Effect of processing on antioxidative capacity of seafood muscle during a simulated gastrointestinal digestion

by

Hogne Abrahamsen

MASTER THESIS IN FISHERY SCIENCE

SEAFOOD SCIENCE

60 CREDITS

Department of Marine Biotechnology

Norwegian College of Fishery Science

University of Tromsø

May 2009

Acknowledgements

The work behind this master thesis was carried out at the Institute of Marine Biotechnology at the Norwegian College of Fishery Science, marking the end of six exciting years.

The greatest gratitude goes to my supervisor Edel O. Elvevoll for her inspiration, all-embracing knowledge and her genuine interest and contagious good humor. All the good advice, both professional and life in general, will be kept close. Thank you.

Special thanks go to Ida-Johanne Jensen, Hanne Mæhre, Birthe Vang and Rune Larsen who relentlessly helped me with methods and other obstacles in the lab. I would also like to thank Ida, Renate, Thomas and Ole Håkon, my officemates, for their friendship and off-topic conversations.

Finally, I would like to thank my family and friends who knew what to do when I was absent-minded.

Tromsø, May 2009

Hogne Abrahamsen

Sammendrag

Reaktive oksygenforbindelser (ROS) er en fellesbetegnelse for frie radikaler og reaktive oksygenforbindelser. Disse produseres kontinuerlig i kroppen. Oksidativt stress er en tilstand som representerer en ubalanse mellom antioksidanter og pro-oksidanter i favør pro-oksidantene. I levende organismer og kan denne tilstanden føre til skader på fettsyrer, proteiner og DNA. Den klart viktigste forsvarsmekanismen i forhold til oksidering er antioksidanter.

Helseaspektet i forbindelse med sjømat har primært vært knyttet opp mot langkjedede fettsyrer. I løpet av den siste tiden har imidlertid flere studier bekreftet at det finnes andre helsefremmende stoffer i sjømat, blant annet antioksidanter.

Formålet med denne oppgaven var å undersøke antioksidativ kapasitet (AOK) i muskel fra sei og reke gjennom en simulert fordøyelse, og undersøke om AOK forandret seg ved tradisjonell og industriell tilberedning. Dette ble gjort ved hjelp av de to metodene ORAC (oxygen radical absorbance capacity) og FRAP (ferric reducing antioxidant power). I tillegg ble hydrolysegraden gjennom fordøyelsen undersøkt. Frie og totale aminosyrer ble også analysert på utvalgte tidspunkt i fordøyelsen.

AOK i sei og reke økte gjennom hele den simulerte fordøyelsen. Den totale økningen ved fullført fordøyelse var på mellom 20 og nesten 40 ganger målt ved hjelp av ORAC. En lik trend var sett når prøvene var målt ved hjelp av FRAP, bare med signifikant lavere verdier. Hydrolysegraden hadde som ventet en tilsvarende økning gjennom fordøyelsen. Sammensetningen av aminosyrer i muskel er av stor betydning for frigjøring og aktivitet av antioksidative peptider. Denne studien konkluderer med at AOK i sei og reker øker gjennom en simulert fordøyelse. Effekten av tradisjonell varmebehandling av sei var minimal i forhold til AOK, og effekten av prosessering av reker var nærmest ikke-eksisterende i forhold til AOK.

Summary

Reactive oxygen species (ROS) are free radicals and non radical oxygen species which are unavoidably produced in the body. Oxidative stress represents a disturbance in the equilibrium status of pro-oxidants and antioxidants reactions in favor of the pro-oxidants. In living organisms and the condition can damage cellular lipids, proteins, or DNA. By far the most important defense mechanism against oxidation is the presence of antioxidants.

The health aspects of seafood have primarily been linked to long-chained polyunsaturated fatty acids. However, numerous studies have reported that seafood contains additional beneficial bioactive compounds, such as antioxidants.

The objectives of this study were to investigate changes in antioxidative capacity (AOC) of saithe and shrimp muscle during a simulated gastrointestinal digestion, in particular the effect of traditional household and industrial processing. The AOC of seafood muscle was evaluated by two the methods ORAC (oxygen radical absorbance capacity) and FRAP (ferric reducing antioxidant power). Additionally, the degree of hydrolysis during digestion was determined. Levels of free and total amino acids were also measured at selected time points.

AOC of seafood muscle increased throughout the gastrointestinal digestion. The total increase at the end of digestion of the samples was between 20- and near 40- fold when measured by ORAC. The FRAP assay revealed a similar trend, only with significantly lower values. A concurrent rise in degree of hydrolysis was also recorded. The composition of amino acids in the muscles may be an important feature for the release and activity of the antioxidative species. The current study concludes that AOC of saithe and shrimp muscle increase throughout gastrointestinal digestion. Effects of household preparation of saithe were minimal to levels of AOC, and the processing of shrimps had virtually no effect to levels of AOC.

KEYWORDS: saithe, shrimp, digestion, antioxidative, peptides, processing.

Abbreviations commonly used

AOC	Antioxidative capacity
DH	Degree of hydrolysis
DW	Dry weight
FRAP	Ferric reducing antioxidant power
GI	Gastrointestinal
OPA	o-phthaldialdehyde
ORAC	Oxygen radical absorbance capacity
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
TE	Trolox equivalents

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1 Introduction

1.1 Seafood and health

Today industrialized societies experience a decrease in protein and antioxidants intake. Along with an increased intake of saturated fat, n-6 fatty acids and trans-fatty acids, the Western diet promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, inflammatory and autoimmune diseases (Simopoulos, 1991; O'Keefe Jr. and Cordain, 2004). Seafood is an important constituent in the human diet and is considered to be desirable for health promotion, particularly because of its inverse association in observational studies with the risk of cardiovascular disease (He *et al.*, 2004; Mozaffarian and Rimm, 2006; Virtanen *et al.*, 2008). Research in this subject started more than thirty years ago when it was reported that Greenland Eskimos living on their traditional diet had a lower incidence of coronary heart disease (Dyerberg *et al.*, 1978). Since that observation, marine polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), quickly became the focal point in studies regarding beneficial health effects of seafood. Lately these health effects have extended to include benefits related to rheumatoid arthritis (Kremer *et al.*, 1985), neurodegenerative disorders (Dyall and Michael-Titus, 2008) and suggested positive effect on psychiatric conditions (Buydens-Branchey *et al.*, 2008). Seafood also seems to prevent development of certain cancers (Sasaki *et al.*, 1993; Caygill and Hill, 1995), particularly those of breast and colon (Bourre, 2007). Clinical trials have confirmed that marine n-3 PUFAs are indeed reducing the risk of coronary heart disease (Schmidt *et al.*, 2005a; 2005b). The amount of marine n-3 PUFAs used in such clinical trials is much higher than the amount typically found in the diet, yet consistent evidence indicates that modest consumption of both lean and fatty fish lowers the risk of cardiac death and other cardiovascular events (Gillum *et al.*, 1996). This may suggest that fish have additional beneficial nutrients to those present in fish oil.

From a nutritional point of view, seafood is a well balanced source of proteins (Friedman, 1996; Njaa, 1990). It contains high level of minerals and trace elements, such as selenium (Zhang *et al.*, 1993) and iodine (Dahl *et al.*, 2004) and high levels of vitamins such as A, D and B_{12} (Braekkan, 1958; Sidwell *et al.*, 1978; Lie, 1994). In recent years there has been growing interest in biological active compounds in food, which are not regarded as essential nutrients, but beneficial under certain circumstances. Antioxidants are examples of such compounds.

Reactive oxygen species (ROS) are products of normal cellular metabolism. At moderate concentrations ROS plays a role e.g. in defense against infectious agents and induction of mitogenic response (Valko *et al.*, 2006). At high concentrations ROS are potentially harmful and the condition is termed oxidative stress (Kovacic and Jacintho, 2001). This occurs in biological systems when there is an overproduction of ROS on one side, and a deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress represents a disturbance in the equilibrium status of pro-oxidants and antioxidants reactions in living organisms. The condition can damage cellular lipids, proteins, or DNA. Thus, oxidative stress has been related to a number of human diseases as well as ageing. Higher intakes of antioxidants has been linked to a lower incidence of oxidative stress (Cemeli *et al.*, 2009).

Seafood, with its PUFAs, is very susceptible to oxidation. Hence, seafood might have a stronger antioxidative defense system. Antioxidative capacity (AOC) of peptides from seafood have been reported in several species of fish including hoki (Kim *et al.*, 2007), yellow stripe trevally (Klompong *et al.*, 2009) and shrimp (Binsan *et al.*, 2008). These studies measured AOC after digestion by different pepsin and enzyme mixtures, and further purifications. Changes in AOC of seafood in samples collected at different time points during a simulated gastrointestinal (GI) digestion have been investigated by Sannaveerappa *et al.* (2007) and Jensen (2008). However, changes in AOC of processed seafood during GI digestion are studied to a lesser extent. *In vivo* AOC of fish proteins have previously been discussed. 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 accurate method to study biological activities is such clinical trials in humans. If performed with a firm control of diet, study length, subjects, gender and age this would give the highest level of evidence. This is, however, time consuming and not really applicable in early stages of assessments. In contrast, *in vitro* techniques are important in the early phases as they are inexpensive, rapid and may serve as efficient tools for screening. The results may be hard to extrapolate to humans but may be used to choose directions for further work.

Two common seafood items were selected for this study. Saithe (*Pollacius virens*), a fish abundant in the North-East Atlantic was chosen because it is the most active swimmer among the gadoid species. It is not a fatty fish, but has a pelagic lifestyle and therefore the largest portion of dark muscle. Dark muscle is more prone to lipid oxidation than light muscle due to high fat content and haematin compounds that catalyze lipid oxidation (Castell and Maclean, 1964). Shrimp (*Pandalus borealis*) was chosen because it is rich in amino acids known to

exhibit AOC like arginine, glycine and proline together with proteins and vitamins (Lie, 1994).

The objectives of this study were to investigate changes in AOC of seafood muscle during a simulated GI digestion, and whether traditional household (saithe) and industrial (shrimp) thermal processing had an effect on AOC in seafood.

The specific aims of this study were limited to:

- Validate an *in vitro* GI digestion protocol with use of seafood muscle.
- Study the course of hydrolysis during an *in vitro* GI digestion of processed and unprocessed seafood muscle.
- Investigate AOC during *in vitro* GI digestion of processed and unprocessed seafood muscle.

1.2 Food processing

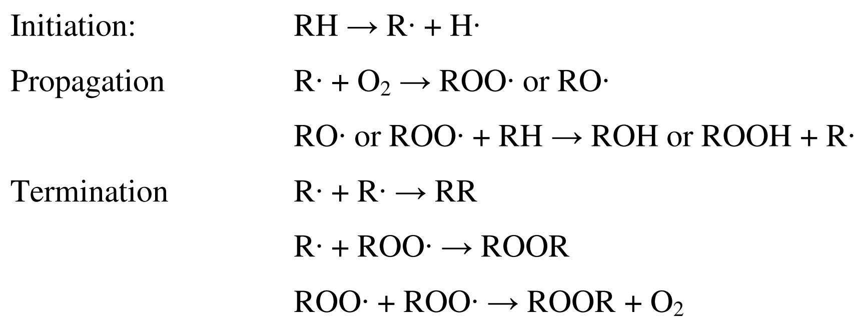
There is a growing awareness that dietary source and form of food may affect the overall health of the consumer. As aforementioned, the observations of Greenland Eskimos inspired researchers to investigate the health aspect of a seafood diet. Other aspects of the Eskimo diet are not fully elucidated. Traditionally, the Eskimos consume the main part of their food raw or dried, seldom boiled or exposed to excessive heat (Bang *et al.*, 1980). There are both positive and negative effects of thermal processing of food, and the degree of heat-induced changes depends upon treatment applied. The advantages include killing of pathogens, inactivation of antinutrient enzymes and an increase in digestibility and bioaviability (Finley *et al.*, 2006). Thermal processing may also give the product a favorable taste. Conversely, processing may alter, destroy or remove components with beneficial effects from the product. Sarcoplasmic proteins, peptides, amino acids and other water-soluble components are in various degree transported out of the product with drip, and when subjected to processing these losses are generally amplified (Ofstad *et al.*, 1996). Purchas *et al. (2004)* and Dragnes *et al.* (2009) showed that thermal processing of beef muscle and seafood muscle respectively, led to a significantly decline in several bioactive compounds.

1.3 Oxidation

The term oxidation applies to chemical reactions in which electron transfer occurs, where oxidation is loss of electrons and reduction is gain of electrons. Compounds that undergo reduction readily are oxidizing agents; those that undergo oxidation are reducing agents.

1.3.1 Oxidation in seafood

As previously mentioned, PUFAs have attracted considerable attention due to their beneficial health and physiological effects. PUFAs are however, very susceptible to oxidation and have received some attention because of its potential involvement in damage of biological systems (Garrido *et al.*, 1989; Hu *et al.*, 1989). Enzymatic and photogenic oxidation may play a role in degradation of seafood, but the two major components involved in seafood oxidation are unsaturated fatty acids and oxygen. The process by which unsaturated fatty acids and oxygen interact is a free radical mechanism and involves three main phases:



In the initiation phase, an unsaturated fatty acid (RH) looses hydrogen, resulting in a lipid radical (R·)[1], which in turn reacts with oxygen to form a lipid peroxyl radical (ROO·). In the propagation phase lipid-lipid interaction occurs, and ROO· abstracts hydrogen from an adjacent RH creating a lipid hydroperoxide (ROOH) and a new R·. Interactions of this type continue up to 100 times (Gutteridge and Halliwell, 1990) before two R· combine and terminate the process. Transition metals such as iron (Fe^{3+}) and copper (Cu^{2+}) magnify lipid oxidation through branching reactions. These metals react directly with ROOH creating new radicals such as alkoxy radical (RO·), hydroxyl radical (OH·) and ROO·.

1.3.2 Oxidation in the human body

More than 95% of the air humans breathe is used in mitochondria for efficient energy production by oxidative phosphorylation (Halliwell, 1997). The mitochondrial electron

[1] The radical dot (·) is inserted to indicate the presence of one or more unpaired electrons.

transport chain is a flow of electrons from NADH through a series of electron carriers to cytochrome oxidase which reduces oxygen to water. A small percentage of the electrons escape directly to oxygen from the electron carriers, and forms the one-electron reduction product of oxygen, superoxide radical ($O_2\cdot^-$) and the two-electron reduction product of oxygen, hydrogen peroxide (H_2O_2). As long as humans breathe, these ROS are constantly and unavoidably produced. In contact with transition metals, $O_2\cdot^-$ and H_2O_2 make a very reactive free radical species, OH·, which attack and damage cell structures, nucleic acids, lipids and proteins. The reason for this reactivity of ROS is that they have unpaired electrons in the outer orbit, and the solitary electron seeks stability by abduction of electrons from other sources.

ROS is a collective term and includes oxygen free radicals and several non-radical agents (table 1). A free radical in this context has been defined as: ”any species capable of independent existence (hence the term “free”) that contains one or more unpaired electrons” (Halliwell and Gutteridge, 2007). Some ROS ($O_2\cdot^-$ and H_2O_2) are produced on purpose in the human defense system as one of the mechanisms used to kill bacteria and fungi and to inactivate virus. As explained in chapter 1.1, problems arise when there is an unbalance between oxidants and antioxidants.

Table 1. Examples of reactive oxygen species, ROS.

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

1.4 Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, 1997). By far the most important defense mechanism against oxidation is the presence of antioxidants. Inhibition may take two forms; a reduction in the rate at which the maximal level of oxidation is approached and a reduction in the maximal level of oxidation. The term antioxidant is broad and has 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 (Halliwell *et al.*, 1995). In biological systems the definition of an antioxidant is broader than in food sciences. Halliwell and Gutteridge (2007) have defined antioxidants as “any substance that delays, prevents or removes oxidative

damage to a target molecule". This definition includes compounds of an enzymatic as well as a non-enzymatic nature.

1.4.1 Antioxidants expected to be present in seafood

Seafood exhibits a variety of antioxidative properties. In this study it is chosen to focus on proteins and derivates thereof; such as peptides and amino acids, together with astaxanthin, an antioxidant abundant in shrimps.

Proteins themselves may act as multifunctional antioxidants that can inhibit several different oxidation pathways due to complex interactions between their ability to scavenge free radicals, chelate prooxidative transition metals, inactivate ROS and reduce hydroperoxides. All amino acids found in proteins have potential to interact with ROS if the energy of the radical is high, such as an OH·. The ability of a protein to behave as an antioxidant depends upon the circumstance that the resulting protein radical has insufficient energy to initiate or propagate further oxidation reactions. Ostdal *et al.* (2002) suggested that the antioxidant activity of proteins may be due to their ability to act as radical trapping devices.

Additionally, enzymes are present in muscle tissues that may be classified as preventive antioxidants. Catalase converts $H_2O_2$to water and O_2, and its activity in saithe muscle have reported to be approximately 700 µmol/min/g tissue (Aksnes and Njaa, 1981). Enzymes of the superoxide dismutase (SOD) family is located in the cytosol, but also in lysosomes and nucleus, and are highly efficient in dismutating $O_2^{\cdot-}$ into H_2O_2 and O_2, thereby preventing further production of O_2^1. The peroxide removing enzyme glutathione peroxidase (GPx) is widely distributed in seafood muscle, and its electron donor, the reduced form of glutathione (GSH), is found at intracellular concentrations that often are in the millimolar range (Halliwell and Gutteridge, 2007). However, selenium is needed in within biological systems to maintain GPx activity. Passi *et al.* (2002) reported a level of GPx between 0.16 and 0.40 units/mg proteins, and a level of SOD of 1.9-9.7 units/mg protein in different Mediterranean fish species. One of the important antioxidants found in shrimp is the carotenoid astaxanthin. Hosokawa *et al.* (2009) has described astaxanthin to be a strong antioxidant by quenching singlet oxygen and by acting as a free radical scavenger, hence it should affect AOC of shrimp. Other antioxidants such as ascorbic acid, α-tocopherol, polyphenols and flavonides are expected to be present in seafood as well.

1.5 Methodological considerations

1.5.1 General comments

Analysis conditions, substrate, and antioxidant concentration must be evaluated when selecting AOC methods. The total AOC value should include methods applicable to measure scavenging of OH· and R·, removal of ROS and reactive nitrogen species and chelating of transition metals, with regards to the similarity and differences of both hydrogen atom transfer and electron transfer.

1.5.2 Methods used to measure antioxidative capacity

No single assay accurately reflects the mechanisms of action of all radical sources or all antioxidants in a complex biological system (Prior et al., 2005). On the basis of the inactivation mechanisms involved, major AOC methods have been divided into two categories: hydrogen atom transfer (HAT) reaction- and electron transfer (ET) reaction-based methods.

HAT-based methods measure the ability of an antioxidant to scavenge free radicals by hydrogen donation to form stable compounds, thus making the methods more relevant to the chain-breaking antioxidant capacity. The oxygen radical absorbance capacity (ORAC) assay is such method. HAT-based methods are generally composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. The protective effect of an antioxidant in the ORAC assay is measured by the net integrated area under a fluorescence decay curve of the sample. The area is compared to that of the blank and stands for lag time, initial rate, and total inhibition in a single value (Prior *et al.*, 2005). The ORAC assay using AAPH as a peroxyl generator measures all traditional antioxidants including ascorbic acid, α-tocopherol, β-carotene, GSH, bilirubin, uric acid and melatonin.

The ferric reducing antioxidant power (FRAP) assay is an ET-based assay. ET-based methods involve two components in the reaction mixture, antioxidants and oxidants. In the FRAP protocol, a ferric tripyridyltriazine complex is used as an oxidant which is reduced to a blue ferrous complex by abstracting electrons from antioxidants. The degree of color change is proportional to the antioxidant concentration and measured 30 minutes after initiation. To make the correlation between the results and AOC it is accepted that AOC is equal to reducing capacity.

1.5.3 The gastrointestinal tract

The gastrointestinal tract consists of the oral cavity, esophagus, stomach, small intestine, and finally the large intestine. These organs together with liver, gallbladder, gall-ducts, and pancreas are responsible for the digestion of foods. Digestion is the mechanical and enzymatic breaking down of food into entities that can be absorbed by the human body, and generally lasts four hours. During digestion, the physiological conditions, such as pH, are regulated to ensure optimal environment for the different enzymes. Within the confinement of a laboratory, some considerations must be made when designing a method that mimic the digestion in humans. A rather coarse assumption is made when assuming that the whole amount of food pass through digestion from one phase of digestion to another. Additionally, temperature and simulated mechanical muscle movement must be imitated along with gradient pH adjustment. Finally, a selection of digestive enzymes must be added at appropriate time points. In this study all samples were sealed with film during digestion, as oxygen exposure should be minimized.

1.5.4 Digestion of proteins

Proteins are very large molecules formed by multiple amino acids linked together by peptide bonds. The peptide bond is a chemical bond between two molecules when the carboxyl group of one molecule reacts with the amine group of the other molecule, and thereby releasing one molecule of water. This condensation reaction usually occurs between amino acids. Digestion reverses the condensation reaction by hydrolysis. 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).

The initial phase in the assimilation of dietary proteins includes homogenization of food by chewing. In the stomach, pepsinogen excreted by the chief cells is transformed to the active protein-cleaving enzyme pepsin in the acidic environment created by the gastric acid. The optimal pH for pepsin is between 2.0 and 3.5, and it is inactivated if pH exceeds 5.0. Pepsin initiates digestion of proteins up to 20 % of the total protein digestion by cleaving preferentially after the N-terminal of aromatic amino acids (Nelson *et al.*, 2008). Immediately after entering the small intestine, pH is neutralized (pH 6.5) and the pancreatic enzymes are excreted; trypsin and chemotrypsin being the most important ones. Trypsin cleaves the dietary proteins at arginine and lysine residues. Chymotrypsin catalyses hydrolysis of aromatic amino acid residues (Brody, 1999).

2 Materials and methods

2.1 Chemicals

Pepsin (P6887), pancreatin (P1750), bile extract (B8631), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2´-azobis (isobuttersaüreamidin) dihydrochloride (AAPH), fluorescein sodium salt, Iron III Chloride 6-hydrate (Fe), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), $C_{12}H_{25}NaO_4S$ (SDS), $C_4H_{10}O_2S_2$ (DTT) and o-phthaldialdehyde 97 % (OPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other solvents and chemicals used were from Merck (KGaA, Darmstadt, Germany) and of analytical grade. MilliQ-water (Milli-Q Academic. Millipore, Molsheim, France) was used for all solutions and preparations.

2.2 Materials

2.2.1 Shrimp

The shrimps were caught at Hopen, South-East of Spitsbergen in May 2008, and acquired from Stella Polaris AS (Finnsnes, Norway) in December 2008. Processed and raw shrimp used in this study were all from the same batch, and were block frozen within four hours at sea.

2.2.2 Saithe

Gutted and beheaded saithe were acquired in October 2008 from Dragøy AS (Tromsø, Norway), and the fish were caught by fishing net in Kvaløyvågen outside Tromsø the day before purchase.

2.3 Preparation of materials

2.3.1 Shrimp

Raw shrimps were thawed at 4 °C over night and peeled. The peeled shrimps were divided into three batches (n = 100), and homogenized at medium speed for 10 seconds in a food processor (Electrolux, Germany).

The processing of shrimps is a highly automated and multiple unit operation and took place at Stella Polaris AS. The shrimps are thawed and either kept on ice or in a weak brine to ease peeling. During peeling, the shrimps are constantly flushed with water before boiling takes place. The boiled shrimps were glazed with fresh water (9.2 – 12 %) and frozen as singles. At the laboratory, the processed shrimps were thawed at 4 °C over night. The melted water used

as glazing were removed before the shrimps were divided into three batches (n = 100), and homogenized at medium speed for 10 seconds in a food processor. All homogenized batches were labeled and frozen at – 55 °C in PE zip-bags until analysis.

2.3.2 Saithe

The fish (n=6, 1833 ± 283 g) were wrapped in plastic and stored on ice for three days. After resolution of rigor the fish were manually filleted, skinned and labeled. The lengths of the fillets (23 ± 1.5 cm) were 50 % the length of the beheaded fish.

The fillets (n = 9) were randomly divided into three groups with three fillets in each group. The fillets in two groups were either boiled or baked, while the fillets in the third group were not subjected to any preparation. The cooking time and temperature were based on traditional Norwegian preparation techniques, and the procedure ensured an internal temperature above 63 °C for 15 seconds, which is necessary for food safety reasons (FDA, 2005). Boiling was performed by adding fillets (n = 3) to a boiling 5 % NaCl (w/v) solution and simmered for 10 min. Baked fillets (n = 3) were prepared by wrapping pieces in aluminum foil and put in a preheated oven for 20 min at 175 °C. All fillets were considered properly cooked by visual inspection of its flakiness and opaqueness. Following cooking, the fillets were cooled and each fillet was homogenized at medium speed for 10 seconds in a food processor. The homogenized samples were labeled and frozen at – 55 °C in polyethylene (PE) zip-bags until analysis.

2.4 Study design

Saithe and shrimp were prepared in triplicate as described in chapter 2.3. Caution is necessary when sampling fish for AOC as local differences within each fillet exists (Love, 1970; Undeland *et al.*, 1998a; Undeland *et al.*, 1998b; Undeland *et al.*, 1999; Larsen *et al.*, 2007), thus all whole fillets and batches of shrimp were properly homogenized before subjected to GI digestion. Each type of sample was digested in triplicate. During digestion, aliquots from the samples were collected at five different time points (figure 1). Analysis of AOC (FRAP and ORAC), quantification of released amino groups (OPA) and levels of free and total amino acids, were performed on the aliquots. Measurements of AOC were performed in triplicate for each sample and method and the results were reported as the average of three parallels, each composed of three measurements. Quantification of released amino groups and determining levels of amino acids were performed on pooled samples. In this case pooled samples were made from equal amounts of one specific sample (raw, baked or boiled saithe, raw or processed shrimp) from one specific time point (0, 30, 75, 105 or 165

minutes of digestion). Amino acid analysis was only performed on aliquots from the start and after 75 minutes of digestion. All values, except OPA are given on basis of dry weight.

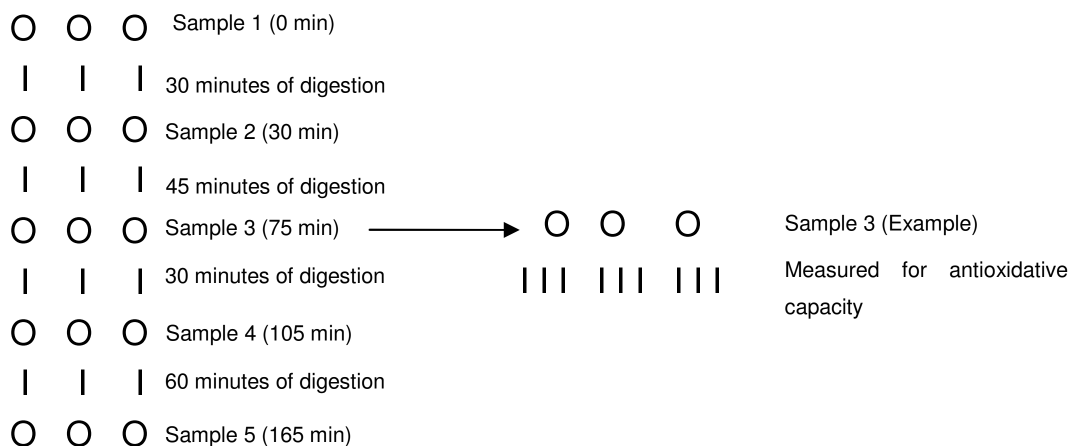


Figure 1. Study design of the digestion and measurement of antioxidative capacity (AOC). Raw saithe and shrimp, and processed shrimp were subjected to gastrointestinal (GI) digestion in triplicate. Baked, boiled saithe were prepared in triplicate before subjected to GI digestion in triplicate. At each time point a sample was collected from each of the triplicates. For each sample, AOC was measured in triplicate and the results were reported as the average of three parallels, each composed of three measurements. The figure is copied from Jensen (2008) with permission.

2.5 Proximate composition

Moisture, ash and nitrogen content were assayed by slightly modified versions of the AOAC International methods 925.04, 938.08 and 981.10, respectively (Cunniff, 1995).

2.5.1 Moisture and ash

Water content was determined by drying approximately 3 g of minced muscle at 105 °C to constant weight using, a Termaks laboratory oven (Termaks, Bergen, Norway). Next, the reduced weights of the samples were determined gravimetrically before the samples were combusted at 500 °C for 24 hours to determine ash content.

2.5.2 Nitrogen

Nitrogen content was determined using the Kjeldahl method. Briefly, 1 g muscle, 1 Kjeltec catalyst tablet and 10 ml H_2SO_4 were put into a Kjeldahl tube and digested for two hours at 420 °C. The product was then made basic by adding 30 % NaOH (w/v), before distillation into 0.1 M HCl and titration against 0.25 M NaOH. Dietary fats and carbohydrates do not contain nitrogen, and nearly all the nitrogen in the diet is present as amino acids in proteins. Furthermore, the average nitrogen (N) content in proteins is 16 percent; hence, protein

quantity can be calculated as N × 6.25 (1/0.16 = 6.25) (Jones, 1941). However, different foods have specific amino acid composition implying that a global conversion factor (Jones factor) is wrong. FAO (2003) recommends the use of specific Jones factors when determining protein content in food, and seafood has a Jones factor of 5.6 (Mariotti *et al.*, 2008). These recommendations is however not widely used in published reports, thus for comparison reasons the traditional Jones factor of 6.25 is used in this study.

2.5.3 Fat

Fat content was determined according to a protocol previously described by Folch *et al.* (1957). In brief, samples were homogenized with chloroform (2)/methanol (1) (v/v) to a final volume 20 times the volume of the samples, before the whole mixture was agitated during 30 minutes in an orbital shaker (Heidolph Multi reax, Heidolph Elektro GmbH & Co, Kelheim, Germany) at room temperature. The homogenate was filtrated to recover the liquid phase. Next, the solvent was washed with 0.2 volumes of 0.9 % NaCl (w/v) solution and mixed with a vortex mixer. After centrifugation of the mixture for 10 minutes at low speed and siphoning of the upper phase, the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator (Heidolph Laborota 4000, with Büchi vacuum controller B-72, BÜCHI Labortechnik AG, Postfach, Switzerland) to dryness. An amount of 5 ml heptane was added to the dried oil and the mixture was evaporated under nitrogen until the tube was completely dry, and fat content of the samples was determined gravimetrically.

2.6 *In vitro* gastrointestinal digestion

A slightly modified protocol from a previously described *in vitro* GI digestion procedure (Sannaveerappa *et al.*, 2007) was used in this study. Typically, 1 g of one of the following test samples; raw shrimp, processed shrimp, raw saithe, boiled saithe or baked saithe, and 1 ml 50 mM phosphate buffer with 0.9 % NaCl (w/v), pH 6.75 (control) was accurately weighed and mixed with 14 ml 50 mM phosphate buffer (pH 7.4) and 15 ml of 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. The pH was adjusted to 5.5 with 1 M HCl, and 6 ml of sample was collected at 0 minutes (sample 1). To minimize oxygen exposure, the samples were sealed with film before they were incubated in an Innova 4300 Incubator shaker (New Brunswick Scientific Co., Edison, New Jersey, USA) at 220 rpm for 165 minutes at 37 °C. After 30 minutes of incubation 8 ml of sample was collected (sample 2) and pH was adjusted to 3.8 with 1 M HCl. After 60 minutes pH was adjusted to 2.0 with 1 M HCl, and after 75 minutes 6 ml of sample was collected (sample 3). Subsequently, 1.5 ml of a pancreatic and bile solution

containing 4 g/l pancreatin, 25 g/l bile extract, and 0.1 M $NaHCO_3$ was added. The pH was then adjusted to 5.0 with 1 M NaOH. By adding the bile/pancreatin solution, the samples were diluted 1.15 times, which were taken into account during the calculations of the results. After 105 minutes of digestion, 6 ml sample was collected (sample 4), and pH was adjusted to 6.0 with 1 M NaOH. Finally, after 165 minutes of digestion, the remaining content was collected (sample 5). All samples (1-5) were immediately put on ice to stop the reactions and they were centrifuged at 4500*g* (Multifuge 1 S-R; Kendro Laboratory Products, Osterode, Germany) for 10 minutes to remove large particles. The supernatants were centrifuged once more at 4000*g* for 15 minutes to remove added enzymes. All filtrates were kept at -55 °C until further analysis. The digestion trials were repeated three times for each type of sample material.

2.7 Amino acid analysis

Amino acid analysis was carried out on pooled samples at selected time points during digestion (at start and after 75 minutes of digestion). Levels of free amino acids (FAA) were determined by loading an amount of 200 µl sample together with 100 µl 20 mM norleucine. MilliQ-water was added until a suitable concentration before proteins and peptides were removed by adding 100 µl 35 % sulphosalicylic acid. The mixtures were homogenized and centrifuged at room temperature, and an aliquot of the supernatants was diluted with a lithium citrate buffer (pH 2.2) to a suitable concentration before analysis. For determination of the total amino acids (TAA) in the samples, 1 ml sample was added to 200 µl 20 mM norleucine and 1200 µl HCl and hydrolysed for 24 hours at 110 °C. The hydrolysate (100 µL) was dried under nitrogen and diluted with 1 ml of a lithium citrate buffer (pH 2.2) before analysis.

All samples were analyzed using a Biochrom 30 Amino Acid Analyzer (Biochrom Limited, Cambridge, UK) with a lithium citrate equilibrated column and post column derivatization with ninhydrine. Norleucine served as an internal standard and the signal was analyzed with Chromeleon software (Dionex, Sunnyvale, CA, USA) and compared with A9906 physiological amino acid standard (Sigma Chemical Co, St. Louis, MO).

2.8 OPA method for the quantification of released amino groups

Released proteinous components in each sample during digestion were determined by analyzing the degree of protein hydrolysis based on the reaction of primary amino groups with o-phthaldialdehyde (OPA). The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved. The OPA method was carried out as described by Church *et al.* (1983), including the recommended modifications suggested by Nielsen *et al.* (2001).

The OPA reagent was made by completely dissolving $B_4Na_2O_7$ (0.133 M) and SDS (4.6 mM) in milliQ-water (solution 1). Separately, OPA was dissolved in ethanol to a concentration of 0.3 M and transferred to solution 1. Finally, 7.33 mM DTT was added to the solution before milliQ-water was added to make the final concentration of $B_4Na_2O_7$, SDS, OPA and DTT 99.9 mM, 3.46 mM, 5.96 mM and 5.7 mM respectively. The samples were suitably diluted according to protein content and expected DH. For the analysis procedure, a tube was loaded with 3 ml OPA reagent before 400 µl sample solution, milliQ-water (blank) or serine standard (0.95 mM $C_3H_7NO_3$) was added (time 0) and mixed on a vortex mixer. After exactly 2 minutes the mixture was measured spectrophotometrically at 340 nm using a Genesys 20 (Thermo Fisher Scientific Inc. USA).

Calculation

Determination of h:

$$\text{Serine NH}_2 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times 0.9516 \text{ meqv/l} \times \frac{0.1 - 100}{\text{X} \times \text{p}} \text{l/g protein}$$

Where serine-NH_2 = meqv serine NH_2/g protein.

X = µl sample.

P = protein percent in sample.

0.1 is the sample volume in liter.

h is then:

$$\text{h} = \frac{\text{serine NH}_2 - \beta}{\alpha} \text{meqv/g protein}$$

Calculation of DH:

$$\text{DH} = \frac{\text{h}}{\text{h}_{\text{tot}}} \times 100\%$$

Where α, β and h_{tot} are constants. In the case of fish α, β and h_{tot} are 1.00, 0.40, and 8.6 respectively (Adler-Nissen, 1986). The DH was expressed in percent of total hydrolysis of fish proteins. For each specific sample, triplicate measurements were performed.

2.9 Antioxidative capacity

2.9.1 ORAC

The ORAC assay was carried out according to Dávalos *et al.* (2004) on a Spectramax Gemini EM Fluorimeter (Molecular Devices, Sunnyvale, USA), which was equipped with an incubator and wavelength adjustable fluorescence filters. An excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. AAHP was used as a synthetic free radical generator and Trolox as a standard (0 – 100 µM) and the antioxidant capacity was quantified by calculating the net protection area under the time recorded fluorescence decay curve. The standard reaction of Trolox, aliquots of samples and control were carried out in a 75 mM phosphate buffer (Na_2HPO_4) at pH 7.4. Twenty microliters of suitably diluted sample and Trolox calibration solutions together with 120 µl of fluorescein (70 nM, final concentration) were loaded in black flat bottom 96-well microplates. The microplates were pre-incubated in the plate reader at 37 °C for 15 minutes, before 60 µl of AAPH reagent (12 mM, final concentration) was added to initiate oxidation. As blank, 200 µl of phosphate buffer was used. The measurements were carried out at 37 °C and the microplates were shaken prior to each reading. The kinetic readings were recorded every 30 seconds for 3 hours.

The ORAC value was calculated and expressed as Trolox equivalents (TE)/g DW sample. Three different aliquots of each specific sample were assayed.

2.9.2 FRAP

A slightly modified protocol described previously by Benzie and Strain (1996) was used for the FRAP assay. The FRAP reagent (Ferric – TPTZ solution) was prepared by mixing 2.5 ml of 10 mM TPTZ solution in 40 mM HCl, 2.5 ml 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ml of 300 mM acetate buffer at pH 3.6. An amount of 10 µl sample and 30 µl milliQ water were mixed with 300 µl FRAP reagent in transparent 96 well microplates and incubated at 37 °C for 30 minutes. The absorbance of the colored complex formed with Fe^{2+} and TPTZ was determined spectrophotometrically at 593 nm using an ASYS UVM 340 (Asys hitech GmbH, Eugendorf, Austria) and compared to a standard curve. Trolox was used as standard for the calibration curve (0 - 1000 µM), and results was expressed as µmol TE/g DW sample. Three different aliquots of each specific sample were assayed.

2.10 Statistical analysis

Values are given as mean ± standard deviation. SPSS 16.0 (SPSS inc., Chicago, IL, USA) was used to perform statistical analysis of the data between specific time points during the simulated GI digestion. A test for homogeneity of variance was performed. This was inconclusive, thus a Dunnett's T3 test was chosen as a post hoc test for comparison between groups. The significant level was set to $p < 0.05$. No extreme values were removed. A T-test was used for comparison between samples.

It should be noted that each digestion procedure were repeated only three times for each specific sample, hence the statistical analysis may be on insufficient grounds.

3 Results

3.1 Proximate composition

The protein, fat and ash content increased in all cooked fillets as the moisture content decreased (table 2). Moisture and ash increased in processed shrimp, whereas protein and fat decreased.

Table 2
Proximate composition of analyzed samples[1] (per 100 g wet weight seafood)

Sample	Moisture (g)	Fat (g)	Ash (g)	Protein (g)
Raw saithe	80.0 ± 0.03	0.53 ± 0.07	1.3 ± 0.02	17.6 ± 1.7
Baked saithe	78.6 ± 0.35	0.71 ± 0.08	1.4 ± 0.04	20.4 ± 0.7
Boiled saithe	77.0 ± 0.86	0.66 ± 0.06	1.9 ± 0.07	21.5 ± 1.1
Raw shrimp	77.5 ± 0.14	1.7 ± 0.10	1.7 ± 0.07	20.2 ± 0.4
Processed shrimp	83.6 ± 0.14	1.2 ± 0.05	2.3 ± 0.30	13.9 ± 0.1

[1]Mean ± standard deviation for all samples (n=3).

3.2 The OPA method

Figure 2 illustrates the increase in the amount of hydrolyzed amino groups at different time points during a simulated GI digestion. OPA reacts primarily with primary amino groups in proteins and amino acids but can also react with ammonia (Zuman *et al.*, 2008). However, protein hydrolysis will inevitably create more OPA reactive primary amino groups implying that the colorimetric response will increase with increasing protein degradation. It can thus be assumed that the data in figure 2 reflects the release of free amino groups. All analyzed aliquots from samples of fillets (raw and prepared), showed an increase in DH from the start of GI digestion up until the stage simulating the small intestine. In fact, DH decreased at the start of the simulated small intestine stage (105 min), possibly due to the fact that the sample at this time were diluted 1.15 times owed to addition of 1.5 ml bile/pancreatin solution at the end of the simulated stomach stage (75 min). Furthermore, pH was raised from 2.0 to 5.0 and thereby pepsin was inactivated at this point. Samples from the digestion of raw shrimp displayed a considerable higher DH than processed shrimp throughout the duration of GI

digestion, however both raw and processed shrimp showed a consistently increase in DH from start to the end of GI digestion.

Measurements were performed on pooled samples due to the amount sample needed to accurately determine quantification of released amino groups; however quantifications in individual samples were consistent with those in pooled samples with respect to both the direction and the absolute changes in DH.

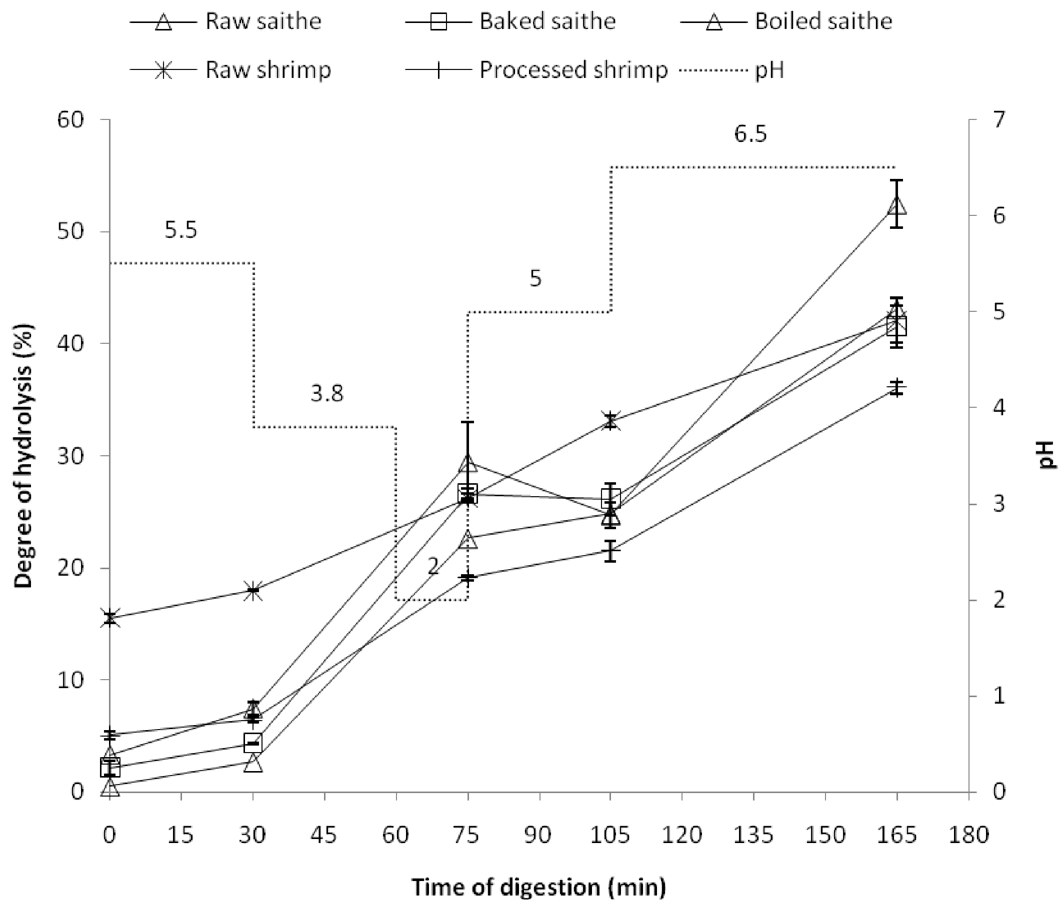


Figure 2. Degree of hydrolysis in raw, baked and boiled saithe, and raw and processed shrimp during an *in vitro* gastrointestinal digestion. The measurements were performed on pooled samples, and were measured in triplicate. The results are presented as the mean ± standard deviation (n = 3).

3.3 Antioxidative capacity

3.3.1 Improving the *in vitro* gastrointestinal procedure

The *in vitro* GI digestion protocol was initially adopted from Sannaveerappa *et al.* (2007) and used for determination of development of AOC during digestion of press juices (PJ). When extrapolated to muscles the validity of the procedure was unfortunately not proven. As this work did not include PJ, the protocol was modified by replacing PJ with 1 g muscle tissue in 15 ml pepsin solution supplemented with 1.5 ml bile/pancreatin solution after 75 minutes.

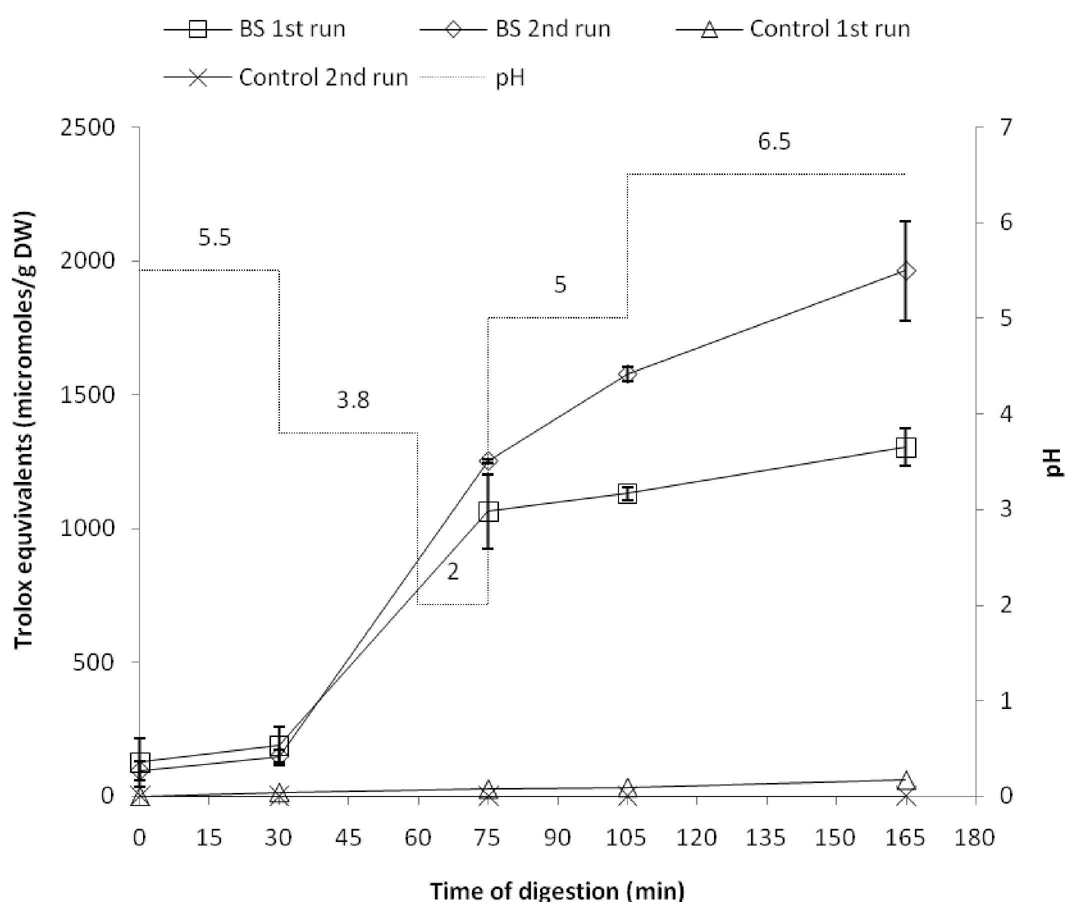


Figure 3. Antioxidative capacity (ORAC) of boiled saithe (BS) 1st and 2nd run with respective controls during an *in vitro* gastrointestinal digestion. Samples (n = 3) were measured in triplicate and the results were presented as the mean ± standard deviation.
1st run sample (□): 1 g muscle tissue and 15 ml pepsin solution.
1st run control: 1 ml phosphate buffer and 15 ml pepsin solution.
2nd run sample (◊): 1 g muscle tissue, 15 ml pepsin solution and 14 ml phosphate buffer (pH 6.75).
2nd run control: 15 ml phosphate buffer, and 15 ml pepsin solution.
ORAC: oxygen radical absorbance capacity.

Digestions of the control were performed in the same manner, only replacing muscle tissue with 1 ml phosphate buffer (pH 6.75). During analysis of AOC an unexpected result was discovered; the control had an increase in AOC during GI digestion. The ORAC values increased from no AOC at the start of digestion, to 60 micromoles TE after 165 minutes. The FRAP values increased from no AOC at the start of digestion, to 5 micromoles TE after 165 minutes. Hence, the pepsin and bile/pancretin solutions used to simulate digestion could influence AOC values of the investigated samples.

To validate the AOC of the samples, a second run of *in vitro* digestions were performed on all samples. This time, 1 g sample, 14 ml phosphate buffer (pH 6.75) and 15 ml pepsin solution were subjected to GI digestion. The control consisted of 15 ml phosphate buffer (pH 6.75) and 15 ml pepsin solution. The second run of digestion displayed a stable control; hence the latter was used in this study.

The difference of GI digestion with 1 g muscle (1st run), and GI digestion with 1 g muscle suspended in 14 ml phosphate buffer (2nd run) and respective controls when analyzed by ORAC are illustrated in figure 3. Although boiled saithe is used as an example, the pattern was similar in raw and baked saithe and raw and processed shrimp as well. The second run of GI digestions with a stable control had significantly higher ORAC values after 105 and 165 minutes of digestion for all samples compared to samples from the first run of GI digestions.

Figure 4 illustrates the difference between GI digestion with 1 g muscle (1st run), and GI digestion with 1 g muscle suspended in 14 ml phosphate buffer (2nd run) and respective controls when analyzed by FRAP. Raw saithe is used as an example, however the pattern was similar in baked and boiled saithe and raw and processed shrimp as well. FRAP values of the first and second run concurs, only differentiated by an elevated level of AOC in the first run.

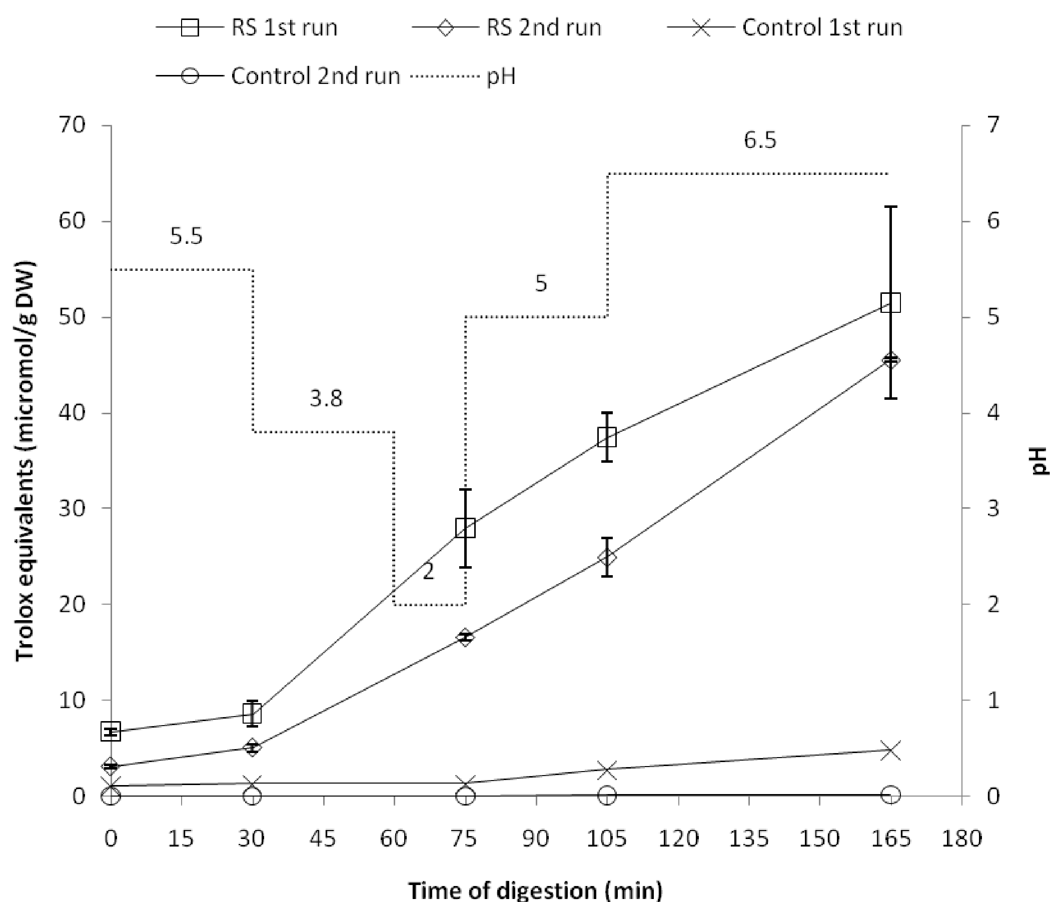


Figure 4. Antioxidative capacity (FRAP) of raw saithe (RS) 1st and 2nd run with respective controls during an *in vitro* gastrointestinal digestion. Samples (n = 3) were measured in triplicate and the results were presented as the mean ± standard deviation.
1st run sample (□): 1 g muscle tissue and 15 ml pepsin solution.
1st run control: 1 ml phosphate buffer and 15 ml pepsin solution.
2nd run sample (◊): 1 g muscle tissue, 15 ml pepsin solution and 14 ml phosphate buffer (pH 6.75).
2nd run control: 15 ml phosphate buffer, and 15 ml pepsin solution.
FRAP: ferric reducing antioxidant power.

3.3.2 ORAC

The general development in the ORAC values of digested seafood muscle (figure 5) seemed to follow the development of released amino groups determined by the OPA method (figure 2). At the start of digestion, saithe (raw, baked and boiled) and shrimp (raw and processed) exhibited ORAC values of approximately 94 and 40 micromoles/g DW, respectively. After 30 minutes of digestion, a tendency of increase in ORAC values was observed. The increase was, however, not significant. In the third sample collected (75 min), the increase in ORAC values were significant ($p < 0.01$) to samples collected earlier in the digestion for both saithe (raw, baked and boiled) and shrimp (raw and processed). There were not observed any significant differences between raw, baked and boiled saithe after 75 minutes of digestion, however, raw and processed shrimp displayed a significant difference ($p < 0.001$). Furthermore, the difference between saithe (raw, baked and boiled) and shrimp (raw and processed) remained significantly different ($p < 0.05$) up until 165 minutes of digestion. After 105 minutes of digestion, ORAC values of all samples continued to increase; however a significant increase was only observed in raw saithe and processed shrimp. From the start of digestion, ORAC values of raw, baked and boiled saithe increased 22, 27, and 20 times, respectively. ORAC values of raw and processed shrimp had increased 38- and 32-fold.

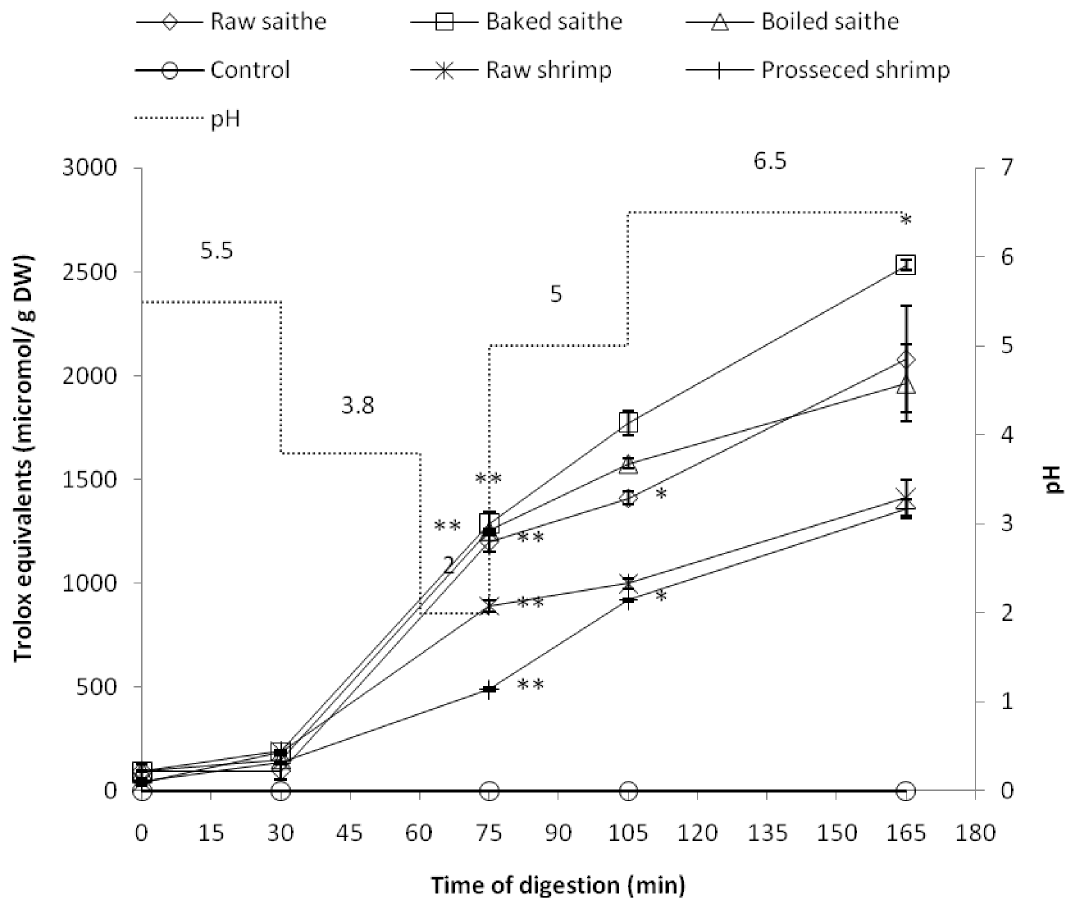
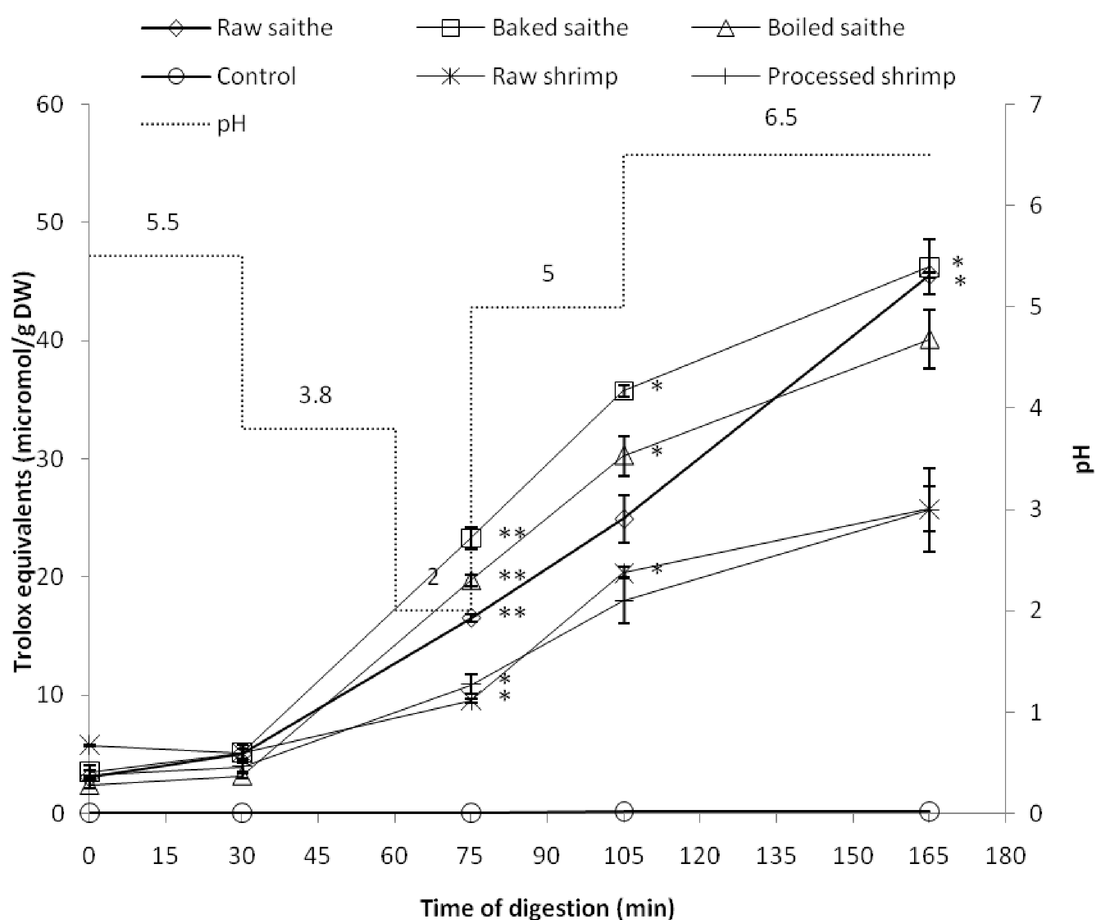


Figure 5. Antioxidative capacity (ORAC) of seafood muscle during an *in vitro* gastrointestinal digestion of 1 g muscle. The samples (n = 3) were measured in triplicate and the results were presented as the mean ± standard deviation.
ORAC: oxygen radical absorbance capacity, *: significant difference from samples earlier in digestion at $p < 0.05$, **: significant difference from samples earlier in digestion at $p < 0.01$.

3.3.3 FRAP

The FRAP values obtained from digested seafood muscle (figure 6) showed a similar trend as for the ORAC values. At the start of digestion, all samples exhibited an FRAP value of approximately 3 micromoles TE/g DW, except raw shrimp which exhibited 6 micromoles TE/g DW. After 30 minutes of digestion the values had a tendency to increase, although not significant in any sample, and the FRAP values of raw shrimp had decreased at this point. After 75 minutes of digestion, the FRAP values of all samples had increased significantly ($p < 0.001$) from samples previously collected. Samples of raw, baked and boiled saithe exhibited values of 17, 23, and 20 micromoles TE/g DW, respectively. At this point there were significant differences ($p < 0.001$) between raw, baked and boiled saithe as well. The FRAP value of raw and processed shrimp had increased to 10 and 11 micromoles TE/g DW,

respectively. There were no significant differences between raw and processed shrimps throughout GI digestion. From 75 minutes of digestion to the end of digestion, the difference between saithe and shrimp remained significant ($p < 0.0001$). After 105 minutes of digestion, FRAP values of all samples continued to increase, however a significant increase was only seen for baked and boiled saithe together with raw shrimp. As for the fourth collection point (105 min), there were significant differences ($p < 0.001$) between raw, baked and boiled saithe. The trend of a continuously increase in FRAP values was confirmed for all samples after 165 minutes of digestion, however only baked saithe had increased significantly. The difference between the samples of saithe had decreased, and only boiled saithe were significantly different ($p < 0.05$) from the others. From the start of the digestion, FRAP values of saithe (raw, baked and boiled) had increased between 15 and 20 times. FRAP values of raw and processed shrimp had increased 4- and 9- fold, respectively.



Figur 6. Antioxidative capacity (FRAP) of seafood muscle during an *in vitro* gastrointestinal digestion of 1 g muscle. The samples (n = 3) were measured in triplicate and the results were presented as the mean ± standard deviation.
FRAP: ferric reducing antioxidant power, *: significant difference from samples earlier in digestion at $p < 0.05$, **: significant difference from samples earlier in digestion at $p < 0.01$.

3.4 Amino acid composition

Levels of FAA (table 3) and TAA (table 4) were measured at the start and after 75 minutes of GI digestion. The selected time points were based on the results of ORAC and FRAP, which showed a significant increase in AOC for all samples at 75 minutes of digestion compared to collections made earlier during GI digestion. The use of pooled samples was based on the relative small standard deviations at the specific time points for all samples, suggesting that the digested materials were equal in composition. Tryptophan is known to be present in seafood, however acidic hydrolysis, as is performed in this study, destroys this amino acid (Sanni *et al.*, 2002), hence it was not detected in this study.

Table 3.
Levels of free amino acids (FAA) of analyzed samples (mg/g dry weight (DW))[1]

FAA (mg/g DW)	Type of processing and time of digestion									
	Raw saithe		Baked saithe		Boiled saithe		Raw shrimp		Processed shrimp	
	0 min	75 min	0 min	75 min	0 min	75 min	0 min	75 min	0 min	75 min
Indispensible										
Histidine	0.4	0.3	0.4	0.4	0.5	0.8	0.4	0.7	0.4	0.3
Isoleucine	1.5	2.0	1.4	2.2	1.2	2.3	1.7	2.7	1.9	2.0
Leucine	2.9	4.9	2.7	6.2	2.4	5.6	4.3	6.8	3.5	4.3
Lysine	1.5	2.2	1.6	2.4	1.1	2.3	2.2	3.2	1.5	2.0
Methionine	0.5	1.5	0.6	2.0	0.4	1.6	1.4	2.3	0.8	1.2
Phenylalanine	1,6	5.4	1.6	7.4	1.2	6.6	3.4	5.0	2.3	4.6
Threonine	1.1	1.2	1.1	1.5	0.9	1.4	1.6	2.1	1.4	1.6
Valine	0.7	1.0	0.7	0.9	0.7	0.8	1.2	1.7	1.2	1.0
Conditionally indisbensible										
Arginine	n.d	0.4	0.3	0.4	0.3	0.3	18.9	26.3	4.6	5.4
Cysteine	n.d	n.d	n.d	n.d	n.d	n.d	0.4	n.d	n.d	n.d
Glutamine	0.3	0.5	0.4	0.2	0.3	0.3	1.7	2.5	0.9	1.1
Glycine	1.3	1.6	1.6	1.9	1.3	1.8	47.5	68.1	16.2	16.3
Proline	n.d	n.d	n.d	n.d	n.d	n.d	7.2	12.5	1.2	1.1
Tyrosine	1.2	2.5	0.9	3.2	1.1	2.7	1.4	2.4	1.4	1.6
Other										
Alanine	2.2	3.4	2.9	4.6	2.3	4.4	7.5	10.3	4.2	4.5
Anserine	7.6	12.2	9.7	13.6	11.9	16.2	n.d	n.d	n.d	n.d
Aspartic Acid	2,2	2.0	2.3	2.6	1.3	1.7	2.1	2.3	2.4	2.4
Asparagine	n.d	n.d	n.d	n.d	0.9	2.1	2.2	2.5	1.9	2.1
Glutamic Acid	2,4	2.6	2.5	3.8	2.2	4.4	3.9	4.9	3.7	4.3
Serine	1.2	1.6	1.3	1.7	1.1	1.7	2.3	2.8	1.9	2.2
Taurine	4.9	5.9	6.4	8.4	4.7	7.6	5.9	8.6	2.6	2.6
Ammonia	0.7	0.9	0.8	1.2	0.6	1.0	1.0	1.5	0.7	1.0

[1] Analysis were performed on pooled samples for all samples.
n.d: Not detected.

The general trend after 75 minutes of digestion was that levels of all amino acids in processed shrimp were lower than in their unprocessed counterparts. Among saithe,

observations in table 3 reveal that amino acids with known antioxidative and synergist effects, such as histidin, leucine, methionine, tyrosine, taurine, and also the dipeptide anserine were present at higher levels in baked and boiled fillets compared to raw fillets.

The observations in table 4 show TAA to follow the same trend as FAA in table 3. At the start of GI digestion, baked and boiled saithe together with processed shrimp exhibited lower levels of all amino acids than their raw counterparts.

Table 4.
Levels of total amino acids (TAA) of analyzed samples (mg/g dry weight (DW))[1]

TAA (mg/g DW)	Type of processing and time of digestion									
	Raw saithe		Baked saithe		Boiled saithe		Raw shrimp		Processed shrimp	
	0 min	75 min	0 min	75 min	0 min	75 min	0 min	75 min	0 min	75 min
Indispensible										
Histidine	4.7	24.0	4.1	32.4	3.7	34.2	9.2	16.4	3.7	17.8
Isoleucine	13.1	58.2	12.9	78.8	9.7	78.1	25.3	42.1	12.3	48.7
Leucine	16.1	95.5	15.0	130.2	11.1	124.7	36.5	64.2	12.8	73.0
Lysine	18.6	109.7	17.4	149.6	11.8	151.5	39.4	69.7	16.1	80.3
Methionine	3.4	33.7	2.6	49.0	1.7	45.5	11.2	20.4	2.9	23.5
Phenylalanine	9,1	42.0	7.5	59.6	4.2	58.7	18.9	32.0	7.5	37.3
Threonine	12.1	52.5	10.9	72.7	7.9	67.5	20.0	32.6	11.7	35.6
Valine	9.8	55.1	8.4	82.3	6.0	82.8	18.3	32.8	7.7	42.6
Conditionally indispensible										
Arginine	8.0	57.5	6.2	114.4	5.7	98.2	24.6	94.1	5.7	93.7
Cysteine	3.1	10.9	2.9	15.6	1.8	11.3	4.6	6.5	2.7	9.7
Glutamine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Glycine	28.7	66.0	29.1	83.2	23.1	74.6	77.1	111.1	41.4	69.2
Proline	3.8	35.0	5.2	48.2	2.8	43.2	19.0	32.2	10.4	25.9
Tyrosine	7.2	43.5	n.d	58.4	4.1	18.8	2.8	17.5	5.7	28.2
Other										
Alanine	18.9	81.3	18.2	108.1	12.5	103.7	36.4	60.8	18.2	64.4
Anserine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Aspartic Acid	25,2	103.4	22.4	141.1	16.5	136.5	48.6	79.7	25.0	89.5
Asparagine	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Glutamic Acid	31,1	167.2	27.2	225.6	20.6	227.5	72.7	123.0	30.2	140.9
Serine	15.4	53.9	14.3	72.5	10.9	61.4	24.2	37.8	16.2	41.1
Taurine	5.8	7.5	6.0	7.7	5.5	6.5	5.1	7.6	2.2	2.2
Ammonia	4.8	10.9	n.d	14.1	3.2	16.7	8.0	9.0	3.5	8.8

[1] Analysis were performed on pooled samples for all samples.
n.d: Not detected.

After 75 minutes of digestion, the trend was mostly reversed. Baked and boiled fillets exhibited higher levels of all amino acids compared to raw fillets. Exceptions were the two antioxidant amino acids tyrosine and taurine, which had decreased. Most of the amino acids in processed shrimp were present in higher concentrations than in raw shrimp, with the exception of proline, tyrosine and taurine.

4 Discussion

4.1 General discussion

In recent years, great academic and industrial interest has been focused on food-derived natural antioxidants. The search for bioactive peptides, such as antioxidants in hydrolysates has spurred numerous publications and AOC has been measured and reported from different marine species. Protein hydrolysates of Alaska pollack (Je *et al.*, 2005), shrimp (Pasquel and Babbitt, 1991), bighead carp (Li *et al.*, 2006), tuna (Je *et al.*, 2007), giant squid (Rajapakse *et al.*, 2005), saithe (Chabeaud *et al.*, 2006) and several other species have been investigated for AOC. Guerard (2007) investigated AOC of shrimp processing discard, however AOC of processed seafood has been investigated to a lesser extent. The objective of the aforementioned studies were to develop hydrolysates enriched in tailored peptides suitable for the production of specific foods with active biological compounds by careful optimization of hydrolysis (e.g. choice of specific enzymes, pH and temperature).

The current study reflects research performed by Sannaveerappa *et al.* (2007), Qian *et al.* (2008) and Jensen (2008) who investigated levels of AOC of seafood during and after GI digestion. These studies showed that AOC of seafood or fractions of seafood, such as press juice (Sannaveerappa) or extracted peptides (Qian) are elevated when subjected to *in vitro* GI digestion. The current study focuses on the effect of household preparation and industrial processing on AOC of seafood muscle during a simulated GI digestion.

As previously mentioned, proteins may act as antioxidants. However, protein antioxidant mechanisms are dependent on amino acid composition. As such, the antioxidative activities may be limited by the tertiary structure of the protein since many amino acids with antioxidative potential are buried within the protein, thus inaccessible to ROS. Hence, the DH of the proteins is important (Jeon *et al.*, 2000) as the proteins are unfolded by hydrolysis. Many amino acids that are known to inhibit oxidation, such as methionine, alanine, proline, leusine and glycine (Fang *et al.*, 2002; Marcuse, 1960) are present in seafood muscle. Shrimp muscle is known to have high levels of proline and glysine, in addition to taurine and arginine (Lie, 1994).

When compared, the ORAC and FRAP assays illustrated similar trends. Levels of AOC increased continuously throughout GI digestion for all samples, and there were significant difference between species. However, AOC of seafood muscle was poor at low pH. AOC of

most amino acids are pH dependent (Marcuse, 1962). The FRAP assay was performed in a buffer solution at pH 3.6, which may resemble the environment of the human stomach. Measured by FRAP, the AOC raw and baked saithe reached a maximum of 46 ± 2.3 micromoles TE/g DW. In contrast, the maximum level of baked saithe when measured by ORAC, which was performed in a buffer solution at pH 7.4, were 2533 ± 26 micromoles TE/g DW. In other words, all AOC measured by FRAP were in an area situated below the lowest AOC values obtained by ORAC. It should be noted that under acidic conditions, such as in the FRAP assay, carboxyl groups of the amino acids are protonated. Thus detection of AOC using this assay may not be optimal. The rationale by including the FRAP assay in this study was that FRAP values in some food items are very high (Serafini *et al.*, 2009; Papandreou *et al.*, 2009), however, AOC will for the remains of this discussion refer to the values obtain by ORAC at physiological pH.

As illustrated in figure 5 it was apparent that all samples increased AOC during GI digestion and saithe more than shrimp. Heat treatment to improve sensory attributes or safety of marine dietary items may disrupt cellular integrity, extricate transition metals, such as iron and copper from proteins and may destroy or use potent antioxidants. The heat itself may initiate chemical reactions such as oxidation. Thus it was expected that thermal preparation and processing would have a negative impact of AOC compared to raw counterparts. Modern meal preparing techniques may also lower the content of compounds with antioxidative activities as losses of low molecular weight compounds, due to preparing techniques are well known. Studies in our laboratories indicate losses up to 70 % loss of for example taurine when preparing traditional (Norwegian) fish products (Dragnes *et al.*, 2009).

It seemed however, that thermal treatment had little or no effect of AOC in the case of saithe and shrimp. This outcome was unexpected, but can be explained. In a study of skim milk (Taylor and Richardson, 1980), an elevated AOC was found after heating due to partially unfolding of proteins, hence exposing for instance cysteine's sulfhydryl groups. In a study of chicken breast meat, Smith *et al.* (1987) determined that acceleration of oxidation with increasing temperature occurred only above a threshold temperature, which in the case of chicken breast was 74°C. Fillets of saithe were heated up until an internal temperature of 63 °C could be maintained for 15 seconds, and an existence of such threshold temperature in seafood could explain the results of the current study.

As explained earlier, all samples were buffered to pH 7.4 before analysis when AOC was measured by ORAC. Thus there were no differences in pH that could explain the increase of AOC during GI digestion. Furthermore, there was no substantial difference between raw,

baked and boiled saithe, neither between raw and processed shrimp. There was, however, a considerable difference between the two species.

The difference between different phases during digestion can be explained by the amount, quality and size formed as a consequence of digestion by pepsin and bile/pancreatin enzymes. Bioactive peptides usually contain 3-20 amino acid residues and their activity is based on the amino acid sequence (Pihlanto-Leppälä, 2000). Furthermore, the low molecular weight peptides (< 1 kDa) are shown to exhibit the highest antioxidative activity (Je *et al.*, 2005; Kim *et al.*, 2007). The size of the peptides are reduced during digestion, thus AOC increases during GI digestion. The difference between the two species however, is most likely due to structural differences such as amino acid composition and the exposure pattern of them. During digestion, antioxidative peptides with known antioxidative effect such as the dipeptides carnosine (Decker *et al.*, 1992) and anserine (Kohen *et al.*, 1988; Kurihara *et al.*, 2009), and in the case of shrimp the protein combined astaxanthin (Nakano *et al.*, 2008) is released due to increased DH. The amounts of these peptides together with the tripeptide glutathione are likely to increase during digestion of sarcoplasmic proteins, hence increase levels of AOC (Sannaveerappa *et al.*, 2007). Figure 2 illustrate differences between DH of raw and processed shrimp. After 75 minutes of digestion the DH of raw and processed shrimp about 25 and 20 % respectively. The difference in AOC between raw and processed shrimp at this point of digestion was also significant. This difference might indicate that some of the antioxidative entities, released during GI digestion, were buried in connective tissue of shrimp, and only released when the muscle tissue were further hydrolyzed by pancreatin and bile solution as the difference was diminished or nonexistent at the next sample collection point. As explained in section 2.3.1, modern shrimp processes are highly automated and the shrimps are constantly in contact with large volumes of water, which in turn results in loss of water-soluble compounds. Raw shrimp have a higher content of for instance myofibrillar proteins that is digested by pepsins.

As mentioned above, exposed sulfhydryl groups increased the level of AOC in skim milk when heated. Atmaca (2004) reported that sulphur containing amino acids such as cysteine, methionine and taurine exhibit elevated levels AOC the more reduced they are. After 75 minutes of GI digestion, the DH of all samples was between 20 and 30 %. Enzymatic hydrolysis unfolds proteins, thereby exposes previously hidden amino acid residues and side chains and releases peptides of various composition and size. Hence, the increase of AOC in hydrolyzed proteins may result directly from increased solvent exposure of amino acids, which in turn leads to increased metal chelation capacity (Elias *et al.*, 2006; Wang and Xiong,

2005), and free radical scavenging activity (Ostdal *et al.*, 1999; Rival *et al.*, 2001; Park *et al.*, 2005).

Tyrosine, methionine, histidine, lysine and tryptophan are amino acids that are readily oxidized, and are shown to be present in antioxidative peptides (Chen *et al.*, 1996). Figure 7 represents TAA in the analyzed samples after 75 minutes of digestion, and includes these amino acids, and others known to have antioxidative activity.

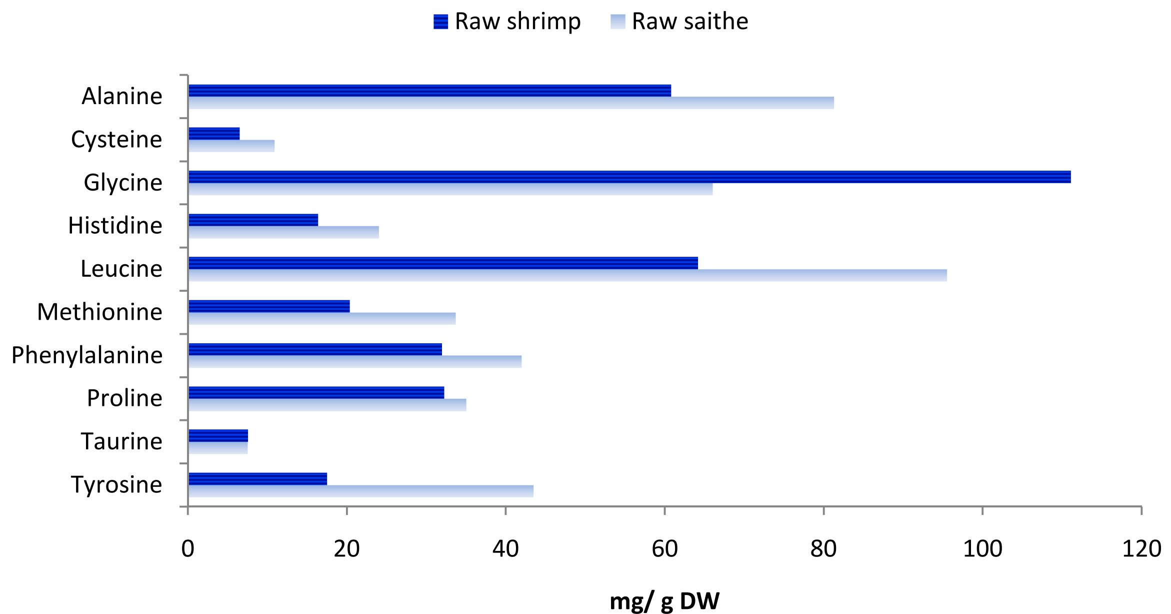


Figure 7. Levels of total amino acids with known antioxidative activities measured on pooled samples from aliquots of raw saithe and shrimp after 75 minutes of an *in vitro* gastrointestinal digestion. Levels were given as mg/ g dry weight (DW) muscle.

Prior to the analysis, all samples were centrifuged to remove large protein particles, thus all analyses were performed on soluble protein fractions of the digested material. Hence, proteins would not influence levels of AOC, only peptides and FAAs. As already mentioned, the size of the peptides is important for AOC, and DH equally so. Figure 2 reveals that DH does not exceed 40 %, except raw saithe at 50 % at the end of digestion. As explained earlier, Pihlanto-Leppälä (2000) determined that the bioactivity of peptides was based on the amino acid sequence. High amount of histidine and hydrophobic amino acids contributes to antioxidative potency (Chen *et al.*, 1998; Pena-Ramos *et al.*, 2004). Furthermore, Chen *et al.* (1996) proved peptides with Pro-His-His sequence to be a very potent antioxidant. However, these findings can, at best, only suggest the reasons why levels of AOC increased during digestion in the current study, as the structure and composition of peptides were not investigated.

Nevertheless, figure 7 illustrates that the soluble protein fractions of raw saithe exhibits higher levels of all amino acids with known synergist and antioxidative effect, except for glycine. Figure 8 illustrates levels of TAA in raw, baked and boiled saithe after 75 minutes of digestion. When figures 7 and 8 were compared, it seemed that baked saithe, which reached the maximum AOC measured in this study, exhibited the highest levels of all antioxidative amino acids, except histidine. Figures 7 and 8 suggest that amino acids with antioxidative activities are in fact contributing to an increase in the overall levels of AOC during digestion.

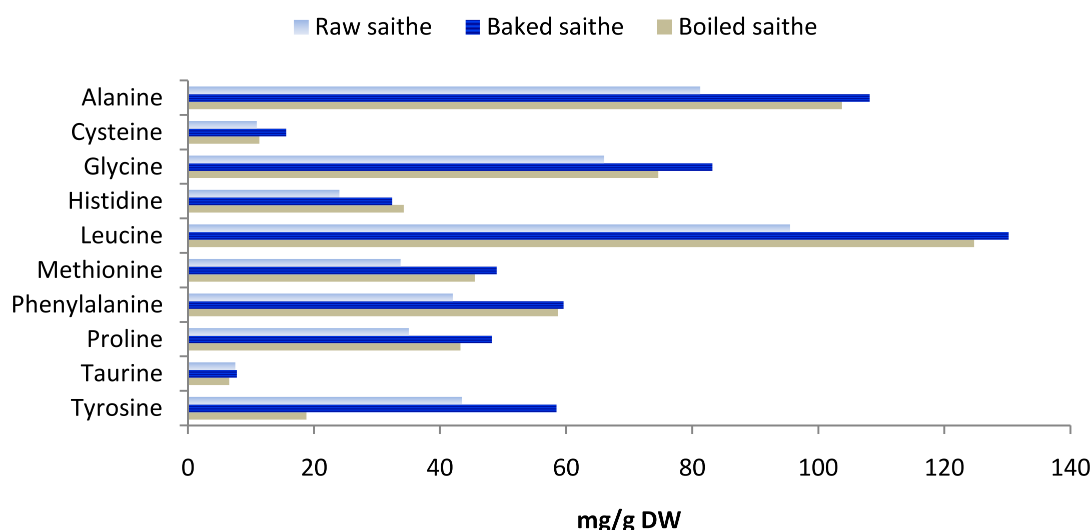


Figure 8. Levels of total amino acids with known antioxidative activities measured on pooled samples from aliquots of raw, baked and boiled saithe 75 minutes of an *in vitro* gastrointestinal digestion. Levels were given as mg/ g dry weight (DW) muscle.

Figures 7 and 8 lists levels of TAA in soluble protein fractions after 75 minutes of digestion. However, one particularly interesting find were discovered when tables 3 and 4 were investigated. There were almost insignificantly small amounts of FAA in the samples of the digested materials compared to TAA. Analysis of FAA will detect FAA and some dipeptides, which suggests that the increase of AOC during digestion is mainly contributed to tripeptides and larger molecules.

4.2 Limitations of the study

It should be pointed out that muscle from marine organisms, e.g. saithe and shrimp which is studied in this report, is noticeably different from avian or mammalian muscle. Fish muscles contain a larger percentage of myofibrillar protein than mammalian skeletal muscle and are characterized by a large percentage of unsaturated fatty acids. Furthermore, differences in muscle composition due to feed, temperature, season, and in particular spawning are well known. For example, fish that live in cold regions have a larger fraction of dark muscle than fish that live in warmer regions (Love, 1970). Hence, future studies of antioxidants derived from muscle tissue during digestion should also include muscle from avian, mammalian and marine organisms.

5 Conclusion

Initially the model of *in vitro* GI digestion was validated and an improved method was adopted. The DH was studied by the use of a method using OPA as a reactant.

Sannaveerappa *et al.* (2007) and Jensen (2008) reported that there was a significant increase in AOC of seafood during GI digestion. The current study confirms that AOC of seafood muscle does increase throughout GI digestion using an improved *in vitro* GI digestion method. Effects of household preparation of saithe were minimal to levels of AOC, and the processing of shrimps had virtually no effect to levels of AOC. One should however recognize that thermal treatment of seafood muscle is detrimental on other beneficial elements of a seafood diet such as destruction of heat labile vitamins such as thiamine and losses of water-soluble compounds.

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