Changes in the antioxidative capacity of saithe (Pollachius virens) and shrimp (Pandalus borealis) press juice and muscle during a simulated gastrointestinal digestion.

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Sammendrag

Reaktive oksygenforbindelser (ROS) brukes ofte som en fellesbetegnelse for frie radikaler og reaktive oksygenforbindelser. Disse produseres kontinuerlig i kroppen. Kroppen har differensierte systemer for å beskytte seg mot skader fra slike forbindelser; antioksidanter. Dersom forholdet mellom ROS og antioksidanter blir høyt, kan det oppstå en tilstand som kalles oksidativt stress. ROS kan reagere med lipider, proteiner og DNA, og dette er satt i sammenheng med sykdommer som kreft, depresjon og åreforkalkning.

Fram til i dag har helseeffekten av sjømat i stor grad vært tillagt de lange, umettede fettsyrene. De senere år er også andre bioaktive komponenter i sjømat blitt viet større oppmerksomhet. Frigjøring av peptider med antioksidativ kapasitet, under fordøyelse av proteiner, er temaet for dette arbeidet.

Målet med denne oppgaven var å studere betydningen av fordøyelsen for den antioksidative kapasiteten til muskel fra sei og reke, samt den vannløselige fraksjonen i disse. Dette ble gjort ved å bruke en modell av mage- og tarmkanalen, målt ved metodene ORAC (oxygen radical absorbance capacity), FRAP (ferric reducing ability of plasma) og ABTS (2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay. Innledende studier av effekten av den vannløselige fraksjonen fra sei på LDL-oksidasjon ble også gjennomført.

Gjennom fordøyelsen på cirka tre timer, økte den antioksidative kapasiteten fram til fasen som simulerte tynntarmen, etter cirka 75 minutter. Målt ved ORAC, var den totale økningen i antioksidativ kapasitet i prøvene etter fullført fordøyelse mellom 3 og 12 ganger. FRAP viste en lignende utvikling i antioksidativ kapasitet gjennom fordøyelsen, som målt ved ORAC, men med signifikant lavere verdier. Resultatene av ABTS-målingene var inkonsekvente og hadde store standardavvik. Innholdet av protein i prøvene korrelerte med den antioksidative kapasiteten målt ved både ORAC og FRAP. Prøver av den vannløselige fraksjonen fra sei, tatt 30 og 75 minutter etter påbegynt fordøyelse, viste hemming av LDL oksidasjon. Denne var dog ikke signifikant.

Resultatene målt ved ORAC samsvarte med tidligere publikasjoner. ABTS viste seg ikke å være en egnet metode for å måle antioksidativ kapasitet av proteiner, peptider og aminosyrer i denne modellen hvor pH varierer. ABTS krever derfor videre utvikling før den kan benyttes til slike målinger. Den antioksidative kapasiteten til fordøyd muskel fra sei og reke viste seg å være omtrent 10 ganger høyere enn den antioksidative kapasiteten til den vannløselige fraksjonen.

Summary

Reactive oxygen species (ROS) are free radicals and non radical oxygen species, produced constantly in the body. The body has differentiated systems to minimize damages from these ROS, namely antioxidants. When the balance between radicals/pro-oxidants and antioxidants shifts in favour of the former, a state called oxidative stress occurs. The oxidants can react with lipids, proteins and DNA and these reactions are linked to diseases like cancer, depression and atherosclerosis.

Up until recently, the health aspects of seafood have primarily been linked to the long chained polyunsaturated fatty acids. In the later years, however, other bioactive compounds in seafood have been devoted more attention. Exposure of peptides with antioxidative capacity, after digestion of proteins, has been the topic for this work.

The aim of this thesis was to study the impact of digestion on the antioxidative capacity of saithe and shrimp muscle and their water soluble fractions (press juice). This was evaluated with three methods; ORAC (oxygen radical absorbance capacity), FRAP (ferric reducing ability of plasma) and ABTS (2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay. Preliminary studies on the capacity of press juice of saithe to inhibit LDL oxidation were also performed.

During the gastrointestinal digestion, approximately three hours, the antioxidative capacity increased up until the phase simulating the small intestine, after approximately 75 minutes. The total increase after complete digestion of the samples was between 3- and 12-fold measured by ORAC. The FRAP assay showed a similar trend in the development of the antioxidative capacity as the ORAC assay, only with significantly lower values. The results obtained from the ABTS assay were inconclusive and had high standard deviations. The protein content measured in the samples correlated well with the antioxidative capacity measured both with ORAC and FRAP. The samples of press juice of saithe, collected after 30 and 75 minutes of digestion, showed an inhibition of LDL oxidation. The results were however not significant.

The results obtained from the ORAC assay were in accordance with previous publications. The ABTS assay did not prove to be an adequate method to measure antioxidative capacity in proteins, peptides and amino acids in this model with pH variations. ABTS assay therefore needs further development before used for these measurements. The muscle from seafood exhibited approximately ten-fold more antioxidative capacity compared to the press juice.

Abbreviations commonly used

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensine-converting-enzyme
ALA	α-linolenic acid
AOC	Antioxidative capacity
CVD	Cardio vascular diseases
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ET	Electron transfer
FRAP	Ferric reducing ability of plasma
GI	Gastrointestinal
HAT	Hydrogen atom transfer
LA	Linolic acid
LDL	Low-density lipoprotein
LMPJ	Light muscle press juice of saithe
LMS	Light muscle of saithe
ORAC	Oxygen radical absorbance capacity
PJ	Press juice
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SPJ	Press juice of shrimp
SWM	Whole muscle of shrimp
TE	Trolox equivalents
WMPJ	Whole muscle press juice of saithe
WMS	Whole muscle of saithe

Content

1. Introduction

During recent decades, public concern about health and food as a health promoter, has increased. It is now common knowledge that a balanced diet can reduce the risk of lifestyle diseases such as obesity and diabetes, as well as cardiovascular diseases (CVD). Seafood, due to its adequate composition of essential amino acids, is an excellent source of protein (Friedman, 1996). In addition, seafood is also a beneficial source of vitamin A, D and B_{12} together with selenium and iodine (Lie *et al.*, 1994). The health aspects of seafood consumption have, since the discovery of the low incidence of coronary vascular disease in Greenland Eskimos (Dyerberg *et al.*, 1978), primarily been linked to the marine polyunsaturated fatty acids (PUFAs); in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Evidence from epidemiological data based on consumption of seafood and clinical trials with marine n-3 PUFA, confirm the association of increased dietary intake with a reduced risk of coronary heart diseases (Schmidt *et al.*, 2006). However, the amount of n-3 used in such clinical trials is much higher than the amount typically found in the diet. It is nevertheless reported that consumption of even small amounts of both lean and oily fish significantly reduces the risk of ischemic stroke, suggesting that fish have other beneficial nutrients, not present in pure fish oil (He *et al.*, 2004). Up until recently, contributions from other beneficial substances have, at least partly, been neglected. Now there is a growing interest in other biologically active compounds that are not regarded as essential nutrients but are likely to be beneficial under certain circumstances. Taurine and ubiquinon are examples of such compounds. Peptides with angiotensine-converting-enzyme (ACE) inhibiting effect and/or antioxidative properties are examples of other biologically active compounds acquired by seafood consumption.

Oxidative stress is a condition resulting from an unbalance between oxidants and antioxidants in favour of oxidants. The condition promotes damage to proteins, lipids and DNA, and is connected to several diseases such as degenerative diseases and CVD. A higher intake of antioxidants has been linked to a lower incidence of oxidative stress. Seafood, with its PUFAs, is very susceptible to oxidation which can lead to off-flavours, rancidity and toxic compounds like alcohols, ketones and aldehydes (Olsen, 2007). Because of this susceptibility to oxidation, seafood might be equipped with a stronger antioxidative defence system. Antioxidative capacity (AOC) of peptides from seafood has been reported in e.g. hoki (Kim *et al.*, 2007), tuna (Je *et al.*, 2007), yellow stripe trevally (Klompong *et al.*, 2007), Alaska

pollack (Je *et al.*, 2005) and shrimp (Binsan *et al.*, 2008). These studies measured the AOC after digestion by different pepsin and enzyme mixtures, and further purification. However, the changes in AOC during digestion are studied to a lesser extent. This could be one of the reasons for the poor correlations seen between *in vitro* assays and clinical trials on antioxidants (Becker *et al.*, 2004). Sannaveerappa *et al.* (2007b) investigated the AOC of herring press juice (PJ) in samples collected at different time points during a simulated gastrointestinal (GI) digestion. *In vivo* AOC of fish proteins have been reported and discussed in a recent paper by Parra *et al*. (2007). In an energy-restricted diet to treat obesity Parra *et al. (2007)* found that a cod-based diet resulted as the most effective strategy to reduce oxidative stress. The most correct method to study changes in AOC of foods in humans, would be such clinical trials. This is however expensive and time consuming. In comparison *in vitro* methods are inexpensive, rapid and may serve as efficient tools for screening of AOC of foods.

Saithe (*Pollachius virens*) is not per definition a fatty fish. Among the gadoid species however, it is the most active swimmer with pelagic lifestyle and therefore has the largest proportion of dark muscle. Dark muscle is more prone to lipid oxidation than light muscle because of higher fat content and haematin compounds that catalyze lipid oxidation (Castell and MacLean, 1964). Therefore, lipids in saithe are more exposed to oxidation than lipids from other gadoid species and the antioxidative defence could therefore be elevated. Shrimp (*Pandalus borealis*) is rich in amino acids like arginine, taurine, glycine and proline, known to exhibit AOC, together with proteins and vitamins (Lie *et al.*, 1994).

The overall aim of this thesis was to investigate the changes in AOC of saithe and shrimp muscle and their water soluble extracts, PJ, during a simulated GI digestion. Specific goals:

1. Establish three different methods to measure antioxidative capacity and study the changes in the AOC of PJ during a simulated *in vitro* GI digestion.
2. Study of the PJ's capacity to inhibit oxidation of low-density lipoprotein (LDL), in order to get closer to the possible mechanisms *in vivo*.
3. Study of muscle from saithe and shrimp with the before mentioned methods and comparison to the results obtained from PJ.

2. General background

2.1 Seafood and health

α-linolenic acid, 18:3n-3 (ALA) and linolic acid, 18:2n-6 (LA) are the two fatty acids essential to humans because of our disability to elongate and desaturate fatty acids longer than nine carbon atoms from the methyl end. From these two fatty acids humans can synthesize EPA; 20:5n-3, DHA; 22:6n-3 and aracidonic acid; 20:4n-6 (ARA). EPA, DHA and ARA are precursors to fatty acid hormones called eicosanoides. These eicosanoides are central in development of inflammatory reactions, allergic reactions and cell division (Psota *et al.*, 2006). Eicosanoides from ARA are proinflammatory and triggers to aggregate the production of phospholipase A_2 and trombocytes which can lead to coronary heart diseases, while eicosanoides from EPA/DHA are less inflammatory. Thus they exhibit antiarrythmic and anti-atherothrombogenic effects. The same set of enzymes used to elongate and desaturate ALA is also used to elongate and desaturate LA. The western diet consists of 10 times more n-6 than n-3 fatty acids and the result is that production of ARA is favoured (Simopoulos, 2004). Recommendations are that the relative ratio between omega-6 and omega-3 fatty acids in the diet should be 2-5:1, depending on the disease in question (Simopoulos, 2004). Positive health benefits from a higher intake of omega-3 fatty acids are reviewed by Ruxton *et al.* (2007). Omega-3 fatty acids from seafood are necessary for neurodevelopment and later cognitive development. An observational study, made by Hibbeln *et al.* (2007) showed that maternal seafood consumption during pregnancy was significantly associated with higher verbal IQ of the child. One of the more important qualities of the omega-3 PUFAs is their significant impact on reducing serum triacylglycerols (TG). Harris (1997) reviewed the effect of n-3 PUFAs on serum lipids, and fish oils were considered the most effective in reducing TG. Elevated levels of serum TG are thought to enhance atherogenesis. The best way for humans to reach a sufficient level of omega-3 fatty acids is by eating seafood. The high amount of EPA and DHA in fish is due to phytoplankton being a major component in their diet and phytoplankton efficiently synthesizes EPA and DHA. A general opinion has been that fish oil concentrates can substitute n-3 from seafood consumption. Visioli *et al*. (2003) and Elvevoll *et al*. (2006) have reported that n-3 fatty acids from salmon are more efficiently incorporated into serum lipids, than when administrated in capsules or a natural oil supplement. This could

be due to an enhanced uptake of n-3 from seafood compared to that from capsules in addition to the previously mentioned beneficial components in seafood.

The nutritional value of proteins from different food sources varies and is governed by amino acid composition, ratios of essential amino acids, susceptibility to hydrolysis during digestion, and the effects of processing. As reviewed by Friedman (1996), the nutritional value of seafood as a protein source may exceed the quality of meat and be equal to that of an ideal protein source such as lactalbumin. Recently there has also been an increased focus on the more specific role of seafood protein. Seafood muscle is generally rich in glycine, taurine, proline, threonine, methionine and arginine. Peptides (two or more amino acid residues) that are released during digestion are reported to exhibit beneficial activity. Kim and Mendis (2006) have reviewed some of the activities of peptides from hydrolysed fish proteins being antihypertensive, antioxidative, antithrombotic and immunomodulating. The antihypertensive activity of marine peptides inhibiting the ACE has been of particular interest the last years. ACE inhibiting effect of cod hydrolysates has recently been studied in the laboratory of IMAB. These results have however not yet been published. Fujita *et al. (2001)* performed a human study on the ACE inhibiting effect of dried bonito (katsuobushi), a traditional Japanese seasoning. A significant effect was found in lowering of the blood pressure of both spontaneously hypertensive rats and in borderline and mildly hypertensive subjects.

Peptides derived from fish proteins/fish protein hydrolysates have shown AOC with different assays. AOC has been documented in, for instance, herring (Gunnarsson *et al.*, 2006), Alaska pollack (Je *et al.*, 2005) and shrimp (De Rosenzweig Pasquel and Babbitt, 1991). There is an increasing interest among researchers to search for natural antioxidants without side effects as an alternative to the synthetic antioxidants. Despite few clinical trials there is a growing awareness that antioxidants, in particular natural ones, may contribute to lower the incidence of LDL oxidation and thus prevent development of CVD through reduced atherosclerosis.

2.2 Oxidation

Oxidation is a chemical reaction where a substance undergoes a gain in oxygen or loss of electrons or hydrogen. Oxidation is always coupled with a reduction of the other chemical substance which loses oxygen or gains electrons or hydrogen. The reduced substance is called an oxidizing agent and the oxidized substance is called a reducing agent.

2.2.1 Oxidation in seafood

Oxidation of lipids is one of the main causes of deterioration in frozen food. Seafood, because of its long chained PUFAs, is very susceptible to oxidation. Due to reduced bond dissociation energy of the C-H bond, abstraction of hydrogen takes place very rapidly at the methylene group between two alkene groups. The higher unsaturation, the more points to attack and the reaction may be accelerated. Lipid oxidation involves three steps; initiation, propagation and termination (table 1) (Olsen, 2007). The reaction is initiated by energy, transition metals (Fe^{3+} and Cu^{2+}), reactive oxygen species (ROS) or free radicals. In the initiation phase, a fatty acid (RH) looses its hydrogen atom and a lipid radical ($R^{\cdot}$) is formed[1]. This is followed by addition of oxygen to $R^{\cdot}$, yielding a peroxyl radical ($ROO^{\cdot}$). $ROO^{\cdot}$ will in turn propagate the peroxidation chain reaction by abstracting a H-atom from a nearby intact RH, leaving yet another $R^{\cdot}$ and a lipid hydroperoxide (ROOH). Transition metals (Fe^{3+} and Cu^{2+}) can react directly with ROOH and lead to a branching process wherein new radicals such as alkoxy radical ($RO^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and $ROO^{\cdot}$ are formed. In the termination of lipid oxidation, radicals react with each other or other molecules to form molecules with a full complement of electrons, thus decreasing the rate by which new oxidation reactions occur.

Table 1. Lipid oxidation is a chain reaction; involving initiation, propagation and termination.

Step	**Reaction**
Initiation:	$RH \rightarrow R^{\cdot} + H^{\cdot}$
Propagation:	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$ or $RO^{\cdot}$
	$RO^{\cdot}$ or $ROO^{\cdot} + RH \rightarrow ROH$ or $ROOH + R^{\cdot}$
Chain brancing:	$ROOH + Fe^{3+}/Cu^{2+} \rightarrow ROO^{\cdot} + H^{+} + Fe^{2+}/Cu^{+}$
	$ROOH + Fe^{2+}/Cu^{+} \rightarrow RO^{\cdot} + OH^{-} + Fe^{3+}/Cu^{2+}$
Termination:	$R^{\cdot} + R^{\cdot} \rightarrow RR$
	$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$
	$ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$

2.2.2 Oxidation in humans

The process of extracting energy from a nutrient involves a transfer of electrons. The mitochondrial electron transport chain is a flow of electrons from nicotinamide adenine

[1] The radical dot ($^{\cdot}$) is inserted to indicate the presence of one or more unpaired electrons.

dinucleotide (NADH) via cytochrome oxidase to oxygen which is reduced to water. A small percentage of the electrons sometimes do not make it to the cytochrome oxidase, but escape directly to oxygen, making superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and $OH^{\cdot}$, corresponding to reduction of one, two or three electrons respectively (Sies, 1997). These ROS are constantly and unavoidably produced. ROS is a collective term used to include oxygen free radicals and several non-radical agents (table 2). Halliwell and Gutteridge (2007) defined a free radical as "any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons". The presence of an unpaired electron in the outer orbit increases reactivity, as the solitary electron seeks a partner for stability. It can therefore be potentially damaging to DNA, lipids and proteins. ROS are also produced on purpose in the human defence system ($O_2^{\cdot -}$ and H_2O_2), namely as killing mechanisms (Halliwell, 1997). Singlet oxygen (O_2^1) has no unpaired electron but is known to be a powerful oxidizing agent, able to combine directly with many molecules that are unreactive with ground-state oxygen (O_2). O_2^1 can be formed in foods and in the skin as a result of photosensitization reaction. Some foods contain sensitizers, most often pigments that under influence of light are excited to a higher state of energy. Examples of such sensitizers are riboflavin, chlorophyll, haematine and myoglobine (Olsen, 2007). In a healthy person, production of these ROS causes no severe harm as the antioxidant defence system is appropriately balanced to the production.

When the balance between radicals/prooxidants and antioxidants shifts in favour of the former, a state called oxidative stress occurs. In most human diseases ROS are not the primary cause of the disease. However there is evidence that ROS probably contribute significantly to the disease pathology in e.g. cancer, depression, Alzheimer's disease and Parkinson's disease (Halliwell and Gutteridge, 2007). CVD are directly linked to the oxidation of LDL by ROS. The relation between oxidative stress and atherosclerosis has been reviewed by Bonomini *et al.* (2008). Atherosclerosis is characterized by the accumulation of plaque caused by repair mechanisms after a tissue injury in large and medium arteries. ROS directly injure cell membranes leading macrophages to attach to the adhesion molecule on the damaged endothelial cell and migrate to the intimate layer of the arterial wall. Here they digest oxidized LDL becoming foam cells. The LDL is oxidized by ROS (Young and McEneny, 2001). The foam cells release substances that cause inflammation and growth of the intimate layer. Eventually the plaque could occlude the vessel or rupture causing the blood in the artery to coagulate and form a thrombus (Guyton and Hall, 2006).

Table 2. Examples of reactive oxygen species, ROS.

Radicals	**Non-radicals**
Superoxide radical, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl radical, $OH^{\cdot}$	Hypochloric acid, HOCl
Peroxyl radical, $RO^{\cdot}$	Singlet oxygen, O_2^1
Alkoxyl radical, $RO_2^{\cdot}$	Organic peroxides, ROOH

2.3 Antioxidants

The word "antioxidant" has gained increased attention lately due to mass media coverage of its health benefits. The term "antioxidant" is broad and can have different meanings depending on the field of discussion. In food science the definition is often limited to the chain breaking antioxidant inhibition of lipid peroxidation, such as α-tocopherol (Halliwell *et al.*, 1995). In biological systems the definition of an antioxidant encompasses a broader meaning than in food science. Halliwell and Gutteridge (2007) defined an antioxidant as "any substance that, when present in low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". Or simpler: "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge, 2007). This definition includes compounds of an enzymatic as well as a non-enzymatic nature (table 3).

Table 3. Examples of enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants	**Non-enzymatic antioxidants**
Superoxide dismutase (SOD)	Transition metal chelators, e.g. ascorbic acid
Catalase	Radical scavengers, e.g. α-tocopherol
Glutathione (GSH) peroxidase	Oxidative enzyme inhibitors, e.g. carotenoides
	Antioxidant enzyme cofactors, e.g. selenium

2.3.1 Antioxidants of enzymatic nature

Antioxidants of enzymatic nature are naturally present in living organisms. The family of superoxide dismutase (SOD) enzymes is highly efficient in dismutating $O_2^{\cdot -}$ quickly into H_2O_2 and O_2 (reaction 1), and by this preventing the production of O_2^1. The enzyme catalase catalyzes the direct removal of H_2O_2 into O_2 and H_2O (reaction 2). A third and important

antioxidant of enzymatic nature is glutathione peroxidase (GPx), a selenium dependent enzyme. This enzyme catalyzes the conversion of ROOH into a fatty acid alcohol and by this inhibits the chain reaction in lipid peroxidation. Glutathion, GSH, a tripeptide made up of the amino acids glutamic acid, cysteine and glysine, functions as an electron donor (reaction 3) (Olsen, 2007). Passi *et al.* (2002) reported a level of GPx of 0.16-0.40 units/mg protein and a level of SOD of 1.9-9.7 units/mg protein in different Mediterranean marine fish species.

$2O_2^{\cdot -} + 2H^+ \rightarrow$ (Superoxide dismutase) $\rightarrow H_2O_2 + O_2$ (Reaction 1)

$2H_2O_2 \rightarrow$ (Catalase) $\rightarrow 2H_2O + O_2$ (Reaction 2)

$ROOH + 2GSH \rightarrow$ (Glutathione peroxidise) $\rightarrow ROH + H_2O + GSSG$ (Reaction 3)

2.3.2 Antioxidants of a non-enzymatic nature

Antioxidants of a non-enzymatic nature are not sufficiently synthesized in humans and have to be supplemented from the diet. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), polyphenols, flavonides and carotenoides are examples of such. Ascorbic acid and α-tocopherol, together with carotenoides and polyphenols are radical scavengers and can also quench singlet oxygen. Ascorbic acid is also a transition metal chelator. The level of ascorbic acid in Mediterranean fish muscle has been reported to 2-20 μg/g (Passi *et al.*, 2002). An important function of ascorbic acid can be to regenerate α-tocopherol from α-tocopherol radical. GSH is then oxidized to diglutathion (GSSG) to regenerate functional ascorbic acid from its radical. Synthetic antioxidants such as butylhydroxyanisol (BHA), butylhydroxytoluen (BHT) propylgallate (PT) and t-butylhydroquinione (TBHQ) are used as food additives to retard lipid oxidation, the latter one legal in USA only. However, the use of such synthetic antioxidants is under strict regulation due to potential health risks, and the search for natural antioxidants with potential benefits to the consumer, in addition to retard lipid oxidation, has been of great interest to researchers in recent years.

2.3.3 Antioxidants expected to be present in press juice

PJ is basically the intra- and extracellular fluids of muscle tissue recovered by centrifugation of muscle mince. Therefore the composition is highly complex, containing both pro-oxidants and antioxidants. Undeland *et al.* (1998) reported that washed samples of minced herring oxidized faster than unwashed samples, and thus suggested that the aqueous fractions of fish muscle contain strong antioxidants that are diluted in the washing process. Later

Undeland *et al.* (2003) showed that addition of PJ from cod, haddock, dab sole, black back and herring to minced and washed cod muscle inhibited Hb-mediated oxidation. Various antioxidative enzymes (SOD, catalase, peroxidises, etc.) can attribute to these antioxidative properties. Seafood; fish and in particular invertebrates, are rich in taurine, an exclusively free amino acid (Roe and Weston, 1965) which together with arginine, histidine and glycine are known to scavenge oxygen free radicals (Fang *et al.*, 2002). In addition ascorbic acid would be present in PJ together with sarcoplasmatic proteins. Other sulphur-containing amino acids e.g. cysteine and methionine exhibit some AOC. The general trend of the AOC of these amino acids is that the highly reduced forms are stronger antioxidants (Atmaca, 2004). Carnosine, a dipeptide made of the two amino acids histidine and β-alanine, is known to have AOC. It is a free radical scavenger and can interact with molecular products of lipid peroxidation as well as superoxide anion radicals and hydroxyl radicals (Guiotto *et al.*, 2005).

2.3.4 Antioxidants expected to be present in muscle

While PJ consists of the sarcoplasmatic proteins together with the water soluble antioxidants like taurine and ascorbic acid, the whole seafood muscle would consist of sarcoplasmic proteins in addition to myofibril proteins, together with water soluble antioxidants and antioxidant which are not water soluble, like α-tocopherol.

2.3.5 Antioxidants expected to be present in digested press juice and muscle

It is known that the molecular size and composition of the peptides influence the AOC, the smaller peptides being the most potent (Je *et al.*, 2005; Kim *et al.*, 2007). The proteolytic activity of pepsin, bile and pancreatic enzymes will affect the amount and size of peptides and free amino acids, thus influencing the AOC. Amino acids with hydrophobic residues are exposed as the proteins are digested, and an increase in hydrophobic residues would lead to an increase in the AOC. Other amino acids with sulphuric residues will be exposed and increase the AOC further. Consequently, an increase in AOC after addition of the proteolytic enzymes should be seen.

2.4 Methods

2.4.1 General comments

The scavenging of $OH^{\cdot}$ and lipid free radicals, removal of ROS and reactive nitrogen species and chelating of transition metals are mechanisms that must be considered to evaluate the antioxidative properties of foods/food extracts. Suitable oxidation substrates, oxidation initiators, relevant test conditions and the specificity of the method employed to analyse the progress of oxidation must thus be carefully chosen.

2.4.2 Methods used to measure antioxidative capacity

Major AOC assays can be roughly divided into two categories: hydrogen atom transfer (HAT) reaction based assays, and single electron transfer (ET) reaction based assays (Huang *et al.*, 2005). Both HAT- and ET- based assays are intended to measure the radical (or oxidant) scavenging activity.

The oxygen radical absorbance capacity (ORAC) assay is a HAT-based method. HAT-based methods are generally composed of a synthetic free radical generator, here 2,2´ -Azo-bis-(isobuttersaüreamidin)-Dihydrocholorid (AAPH), an oxidizable molecule (here fluorescein sodium salt) as fluorescence, and an antioxidant. As the reaction progresses, fluorescein is consumed and fluorescence intensity decreases. In the presence of an antioxidant the fluorescence decay is inhibited. The area under the kinetic curve of the sample minus the area under the blank kinetic curve, is compared with the net area under the kinetic curves for known concentrations of 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The advantage of this approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and antioxidants that have no lag phase (Huang *et al.*, 2005).

The ferric reducing ability of plasma (FRAP) assay and 2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay are ET-based assays. These assays involve two components in the reaction mixture; an antioxidant and an oxidant. The assays are based on an electron being transferred from the antioxidant to the oxidant, resulting in a reduced oxidant and an oxidized antioxidant. The oxidant abstracts an electron from the antioxidant, causing colour changes. The reaction end point is reached when the colour change stops. ET-based assays resemble the redox reactions in classical chemistry. To make the correlation between the results and the antioxidant capacity it is assumed that antioxidant capacity is equal to reducing capacity (Benzie and Strain, 1996).

In vitro inhibition of LDL oxidation is also used as a tool to measure AOC. LDL is most often isolated from plasma following lengthy centrifugations. In the assays LDL is usually oxidized by incubation with Cu^{2+} (e.g. $CuSO_4$). The oxidation is a free-radical lipid peroxidation. The removal of a hydrogen-atom from the fatty acid results in a molecular rearrangement of the unstable carbon radical, which in turn results in a more stable configuration, a conjugated diene (Young and McEneny, 2001). The conjugated dienes have an absorbance at 234 nm. The use of Cu^{2+} ions in these assays is questioned since it does not directly parallel a biological system as circulating copper in human blood is scarce (Burkitt, 2001). In contrast to copper, iron in the form of haemoglobin is abundant in blood and can be released under stress conditions. However, iron is less frequently used in such assays because of its poor solubility in phosphate buffers (Kuzuya *et al.*, 1991). The inhibition of oxidation can be characterized by several parameters; a delay in the lag phase, the maximum rate of oxidation (Vmax) or maximum accumulation of oxidation products (Amax).

2.4.3 The gastrointestinal tract

The path through the digestive tract begins at the mouth, proceeds trough the oesophagus to the stomach, and through the pyloric sphincter into the small intestine and then the large intestine (figure 1). The entire digestion of foods lasts four to five hours. During this pathway the physiological conditions, among these pH, are regulated in order to ensure optimal environment for the enzymes.

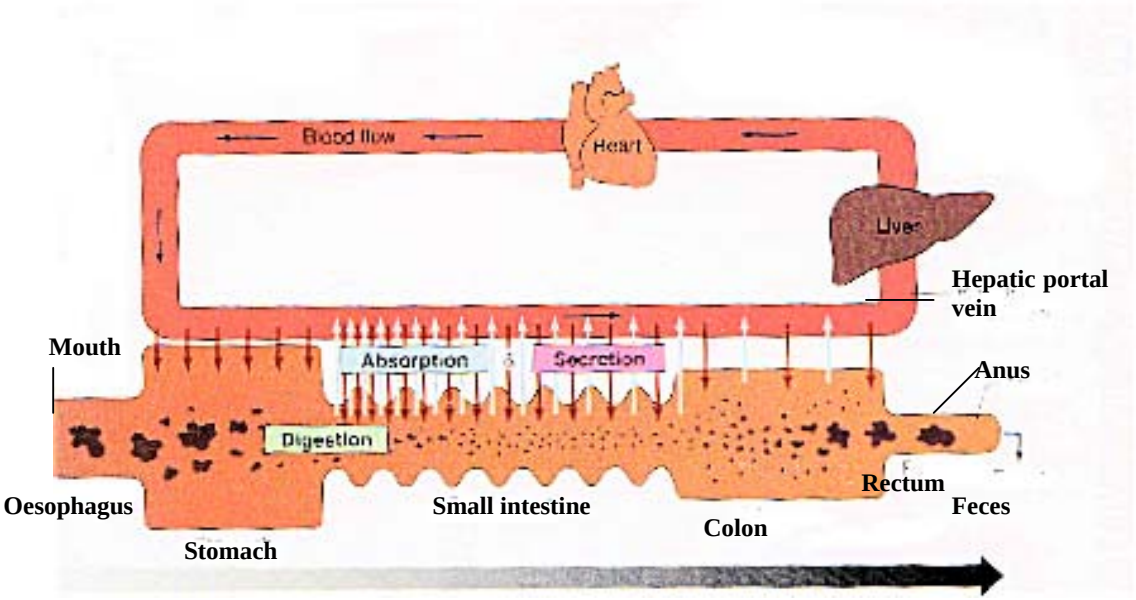

Figure 1. Schematic presentation of the gastrointestinal tract showing mouth, oesophagus, stomach, small intestine, colon and rectum (Widmaier *et al.*, 2004).

2.4.4 Digestion of proteins

Proteins are formed from multiple amino acids linked together by peptide bounds (figure 2). At each linkage, a hydroxyl ion has been removed from one amino acid and a hydrogen ion has been removed from the succeeding one. Thus, the amino acids in the protein chain are bound together by condensation, and digestion occurs by the reverse effect: hydrolysis. That is, the proteolytic enzymes return hydrogen and hydroxyl ions from water molecules to the protein molecules to split them into peptides, and at the end their constituent amino acids (Guyton and Hall, 2006).

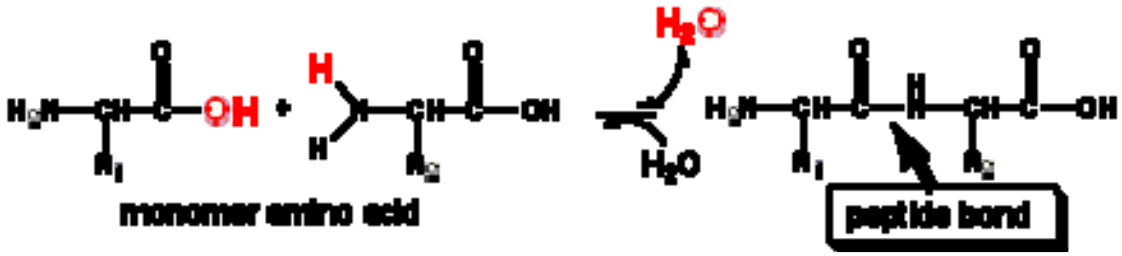

Figure 2. A peptide is formed from condensation of water from two amino acids and can be digested to amino acids by hydrolysis of water.
(Copied from www.chemical-universe.com/biochemistry.html 27.04.08).

The initial phase in the assimilation of dietary proteins includes homogenization of food by chewing. Thereafter, in the stomach: denaturation of proteins by pepsin enzymes. Both pepsin and gastric acid are required for the digestion of dietary proteins in the stomach. The optimal pH for pepsin is between 2.0 and 3.5, and if the pH exceeds 5.0, pepsin is inactivated. One of the important features of pepsin is the ability to digest collagen; a major constituent of connective tissue of meats. Pepsin only initiates the digestion of proteins, usually 10-20 per cent of the total protein digestion. It is not specific in its site of action, and catalyzes the partial digestion of proteins by cleaving them at a variety of amino acid residues into proteoses, peptones, and a few polypeptides. Immediately after entering the small intestine, the pH is neutralized (pH 6.5) and the pancreatic enzymes are excreted; trypsin and chymotrypsin being the most important ones. Trypsin is quite specific, in contrast to pepsin, and cleaves the dietary proteins at arginine and lysine residues. Chymotrypsin cleaves proteins at aromatic amino acid residues (Brody, 1999; Sand *et al.*, 2005).

3. Materials and methods

3.1 Raw materials

3.1.1 Saithe

Fresh fillets of saithe were obtained from Dragøy AS (Tromsø, Norway) and were prepared in the laboratory within 24 hours after landing. The supplier informed that the saithe used in the experiment was caught outside Vengsøya in Troms, October (batch 1) and November (batch 2) 2007, and kept alive in sea cages until slaughtering.

3.1.2 Shrimp

The shrimps were obtained from Stella Polaris AS (Tromsø, Norway). The supplier informed that the shrimps were caught in the Norwegian Sea outside the Faroe Islands and were block frozen within four hours. Prior to the analysis the shrimps were thawed at 4 ^{o}C overnight.

3.2 Preparation of raw materials

3.2.1 Press juice

- Saithe
  - Light muscle only (dark muscle discarded)
  - Whole muscle (light muscle and dark muscle)
- Shrimp

The muscles of saithe (100 g) were homogenized in a food processor (Braun electronics) for one minute at medium speed, and thereafter centrifuged at 18250 G for 2 hours at 4 ºC as described by Gunnarsson *et al.* (2006). The supernatant was filtered trough a Schleicher & Schuell folded filter and resulted in 20 mL light muscle press juice (LMPJ) and 23 mL whole muscle press juice (WMPJ). The PJs were stored at -55 ºC until use.

The muscles of shrimp (300 g) were peeled half frozen and cut in the food processor for five seconds at medium speed. To be able to extract the water soluble components from shrimp it was necessary to add 300 mL of water. The muscles were stirred at room

temperature for 1 hour and centrifuged at 18250 G for 2 hours at 4 ºC as described by Gunnarsson *et al.* (2006). The supernatant was filtered trough the folded filter and resulted in 120 mL PJ (SPJ). The SPJ was stored at -55 ºC until use. The abbreviations used in the text are shown in table 4.

3.2.2 Muscle

- Saithe
  - Light muscle only (dark muscle discarded)
  - Whole muscle (light muscle and dark muscle)
- Shrimp

The muscles of saithe were homogenized in the food processor for one minute at medium speed and stored at – 55 ºC until use.

The muscles of shrimp were peeled half frozen and cut in the food processor for five seconds at medium speed and stored at – 55 ºC until use. The abbreviations used in the text are shown in table 4.

Table 4. Raw materials and abbreviations used in the text.

Raw materials	**Abbreviation**
Light muscle press juice of saithe	LMPJ
Whole muscle press juice of saithe	WMPJ
Press juice of shrimp	SPJ
Light muscle of saithe	LMS
Whole muscle of saithe	WMS
Whole muscle of shrimp	SWM

The samples were prepared in duplicate.

3.3 Study design

Six "raw materials" were prepared in duplicate as described in chapter 3.2 and will be referred to as shown in table 4. Each of them was then subjected to GI digestion in triplicates. During the digestion, samples were collected at five different time points; 0, 30, 75, 105 and 165 minutes after the start of digestion (figure 3). At each time point, analysis of AOC was

carried out using 3 methods: ORAC, FRAP and ABTS. The Copper induced LDL oxidation assay was used to measure the inhibiting effect of LMPJ. The measurements were performed in triplicate for each sample and the results were reported as the average of six parallels, each composed of three measurements. Analysis of the protein content was performed in duplicate from each sample and the results were reported as the average of six parallels, each composed of two measurements.

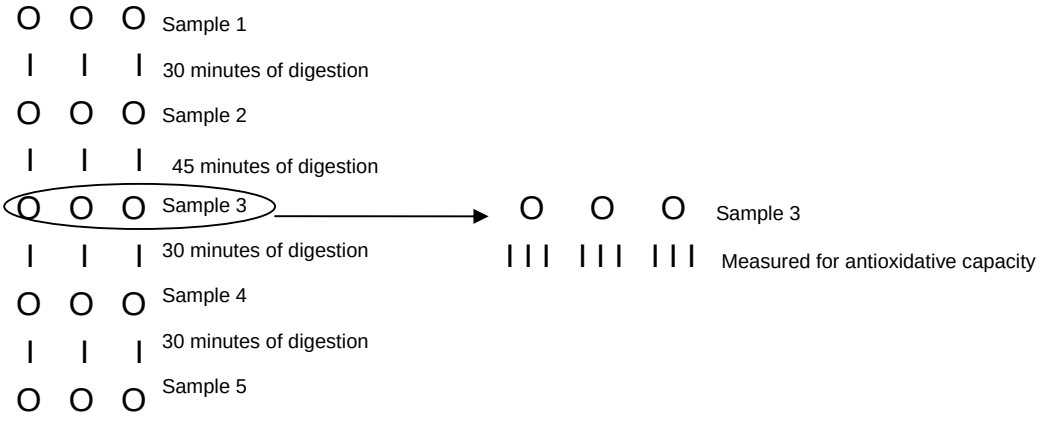

Figure 3. Study design of the digestion and measurement of the antioxidative capacity (AOC). The "raw materials" were prepared in duplicate and subjected to the gastrointestinal (GI) digestion in triplicate. At each time point a sample was collected from each of the triplicates. From each sample, AOC was measured in triplicate and the results were reported as the average of six parallels, each composed of three measurements. The protein content was measured in duplicate and the results were reported as the average of six parallels, each composed of two measurements.

3.4 *In vitro* gastrointestinal digestion procedure

Unless stated otherwise, all solvents and chemicals used were from Merck (KGaA, Darmstadt, Germany) and of analytical grade. Pepsine crystalline, pancreatin (P1750) and bile extract (B8631) were bought from Sigma Chemical Co. (St. Louis, MO, USA). The shaking bath used was an Innova 4300 Incubator shaker, from new Brunswick Scientific Co. (INC, Edison, New Yersey, USA) and the centrifuge was a multifuge 1 S-R, from Kendro Laboratory Products (GmbH, Osterode, Germany).

The human digestion was simulated by adding pepsin solution representing the gastric phase and bile/pancreatic solution representing the intestinal phase, along with gradient pH adjustment. The method was performed as described by Sannaveerappa *et al.* (2007b) with

modifications in the case of the muscle "raw materials" (LMS, WMS and SWM). The PJs were thawed in cold water for 30 minutes. A volume of 15 mL of one of the following PJs; LMPJ, WMPJ, SPJ, and 50 mM phosphate buffer (Na_2HPO_4) with 0.9 % NaCl, pH 6.75 (control) were mixed with 15 mL of a pepsin solution containing 49 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 2.4 mM $MgCl_2$, 3.5 mM K_2HPO_4 and 0.462 % pepsin crystalline. In the case of muscle of saithe and shrimp, LMS, WMS and SWM, 1 g of muscle was mixed with 15 mL of the pepsin solution. The reaction volume was 30 mL for the PJs and 16 mL for the muscles. The pH was adjusted with 3 M HCl to pH 5.5 and a PJ/muscle sample (6 mL/3 mL) was collected (sample 1). The reaction mixtures were then incubated in a shaking bath at 220 rpm and 37 ºC for 30 minutes. Another PJ/muscle sample (8 mL/4 mL) was then collected (sample 2). The pH was adjusted to pH 3.8 and the reaction mixtures were incubated for 30 minutes. A pH-adjustment was performed to pH 2.0 and after 15 minutes in the incubator shaker, a PJ/muscle sample (6 mL/3 mL) was collected (sample 3). After this, 1.5 mL of a bile/pancreatic solution was added. The solution contained 50 mL distilled water, 0.2 g pancreatine, 1.25 g bile extract and 0.1 M $NaHCO_3$. The reaction mixtures were thereby diluted 1.15/1.30 times. The pH was adjusted to pH 5.0 with 3 M NaOH and the reaction mixtures were incubated for 30 minutes before a PJ/muscle sample (6 mL/3 mL) was collected (sample 4). The pH was adjusted to pH 6.5 and the reaction mixtures were incubated for 60 minutes. The remaining amount of the reaction mixtures was collected (sample 5). All of the samples were immediately frozen at -55 ºC to stop the reaction. The samples were then thawed and centrifuged at 4500 G at 4 ºC for 15 minutes to remove large particles. The supernatant was again centrifuged at 4000 G and 4 ºC for 15 minutes to remove added enzymes. The centrifugation, removing of the supernatant and subsequent centrifugation were time consuming, and consequently the samples were frozen again and kept frozen at – 55 ºC until analysis of AOC.

Table 5. Schedule of the *in vitro* gastrointestinal (GI) digestion procedure.

			Enzymatic solutions		
Simulated digestive part	pH	Digestion (minutes)	Pepsin, mL	Pancreatic and bile, mL	mL PJ/muscle (no)
Stomach	5.5	0	15		6/3 (1)
	5.5	30			8/4 (2)
	3.8	60			
	2.0	75			6/3 (3)
Small intestine	5.0	105		1,5	6/3 (4)
	6.5	165			5/3 (5)

The samples were centrifuged at 4500 G for 15 minutes and the supernatants were ultrafiltered at 4000 G for 15 minutes. The supernatants were kept at -55 ^{o}C until analysis.
PJ: press juice. The table was adopted from Sannaverrappa *et al. (*2007b*)*.

3.5 Protein content

The protein content in the samples was determined using the Bio-Rad Detergent Compatible Protein Assay (Bio-Rad, Herkules, CA, USA), using bovine serum albumine (BSA) as standard protein. The method is developed from the original method of Bradford (1976). Spectra max, 190, spectrophotometer (Molecular devices, Sunnyvale, USA) was used to perform the analysis.

In each well of a transparent microplate, 5 µL of sample was added. Thereafter 25 µL of reagent A' (alkaline copper tartrate solution mixed with surfactant solution, ratio 49:1) and 200 µL of reagent B (dilute Folin reagent) was added. The microplate was incubated for 15 minutes before the absorbance was read at 750 nm. The results were presented as mg/mL PJ or sample.

3.6 Antioxidative capacity

Unless stated otherwise, all solvents and chemicals used were from Merck (KGaA, Darmstadt, Germany) and of analytical grade. Trolox (97 %), AAPH, fluorescein sodium salt, Iron III Chloride 6-hydrate (Fe), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), ABTS and potassium persulfate were bought from Sigma Chemical Co. (St. Louis, MO, USA). The fluorimetric

measurements were carried out using a Spectramax Gemini EM fluorimeter (Molecular devices, Sunnyvale, USA), and the spectrophotometric measurements were carried out using a Spectra max, 190, spectrophotometer (Molecular devices, Sunnyvale, USA).

3.6.1 ORAC

The ORAC assay, using a fluorescent probe, was carried out according to Dávalos *et al.* (2004). The principle of the method is to measure the ability of an extract to inhibit the fluorescence decay of fluorescein, according to the attack of an AAPH reagent. Trolox was used as standard and the reaction was carried out in a 75 mM phosphate buffer (Na_2HPO_4), pH 7.4. The AAPH reagent was prepared by dissolving AAPH in phosphate buffer to a concentration of 40 mM (12 mM, final concentration in well) and the fluorescein sodium salt was dissolved in phosphate buffer to a concentration of 117 nM (70 nM, final concentration in well). A black 96 well microplate was used. The test sample, 20 µL, and fluorescein, 120 µL, were placed in a well and preincubated at 37 ^{o}C for 15 minutes, before 60 µL AAPH reagent was added. The readings were performed at 485 and 520 nm excitation and emission respectively. The measurement was carried out at 37 ºC and the microplate was automatically shaken prior to each reading – every 30 seconds for 120 minutes. The net area under curve (area under curve for test sample – area under curve for blank) was calculated and compared to Trolox.

3.6.2 FRAP

The FRAP assay was carried out according to Benzie and Strain (1996) with slight modifications. The principle of the method is to determine the reducing ability of the extract as a measure of its antioxidant capacity. A ferric-tripyridyltriazine complex is reduced to its ferrous form in the presence of a reductant. The reduced form has a blue colour that is measured spectrophotometrically and compared to a standard. The FRAP reagent contained 2.5 mL of 19 mM Fe plus 2.5 mL of 10 mM TPTZ in 40 mM HCl plus 25 mL acetate buffer, pH 3.6. The FRAP mixture was prepared freshly and kept at 37 °C. A volume of 300 µL FRAP reagent, 30 µL distilled water and 10 µL test sample were mixed in a well of a transparent 96 well microplate. The microplate was shaken prior to each reading – every 20 second for 30 minutes at 37 ^{o}C and 595 nm. The absorbance after 1800 seconds was used in the calculations. The values obtained were compared to Trolox.

3.6.3 ABTS

The ABTS assay was carried out as described by Re *et al.* (1999), with some modifications. The principle is to measure the ability of a test sample to reduce the premade radical. The radical has a dark colour and is decolorized as it is reduced. The inhibition of the radical is expressed by a decay in absorbance. The ABTS reagent was prepared the day before the assay was carried out: ABTS was diluted in 2.44 mM potassiumpersulfat to a concentration of 7 mM. The ABTS working reagent was kept dark at ambient temperature overnight. An amount of 1 mL of the ABTS reagent was diluted in 75 mL methanol to an absorbance of 0.70 ± 0.02 at 658 nm and 30 ºC. Too high or too low absorbance was adjusted with methanol or ABTS working reagent respectively. The solution was kept at 30 ºC. ABTS reagent and test sample (solvent as control), 3.9 mL and 0.1 mL respectively, were mixed and 200 µL of this mixture was added to the well. The readings were performed at 658 nm every 30 seconds for 6 minutes at 30 ºC. The net area under the absorbance curve (area under curve for control – area under curve for test sample) was calculated and compared to Trolox.

3.6.4 Inhibition of low-density lipoprotein oxidation assay

The assay was carried out as described by Sannaveerappa *et al.* (2007b) and Kleinveld *et al*. (1992). The LDL stock solution was prepared by diluting human LDL in 50 mM phosphate buffer containing 0.9 % NaCl (pH 7.4) to a concentration of 400 µg LDL/L. To each well in a 96 well transparent microplate, 50 µL of LDL stock solution was added (100 µg LDL/L, final concentration). The test samples, 20 µL, and phosphate buffer, 80 µL, was thereafter added to the wells. The initial absorbance was measured at 234 nm. 50 µL of 40 µM $CuSO_4$ was then added to each well (10 µM, final concentration). The microplate was incubated at 37 ^{o}C for 600 minutes while the absorbance was measured at 234 nm every 20 minute. The sample values were ultimately divided in the initial absorbance value from the respective samples.

3.7 Statistical analysis and calculations of results

Values are given as mean ± standard deviation. SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis of the data. A test for homogeneity of variance was performed, and as this was inconclusive, a Dunnett's T3 test was chosen as a post hoc

test for comparison between groups. The significant level was set to $p < 0.05$. Extreme values that were not within 2 standard deviations were regarded as outliers and removed.

4. Results

4.1 Properties of raw material

The amount of saithe muscle needed to make 1 mL of PJ was 4.8 g LMS and 4.4 g WMS (table 6). The amount of shrimp muscle needed to make 1 mL of PJ was 2.5 g SWM (table 6). The amount of PJ thus calculated to correspond to 1 g of muscle was 0.21 mL, 0.23 mL and 0.40 mL LMPJ, WMPJ and SPJ respectively.

Table 6. The ratio between muscle and press juice (PJ) for 1 mL of PJ and 1 g of muscle.

	Muscle (g) / 1 ml PJ	PJ (ml) / 1 g muscle
Light muscle press juice of saithe (LMPJ)	4.8	0.21
Whole muscle press juice of saithe (WMPJ)	4.4	0.23
Press juice of shrimp (SPJ)	2.5	0.40

The protein content in the undigested PJs is shown in table 7 together with the AOC measured by ORAC, ABTS and FRAP. The protein content in LMPJ and WMPJ was not significantly different from each other. SPJ had the lowest protein content. The ORAC values of the undigested PJs were ranked in following order: LMPJ, WMPJ and SPJ. There was a significant difference between LMPJ and SPJ. LMPJ exhibited the highest value of approximately 19 mmol Trolox equivalents (TE)/L PJ, and WMPJ exhibited a value of 16 mmol TE/L PJ. SPJ exhibited the lowest ORAC value; 6 mmol TE/L PJ. In the FRAP assay the values from all PJs were approximately 1 mmol TE/L PJ, WMPJ exhibiting the highest value and SPJ the lowest. The results from the ABTS assay did not show any difference between the PJs. SPJ showed an activity of 11 mmol TE/L PJ compared to approximately 13 mmol TE/L PJ for LMPJ and WMPJ.

Table 7. Protein content measured by Bio-Rad, and antioxidative capacity (AOC) of press juice (PJ) measured by ORAC, ABTS and FRAP assays.

Raw materials	Peptide content (mg/mL PJ)	ORAC value (mmol TE/L PJ)	FRAP value (mmol TE/L PJ)	ABTS value (mmol TE/L PJ)
LMPJ	66.0 ± 9.6	18.5 ± 2.2[a]	0.9 ± 0.2	13.2 ± 10.4
WMPJ	68.0 ± 5.7	16.1 ± 1.8	1.1 ± 0.1	13.0 ± 7.0
SPJ	48.0 ± 1.8[a]	6.6 ± 1.8[b]	0.7 ± 0.1	11.1 ± 7.3

Six parallels of "raw materials" were measured in duplicate for protein content and in triplicate for AOC. The results were presented as the mean ± standard deviation. Values followed by different letter in the same column were significantly different at $p < 0.05$.
LMPJ: light muscle press juice of saithe, WMPJ: whole muscle press juice of saithe, SPJ: press juice of shrimp, ORAC: oxygen radical absorbance capacity, ABTS: 2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, FRAP: ferric reducing ability of plasma.

4.2 Protein content in press juice and muscle

The protein content was measured in each sample during the digestion (table 8). Due to dilution and centrifugation after collection, the PJ samples collected just before the digestion started (sample 1) had lower protein content than the undigested PJs. For all the samples the trend was that the protein content increased up until the stage simulating the small intestine, 75 minutes after the digestion had started, and thereafter stabilized or decreased.

Table 8. Protein content (mg/mL sample) measured by Bio-Rad in the five samples collected throughout the digestion.

Raw materials	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
LMPJ	32.0 ± 17.6	40.2 ± 8.3[a]	57.1 ± 6.3[b]	49.5 ± 5.6[b]	39.8 ± 4.9
WMPJ	34.6 ± 2.7[a]	34.2 ± 0.7[ab]	71.4 ± 12.9	49.0 ± 3.4[bc]	50.7 ± 0.9[c]
SPJ	29.0 ± 3.2	32.0 ± 7.7	44.4 ± 5.0	38.7 ± 7.0	27.7 ± 5.7
LMS	8.5 ± 1.7[a]	11.9 ± 1.7	22.7 ± 3.6	20.2 ± 2.4[b]	18.7 ± 3.9
WMS	12.2 ± 3.5	10.7 ± 0.7	22.0 ± 4.0	18.5 ± 2.6	22.8 ± 3.7
SWM	7.2 ± 2.7[a]	12.3 ± 0.6[b]	27.6 ± 1.7[c]	21.4 ± 2.2[bc]	23.0 ± 3.8[bc]

The samples ($n = 6$), were measured in duplicate and the results were presented as the mean ± standard deviation. Values followed by different letter in the same row were significantly different at $p < 0.05$.
LMPJ: light muscle press juice of saithe, WMPJ: whole muscle press juice of saithe, SPJ: press juice of shrimp, LMS: light muscle of saithe, WMS: whole muscle of saithe, SWM: whole muscle of shrimp.

4.3 Antioxidative capacity of press juice and muscle

4.3.1 Antioxidative capacity (ORAC) of press juice

The general development in the ORAC values for the PJ samples during the GI digestion (figure 4 and 5) seemed to follow the development of protein content during digestion for the case of LMPJ and WMPJ (table 8). The correlation between the development in ORAC values and protein content was calculated to be 0.802, 0.856 and 0.631 for LMPJ, WMPJ and SPJ respectively. In the first collected sample (sample 1), LMPJ and WMPJ exhibited ORAC values lower than 6 mmol TE/L sample. SPJ exhibited an ORAC value of approximately 10 mmol TE/L sample. After 30 minutes of digestion the values had a tendency to increase, although not significant in any sample. In sample 3, after 75 minutes of digestion, the ORAC values had increased significantly compared to sample 1. At this point the samples displayed ORAC values of between 34 and 38 mmol TE/L sample for LMPJ and WMPJ and approximately 30 for SPJ. Throughout the following digestion the ORAC values remained fairly stable and did not change significantly. After 165 minutes of digestion the ORAC values had increased 8-, 5- and 2,5- fold for LMPJ, WMPJ, and SPJ respectively.

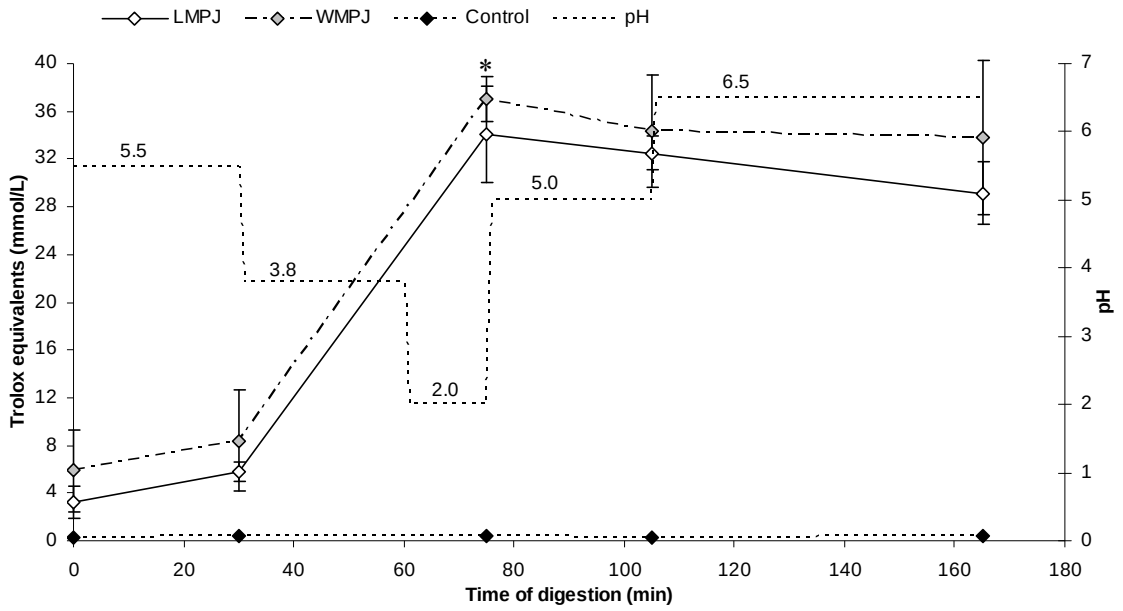

Figure 4. Antioxidative capacity (ORAC) of press juice (PJ) during an *in vitro* gastrointestinal (GI) digestion of 15 mL light muscle press juice of saithe (LMPJ) and whole muscle press juice of saithe (WMPJ). The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviation.
ORAC: oxygen radical absorbance capacity, *: significant difference from samples earlier in the digestion at $p < 0.05$.

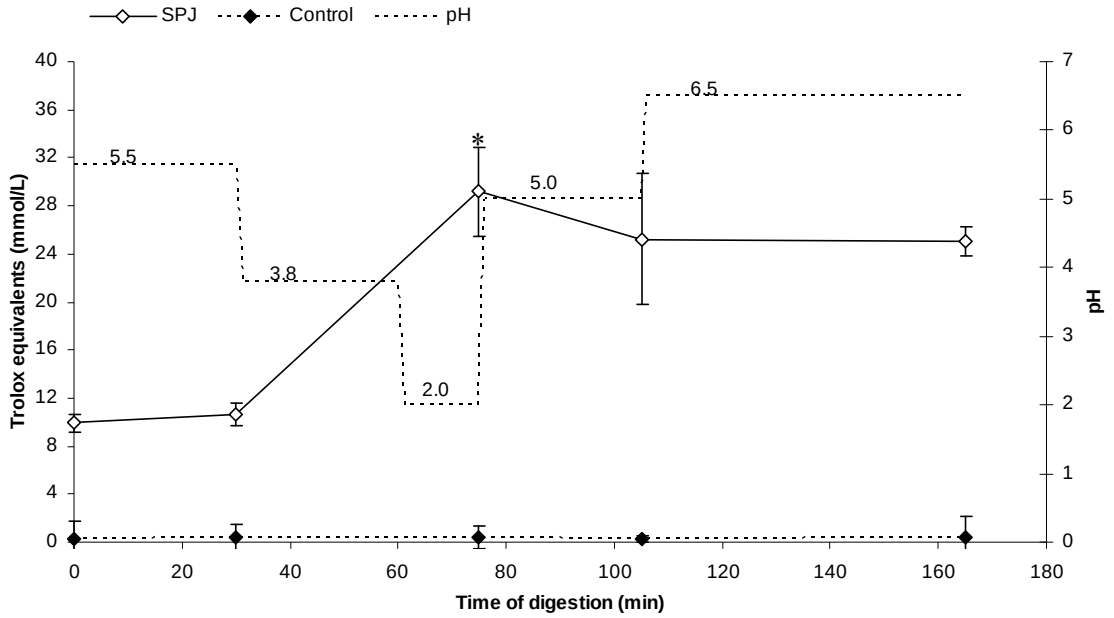

Figure 5. Antioxidative capacity (ORAC) of press juice (PJ) during an *in vitro* gastrointestinal (GI) digestion of 15 mL press juice of shrimp (SPJ). The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviation.
ORAC: oxygen radical absorbance capacity, *: significant difference from samples earlier in the digestion at $p < 0.05$.

4.3.2 Antioxidative capacity (ORAC) of muscle and press juice

In figure 6 the ORAC values for 1 g of muscle together with the ORAC values for the PJ samples from figure 4 and 5, corresponding to 1 g of muscle, are presented. To be able to present this comparison, a rather rough assumption was needed. It was assumed that the whole entity of digested "raw materials" arrived at the points of sample collection. The trend for the changes in the ORAC values of muscle samples followed the trend for the PJ samples and for the protein content in the related samples. The correlation was calculated to be 0.81 for LMS and 0.86 for WMS and SWM. In sample 1, collected before the digestion had started (the pepsin solution was added, the pH was adjusted and the sample was centrifuged) the ORAC values ranged between approximately 20-50 micromoles TE. SWM exhibited the lowest value and LMS the highest. There was a tendency of increase in the AOC during the first 30 minutes of the digestion, but this was not significant. When the pH was adjusted to 2.0 and the samples had been digested until the stage simulating the small intestine, 75 minutes, the increase shown was significant ($p < 0.05$) for all the samples. The ORAC values for the muscles of saithe, LMS and WMS, were approximately 150 micromoles TE. SWM exhibited an ORAC value of approximately 210 micromoles TE. As for the PJ samples, the values for

the muscle samples remained fairly stable after the first 75 minutes of digestion. After 165 minutes of digestion the ORAC values had increased approximately 3-fold for the muscle of saithe and 10-fold for the muscle of shrimp.

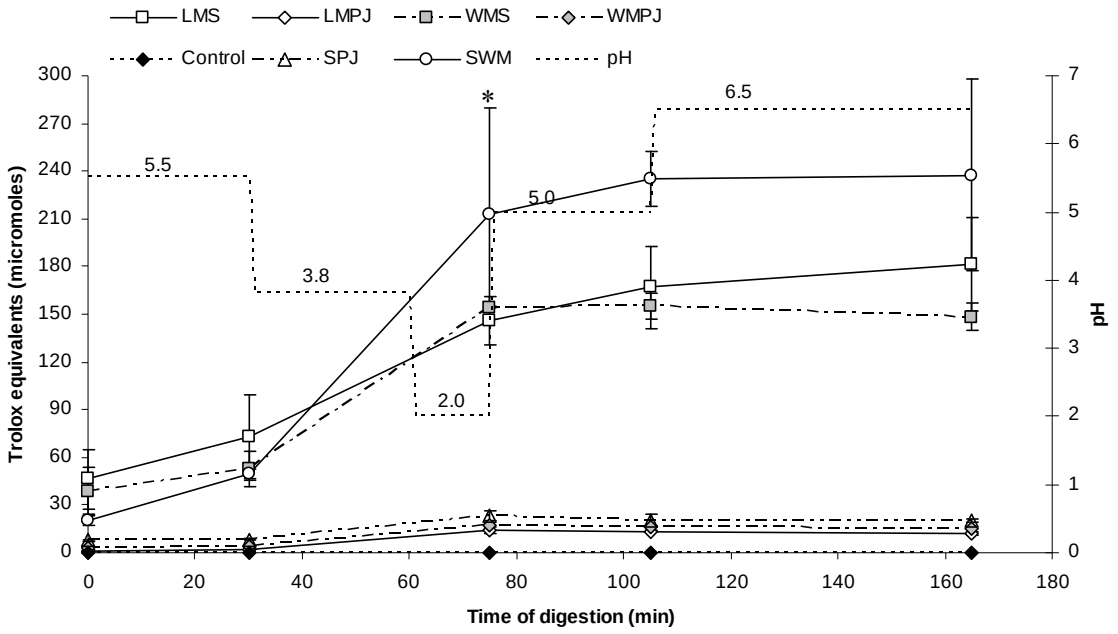

Figure 6. Antioxidative capacity (ORAC) of 1 g of muscle and the corresponding amount of press juice (PJ) (0.21, 0.23 and 0.40 mL of LMPJ, WMPJ and SPJ respectively) during an *in vitro* gastrointestinal (GI) digestion. The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviation.
LMPJ: light muscle press juice of saithe, WMPJ: whole muscle press juice of saithe, SPJ: press juice of shrimp, LMS: light muscle of saithe, WMS: whole muscle of saithe, SWM: whole muscle of shrimp, , ORAC: oxygen radical absorbance capacity, *: significant difference from samples earlier in the digestion at $p < 0.05$.

4.3.3 Antioxidative capacity (FRAP) of press juice

The FRAP values obtained from the PJs (figure 7 and 8) showed a similar trend as for the ORAC values, but lower and with higher standard deviations. Before the digestion had started (when pH was adjusted to 5.5, the pepsin enzymes were added and the samples centrifuged) the samples exhibited FRAP values between 0.3 and 0.5 mmol TE/L sample. In the third sample collected (sample 3), after 75 minutes of digestion, a tendency of increase was observed and the samples exhibited FRAP values between 1.0 and 1.2 mmol TE/L sample for LMPJ and WMPJ respectively, and 0.6 mmol TE/L sample for SPJ. The increase was, however, not significant.

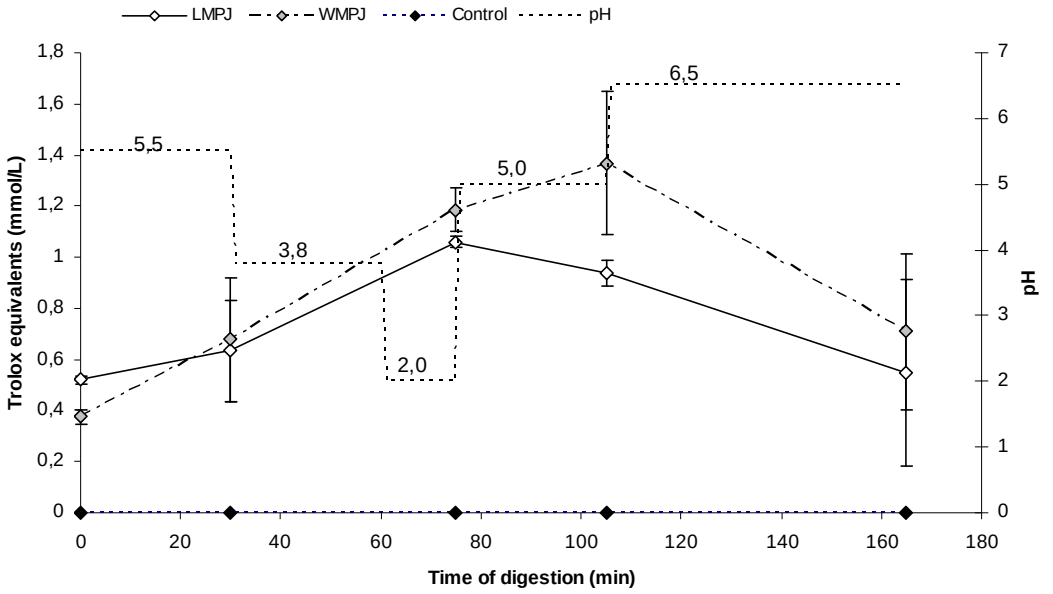

Figure 7. Antioxidative capacity (FRAP) of press juice (PJ) during an *in vitro* gastrointestinal (GI) digestion of 15 mL light muscle press juice of saithe (LMPJ) and whole muscle press juice of saithe (WMPJ). The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviation.
FRAP: ferric reducing ability of plasma.

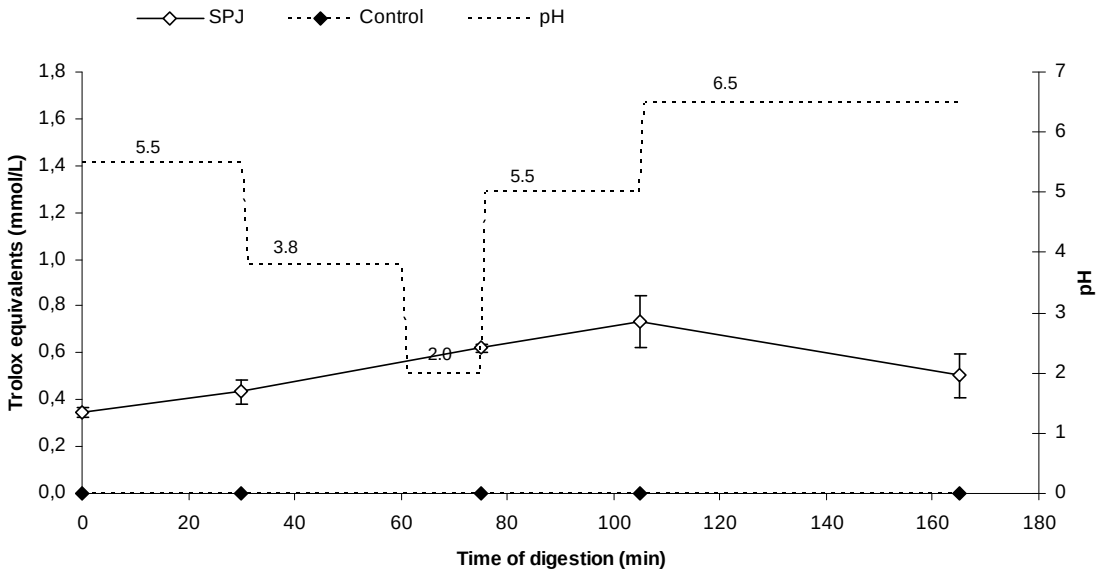

Figure 8. Antioxidative capacity (FRAP) of press juice (PJ) during an *in vitro* gastrointestinal (GI) digestion of 15 mL press juice of shrimp (SPJ). The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviation.
FRAP: ferric reducing ability of plasma.

4.3.4 Antioxidative capacity (FRAP) of muscle and press juice

In figure 9 the FRAP values for 1 g of muscle together with the FRAP values for the PJ samples from figure 7 and 8, corresponding to 1 g of muscle, are presented. As for figure 6, a rather rough assumption was needed to present the comparison between muscles and PJs. It was assumed that the whole entity of digested "raw materials" arrived at the points of sample collection. The muscle samples from saithe (LMS and WMS) exhibited FRAP values of approximately 1 micromoles TE at the beginning of the digestion. SWM exhibited FRAP values of approximately twice as much. After 75 minutes of digestion the FRAP values had increased to approximately 3 micromoles TE for LMS and WMS and 9 micromoles TE for SWM. The FRAP values thereafter showed a tendency to decrease.

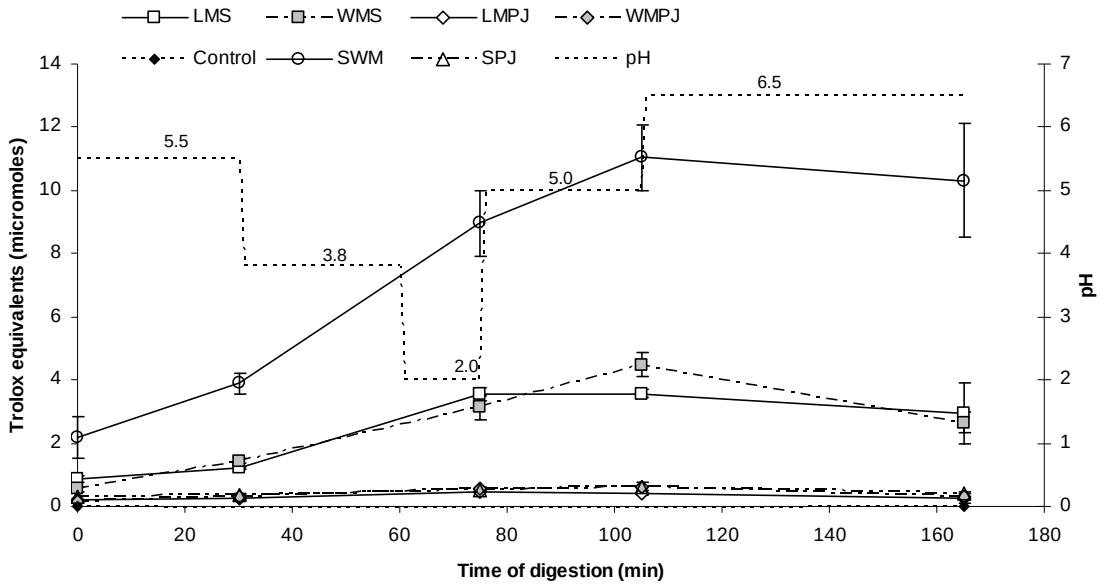

Figure 9. Antioxidative capacity (FRAP) of 1 g muscle and the corresponding amount of press juice (PJ) (0.21, 0.23 and 0.40 mL for LMPJ, WMPJ and SPJ respectively) during an *in vitro* gastrointestinal (GI) digestion. The samples (n = 6) were measured in triplicate and the results were presented as the mean ± standard deviations.
LMPJ: light muscle press juice of saithe, WMPJ: whole muscle press juice of saithe, SPJ: press juice of shrimp, LMS: light muscle of saithe, WMS: whole muscle of saithe, SWM: whole muscle of shrimp, FRAP: ferric reducing ability of plasma.

4.3.5 Antioxidative capacity (ABTS) of press juice

In contradiction to the two previous methods, the samples did not show any clear trend in the ABTS values (figure 10). Before the digestion had started (after addition of pepsine

solution, pH adjustment and centrifugation), the PJ samples exhibited ABTS values between approximately 2-8 mmol TE/L sample. After the first 30 minutes the values from the two digestions differed in the trends resulting in high standard deviations. After 75 minutes and throughout the digestion the samples increased or decreased randomly.

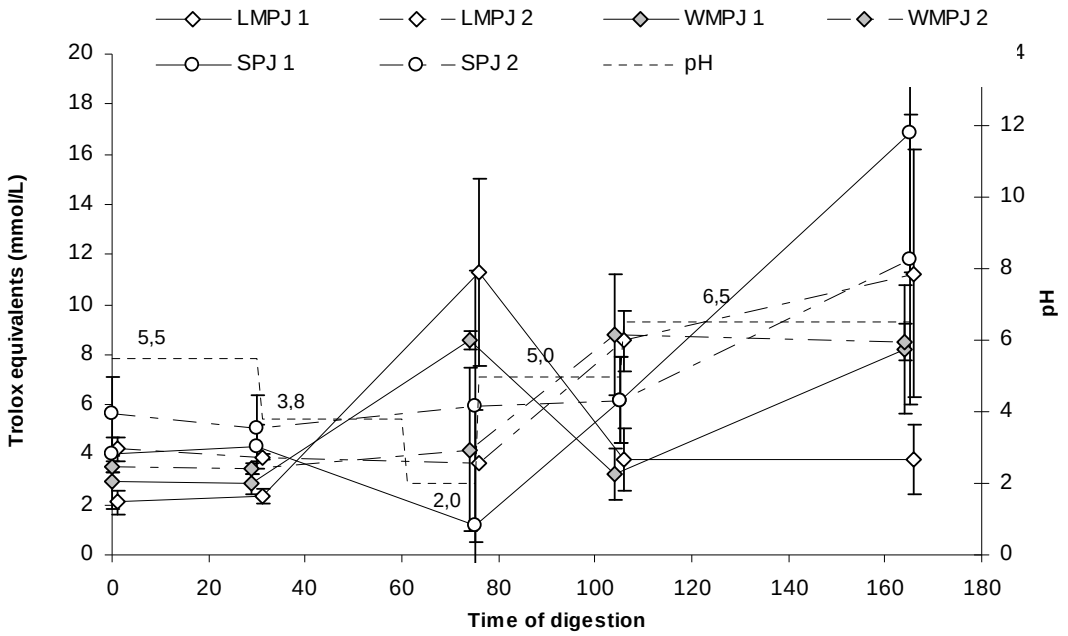

Figure 10. Antioxidative capacity (ABTS) in press juices (PJ) during an *in vitro* gastrointestinal (GI) digestion of 15 mL of light muscle press juice of saithe (LMPJ), whole muscle press juice of saithe (WMPJ) and press juice of shrimp (SPJ). 1 and 2: first and second digestion, ABTS: 2,2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

4.4 Inhibition of low density lipoprotein oxidation

The inhibition of oxidized LDL was measured in samples from LMPJ collected after 30, 75 and 105 minutes of digestion. A typical LDL oxidation curve for control and sample is presented in figure 11. Three phases were seen; a lag phase where diene absorption increased slowly, a propagation phase with a rapid increase in diene absorption, and a diene decomposition phase. No significant difference in lag time for the different samples was observed. As presented in table 9 the control showed a higher value of Vmax than the samples, and time to maximum tended to be longer when sample antioxidants were present, although this was not significant. Since Vmax, time at maximum, Amax and a delay in the lag phase are used to characterize LDL oxidation inhibition; a total measure of the area under the curve is also presented in table 9. Except from the sample collected after 105 minutes of digestion

(sample 4), there was a tendency of inhibition of LDL oxidation, not significant however: The Vmax for the control was 0.32 while it for the samples collected after 30, 75 and 105 minutes of digestion, was 0.26, 0.31 and 0.30 respectively. The time at maximum oxidation was shorter in the control (370 minutes) than when samples were present (390, 400 and 380 minutes for sample 2, 3 and 4 respectively). The total area under the curve was 8850 for the control. For the LMPJs it was 7400, 7700 and 9300 for sample 2, 3 and 4 (that is 30, 75 and 105 minutes after the digestion had started) respectively. The sample collected 105 minutes after the digestion had started (sample 4) did not show inhibition of LDL oxidation when characterized by area under curve.

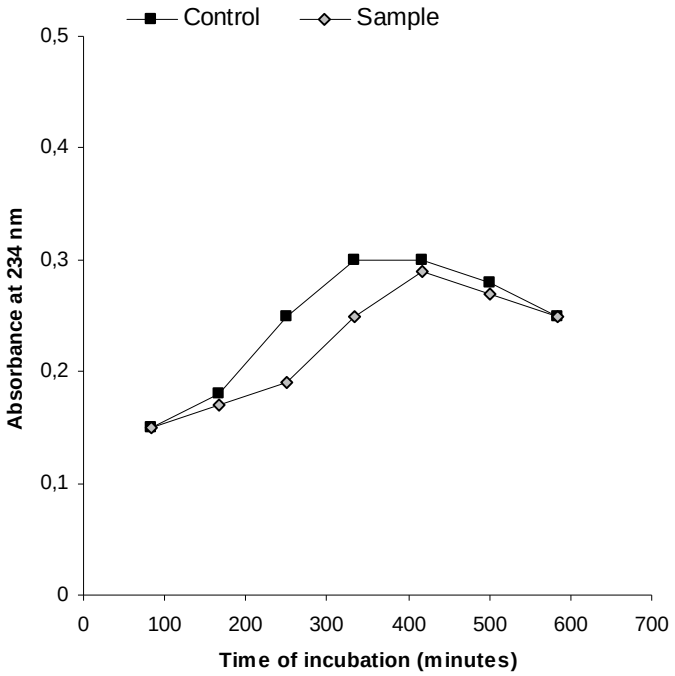

Figure 11. Representative absorbance curve obtained during Cu^{2+}-induced low density lipoprotein (LDL) oxidation. Control (50 mM phosphate buffer) and LMPJ are shown.
LMPJ: light muscle press juice of saithe.

Table 9. Normally used characteristics of Cu^{2+}-induced low-density lipoprotein (LDL) oxidation, in the presence of press juice from light muscle of saithe (LMPJ) samples.

Sample	Vmax (absorbance)	Time at max (min)	Area under curve
Control	0.32 ± 0,0	370 ± 30	8850 ± 1000
Sample 2	0.26 ± 0,0	390 ± 18	7400 ± 100
Sample 3	0.31 ± 0,0	400 ± 28	7700 ± 500
Sample 4	0.30 ± 0,0	380 ± 0	9300 ± 100

The samples (n = 6) were measured in duplicate and the results were presented as the mean ± standard deviation. Vmax: maximum rate of oxidation.

5. Discussion

Academic interest for natural antioxidants, in general and from marine peptides, has increased in recent years, spurring several publications. AOC has been measured and reported in hydrolysates from different marine species such as Alaska pollack (Je *et al.*, 2005), yellow stripe trevally (Klompong *et al.*, 2007), shrimp (De Rosenzweig Pasquel and Babbitt, 1991), tuna (Je *et al.*, 2007), mackerel (Wu *et al.*, 2003), hoki (Kim *et al.*, 2007), herring (Sannaveerappa *et al.*, 2007a) and oyster (Qian *et al.*, 2008). Only the three latter have reported AOC during or after a GI digestion. Digestion by GI proteases could be used as a unit operation in a production process for peptides with antioxidative effects, with the advantage that the formed peptides would resist the physiological digestion after oral intake.

One of three aims of this study was to implement three well known methods to measure AOC of extracts; namely ORAC, FRAP and ABTS. The ORAC assay was used in order to compare the AOC of PJ from saithe and shrimp to an earlier report on AOC of herring PJ during an *in vitro* GI digestion, by Sannaveerappa *et al*. (2007b). As figure 4 and 5 displayed, the ORAC values increased during digestion up until the phase simulating the small intestine. This is in accordance with the results obtained by Sannaveerappa *et al.* (2007b). Sannaveerappa *et al.* (2007b) reported that the highest ORAC value during the digestion of herring PJ was observed after 75 minutes of digestion and was approximately 25 mmol TE/L sample. In this study, the PJ samples of saithe and shrimp, collected after 75 minutes of digestion, exhibited ORAC values of approximately 36 and 30 mmol TE/L sample respectively. 100 g herring was used to obtain 15 mL PJ (Sannaveerappa, 2007). In this study 100 g LMS, WMS and SWM yielded approximately 21, 23 and 40 mL PJ respectively. This indicates that PJ from less than 100 g of saithe and shrimp muscle exhibited elevated AOC compared to PJ from 100 g herring. Reasons for this difference could be due to inherent differences between herring, saithe and shrimp. Many of the amino acids known to inhibit oxidation, such as methionine, alanine, proline, leusine and glycine (Fang *et al.*, 2002; Marcuse, 1960), are more abundant in proteins of saithe than of herring (Lie *et al.*, 1994). Shrimp muscle is known to have even higher levels of proline, glycine, taurine and arginine (Lie *et al.*, 1994). Difference in structural proteins between species e.g. content of amino acids and exposure patterns of them, may explain why the rise in AOC during the *in vitro* GI digestion was not proportional between the species. Shrimps are also known to contain protein combined astaxanthin. Thus the release of astaxanthin from these may also enhance

AOC. The AOC of most amino acids is dependent on pH (Marcuse, 1962). In this assay all samples were buffered to pH 7.4 prior to analysis, so there were no differences in pH that could explain the difference in AOC during the GI digestion. The differences could most likely be explained by the amount, quality and size of the peptides formed as a consequence of digestion by pepsin and bile/pancreatine enzymes. Bioactive peptides usually contain 3-20 amino acid residues and their activity is based on the amino acid sequence (Pihlanto-Leppälä, 2001). The low molecular weights peptides (< 1 kDa) have shown to exhibit elevated AOC (Je *et al.*, 2005; Kim *et al.*, 2007). The results obtained from the ORAC assay, showed that the digested peptides, thus expected to be smaller, from muscle samples exhibited higher AOC. The increase in AOC of muscle was observed throughout the digestion, although not significant after the phase simulating the small intestine. This increase was not seen in the the PJ samples. This may indicate that PJ is easily digested and that the proteins were already digested until a degree where a high proportion of the antioxidative amino acid residues or side chains had been exposed when entering the "small intestine" (Sannaveerappa *et al.*, 2007b). Dipeptides like carnosine and anserine, together with the tripeptide glutathione, are also released during digestion.

The protein content was measured (table 8) and a significant increase up until the stage simulating the small intestine was recorded. Thereafter no significant change was measured. A correlation between the protein content and AOC measured by ORAC ($r = 0.802$ and $r = 0.856$ for LMPJ and WMPJ respectively) was calculated, indicating that an increase in protein content would lead to an increase in the AOC. A lower correlation was calculated for the SPJ ($r = 0.631$). The lower correlation seen between ORAC values of SPJ and the protein contents could be due to high content of the free amino acids arginine and taurine in SPJ. Taurine is an exclusively free amino acid (FAA) and therefore the digestion would not have an impact on the amount of taurine. Arginine is generally located on the outside of the proteins, and would therefore be released already in the first phase of digestion. The increase in protein content did however not match the increase in the ORAC values. This may indicate that the AOC is not exclusively due to the protein content, but also due to the increase in the amount of FAA that might act as peroxyl radical scavengers (Je *et al.*, 2005). It should, however, be emphasized that the Bio-Rad DC assay is not an adequate method to measure the protein, peptide and amino acid content in hydrolysates, but a method developed to measure the protein content only. Previously in our laboratory, another method, O-Phthalaldehyde spectrophotometric assay for proteinases (Church *et al.*, 1985) was used to determine the content of peptides in hydrolysates. The chemicals used in this assay are hazardous and it was

chosen not to use this method. In retrospect, it is seen that an improved ninhydrin method (Solgaard *et al.*, 2008) or an improved method for determining food protein degree of hydrolysates (Nielsen *et al.*, 2001), could have been used with a higher possibility to acquire more information about the peptides and amino acids.

Tyrosine, methionine, histidine, lysine and tryptophan are amino acids that are readily oxidized. These are shown to be present in antioxidant peptides (Chen *et al.*, 1996). Cysteine, methionine and taurine are sulphur containing amino acids that exhibit an elevated AOC the more reduced they are (Atmaca, 2004). The content of FAA acids and total amino acids were examined in both light muscle and LMPJ of saithe in sample 2 and 3, that is 30 and 75 minutes after the digestion had started. This was done to study the possible effect of a difference in amino acid content which could explain the increase in AOC. However, no significant difference in FAA or total amino acids between the samples (results not shown) was observed.

The FRAP assay was also used to measure the AOC of the samples. The trend during the digestion was similar to that observed in the ORAC assay, but with significantly lower values. The correlation between the protein content and the AOC measured by FRAP was calculated ($r = 0.963$, 0.680 and 0.741 for LMPJ, WMPJ and SPJ respectively). The increase seen in the FRAP values was nevertheless insignificant.

The AOC of the samples was significantly higher measured by the ORAC assay than by the FRAP assay. The ORAC assay is an H-atom transfer reaction based assay while the FRAP assay is an electron transfer reaction based assay. This indicates that the H-atom donating capacity of the samples might have been greater than the electron-donating capacity. It should however be taken into account that the ORAC assay is performed at pH 7.4, while the FRAP assay is performed in acidic environment at pH 3.6. Under such acidic conditions, the carboxyl groups of the amino acids are protonated. Thus detection of AOC may not be optimal using the FRAP assay.

The ABTS values for the samples were inconclusive and exhibited large standard deviations. In previous papers the method has been performed in 1 mL quantity (Binsan *et al.*, 2008; Re *et al.*, 1999). Because of the size of the equipment it was necessary to perform the assay in smaller volumes, and this might have been one of the reasons for the large standard deviations. Both ORAC and FRAP assays were performed in buffer solutions with pH 7.4 and 3.6 respectively. All the samples were therefore buffered to the same pH and this would rule out any difference in AOC due to different state of reduction. In the ABTS assay the samples

were not buffered to an equal pH and this may be another reason for this. Thus the method needs further development to be applied.

Both ORAC assay and LDL assay were performed at physiological conditions (pH 7.4 and 37 ^{o}C), and both methods can therefore be considered as more accurate methods than other AOC measuring assays. The fact that the reactions are allowed to go to completion may contribute to the similarity to oxidation reactions in the human body. LDL is present in the human body and consequently the LDL assay can be regarded as similar to *in vivo*. The second aim of this study was to analyse the LDL oxidation inhibiting capacity of the samples. As seen in table 9 there was inhibition of LDL oxidation in the samples collected 30 and 75 minutes after the digestion had started, but not after 105 minutes. Because of extreme standard deviations it was difficult to interpret the results. The variations between each run were large, even though the same LDL batch and the same Cu^{2+} concentrations were used throughout this study (data not shown). This could be due to small variations in room temperature, plate preparation time or storage time (Kleinveld *et al.*, 1992; Sannaveerappa *et al.*, 2007b). The mechanism of action of antioxidants in oxidation of LDL has been reviewed by Pinchuk and Lichtenberg (2002). An increase in the lag phase together with no effect in ODmax and Vmax, suggests that the mechanism of the antioxidant could be quenching and stabilizing free radicals. To interpret the results obtained in this thesis, the lag phase was not significantly prolonged and together with a reduction in Vmax and ODmax, indicating that the mechanism of the antioxidant would be a non-radical decomposition of hydroperoxides. However, one could speculate about the relevance of this method. It is questioned whether haem and iron ions are proatherosclerotic *in vivo*, and evidence for copper being atherosclerotic are even more scarce (Halliwell and Gutteridge, 2007). The principle of the method is, as previously explained, to examine the lag phase of LDL oxidation, the slope of the acceleration phase and the level of conjugated dienes. *In vivo,* the factors influencing LDL oxidation are, among others, the amount of PUFA in the diet, the O_2-concentration in the blood, and the lipid peroxide content of the LDL particle. *In vitro* methods are usually performed at ambient O_2 concentration (21 %), while in blood the concentration is normally as low as 2.5 %. Copper ions appear to decompose peroxides in the LDL to chain-propagating radicals, and therefore the lipid peroxide content of LDL influences the results (Halliwell and Gutteridge, 2007).

Earlier reports have concluded that the AOC of seafood is mainly due to the water soluble fraction (Bhadra *et al.*, 2007; Undeland *et al.*, 1998), indicating that PJ would exhibit a higher AOC than muscle. Thus the third aim of this study was to compare the AOC exerted

from seafood muscle to that of the PJ. In order to compare the muscle with the PJ, it was chosen to calculate and present (table 10) the AOC of 100 g of muscles or the amount of PJ possible to "extract" from 100 g of muscle (21 mL LMPJ, 23 mL WMPJ and 40 mL SPJ (table 6)). It should be emphasized that to make these comparisons, it was necessary to make a rather rough assumption that the whole amount of digested "raw materials", arrived to the "small intestine". The comparisons were exclusively made on sample 3; that is after 75 minutes of digestion. The reason for this is that at this point, the difference between the "raw materials" was the greatest. In addition the "raw materials" are entering the stage of uptake and may possible be expected to have a physiological impact.

Table 10. Amount of "raw materials" digested, antioxidative capacity (AOC) measured by ORAC assay and AOC calculated to be in 100 g seafood.

Raw material	Amount digested PJ (mL), muscle (g)	AOC after 75 min mmol TE/L sample	AOC mmol TE/100 g
LMPJ	15	34	1.4
WMPJ	15	38	1.8
SPJ	15	30	2.4
LMS	1	9	14.4
WMS	1	10	16.0
SWM	1	13	21.0
Herring PJ[1]	15	25	0.75
Blueberry[2]	-	-	7

[1]Adopted from Sannaveerappa *et al.* (2007b).
[2]Adopted from Jensen (2007).
LMPJ: light muscle press juice of saithe, WMPJ: whole muscle press juice of saithe, SPJ: press juice of shrimp, LMS: light muscle of saithe, WMS: whole muscle of saithe, SWM: whole muscle of shrimp, ORAC: oxygen radical absorbance capacity assay.

As seen in table 10, the AOC of 100 g of muscle was approximately 10-fold higher than the related PJ for both saithe and shrimp. A muscle contains all the PJ in addition to all the myofibril proteins and water insoluble fractions.

Recent years a strong focus has been directed to, among others, blueberry and its antioxidative potential. Previous studies on the AOC of blueberry measured by FRAP (Halvorsen *et al.*, 2002) reported a capacity of approximately 8 mmol TE/100 g of fresh blueberry. In this study, when 100 g of LMS, WMS and SWM was digested, the FRAP values

after 75 minutes of digestion were 0.4 mmol TE/100 g saithe and 0.9 mmol TE/100 g shrimp. AOC in blueberry measured by ORAC has been reported to 3-7 mmol TE (Jensen, 2007; Zheng and Wang, 2003). In this study the ORAC values obtained after 75 minutes of digestion of saithe and shrimp were 15 and 21 mmol TE/100 g respectively.

Parra *et al*. (2007) measured the malondialdehyde (MDA), the end product of lipid peroxidation, in obese men and women before and after different energy-restricted diet based on cod, salmon and fish-oil supplements. The MDA significantly declined after intake of a cod-based energy-restricted diet. These results may indicate that antioxidative species like the ones found in this thesis may have physiological impact.

5.1.1 Limitations of the study

The method used in this thesis to measure the peptide content in the hydrolysate was not optimal. If further work were to be performed, the protein measurement should have been repeated on more samples and with the improved ninhydrin method explained by Solgaard *et al.* (2008) or the method explained by Nielsen *et al.* (2001), to possibly obtain more information on to what extent the total amount of peptides and amino acids effect the AOC. Thus the effects of exposure of amino acid residues, size of peptides and differences in amino acid sequences may be easier to interpret. A full separation of peptides exhibiting the highest AOC and amino sequences would of course give a more complete understanding of the mechanisms involved in the AOC of the "raw materials".

Different ways of food processing and different ways of household preparations influence the nutrient content in food. Larsen *et al*. (2007) found that traditional cooking methods resulted in a substantial loss of some biologically active compounds, such as taurine, creatine, free glycine and free alanine. The results obtained in this thesis concluded that the majority of the AOC of a seafood muscle was not released from the water fraction. This is due to less protein being in the samples of PJ. It would therefore also be relevant to analyse the AOC of saithe and shrimp in respect to different ways of household preparation; such as boiling, brining and baking. Structure proteins in seafood and terrestrial animal do not differ too much. Meat from terrestrial animals and seafood are generally treated as substitutable protein sources in a household meal. A study of the release of antioxidants during digestion of muscle from both terrestrial animal and seafood, to compare the two sources of proteins, would therefore be of great interest. Many questions remain unanswered and call for future work.

6. Conclusion

ROS are inevitably produced in our body during respiration and in the immune defence system. Antioxidants may help to inhibit development of damages caused by these ROS. This thesis has examined the changes in AOC of muscle from saithe and shrimp and PJ during an *in vitro* simulated GI digestion. The results showed that AOC of both muscle and PJ from saithe and shrimp increased up until the phase simulating the small intestine, after 75 minutes of digestion, before it stabilized. The difference between the results obtained from light muscle of saithe and whole muscle of saithe was insignificant. After addition of pancreatic and bile solutions, the AOC did not increase any further, which may indicate that the peptides containing amino acids with antioxidative residues, had already been digested to a complete degree. Inhibition of copper-mediated oxidation of LDL was measured in LMPJ. The samples collected after 30 and 75 minutes of digestion inhibited the oxidation of LDL. However, due to the high standard deviations, the results obtained cannot be regarded as significant. Muscle from saithe and shrimp showed elevated AOC compared to PJ from the related muscle. Measured by ORAC, the values obtained from seafood muscle exceeded the values obtained from the PJs 10-fold.

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