52]. Thus, to best present the antioxidant activity of a sample, it is necessary to combine several antioxidant assays to measure different antioxidant mechanisms. In this study, the antioxidant activity was measured by ABTS, FRAP, and ORAC assays. The reference compounds used were PG and Trolox and the antioxidant activities were calculated as PG/Trolox equivalent antioxidant activity concentration per gram of SPH (μmol/g). The calculations of antioxidant activity and detailed information can be found in Appendix F-H. The SPHs and the <4 kDa fraction were analysed for antioxidant activity in the specialization project [1].

ABTS assay

The radical scavenging activity of the SPHs and the UF fractions from UF1 and UF2 was measured using the ABTS assay (Figure 3.3, Appendix F). The crude SPHs had the highest radical scavenging activity (59.8-65.8 μmol/g), and generally, with some exceptions, the 150-2 kDa, <4 kDa, and <2 kDa fractions showed significantly ($p \leq 0.05$) lower antioxidant activity (31.7-54.84 μmol/g) than the SPHs. The fraction with the lowest radical scavenging activity was <2 kDa (31.7-46.6 μmol/g). The hydrolysate, BJII, exhibited the highest radical scavenging activity in all fractions except in the >150 kDa and 150-2 kDa fraction.

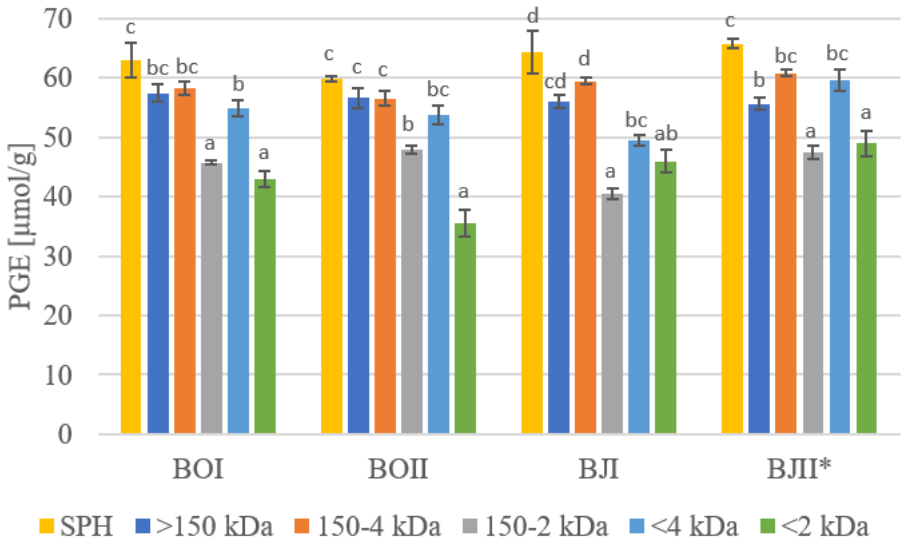

Figure 3.3: Antioxidant activity (µmol propyl gallate equivalent (PGE)/g) measured with ABTS assay in saithe protein hydrolysates (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa, <2 kDa) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II) (mean ± SD, n=3). Different letters (a-d) indicate significant ($p \leq 0.05$) difference between the fractions within the hydrolysate. *Only filtrated with a 2 kDa molecular weight cut-off membrane in UF2.

In another thesis, by Monslaup (2018) [39], cod head hydrolysates were produced under the same conditions as in this project and ultra-filtrated with a 4 kDa MWCO membrane. She also found that the crude hydrolysates exhibited the highest radical scavenging activity. On the other side, Monslaup (2018) found that the permeates had higher activity than the retentates, which is the opposite of what is found in this study. In another study, yellowfin tuna viscera were separated by UF into four size fractions (<3 kDa, 3-10, 10-30, and 30 kDa <) [93]. They found that the <3 kDa fraction had the highest scavenging activity measuring with ABTS [93]. The MW distribution showed that the SPHs had the highest concentration of peptides with MW size of 1-5 kDa, and that the <4 kDa had the highest concentration of peptides with MW size from 2 kDa to below 0.2 kDa. In Monslaup's thesis [39], the MW distribution of the crude FPH had most peptide sizes below 12.4 kDa, the permeates had majority of peptides sizes below 6.5 kDa, and the retentates have

most peptide sizes of 6.5-34.0 kDa. Therefore, by comparing the MW distribution, it can be observed that the <4 kDa fraction in this study had lower MW peptides compared to the permeate in Monslaup's thesis. The difference in peptide sizes between Monslaup's permeate and the permeate in this study, might explain why the retentates had higher radical scavenging activity than the permeate in this study. Comparable, a study of saithe head hydrolysates from the same project (OPTiMAT) [82], found no difference between the retentates and permeates in radical scavenging activity. Nevertheless, the crude hydrolysates from saithe heads had the highest ABTS radical scavenging activity (57.5-60.3 μmol/g) as this study and in Monslaup's thesis.

It is expected that HAA and AAA contribute with antioxidant activity [12]. However, it was found no significant ($p>0.05$) correlation between ABTS radical scavenging activity and the total amount of AAA (mg/g) (r=-0.36) and HAA (mg/g) (r=-0.39). In fact, the fraction <4 kDa had the highest content of HAA, but one of the lowest ABTS radical scavenging activity in this case.

The <2 kDa had a significant higher content of FAA, and a significant lower ABTS radical scavenging activity. Even so, it was found no significant ($p>0.05$) correlation (r=-0.36) between ABTS radical scavenging activity and FAA content. In comparison, another studied suggested both FAA and low MW peptides contributed antioxidant activity in cod protein hydrolysates [62]. Also, it was found no correlation between the ABTS radical scavenging activity and the amount of free AAA (r=-0.3) and the amount of free HAA (r=-0.32). Interestingly, it was a significant ($p\leq0.05$) correlation if the data for the <2 kDa was removed (which had a significant ($p\leq0.05$) higher FAA content) respectively r=0.7 and r=0.7 for free AAA and HAA. Stensen (2019) [38] reported that the amount of free AAA and ABTS radical scavenging activity had a significant ($p\leq0.05$) correlation (r=0.83). This might imply that the amount of free AAA can contribute with ABTS radical scavenging activity.

FRAP assay

The ferric reducing ability of the SPHs and the UF fractions from UF1 and UF2 was measured by using FRAP assay (Figure 3.4, Appendix G). The retentates had generally higher FRAP (11.7-16.7 μmol/g), and the permeates had generally lower FRAP (7.9-11.7 μmol/g) than the crude SPHs (11.4-13.5 μmol/g). The >150 kDa fraction had the highest FRAP, except for BJII, and it was significantly ($p\leq0.05$) different from the crude SPHs for BOI and BOII. The fraction <2 kDa had a significantly ($p\leq0.05$) lower FRAP activity than crude SPHs for hydrolysate BOI, BJI, and BJII. The hydrolysate, BJII, was only filtrated with a 2 kDa MWCO membrane in UF2 and thus explain the small difference (0.5 μmol/g) between fraction >150 kDa and 150-2 kDa.

In another master thesis from the same project (OPTiMAT) analysing saithe head hydrolysates [82], the highest FRAP activity was also found in the >150 kDa fraction (12.4-16.9 μmol/g). However, in that thesis, the <4 kDa was found to exhibit higher FRAP activity than the crude SPH. One reason for this difference could be the different protein content in protein hydrolysates from saithe head (87-91 %) compared to saithe backbone (94-96 %). However, still after comparing the SPHs on a protein basis, the SPHs from head showed lower FRAP activity than the SPHs from backbone, respectively 4.2-6.8 μmol/g

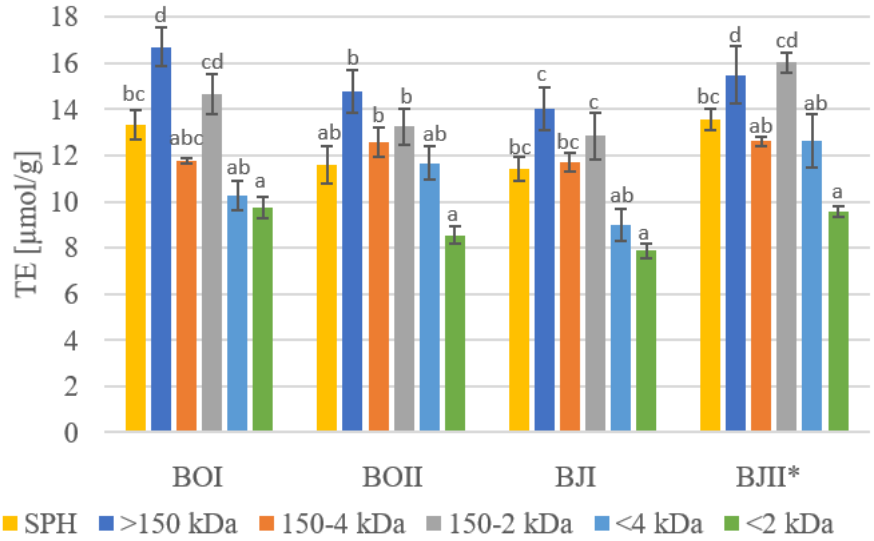

Figure 3.4: Antioxidant activity (µmol Trolox equivalent (TE)/g) measured with FRAP assay in saithe protein hydrolysate (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa, <2 kDa) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II) (mean ± SD, n=3). Different letters (a-d) indicate significant ($p \leq 0.05$) difference between the fractions within the hydrolysate. *Only filtrated with a 2 kDa molecular weight cut-off membrane in UF2.

protein and 11.4-14.68 μmol/g protein. Further, the SPHs from head and backbone had the same abundance of TAA, no significant ($p > 0.05$) difference in FAA, and apparently the same MW distributions. Nevertheless, the hydrolysates from saithe heads had significantly ($p \leq 0.05$) lower DH than backbone SPH, (14.8 % and 16.3 %, respectively) which is reflected in the MW distribution by having a lower content of 2-0.5 kDa peptides. Studies have shown that FRAP increases with the DH [105, 106] and thus, the difference in DH might explain the difference between the FRAP of the SPHs. However, the fraction >150 kDa, which have the highest FRAP in both theses, is assumed to have a higher content of peptides with a high MW which contradicts this assumption. A significant ($p \leq 0.05$) correlation ($r = -0.58$) between the FAA content (mg/g) and FRAP was found. Thus, the negative effect on FRAP with FAA content might explain why the retentates, which contain less FAA, have a higher FRAP. However, it is important to remember that the retentates with a low FAA content have larger peptides, and the permeates with a high FAA content have smaller peptides. Thus, this correlation can reflect the peptide sizes as well. Another explanation could be that the retentates were measured to have higher TAA content, and it was found a significant ($p \leq 0.05$) correlation ($r = 0.86$) between FRAP and TAA content (mg/g). Further, Arg, Tyr, Phe, Glu, and Asp rich peptides are suspected to have higher reducing power [62, 64]. The amino acids showed significantly ($p \leq 0.05$) correlations (Arg: $r = 0.72$, Tyr: $r = 0.55$, Phe: $r = 0.62$, Glu: $r = 0.88$, Asp: $r = 0.93$). However, it must be emphasized that the correlations may reflect the correlation found between FRAP values and the TAA content.

ORAC assay

The ORAC of the SPHs and the UF fractions from UF1 and UF2 was measured by using ORAC assay (Figure 3.5, Appendix H). In general, with some exceptions, the retentates exhibited a significantly ($p \leq 0.05$) lower ORAC (165-349 μg/mol) than the crude SPH (454-695 μg/mol) and the permeates (400-673 μg/mol). The <4 kDa fraction had the highest ORAC, except in BJII.

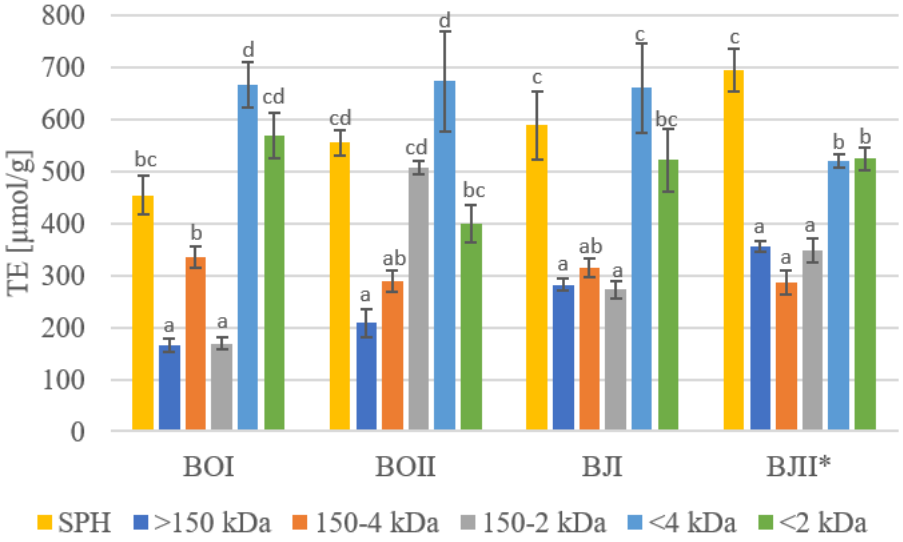

Figure 3.5: Antioxidant activity (µmol Trolox equivalent (TE)/g) measured with ORAC assay in saithe protein hydrolysate (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa, <2 kDa) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II) (mean ± SD, n=3 except for SPH and <2 where n=2). Different letters (a-d) indicate significant ($p \leq 0.05$) difference between the fractions within the hydrolysate. *Only filtrated with a 2 kDa molecular weight cut-off membrane in UF2.

Previous studies have shown a positive correlation between ORAC and HAA content [107, 108]. In this thesis, it was found a significant ($p \leq 0.05$), although negative correlation (r=-0.68) between ORAC and HAA content (mg/g). On the other hand, the <4 kDa fraction had generally a higher ORAC (except BJII) and a significantly ($p \leq 0.05$) higher proportion of HAA (36-40 %). In another master thesis from the same project (OPTiMAT), the same relationship between high ORAC and high content of HAA in the <4 kDa fraction was found [82]. Thus, HAA might contribute with higher ORAC. It must, however, be emphasized that it was no significant ($p > 0.05$) correlation (r=0.32) between ORAC and the HAA proportion for all fractions. Thus, the correlations based on the TAA content results (mg/g) may require further investigation.

The retentates are assumed to contain peptides with higher MW than the crude SPHs and the permeates. They showed lower ORAC than the crude SPHs and the permeates. It was on the other hand, no significant difference in HAA or AAA between the SPHs and the retentates. However, it was found a significant ($p < 0.05$) correlation (r=0.58) between

amount of FAA and ORAC. The positive correlation might imply that FAA can contribute with ORAC, but also that the fraction with smaller peptides contribute with ORAC. The <4 kDa fraction showed higher ORAC, but the <2 kDa fraction showed no significant difference or significant lower ORAC (BJII). The total HAA was not analysed for that fraction. So, the difference between the ORAC of the permeates can be because the <2 kDa contains smaller peptides or because of HAA content. In short, it might imply that peptides with lower MW or the synergistic effect of peptides with a wide MW distribution contribute more to ORAC than high MW peptides. In a study by Theodore et al. (2008) [109] of alkaline-aided channel Catfish protein isolates, they found that generally low MW peptides had high ORAC values.

3.4.3 Comparison of antioxidant activity analyses

There is no standardized method to measure total antioxidant activity, and most methods analyse one specific mechanism of action [52]. The ABTS assay is set up to measure the ability of a compound to scavenge free radicals with both ET and HAT mechanisms, while the FRAP assay measure the antioxidant ability to reduce Fe(III) to Fe(II) by ET [52]. The ORAC assay is a nonenzymatic chain-breaking antioxidant activity that measure the ORAC, and its ability to HAT [52]. The ORAC assay is a competitive method, while ABTS and FRAP are non-competitive methods. The advantage of ORAC is that the peptides are tested as antioxidant against physiologically important oxidants such as ROS, compared to ABTS and FRAP assays [52]. Hence, the ORAC assay can represent a more physiological relevant antioxidant action for the bioactive peptides. Further, the antioxidant activity depends on pH, solvent, substrate, and other reaction conditions [52]. It is discussed that most ET reactions occur at higher rate at higher pH. One reason for this is that phenolic compounds are not dissociated in acid, and the corresponding phenolates are oxidized more rapidly [52]. The FRAP assay is performed in acidic conditions to suppress Fe(III) hydrolysis, while the ABTS and ORAC assays are performed around neutral pH. Hence, the results from the FRAP assay can imply a lower ability to reduce, than the peptides would have under neutral pH. The differences between the reaction´s mechanisms, substrate, and reaction conditions reflect the importance to due several assays to see the whole picture of the peptides' antioxidant capacity. It also reflects why results from different antioxidant assays are difficult to compare.

To summarize, the results from the antioxidant activity measurements have shown that the SPHs can work as radical scavengers by reducing the free $ABTS^{+\bullet}$ radical by ET and HAT, that the SPHs have reducing ability of free radicals by ET, and that the SPHs have ORAC by HAT. Thus, the SPHs might contribute with reducing oxidative stress and lipid oxidation by ending free radical chain reactions.

Whether the fractions from the UFs showed the higher antioxidant activity or not was depending on the assay used. For the ABTS assay, the UFs did not improve the free radical scavenging activity which might imply that an additive effect of peptides with different sizes improved the radical scavenging activity. For the FRAP assay, the UFs improved the FRAP activity for retentates, where the removal of FAA and/or the high TAA content in the retentates might be one reason for the improvement. At last, the UFs improved the ORAC to some extent. The retentates had significant lower ORAC. Thus,

the crude SPHs and the permeates had the highest ORAC. The ORAC value might be affected by FAA content and HAA content.

3.5 In vitro digestion

An *In vitro* simulated GI digestion was performed on the SPHs to analyse the effect of further digestion on the antioxidant capacity of the bioactive peptides. After digestion, the SPHs were further analysed for DH, FAA, and for antioxidant activity (Table 3.9, Figure 3.6). The antioxidant activity results of the SPHs and the <2 kDa fraction are included in the figures for comparison.

All GI digested SPHs, except BJII, had a significantly ($p \leq 0.05$) higher DH (19.4-21.6 %), compared to the DH of undigested SPHs (17.5-18.0 %). Further, digested BJI had the highest FAA content (55.27 mg/g), and digested BOII had the lowest FAA content (20.2 mg/g). The relationship between DH and FAA was not as expected since increased DH should result in more FAA. Also, the digested SPHs had generally lower FAA content than the undigested SPHs when it was expected to be higher. However, only BOII had a significant ($p \leq 0.05$) lower FAA. One reason for the unexpected results can be the content of high MW enzymes.

Table 3.9: The degree of hydrolysis (DH) and free amino acid content (FAA) of *in vitro* simulated gastrointestinal digestion of hydrolysates (I and II) of backbone (B) of saithe caught in October (BOI and BOII) and in January (BJI and BJII) (mean $\pm$ SD, n=2 for DH and n=3 for FAA). It was assumed that the protein content in the enzymes added to the hydrolysates was 100 % (Equation 2.1 and 2.2).

	BOI	**BOII**	**BJI**	**BJII**
DH [%]	21.2 ± 0.2*	21.6 ± 0.0*	19.7 ± 0.4*	19.4 ± 0.4
FAA [mg/g]	39.9 ± 9.2	20.2 ± 0.3*	55.27 ± 1.2	32.8 ± 11.0

*Denotes a significant ($p \leq 0.05$) difference between GI digested SPHs and SPHs.

The human GI digestion of proteins can alter the properties of FPH during digestion because of proteases like pepsin, trypsin, and chymotrypsin. In a previous study by Jensen et al. (2009) [81], they performed *in vitro* GI digested of saithe muscle. The DH went from around 5 % to around 30 % after two hours of digestion. In another study, it was simulated digestion of cooked meats, where the DH increased from around 10 % to 80 % within 165 minutes [110]. Hence, a higher DH was expected after *in vitro* GI digestion of the SPHs. However, small peptides are less susceptible to GI digestion since some are too small to be substrates [103, 104]. Also, the peptides' ability to resist the proteases depends on the amino acid composition [111, 112], but no relationship has appeared in the literature. Nevertheless, it is suggested that peptides that contain Pro residues are more resistant to digestive enzymes [103, 112]. Proline was not detected in this project, but it has been shown in another study that saithe contained Pro [81]. Thus, two reasons for the unexpected small increased in DH can be because of the peptide sizes and because of the amino acid composition.

The effect of *in vitro* GI digestion of the SPHs on antioxidant capacity was measured with ABTS, FRAP, and ORAC assays (Figure 3.6). A significant ($p \leq 0.05$) decrease in radical scavenging activity measured with ABTS assay was observed in the digested SPHs (38.0-43.9 μmol/g) compared to the undigested SPHs (59.8-65.8 μmol/g). From the FRAP assay results, a similar significant ($p \leq 0.05$) decrease was observed for BOI (10.4 μmol/g compared to 13.3 μg/mol) and BJII (9.4 μmol/g compared to 13.5 μmol/g). In the ORAC assay, no significant ($p > 0.05$) difference between digested SPHs (481-645 μmol/g) and undigested SPHs (454-695 μmol/g) was observed. The previous results from the UFs can confirm the trends between digested and undigested SPHs because digested SPHs contain smaller peptides like the permeates from the UFs. The permeates from the UFs had lower radical scavenging activity measured with ABTS assay and lower FRAP activity. In the ORAC assay, 4/8 permeates were higher and 4/8 were lower in ORAC compared to the SPHs, but only 3/8 permeates were significantly ($p \leq 0.05$) different. Thus, the antioxidant activity results imply that in general, the SPHs will lose some of its antioxidant activity properties after digestion. Comparable, a study of Pacific hake FPH showed that digested FPH, 1-3 kDa, and <1 kDa fractions had a higher ABTS radical scavenging activity compared to undigested FPH and fractions [113].

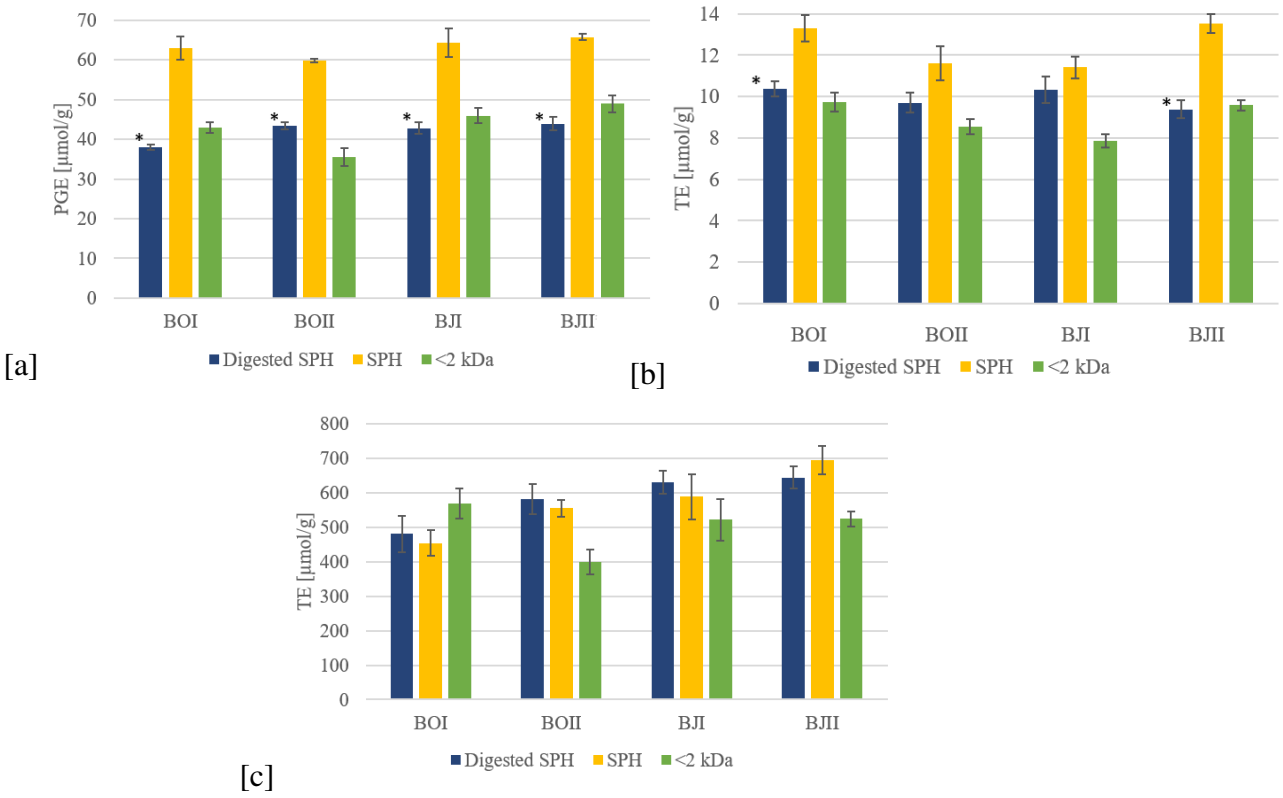

Figure 3.6: Antioxidant activity measured with ABTS assay (a), FRAP assay (b), and ORAC assay (c) in saithe protein hydrolysates (SPH), digested SPH, and in fraction <2 kDa of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II) (mean ± SD, n=2-3, except FRAP results of digested SPH where n=6). The results are presented as propyl gallate equivalent (PGE) or as Trolox equivalent (TE). *Denotes a significant ($p \leq 0.05$) difference between GI digested SPHs and SPHs.

From MW distribution of the SPHs, the peptides were ranging from 15 kDa to <0.2 kDa, which is equivalent to 136-2 amino acids in the sequence. The most abundant lengths of the peptides were 18-9 amino acids in the sequence. Small peptides, especially hydrophobic peptides, have increased probability to cross the intestinal barrier [103, 104]. A study of simulated Human adenocarcinoma colon cancer cell permeation of Pacific hake FPH showed that some peptides passed through and had ABTS radical scavenging activity [113]. Thus, the SPHs may have a higher probability to perform their antioxidant activity than the native proteins. Also, the <4 kDa fraction with the highest HAA content and smaller peptides would most likely have a higher probability to carry out their antioxidant properties *in vivo*. Hence, the SPHs could have a potential as a nutraceutical to reduce oxidative stress, but more *in vivo* studies are needed to confirm these simulations.

4 Conclusion

To investigate the potential for utilization of by-products from white fish, SPHs were produced by enzymatic hydrolysis of backbone from saithe. Functional properties and the effect of two different UFs (UF1 and UF2) and *in vitro* GI digestion on antioxidant properties in SPHs were explored. The SPHs and the UF fractions were analysed for amino acid content and antioxidant activity with ABTS, FRAP, and ORAC assays. To characterize the size of the peptides and to investigate the degree of separation for UF1, the SPHs and the <4 kDa fraction were analysed for MW distribution.

The SPHs consisted of different peptides sizes ranging from 15 kDa to <0.2 kDa, where most of the peptides had a size of 2-1 kDa. The abundance of small peptides was reflected in the high protein solubility of around 100 %. Further, the SPHs had a DH of around 18 %, and poor WHC, EC, and ES were found compared to other FPH. Too extensive hydrolysis can be one reason for the poor functional properties. The MW distribution showed that UF1 was able to separate peptides into fractions based on size, where the <4 kDa fraction contained a higher concentration of smaller peptides. The SPHs and the fractions were found to contain a high proportion of EAA, and the <4 kDa fraction had a significantly ($p \leq 0.05$) higher proportion of HAA.

The results from the antioxidant activity assays showed that the SPHs can work as radical scavengers by reducing the free ABTS+• radical, that the SPHs have reducing ability of free radicals, and that the SPHs have ORAC. The ABTS assay showed that the UFs did not improve the free radical scavenging activity and imply that an additive effect of peptides with different sizes improves radical scavenging activity. Free AAA might contribute to ABTS radical scavenging activity. The FRAP assay showed that the UFs improved the FRAP activity for retentates, where the removal of FAA and/or the high TAA content in the retentates might be a reason for the improvement. Lastly, the UFs improved the ORAC to some extent for the <4 kDa fraction, where the increased ORAC value for <4 kDa fraction might be because of a higher proportion of HAA. The ORAC might also be positively affected by FAA content and content of smaller peptides. In short, the results in this thesis indicate that UF is probably not necessary for concentrating bioactive peptides to increase antioxidant properties.

The *in vitro* GI digestion of the SPHs had a significantly ($p \leq 0.05$) increased DH (19-22 %), but a smaller increase than expected based on the original DH of the SPHs. Two reasons for the small increase in DH can be because the peptides were too small to be substrates for the enzymes or because of the amino acid composition. The GI digestion decreased ABTS and FRAP activities, and increased ORAC activity, except for BJII. In general, the *in vitro* GI digestion showed that the SPHs will lose some of its antioxidant activity properties after digestion. Thus, the SPHs have shown that hydrolysates of white fish RRM may have the potential as a nutraceutical to reduce oxidative stress, but more *in vivo* studies are needed to confirm these simulations. Therefore, to utilize white fish RRM, FPHs from white fish RRM most likely have a higher potential as a multi-functional food ingredient that can contribute with functional properties, a higher nutritious value, and antioxidant activity in food.

4.1 Future work

The functional properties of the SPHs were found to be poor. Changing the hydrolysis conditions to yield larger peptides could improve the properties. Further, the FPH from white fish RRM may have the potential to be used as a functional food ingredient, but more research on the functional and antioxidant properties of FPH should be done in food model systems. The bitterness of the SPHs should also be investigated.

The retentates from the UFs contain larger peptides and hence, it would be interesting to test the functional properties and to characterize the MWs in the retentates. More evaluations of antioxidant properties of FPH after digestion are also needed especially *in vivo* digestion studies.

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Appendix

A Solubility

A series of standard solutions with BSA were prepared and measured to determine the solubility of SPH with the Lowry method (Section 2.8.1). Standard curves were obtained by plotting the absorbance at 750 nm against the concentrations (μg/ml) of BSA to determine solubility. Further, the equation was obtained by linear regression in excel (Figure A.1).

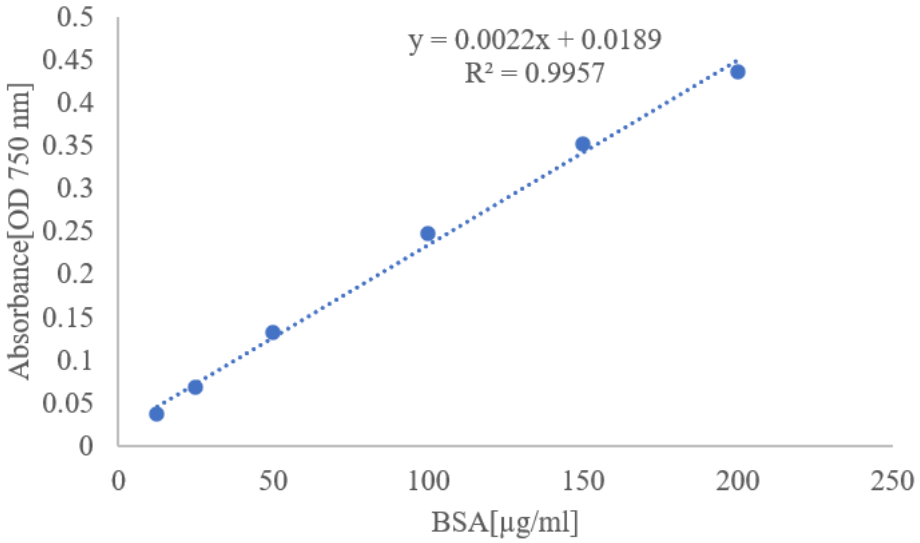


Figure A.1: Linear regression of standard curve of bovine serum albumin (BSA) concentration plotted against the absorbance at 750 nm. The equation given from linear regression was y=0.0022x+0.0189.

From the linear equation (Figure A.1) where y is the absorbance and x is the concentration (µg/ml) of BSA, the solubility of the SPHs was calculated. An example calculation of solubility of BOI SPH are shown in equation A.1 and A.2. In equation A.2, the average absorbance from Table A.1 is used to find the percentage of soluble proteins.

$$x = \frac{0.2400 - 0.0189}{0.0022} = 100.5\mu g/ml \tag{A.1}$$

Further, to find the percentage of soluble proteins in the SPH:

$$\frac{\text{Average conc.} * DF * \text{V(SPH)} * 100\%}{1000\frac{\mu g}{mg} * 1000\frac{mg}{g} * \text{m(SPH)}} = \frac{100.5\mu g/ml * 100 * 5mL * 100\%}{1000\frac{\mu g}{mg} * 1000\frac{mg}{g} * 0.0503g} = 99.9\% \tag{A.2}$$

The results from all SPH are shown in Table A.1.

Table A.1: Absorbance at 750 nm measured for all saithe protein hydrolysates (SPH) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II). Concentration and % soluble proteins are calculated from the equation from the standard curve in excel (mean ± SD, n=3 except BOI where n=2). (Figure A.1 and equation A.1 and A.2 for example calculations.)

SPH	Average absorbance	Protein[μg/ml]	Protein [%]
BOI	0.2400	100.500	99.90 ± 0.45
BOII	0.2447	102.621	101.20 ± 0.54
BJI	0.2400	100.500	99.70 ± 3.38
BJII	0.2640	111.409	109.22 ± 1.43

B Water holding capacity

Water holding capacity was determined by measuring weight loss after centrifugation (Δr) and the water content (V1) in the cod (Equation B.1). The water content in the minced cod muscle was decided to be 81.7 % but adjusted for the amount of SPH added to the cod. The respectively water contents for cod muscle with added 1 %, 2 %, and 3 % SPH concentrations were 79.6 %, 77.9 %, and 75.6 %. Three different WHC for pure cod muscle is used when it turned out that the same minced cod, but separated into different plastic bags, had different WHC. The average weight loss and WHC were calculated (Table B.1).

$$WHC(\%) = \frac{V1 - \Delta r}{V1} * 100\%, \tag{B.1}$$

Table B.1: The weight difference (Δr) after centrifugation and calculated water holding capacity (WHC) after adding hydrolysates of backbone of saithe caught in October (BOI and BOII) and in January (BJI and BJII) to minced cod muscle (mean ± SD, n=4, except BOI 1 % where n=3) (Equation B.1).

SPH conc.	Δr [%]	WHC [%]
Pure cod	29.7 ± 0.4	63.6 ± 0.5
BOI 1 %	26.5 ± 0.0	66.7 ± 0.3
BOII 1 %	26.2 ± 0.6	67.0 ± 0.7
BJI 1 %	25.3 ± 0.4	68.2 ± 0.6
BJII 1 %	24.9 ± 0.7	68.8 ± 0.9
Pure cod	35.7 ± 0.0	56.3 ± 0.6
BOI 2 %	32.9 ± 0.0	59.7 ± 0.4
BOII 2 %	33.7 ± 0.0	58.7 ± 1.1
BJI 2 %	34.9 ± 0.0	57.3 ± 0.8
BJII 2 %	34.8 ± 0.0	57.4 ± 0.9
Pure cod	31.5 ± 0.4	61.4 ± 0.8
BOI 3 %	30.7 ± 0.0	59.4 ± 0.9
BOII 3 %	33.6 ± 0.0	55.6 ± 1.2
BJI 3 %	31.5 ± 0.0	58.3 ± 0.3
BJII 3 %	30.0 ± 0.0	60.3 ± 1.3

C Ultrafiltration

The SPH were ultra-filtrated with a 150kDa cut-off membrane followed by a 2kDa cut-off membrane. Each filtration resulted in two fractions, namely retentate (>150 kDa and 150-2 kDa) and permeate (<150 kDa and <2 kDa), where the permeate from the first filtration (<150kDa) was filtrated with a cut-off of 2kDa as well. The amount of SPH in the four different fractions and the loss was calculated by using the measured volumes and DM (%) after UF of the SPH (Table C.1). An example of SPH in BOI >150 kDa is presented in Equation C.1.

$$\frac{1.2\%}{100\%} \cdot 71mL = 0.9g, \tag{C.1}$$

where it is assumed that 1 mL equals 1 g (Table C.2) Further, the loss was calculated by subtracting the amount of SPH dissolved (5 g) with the content in the retentate from both UFs (>150 kDa, 150-2 kDa) and with the permeate content after the last filtration (<2 kDa) (Equation C.2). An example calculation of SPH lost from filtration of 5 g BOI is presented in Equation C.3.

$$\text{SPH loss} = SPH- > 150kDa - (150 - 2kDa) - < 2kDa \tag{C.2}$$

$$\text{BOI SPH loss} = 5g - 0.9g - 2.3g - 0.4g = 1.4g \tag{C.3}$$

Table C.1: Volumes and dry matter (DM) in fractions from ultrafiltration 2 (>150 kDa, <150 kDa, 150-2 kDa, <2 kDa) with a cut-off of 150kDa and 2kDa of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII).

SPH	**Volume[mL]**			
	>150 kDa	<150 kDa	150-2 kDa	<2 kDa
BOI	71	446	73	397
BOII	64	449	67	403
BJI	65	450	70	402
BJII	-	-	90	447
	DM[%]			
BOI	1.2	0.8	3.2	0.1
BOII	1.5	0.7	3.3	0.1
BJI	1.8	0.7	2.9	0.1
BJII	-	-	3.8	0.1

Table C.2: Dry matter (DM) in fractions from ultrafiltration 2 (>150 kDa, <150 kDa, 150-2 kDa, <2 kDa) with a cut-off of 150kDa and 2kDa, and the total loss from the processing of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII).

SPH	**DM[g]**				
	>150 kDa	<150 kDa	150-2 kDa	<2 kDa	Loss
BOI	0.9	3.6	2.3	0.4	1.4
BOII	1.0	3.1	2.2	0.4	1.4
BJI	1.2	3.2	2.0	0.4	1.4
BJII	-	-	3.4	0.4	1.1

D Free amino acid content

To calculate the content of FAA in a sample, an excel sheet with raw data from HPLC analysis was given (raw data from BOI >150 kDa parallel 1: Figure D.1). From the excel sheet, the last column was used to calculate the FAA content (mg/g). An example calculation with BOI >150 kDa parallel 1:

$$BOI > 150_1, Asp = \frac{M_W(Asp) \cdot \frac{\mu mol}{L}}{1000} = \frac{133.10 \frac{g}{mol} \cdot 1.0697 \frac{\mu mol}{L}}{1000} = 0.14 \frac{mg}{L} \quad \text{(D.1)}$$

$$BOI > 150_1, Asp = \frac{0.14 \frac{mg}{L} \cdot 100 \cdot 0.00125 L^2 \cdot 0.001 L}{0.0104 g \cdot 0.001 L^2} = 2.10 \frac{mg}{g} \quad \text{(D.2)}$$

The sample did have precipitated protein after the first round, but the supernatant was used after the second round hence 0.00125 L^2 and 0.001 L^2. The calculated average results for all SPH and fractions are shown in Table D.1-D.4.

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l
1	Asp	1.595	2.019	18.386	8.65	11.99	1.0697
2	Glu	2.637	2.460	9.789	10.54	6.38	1.3279
3	Asn	3.650	0.034	0.220	0.15	0.14	0.0203
4	His	4.578	0.129	0.835	0.55	0.54	0.0952
5	Ser	4.973	0.637	3.947	2.73	2.57	0.4421
6	Gln	5.288	1.378	4.608	5.91	3.01	0.8900
7	Gly/Arg	8.303	3.687	10.541	15.80	6.88	1.2151
8	Thr	9.128	0.787	3.323	3.37	2.17	0.6221
9	Ala	13.607	2.780	16.467	11.91	10.74	2.1032
10	Tyr	14.817	0.539	4.171	2.31	2.72	0.3469
11	Aba	16.550	0.234	1.896	1.00	1.24	0.1337
12	Met	18.337	0.912	8.172	3.91	5.33	0.5135
13	Val	18.635	1.679	13.813	7.20	9.01	0.8838
14	Phe	19.103	0.592	5.795	2.54	3.78	0.3731
15	Ile	20.082	0.661	6.549	2.83	4.27	0.3430
16	Leu	20.422	2.649	24.785	11.35	16.17	1.5729
17	Lys	22.078	2.156	20.029	9.24	13.06	1.3826
Total:			**23.335**	**153.324**	**100.00**	**100.00**	

Figure D.1: Raw data of BOI >150 kDa parallel 1 from analysis of free amino acids with HPLC.

Table D.1: Free amino acids composition (mg/g SPH) of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII)(mean ± SD, n=3).

Amino acids	**BOI** [mg/g SPH]	**BOII** [mg/g SPH]	**BJI** [mg/g SPH]	**BJII** [mg/g SPH]
Asp	4.55 ± 0.07	4.98 ± 0.14	4.64 ± 0.07	4.62 ± 0.07
Glu	1.49 ± 0.30	1.82 ± 0.12	5.52 ± 0.11	5.39 ± 0.11
Asn	0.05 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
His	0.57 ± 0.06	0.41 ± 0.04	0.46 ± 0.02	0.34 ± 0.05
Ser	2.09 ± 0.08	1.67 ± 0.06	2.45 ± 0.04	2.42 ± 0.02
Gln	3.83 ± 0.08	3.49 ± 0.14	3.82 ± 0.05	3.68 ± 0.07
Gly/Arg	4.91 ± 0.09	4.24 ± 0.18	6.02 ± 0.11	5.79 ± 0.07
Thr	2.80 ± 0.06	2.50 ± 0.10	2.76 ± 0.07	2.66 ± 0.03
Ala	6.85 ± 0.22	6.13 ± 0.15	5.02 ± 2.51	7.31 ± 0.14
Tyr	2.36 ± 0.04	2.05 ± 0.06	2.69 ± 0.08	2.70 ± 0.03
Met	2.69 ± 0.02	2.35 ± 0.09	3.25 ± 0.07	3.19 ± 0.08
Val	3.10 ± 0.06	2.79 ± 0.10	3.73 ± 0.07	3.71 ± 0.08
Phe	2.07 ± 0.05	1.79 ± 0.06	2.46 ± 0.07	2.50 ± 0.04
IIe	1.74 ± 0.03	1.55 ± 0.06	2.09 ± 0.03	2.08 ± 0.04
Leu	6.88 ± 0.15	6.07 ± 0.21	8.06 ± 0.15	7.72 ± 0.12
Lys	5.56 ± 0.16	5.10 ± 0.17	5.90 ± 0.11	5.95 ± 0.08
Total	51.53 ± 0.84	46.96 ± 1.54	58.94 ± 3.52	60.10 ± 1.00

Table D.2: Free amino acid content (mg/g >150 kDa) of >150 kDa fraction from ultrafiltration 1 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3 except BJI and BJII where n=2).

Amino acids	**BOI** [mg/g >150 kDa]	**BOII** [mg/g >150 kDa]	**BJI** [mg/g >150 kDa]	**BJII** [mg/g >150 kDa]
Asp	2.17 ± 0.05	1.92 ± 0.06	2.34 ± 0.14	2.64 ± 0.08
Glu	2.95 ± 0.06	2.80 ± 0.07	2.86 ± 0.05	3.27 ± 0.07
Asn	0.03 ± 0.01	0.02 ± 0.00	0.05 ± 0.02	0.03 ± 0.00
His	0.22 ± 0.01	0.16 ± 0.06	0.27 ± 0.06	0.26 ± 0.04
Ser	0.71 ± 0.01	0.79 ± 0.05	1.50 ± 0.46	1.15 ± 0.12
Gln	1.97 ± 0.04	2.03 ± 0.12	1.98 ± 0.03	2.14 ± 0.03
Gly/Arg	2.30 ± 0.03	2.46 ± 0.09	3.20 ± 0.21	5.85 ± 2.61
Thr	1.13 ± 0.01	1.20 ± 0.07	1.19 ± 0.17	1.26 ± 0.05
Ala	2.94 ± 0.08	3.31 ± 0.04	3.58 ± 0.09	3.76 ± 0.09
Tyr	0.94 ± 0.03	0.98 ± 0.02	1.33 ± 0.03	1.37 ± 0.01
Met	1.12 ± 0.02	1.18 ± 0.05	1.45 ± 0.06	1.69 ± 0.02
Val	1.46 ± 0.06	1.53 ± 0.06	1.80 ± 0.09	2.04 ± 0.04
Phe	0.95 ± 0.06	1.02 ± 0.02	1.26 ± 0.05	1.37 ± 0.00
Ile	0.68 ± 0.02	0.72 ± 0.03	0.92 ± 0.08	1.03 ± 0.01
Leu	3.21 ± 0.07	3.37 ± 0.04	3.87 ± 0.07	4.20 ± 0.09
Lys	3.14 ± 0.08	3.40 ± 0.07	3.56 ± 0.11	3.66 ± 0.06
Total	25.91 ± 0.52	26.88 ± 0.71	31.16 ± 1.60	35.72 ± 1.93

Table D.3: Free amino acid content (mg/g 150-4 kDa) of 150-4 kDa fraction from ultrafiltration 1 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3).

Amino acids	**BOI** [mg/g 150-4 kDa]	**BOII** [mg/g 150-4 kDa]	**BJI** [mg/g 150-4 kDa]	**BJII** [mg/g 150-4 kDa]
Asp	1.98 ± 0.19	1.55 ± 0.10	2.78 ± 0.12	2.66 ± 0.04
Glu	2.52 ± 1.05	2.87 ± 0.11	4.19 ± 0.24	3.93 ± 0.11
Asn	0.06 ± 0.04	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
His	0.29 ± 0.06	0.19 ± 0.05	0.44 ± 0.03	0.31 ± 0.02
Ser	1.16 ± 0.15	0.84 ± 0.04	1.51 ± 0.06	1.39 ± 0.03
Gln	2.12 ± 0.85	2.11 ± 0.05	2.63 ± 0.17	2.47 ± 0.08
Gly/Arg	2.12 ± 0.85	1.52 ± 0.68	3.17 ± 0.11	1.60 ± 0.78
Thr	1.55 ± 0.04	0.87 ± 0.29	1.38 ± 0.03	0.68 ± 0.27
Ala	5.23 ± 0.18	2.82 ± 0.96	3.94 ± 1.28	2.60 ± 1.00
Tyr	1.52 ± 0.07	0.75 ± 0.30	1.26 ± 0.45	0.79 ± 0.38
Met	1.81 ± 0.08	0.90 ± 0.18	1.99 ± 0.14	1.82 ± 0.06
Val	2.24 ± 0.07	1.43 ± 0.06	2.46 ± 0.10	2.35 ± 0.04
Phe	1.58 ± 0.08	0.75 ± 0.27	1.84 ± 0.23	1.66 ± 0.08
Ile	1.09 ± 0.06	0.58 ± 0.19	0.99 ± 0.13	1.26 ± 0.05
Leu	4.14 ± 0.19	3.10 ± 0.17	4.33 ± 0.51	4.50 ± 0.17
Lys	3.60 ± 0.08	1.96 ± 0.79	3.64 ± 0.21	3.49 ± 0.24
Total	32.98 ± 3.37	22.28 ± 3.62	36.54 ± 0.25	31.53 ± 1.70

Table D.4: Free amino acid content (mg/g 150-2 kDa) of 150-2 kDa fraction from ultrafiltration 2 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3).

Amino acids	**BOI** [mg/g 150-2 kDa]	**BOII** [mg/g 150-2 kDa]	**BJI** [mg/g 150-2 kDa]	**BJII** [mg/g 150-2 kDa]
Asp	3.41 ± 0.05	3.49 ± 0.04	4.79 ± 0.04	3.93 ± 0.09
Glu	4.52 ± 0.04	4.87 ± 0.08	6.08 ± 0.01	4.94 ± 0.20
Asn	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
His	0.21 ± 0.03	0.16 ± 0.01	0.20 ± 0.01	0.14 ± 0.02
Ser	0.77 ± 0.17	0.70 ± 0.08	0.93 ± 0.00	0.75 ± 0.02
Gln	1.54 ± 0.06	1.56 ± 0.06	1.77± 0.04	1.39 ±0.03
Gly/Arg	1.96 ±0.05	1.84 ± 0.05	2.45 ± 0.00	2.20 ± 0.04
Thr	1.08 ± 0.03	1.10 ± 0.05	1.15 ± 0.01	0.91 ± 0.02
Ala	2.15 ± 0.05	2.09 ± 0.01	2.64 ± 0.07	2.05 ± 0.10
Tyr	0.80 ± 0.02	0.68 ± 0.04	1.02 ± 0.01	0.84 ± 0.03
Met	0.82 ± 0.01	0.78 ± 0.03	1.30 ± 0.03	0.88 ± 0.02
Val	1.20 ± 0.05	1.19 ± 0.02	1.77 ± 0.03	1.32 ± 0.04
Phe	0.72 ± 0.04	0.68 ± 0.01	0.98 ± 0.02	0.76 ± 0.03
Ile	0.63 ± 0.01	0.62 ± 0.01	0.86 ± 0.01	0.68 ± 0.01
Leu	2.70 ± 0.03	2.63 ± 0.06	3.58 ± 0.01	2.88 ± 0.06
Lys	2.94 ± 0.12	2.94 ± 0.02	3.26 ± 0.07	3.02 ± 0.08
Total	25.48 ± 0.50	25.34 ± 0.31	32.81 ± 0.08	26.71 ± 0.74

Table D.5: Free amino acid content (mg/g <2 kDa) of <2 kDa fraction from ultrafiltration 2 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3 except BOI where n=2).

Amino acids	**BOI** [mg/g <2 kDa]	**BOII** [mg/g <2 kDa]	**BJI** [mg/g <2 kDa]	**BJII** [mg/g <2 kDa]
Asp	5.11 ± 0.66	1.49 ± 0.32	3.82 ± 1.38	3.97 ± 1.13
Glu	4.14 ± 1.86	2.44 ± 0.55	3.99 ± 1.63	4.08 ± 1.35
Asn	0.06 ± 0.01	0.08 ± 0.04	0.11 ± 0.05	0.10 ± 0.04
His	1.55 ± 0.52	1.06 ± 0.17	1.22 ± 0.57	1.09 ± 0.41
Ser	7.54 ± 2.90	4.34 ± 1.33	8.21 ± 2.17	7.92 ± 2.89
Gln	14.89 ± 1.65	10.47 ± 3.75	5.30 ± 0.58	9.95 ± 3.47
Gly/Arg	13.48 ± 4.97	11.99 ± 2.26	16.22 ± 4.49	12.37 ± 4.31
Thr	7.17 ± 2.21	6.81 ± 0.95	8.23 ± 1.85	5.07 ± 1.60
Ala	26.88 ± 6.70	11.53 ± 2.31	22.66 ± 5.36	25.97 ± 5.61
Tyr	9.15 ± 0.44	2.68 ± 1.14	5.89 ± 1.53	6.71 ± 2.49
Met	9.70 ± 1.31	3.66 ± 1.34	7.00 ± 2.85	11.47 ± 0.98
Val	7.18 ± 5.25	6.08 ± 3.24	8.25 ± 3.37	12.94 ± 0.65
Phe	6.80 ± 2.73	3.02 ± 0.86	6.29 ± 2.57	10.53 ± 0.62
Ile	5.79 ± 0.32	3.18 ± 1.51	4.51 ± 1.35	4.72 ± 1.98
Leu	24.48 ± 3.40	12.79 ± 2.79	16.92 ± 3.63	19.69 ± 6.26
Lys	13.29 ± 0.44	5.15 ± 1.82	12.01 ± 2.66	9.76 ± 3.70
Total	157.21 ± 4.95	86.75 ± 12.40	130.61 ± 25.23	146.35 ± 6.70

Table D.6: Free amino acid content (mg/g digested SPH) of digested hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3 except BJI where n=2).

Amino acids	**BOI** [mg/g digested SPH]	**BOII** [mg/g digested SPH]	**BJI** [mg/g digested SPH]	**BJII** [mg/g digested SPH]
Asp	3.34 ± 0.87	2.78 ± 0.04	3.25 ± 0.26	2.29 ± 0.79
Glu	3.64 ± 1.36	3.88 ± 0.04	4.71 ± 0.25	3.47 ± 1.15
Asn	0.33 ± 0.23	0.02 ± 0.01	0.25 ± 0.00	0.15 ± 0.06
His	0.98 ± 0.60	0.13 ± 0.01	0.40 ± 0.05	0.41 ± 0.07
Ser	5.07 ± 4.57	0.56 ± 0.07	2.29 ± 0.10	1.60 ± 0.64
Gln	1.69 ± 0.88	1.24 ± 0.03	3.34 ± 0.11	2.25 ± 0.91
Gly/Arg	2.83 ± 0.35	1.46 ± 0.02	5.17 ± 0.19	3.58 ± 1.29
Thr	0.64 ± 0.23	0.88 ± 0.03	1.96 ± 0.09	1.39 ± 0.49
Ala	4.37 ± 1.65	1.67 ± 0.03	5.52 ± 0.04	3.77 ± 1.28
Tyr	2.68 ± 1.39	0.55 ± 0.04	3.36 ± 0.19	1.39 ± 0.58
Met	2.11 ± 1.03	0.62 ± 0.02	3.72 ± 0.31	1.63 ± 1.04
Val	1.73 ± 0.34	0.95 ± 0.01	2.74 ± 0.05	1.36 ± 0.78
Phe	1.91 ± 0.91	0.54 ± 0.01	3.83 ± 0.18	3.21 ± 0.22
Ile	0.71 ± 0.26	0.50 ± 0.00	1.30 ± 0.13	0.84 ± 0.38
Leu	3.40 ± 1.27	2.10 ± 0.01	6.39 ± 0.07	2.65 ± 1.77
Lys	4.41 ± 1.47	2.35 ± 0.06	7.04 ± 0.18	2.81 ± 1.50
Total	39.86 ± 9.17	20.22 ± 0.26	55.27 ± 1.18	32.80 ± 11.01

E Total amino acid content

To calculate the content of TAA in a sample, an excel sheet with raw data from HPLC analysis was given (raw data from BOI >150 kDa parallel 1 FAA analysis: Figure D.1). From the excel sheet, the last column was used to calculate the TAA content (mg/g). An example calculation is presented in equation E.1 and E.2 with BOII parallel 1. From the raw data excel sheet, the total Asp content in BOII parallel 1 was 5.5419 (μmol/L):

$$BOII_1, Asp = \frac{M_W(Asp) \cdot \frac{\mu mol}{L}}{1000} = \frac{133.10 \frac{g}{mol} \cdot 5.5419}{1000} = 0.7371 \frac{mg}{L} \tag{E.1}$$

$$BOII_1, Asp = \frac{0.7371 \frac{mg}{L} \cdot 10 \cdot 500 \cdot 0.01L}{0.046g} = 80.12 \frac{mg}{g} \tag{E.2}$$

The calculated average results for SPH and fractions are shown in Table E.1-E.4.

Table E.1: Total amino acid content (mg/g SPH) of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3 except for BOI where n=2).

Amino acids	**BOI** [mg/g SPH]	**BOII** [mg/g SPH]	**BJI** [mg/g SPH]	**BJII** [mg/g SPH]
Asp	64.8 ± 0.6	47.8 ± 8.5	81.5 ± 13.7	67.6 ± 6.6
Glu	90.4 ± 0.2	66.7 ± 13.1	121.3 ± 20.0	97.8 ± 10.0
Asn	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.2 ± 0.2
His	9.7 ± 0.3	4.0 ± 4.9	10.8 ± 0.9	8.7 ± 0.9
Ser	29.7 ± 1.1	20.6 ± 6.1	37.9 ± 7.4	32.7 ± 4.2
Gln	2.1 ± 0.9	0.9 ± 0.4	3.1 ± 1.9	1.7 ± 0.6
Gly/Arg	67.4 ± 0.7	49.2 ± 11.7	92.9 ± 21.7	71.7 ± 7.4
Thr	28.7 ± 0.3	21.1 ± 3.4	37.6 ± 8.2	29.6 ± 2.9
Ala	47.4 ± 0.1	35.3 ± 6.0	61.1 ± 13.12	48.7 ± 5.1
Tyr	7.4 ± 0.3	2.9 ± 4.2	3.9 ± 1.7	5.5 ± 0.7
Met	16.4 ± 0.1	10.5 ± 4.9	18.8 ± 1.65	17.1 ± 1.7
Val	27.7 ± 0.3	20.7 ± 2.8	33.9 ± 5.4	28.2 ± 2.7
Phe	17.7 ± 0.4	10.7 ± 4.9	21.9 ± 3.2	19.3 ± 1.9
Ile	21.0 ± 0.1	15.3 ± 2.6	26.0 ± 3.7	22.2 ± 2.1
Leu	41.8 ± 0.4	30.8 ± 5.5	51.7 ± 7.5	43.6 ± 4.1
Lys	60.0 ± 0.3	44.7 ± 8.2	77.7 ± 11.3	65.3 ± 6.1
Total	532.2 ± 4.5	572.1 ± 87.0	680.3 ± 121.3	598.8 ± 70.4

Table E.2: Total amino acid content (mg/g >150 kDa) of >150 kDa fraction from ultrafiltration 1 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3 except BJI and BJII where n=2).

Amino acids	BOI [mg/g >150 kDa]	BOII [mg/g >150 kDa]	BJI [mg/g >150 kDa]	BJII [mg/g >150 kDa]
Asp	107.8 ±2.0	109.7 ±1.2	106.8 ± 15.7	122.4 ± 5.3
Glu	179.4 ± 3.0	186.4 ± 2.2	182.0 ±62.7	205.7 ± 7.7
Asn	3.1 ± 0.4	2.7 ± 0.5	0.7 ± 0.5	2.6 ±2.5
His	13.5 ± 0.4	14.6 ±0.4	13.6 ± 4.9	16.3 ± 0.4
Ser	49.6 ± 1.0	50.6 ± 0.6	48.0 ± 16.5	50.8 ±1.3
Gln	0.8 ± 0.3	0.5 ± 0.2	1.1 ±0.4	4.8 ± 4.3
Gly/Arg	91.0 ± 2.3	93.8 ± 1.4	84.1 ± 28.8	98.7 ± 6.1
Thr	42.9 ± 1.6	42.6 ± 2.7	45.5 ±16.2	48.4 ± 0.2
Ala	71.9 ± 0.8	73.3 ± 1.4	68.1 ±23.6	77.3 ± 5.4
Tyr	10.1 ±0.3	11.4 ±0.6	8.5 ± 2.9	9.9 ± 0.4
Met	24.1 ±1.6	26.2 ± 0.5	24.4 ±8.4	28.5 ±0.6
Val	39.3 ± 0.8	41.1 ± 0.4	39.3 ± 13.5	45.0 ± 2.0
Phe	27.1 ± 0.5	27.6 ± 0.2	27.0 ±9.3	31.0 ± 1.1
Ile	32.8 ± 0.3	33.7 ±0.4	33.1 ± 11.3	38.5 ± 1.6
Leu	68.4 ± 1.8	70.2 ± 0.9	67.5 ±23.2	77.6 ± 3.6
Lys	81.1 ± 1.3	83.3 ±1.0	80.6 ± 27.9	93.0 ± 5.2
Total	842.7 ±15.0	867.4 ± 11.3	830.3 ± 126.1	950.6 ± 40.0

Table E.3: Total amino acid content (mg/g 150-2 kDa) 150-2 kDa fraction from ultrafiltration 2 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3).

Amino acids	BOI [mg/g 150-2 kDa]	BOII [mg/g 150-2 kDa]	BJI [mg/g 150-2 kDa]	BJII [mg/g 150-2 kDa]
Asp	97.5 ± 1.2	99.1 ± 1.1	-	100.7 ± 5.4
Glu	143.2 ± 2.8	147.0 ± 2.0	-	144.6 ± 7.6
Asn	0.1 ± 0.0	0.0 ±0.0	-	0.2 ± 0.2
His	16.6 ± 0.4	15.9 ± 0.4	-	10.6 ± 4.2
Ser	49.5 ±1.4	51.7 ± 0.9	-	47.2 ± 2.0
Gln	1.3 ± 0.4	1.4 ± 0.4	-	0.5 ± 0.2
Gly/Arg	96.3 ± 2.0	103.7 ± 1.0	-	93.1 ± 4.2
Thr	43.9 ± 2.4	44.6 ± 1.0	-	44.7 ± 2.3
Ala	67.4 ± 1.5	71.3 ± 1.0	-	66.9 ± 3.8
Tyr	9.4 ± 0.5	7.7 ± 3.1	-	5.7 ± 2.4
Met	22.3 ± 0.2	24.6 ± 0.2	-	21.9 ±0.5
Val	37.2 ± 0.4	38.3 ± 0.0	-	36.2 ± 2.9
Phe	27.5 ±0.3	27.5 ± 0.1	-	26.5 ± 1.5
Ile	28.2 ± 0.3	28.6 ±0.2	-	28.9 ± 2.1
Leu	65.1 ± 0.8	67.5 ± 1.0	-	64.7 ± 3.3
Lys	88.5 ± 1.1	92.5 ± 0.8	-	83.6 ± 4.2
Total	794.1 ± 13.6	821.3 ± 9.8	-	755.3 ± 51.2

Table E.4: Total amino acid content (mg/g <4 kDa) of <4 kDa fraction from ultrafiltration 1 of hydrolysates (I and II) of backbone (B) of saithe (SPH) caught in October (BOI and BOII) and in January (BJI and BJII) (mean ± SD, n=3).

Amino acids	**BOI** [mg/g <4 kDa]	**BOII** [mg/g <4 kDa]	**BJI** [mg/g <4 kDa]	**BJII** [mg/g <4 kDa]
Asp	53.8 ± 1.3	44.3 ± 8.7	49.9 ± 15.3	49.6 ± 8.8
Glu	93.9 ± 2.6	75.1 ± 16.5	77.2 ± 23.0	77.4 ± 13.4
Asn	2.8 ± 0.1	1.5 ± 0.0	2.2 ± 1.0	1.9 ± 0.8
His	12.3 ± 0.2	5.3 ± 3.9	3.5 ± 3.0	8.8 ± 0.3
Ser	36.4 ± 0.9	25.4 ± 6.8	27.0 ± 7.0	31.2 ± 5.1
Gln	1.3 ± 0.3	1.0 ± 0.7	0.2 ± 0.1	0.6 ± 0.1
Gly/Arg	76.7 ± 2.3	56.7 ± 14.2	60.0 ± 16.5	65.2 ± 11.2
Thr	32.6 ± 1.8	26.2 ± 6.0	27.4 ± 8.2	28.9 ± 4.9
Ala	65.2 ± 2.7	53.6 ± 10.6	61.9 ± 19.1	58.8 ± 11.1
Tyr	12.1 v 0.6	3.5 ± 2.4	3.1 ± 2.7	3.8 ± 1.9
Met	21.2 ± 0.7	13.0 ± 4.9	13.9 ± 4.0	18.0 ± 2.9
Val	33.0 ± 1.2	28.9 ± 4.3	33.6 ± 11.5	31.1 ± 5.8
Phe	23.5 ± 1.1	26.7 ± 5.4	22.6 ± 7.8	22.0 ± 4.0
Ile	22.8 ± 0.7	18.3 ± 3.3	21.4 ± 6.7	20.8 ± 4.1
Leu	53.5 ± 1.2	42.5 ± 8.2	48.5 ± 14.5	46.6 ± 8.2
Lys	72.0 ± 2.3	55.4 ± 12.6	59.2 ± 17.3	61.8 ± 10.9
Total	613.2 ± 18.3	477.4 ± 100.1	511.5 ± 150.2	526.5 ± 89.5

F ABTS assay

To determine antioxidant activity in the SPH, digested SPH and in the UF fractions with ABTS assay a series of standard solutions with PG were prepared and measured (2.12.1). The absorbances at 734 nm were plotted against the concentrations (μM) of PG to obtain the standard curves. Further, the equations were obtained by linear regression in excel (Table F.1).

Table F.1: Linear equations from standard curves of propyl gallate (PG) to determine antioxidant activity in saithe protein hydrolysates (SPH), in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH with ABTS assay. The absorbance is y and x is the PG concentration (μM).

Fraction	Linear equation
SPH	y=-0.0075x+0.6619
>150 kDa	y=-0.007x+0.6528
150-4 kDa	y=-0.0072x+0.6633
150-2 kDa	y=-0.0068x+0.6205
<4 kDa	y=-0.0068x+0.6188
<2 kDa	y=-0.0068x+0.622
Digested SPH	y=-0.0074x+0.6529

From the linear equations, the equivalent PG concentration of antioxidant activity in the SPH, digested SPH and UF fractions could be found. An example with average absorbance calculations with BOII SPH are shown in equation F.1 and F.2. In equation F.1, the average absorbance (Table F.2) is used to find the average concentration of PGE antioxidant activity.

$$x = \frac{0.4377 - 0.6619}{-0.0075} = 28.893\mu M \tag{F.1}$$

Further, equation F.2 was used to find equivalent μmol PG. For all samples, the dilution factor 20 is used and the sample concentration is 0.05 g sample/5 mL distilled water. The exact sample weight was used.

$$\frac{\frac{\text{Average concentration}\cdot\text{DF}}{1000mL/L}}{\text{BOII concentration}} = \frac{\frac{28.89\mu M\cdot 20}{1000mL/L}}{\frac{0.05g\text{BOII}}{5\text{mL}}} = 57.79\frac{\mu mol}{g} \tag{F.2}$$

The results from all samples are shown in Table F.2.

Table F.2: Absorbance at 734 nm measured for all saithe protein hydrolysates (SPH) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II), and for fractions from ultrafiltrations (UF) 1 (>150 kDa, 150-4 kDa,<4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH. Concentration and equivalent propyl gallate concentrations (PGE) found with ABTS assay are calculated from the equation from the standard curve in excel (mean ± SD, n=3). (Table F.1 and equation F.1 and F.2 for example calculations.)

SPH	**Average absorbance**	**PGE[μM]**	**PGE [μmol/g SPH]**
BOI	0.4355	31.50	63.00 ± 2.93
BOII	0.4377	29.90	59.80 ± 0.49
BJI	0.4330	32.16	64.33 ± 3.52
BJII	0.4153	32.88	65.75 ± 0.79
>150 kDa			
BOI	0.4520	28.69	57.37 ± 1.44
BOII	0.4537	28.45	56.56 ± 1.71
BJI	0.4570	27.97	55.94 ± 1.19
BJII	0.4457	29.59	55.62 ± 0.95
150-4 kDa			
BOI	0.4467	30.09	58.20 ± 1.20
BOII	0.4533	29.16	56.52 ± 1.25
BJI	0.4490	29.96	59.41± 0.55
BJII	0.4293	32.50	60.74 ± 0.57
150-2 kDa			
BOI	0.4647	22.92	45.74 ± 0.43
BOII	0.4533	24.58	47.92 ± 0.75
BJI	0.4790	20.81	40.48 ± 1.00
BJII	0.4537	24.53	47.46 ± 1.12
<4 kDa			
BOI	0.4323	27.42	54.84 ± 1.38
BOII	0.4360	26.88	53.76 ± 1.56
BJI	0.4507	24.73	49.45 ± 0.84
BJII	0.4163	29.77	59.55 ± 1.86
<2 kDa			
BOI	0.4740	21.76	43.01 ± 1.33
BOII	0.5133	15.98	31.71 ± 2.20
BJI	0.4713	22.16	43.28 ± 1.91
BJII	0.4617	23.58	46.60 ± 2.16
Digested SPH			
BOI	0.5110	19.18	38.01 ± 0.71
BOII	0.4883	22.24	43.39 ± 0.93
BJI	0.4923	21.70	42.80 ± 1.43
BJII	0.4900	22.01	43.94± 1.64

G FRAP assay

To determine antioxidant activity in the SPH, digested SPH and in the UF fractions with FRAP assay a series of standard solutions with Trolox were prepared and measured (Section 2.12.2). The absorbances at 593 nm were plotted against the concentrations (μM) of Trolox to obtain the standard curves. Further, the equations were obtained by linear regression in excel (Table G.1).

Table G.1: Linear equations from standard curves of Trolox to determine antioxidant activity in saithe protein hydrolysates, in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH with ABTS assay. The absorbance is y and x is the PG concentration (μM).

Fraction	Linear equation
SPH	y=0.0013x+0.0597
>150 kDa	y=0.0011x+0.0363
150-4 kDa	y=0.0011x+0.0363
150-2 kDa	y=0.0012x+0.038
<4 kDa	y=0.0013x+0.0597
<2 kDa	y=0.0012x+0.038
Digested SPH	y=0.0017x+0.0343

From the linear equations, the equivalent Trolox concentration of antioxidant activity in the SPH, digested SPH and UF fractions could be found. An example with average absorbance calculations with BOII SPH are shown in equation G.1 and G.2. In equation G.1, the average absorbance (Table G.2) is used to find the average concentration equivalent Trolox antioxidant activity:

$$x = \frac{0.2107 - 0.0597}{0.0013} = 116.15\mu M \tag{G.1}$$

Further, equation G.2 was used to find equivalent μmol Trolox. For all samples, the sample concentration is 0.05 g sample/5 mL distilled water. The exact sample weight was used.

$$\frac{\frac{\text{Average concentration}}{1000mL/L}}{\text{BOII concentration}} = \frac{\frac{116.15\mu M}{1000mL/L}}{\frac{0.05g\text{BOII}}{5\text{mL}}} = 11.62\frac{\mu mol}{g} \tag{G.2}$$

The results from all samples are shown in Table G.2.

Table G.2: Absorbance at 593 nm measured for all saithe protein hydrolysates (SPH) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II), and for fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa,<4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH. Concentration and equivalent Trolox (TE) concentrations found with FRAP assay are calculated from the equation from the standard curve in excel (mean ±SD, n=3 except digested SPH where n=6). (Table G.2 and equation G.1 and G.2 for example calculations.)

SPH	**Average absorbance**	**TE[μM]**	**TE [μmol/g SPH]**
BOI	0.2327	133.05	13.31 ± 0.63
BOII	0.2107	116.13	11.61± 0.81
BJI	0.2080	114.08	11.41 ± 0.51
BJII	0.2357	135.36	13.54 ± 0.47
>150 kDa			
BOI	0.2200	167.00	16.70 ± 0.85
BOII	0.2000	148.82	14.79± 0.93
BJI	0.1907	140.33	14.03 ± 0.92
BJII	0.2173	164.58	15.47 ± 1.24
150-4 kDa			
BOI	0.1700	121.55	11.75± 0.11
BOII	0.1790	129.73	12.57 ± 0.66
BJI	0.1653	117.30	11.71 ± 0.40
BJII	0.1847	134.88	12.61 ± 0.22
150-2 kDa			
BOI	0.2143	146.94	14.67 ± 0.86
BOII	0.2010	135.83	13.24 ± 0.77
BJI	0.1963	131.94	12.84 ± 1.02
BJII	0.2370	165.83	16.04 ± 0.43
<4 kDa			
BOI	0.1930	102.54	10.25 ± 0.63
BOII	0.2113	116.64	11.66 ± 0.72
BJI	0.1763	89.72	8.97 ± 0.68
BJII	0.2238	126.21	12.62 ± 1.16
<2 kDa			
BOI	0.1560	98.33	9.72 ± 0.46
BOII	0.1413	86.11	8.54 ± 0.38
BJI	0.1347	80.56	7.87 ± 0.32
BJII	0.1543	96.94	9.58 ± 0.24
Digested SPH			
BOI	0.2992	209.44	10.38 ± 0.37
BOII	0.2802	198.95	9.70 ± 0.49
BJI	0.2912	209.44	10.33 ± 0.66
BJII	0.2665	187.87	9.37 ± 0.44

H ORAC assay

To determine antioxidant activity in the SPH, digested SPH and in the fractions with ORAC assay a series of standard solutions with Trolox were prepared and measured (Section 2.12.3). The net AUC was plotted against the concentrations (μM) of Trolox to obtain the standard curves. The software SPARKCONTROL magellan V1.2 was used to calculate the AUC from the raw data curve of the standards and samples (e.g., Figure H.1). By subtracting the AUC with the AUC of the blank, the net AUC could be calculated for the standards and samples. Further, the equations were obtained by linear regression in excel (Table H.1).

Table H.1: Linear equations from standard curves of Trolox to determine antioxidant activity in saithe protein hydrolysates (SPH) and in fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH with ORAC assay. The net area under the curve is y and x is the Trolox concentration (μM).

Fraction	Linear equation
SPH	y=678891x+10^7
>150 kDa	y=806877x+10^7
150-4 kDa	y=806877x+10^7
150-2 kDa	y=979396x+5*10^6
<4 kDa	y=678891x+10^7
<2 kDa	y=979396x+5*10^6
Digested SPH	y=683904+10^7

From the linear equations, the equivalent Trolox concentration of antioxidant activity in the SPH, digested SPH and UF fractions could be found. An example with average absorbance calculations with BOII SPH are shown in equation H.1 and H.2. In equation H.1, the average net AUC (Table H.2) is used to find the average concentration of TE antioxidant activity:

$$x = \frac{28841667 - 10^7}{678819} = 27.757\mu M \tag{H.1}$$

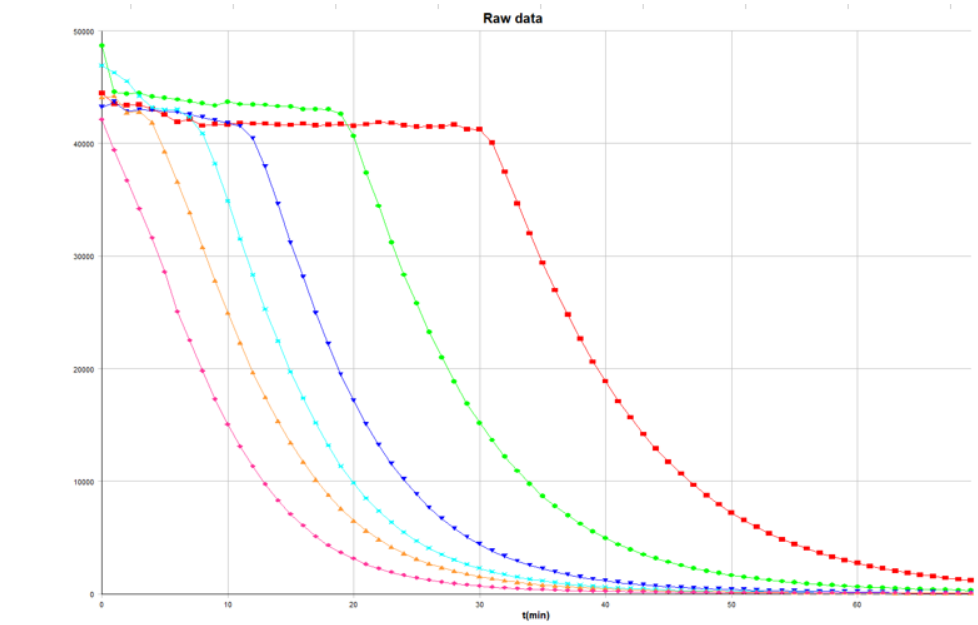
Further, equation G.2 was used to find equivalent μmol Trolox. For all samples, the dilution factor 200 is used, and the sample concentration is 0.05 g sample/5 mL PB. The exact sample weight was used.

$$\frac{\frac{\text{Average concentration} \cdot DF}{1000mL/L}}{\text{BOII concentration}} = \frac{\frac{27.757\mu M \cdot 200}{1000mL/L}}{\frac{0.05g\text{BOII}}{5\text{mL}}} = 555\frac{\mu mol}{g} \tag{H.2}$$

The results from all samples are shown in Table H.2.

Table H.2: Fluorescence with excitation wavelengths of 485 nm and with emission wavelengths of 535 nm was measured for all saithe protein hydrolysates (SPH) of backbone (B) from saithe caught in October (O) and in January (J), hydrolysis 1 (I) and 2 (II), and for fractions from ultrafiltration (UF) 1 (>150 kDa, 150-4 kDa, <4 kDa) and UF2 (150-2 kDa,<2 kDa), and in digested SPH. Concentration and equivalent Trolox (TE) concentrations are calculated from the equation from the standard curve in excel (mean ± SD, n=3 except for SPHs and < 4 kDa where n=2) (Table H.1 and equation H.1 and H.2 for example calculations).

SPH	**Net AUC**	**TE [μM]**	**TE [μmol/g SPH]**
BOI	25415667	22.707	454 ± 38
BOII	28841667	27.754	555 ± 24
BJI	29998167	29.457	589 ± 65
BJII	33581667	34.736	695± 41
> 150 kDa			
BOI	17041333	8.727	165 ± 13
BOII	18745333	10.838	208 ± 27
BJI	22079667	14.971	282 ± 13
BJII	25202667	18.841	355± 10
150-4 kDa			
BOI	23491667	16.721	334 ± 21
BOII	22349333	15.305	289 ± 21
BJI	23735333	17.023	315 ± 18
BJII	22227667	15.154	286± 23
150-2 kDa			
BOI	13465333	8.643	169 ± 11
BOII	30319667	25.852	507 ± 13
BJI	19155667	14.453	273 ± 17
BJII	22438000	17.805	349± 23
< 4 kDa			
BOI	32665667	33.386	668 ± 44
BOII	32855667	33.666	673 ± 96
BJI	32457667	33.080	662 ± 86
BJII	27672667	26.032	521± 13
< 2 kDa			
BOI	35120000	30.754	570 ± 45
BOII	24579000	19.991	400 ± 36
BJI	31588333	27.148	522 ± 61
BJII	31226333	26.778	525± 22
Digested SPH			
BOI	26424667	24.02	481 ± 53
BOII	29869333	29.05	582 ± 43
BJI	32551667	32.97	631 ± 34
BJII	31960667	32.11	645± 31



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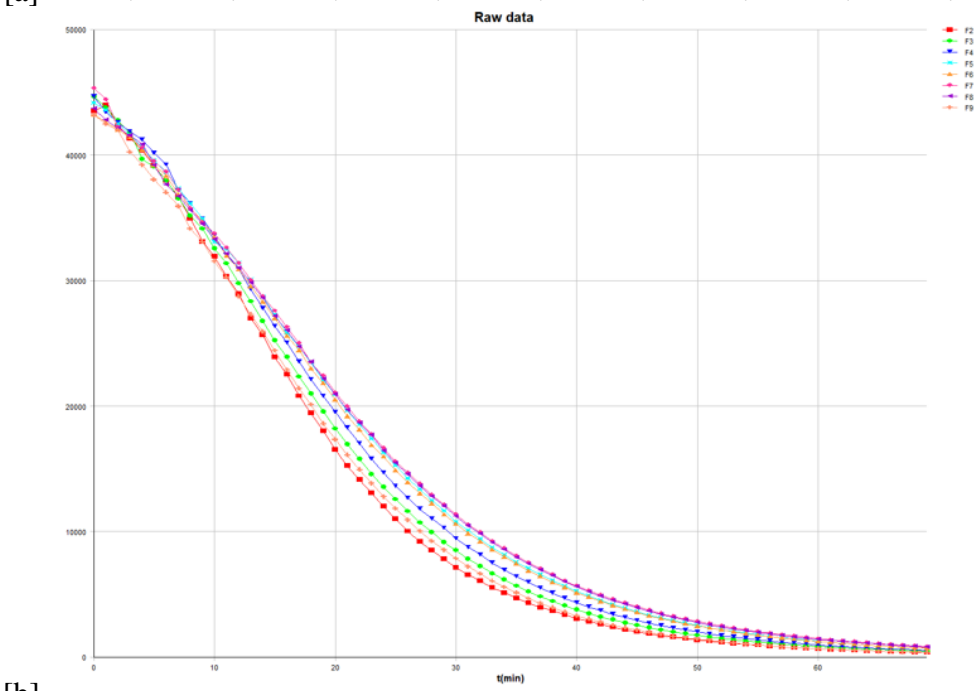
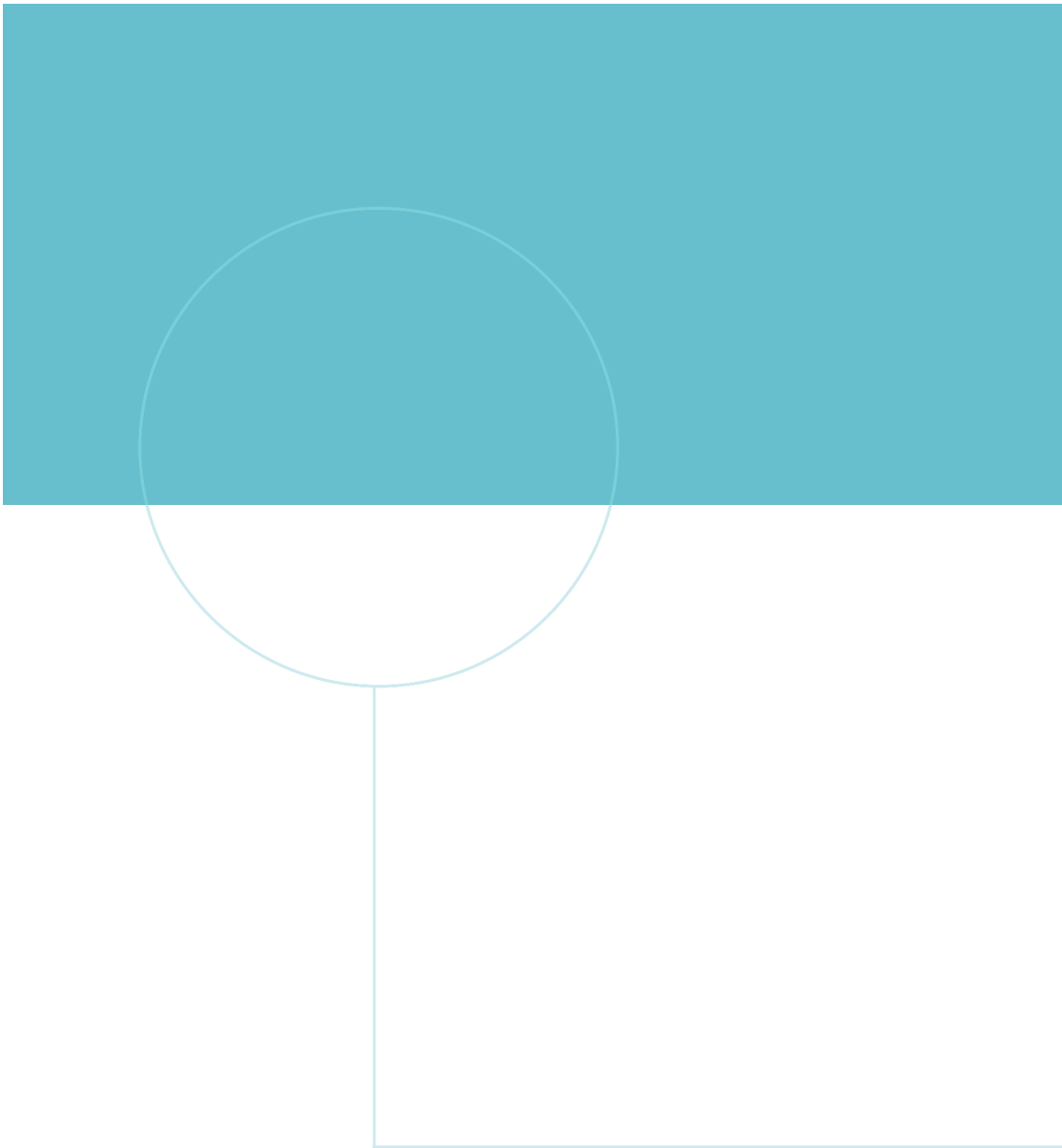


[b]

Figure H.1: Raw data curves of the standards of Trolox and samples obtained from SPARKCONTROL magellan V1.2 software. The y-axis represents the fluorescence measured and the x-axis is in time(minutes). In Figure (a): The raw data curves for Trolox standards. In Figure (b): The raw data curves for the samples. The figure was also presented in the specialization project [1].



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