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ARTICLE

Changes in Antioxidative Capacity of Saithe (*Pollachius virens*) and Shrimp (*Pandalus borealis*) during *in Vitro* Digestion

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The health effects of seafood have primarily been linked to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). These omega-3 fatty acids are highly susceptible to oxidation. Peptides exhibiting antioxidative capacity (AOC) are encrypted in seafood muscle proteins. Such components may protect the fatty acids during digestion and uptake, as well as exhibit antioxidative effects in general. The oxygen radical absorbance capacity (ORAC) assay and the ferric reducing antioxidant power (FRAP) assay were used to study the changes in AOC of fish and shrimp muscle and their aqueous fractions, referred to as press juice (PJ), during a simulated *in vitro* gastrointestinal (GI) digestion. Blueberry (*Vaccinium myrtillus*), well-known for its AOC, was included for comparison. During digestion the AOC increased in all samples. After digestion the AOC of muscle of both autumn saithe and shrimp were higher (130−165 μmol/g), and winter saithe comparable (110 μmol/g) to digested blueberry when measured by ORAC. The AOC of PJ was low in general (5−20 μmol/g). When measured by FRAP, blueberry exhibited ten times the AOC of seafood muscle. Antioxidative compounds in seafood increased significantly during digestion, and may offer protection of other beneficial food components such as EPA and DHA.

KEYWORDS: Antioxidative capacity; ORAC; FRAP; press juice; saithe (*Pollachius virens*); shrimp (*Pandalus borealis*); blueberry (*Vaccinium myrtillus*)

INTRODUCTION

The health aspects of seafood consumption have primarily been linked to the marine polyunsaturated fatty acids, in particular eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Up until now, contributions from other substances have, at least partly, been neglected. Lately there has been a growing interest in other biologically active compounds that are not regarded as essential nutrients but likely to be beneficial. Specific amino acids like taurine (*1*) and bioactive peptides released by enzymes during gastrointestinal (GI) digestion are such compounds. The peptides may typically exhibit antithrombotic, immunomodulatory, antihypertensive and antioxidative effects (*2*, *3*).

During respiration, reactive oxygen species (ROS) are constantly and unavoidably produced. These ROS can be potentially damaging to DNA, lipids and proteins. In our bodies we have natural substances that delay, prevent or repair oxidative damage to a target molecule: antioxidants (*4*). Oxidative stress has been linked to several diseases, e.g., cardiovascular diseases, cancer, Alzheimer's disease and Parkinson's disease (*4*). Oxidative stress can be ameliorated by higher intake of antioxidants. Antioxidants released during digestion of seafood, including peptides with antioxidative capacity (AOC), have recently attracted considerable attention. Early reports concluded that the AOC of seafood is mainly due to the aqueous fraction, referred to as press juice (PJ) (*5*, *6*). In addition, AOC of hydrolysates or peptides from seafood byproduct has been reported in, e.g., hoki (*7*), tuna (*8*), yellow stripe trevally (*9*), Alaska Pollack (*10*), shrimp (*11*, *12*), cod (*13*), saithe (*14*) and blue whiting (*15*). The activities of such hydrolysates are highly dependent on the selection of enzymes, time, temperature and pH (*16*). Sannaveerappa et al. (*17*) measured the changes in AOC during a GI digestion of PJ from herring. The AOC of whole muscle during GI digestion has been studied to a lesser extent.

Aims of This Study. This study initially evaluated the changes in AOC during digestion of PJ of saithe and shrimp for comparison with previous work (*17*). In order to more accurately resemble the AOC we would get from a seafood diet, the AOC of saithe and shrimp during a simulated *in vitro* GI digestion was measured and compared to that of PJ. Blueberry (*Vaccinium myrtillus*) is regarded as a dietary source of polyphenols which recently has been shown to counteract oxidation during digestion (*18*) and was therefore included for comparison.

MATERIALS AND METHODS

Chemicals. Pepsin (P6887), pancreatine (P1750), bile extract (B8631), 2,2′-azobis(isobutyramidine) dihydrochloride (AAPH), fluorescein sodium salt, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), iron(III) chloride 6-hydrate (Fe_3), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), bovine serum albumin, sodium borate, *o*-phthaldialdehyde

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(OPA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and A9906 physiological amino acid standard were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). The solutions used in the protein concentration assay were purchased from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). All other reagents used were of analytical grade.

Materials. Fresh fillets of saithe (*Pollachius virens*) were obtained from Dragøy AS (Tromsø, Norway). The fish (approximately 2 kg) were landed autumn 2008 and winter 2009 and were immediately put on ice and prepared in the laboratory within 24 h after landing. The shrimps (*Pandalus borealis*) were obtained from Stella Polaris AS (Tromsø, Norway). The shrimps were caught in the Norwegian Sea outside the Faroe Islands, autumn 2008, and were block frozen within four hours at sea. Prior to the analysis the shrimps were thawed at 4 °C overnight and peeled. The blueberries were collected during autumn 2006 (Troms, Norway), and were immediately frozen at −55 °C until use.

Preparation of Press Juice. Whole skinless fillets of saithe (100 g) and muscles of shrimp (300 g) were minced in a food processor. To be able to extract the PJ of shrimp, it was necessary to add 300 mL of water prior to the mincing. Thereafter the minces were centrifuged at 18250*g* for two hours at 4 °C as described by Gunnarsson et al. (*19*). The supernatants were filtered through a Schleicher & Schuell folded filter (125 mm) and resulted in 23 mL of PJ of autumn saithe, 20 mL of PJ of winter saithe and 120 mL of PJ of shrimp. The PJs were stored at −55 °C until further use.

Preparation of Muscle. An amount of 100 g of saithe and shrimp was minced coarsely in a food processor and stored at −55 °C until further use.

Preparation of Blueberry. An amount of approximately 30 g of blueberry was homogenized in a food processor and stored at −55 °C until further use.

***In Vitro* Gastrointestinal Digestion Procedure.** Human digestion was simulated by adding a pepsin solution, containing 0.462% pepsin, 49 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 2.4 mM $MgCl_2$, and 2.5 mM K_2HPO_4, representing the gastric phase and a bile/pancreatic solution (containing 0.2 g of pancreatine, 1.25 g of bile extract and 0.1 M $NaHCO_3$ in 50 mL of distilled water) representing the intestinal phase, along with gradient pH adjustment. The method was performed as described by Sannaveerappa et al. (*17*) with minor adjustments, and the pH was adjusted with 3 M HCl and 3 M NaOH for the PJs and 1 M HCl and 1 M NaOH for the muscles. An amount of 15 mL of PJ was mixed with 15 mL of pepsin solution, resulting in 30 mL of reaction mixture. The selected amount of muscles and blueberry was 1 g, thus resulting in a total volume of 16 mL of reaction mixture. A 50 mM phosphate buffer (pH 6.75) containing 0.9% NaCl (15 mL) was used as control. The pH was adjusted to 5.5, and a PJ and muscle sample (6 and 3 mL respectively) was collected (0 min). The reaction mixtures were incubated in an incubator shaker at 220 rpm and 37 °C for 30 min, before another PJ and muscle sample (8 and 4 mL respectively) was collected (30 min). The pH was adjusted to 3.8, and the reaction mixtures were further incubated 30 min before adjusting the pH to 2.0. After 15 min of incubation, a PJ and muscle sample (6 and 3 mL respectively) was collected (75 min). After this, 1.5 mL of a bile and pancreatic solution was added. The reaction mixtures were thereby diluted 1.15 and 1.30 times for the PJ and muscle samples respectively. The pH was adjusted to 5.0 and the reaction mixtures were continuously incubated for 30 min before another PJ and muscle sample (6 and 3 mL respectively) was collected (105 min). The pH was adjusted to 6.5 and the reaction mixtures were incubated for another 60 min. The remaining amount of reaction mixtures was then collected (165 min). All samples were immediately put in ice to stop the reaction. The samples were centrifuged at 4500*g* at 4 °C for 15 min to remove large particles, resulting in a clear solution that could be measured spectrophotometrically in the different assays. Following centrifugation and removal of the supernatant the samples were frozen and kept at −55 °C until analysis of AOC.

Protein Concentration Determination. The protein concentration was measured to evaluate whether the AOC was correlated to the concentration of proteins. The protein concentration in the centrifuged incubated samples was determined according to Lowry et al. (*20*) using the Bio-Rad protein kit assay. The results are presented as mg/mL sample.

Hydrolysis Measurement. Hydrolysis was assessed by analyzing the degree of protein hydrolysis based on the reaction of primary amino groups with OPA. The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved. The OPA assay was carried out according to Church et al. (*21*), including the modifications suggested by Nielsen et al. (*22*). The OPA reagent was made by completely dissolving sodium borate (0.133 M) and SDS (4.6 mM) in water. Separately, OPA was dissolved in ethanol to a concentration of 0.3 M. The OPA solution, 7.33 mM DTT and water were added to the sodium borate−SDS solution, resulting in a final concentration of sodium borate, SDS, OPA and DTT of 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. OPA reagent (3 mL) was mixed with sample (400 μL), water (control) or serine standard (0.97 mM) and measured spectrophotometrically at 340 nm (Genesis 20 spectrophotometer, Thermo Fisher Scientific Inc., USA) after 2 min. Preliminary results on single samples were not accurate due to limited amount of sample volumes, and the measurements were thus performed on pooled samples.

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was carried out according to Dávalos et al. (*23*). The reaction was carried out in a 75 mM phosphate buffer (pH 7.4) which was also used as a blank. The AOC is defined as the net difference between the area under the decay curve of the fluorescence, as a consequence of a radical attack, of the sample and that of the blank. This was compared to a Trolox standard curve (1−8 μM, final concentration). The fluorescence was measured at 485 and 520 nm (Spectramax Gemini EM fluorimeter, Molecular Devices, Sunnyvale, CA). The results are expressed as mmol Trolox equivalents (TE)/L of PJ and μmol TE/g of muscle.

Ferric Reducing Antioxidant Power (FRAP). The FRAP assay was carried out according to Benzie and Strain (*24*). Trolox was used as a standard (15.625−500 μM), and water was used as a blank. The reaction was carried out in acetate buffer (pH 3.6). The color change when the ferric−TPTZ complex was reduced to its ferrous state by the samples was measured spectrophotometrically at 593 nm (ASYS UVM 340 spectrophotometer, Asys, hitech GmbH, Eugendorf, Austria). The results are expressed as mmol TE/L of PJ and μmol TE/g of muscle.

Amino Acid Analysis. To study the effect of GI digestion on the increase in AOC, the contents of free amino acids (FAA) and total amino acids (TAA) were examined in the samples collected after 0 and 75 min of digestion of autumn saithe and shrimp. Between these collection points the increase in AOC was the largest. For determination of FAA an amount of 500 μL of sample was mixed with 100 μL of 20 mM norleucine and adjusted to 1.1 mL with water. An amount of 100 μL of 35% sulfosalicylic acid was added for removal of proteins and peptides. The mixtures were centrifuged, 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 TAA in the samples, 1 mL of sample was combined with 200 μL of 20 mM norleucine and 1200 μL of HCl and hydrolyzed for 24 h at 110 °C. An aliquot of the hydrolysate (100 μL) was dried under nitrogen and diluted with lithium citrate buffer (pH 2.2) to a suitable concentration before analysis. All samples were analyzed using a Biochrom 30 amino acid analyzer (Biochrom Limited, Cambridge, U.K.) with a lithium citrate equilibrated column and post column derivatization with ninhydrin. The amino acids were quantified by using norleucine as an internal standard, and the signal was analyzed with Chromeleon software (Dionex, Sunnyvale, CA). The identification of the amino acids was made by comparison with an A9906 physiological amino acid standard.

Statistical Analysis. The whole *in vitro* digestion procedure was performed three times for each raw material ($n = 3$). Values are given as mean ± standard deviation of three parallels. SPSS 15.0 (SPSS Inc., Chicago, IL) 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 significance level was set to $p < 0.05$.

RESULTS AND DISCUSSION

Protein Concentration. The protein concentrations in the different samples collected during the GI digestion are presented in **Table 1**. After the samples were collected, they were centrifuged to remove large particles, and only the remaining peptides < 15 kDa (*17*) were determined as proteins. The protein concentration was highest at 75 min.

Amino Acid Composition. Tyrosine, methionine, histidine, cysteine, taurine, alanine, proline, leucine and glycine are amino

Table 1. Protein Concentration (mg/mL sample) Measured by Bio-Rad in the Samples Collected after 0, 30, 75, 105, and 165 min of an *in Vitro* Gastrointestinal Digestion of Press Juice (PJ) of Autumn Saithe and Shrimp and Muscle of Autumn Saithe and Shrimp[a]

raw materials	0 min	30 min	75 min	105 min	165 min
PJ of autumn saithe	34 ± 6	34 ± 3	69 ± 18	47 ± 5	50 ± 6
PJ of shrimp	28 ± 7	32 ± 7	44 ± 10	36 ± 9	33 ± 9
autumn saithe	13 ± 6	15 ± 2	24 ± 8	18 ± 6	22 ± 6
shrimp	8 ± 3	21 ± 7	30 ± 6	27 ± 6	29 ± 10

[a] The results were presented as the mean ± standard deviation of three parallels of digestion.

Table 2. Levels of Free Amino Acids (FAA) and Total Amino Acids (TAA) Generally Associated with Known Antioxidative Effects (*25*−*27*) of Analyzed Samples (μg/mL sample)[a]

	autumn saithe				shrimp			
	FAA		TAA		FAA		TAA	
amino acids	0 min	75 min	0 min	75 min	0 min	75 min	0 min	75 min
histidine	0	0	30	140	0	10	60	110
leucine	20	30	100	570	30	50	250	430
methionine	0	10	20	200	10	20	80	140
phenylalanine	10	30	50	250	20	30	130	220
cysteine	nd	nd	20	70	0	nd	30	400
glycine	10	10	170	400	320	460	520	750
proline	nd	nd	20	210	50	80	130	220
tyrosine	10	20	40	260	10	20	20	120
alanine	10	20	110	490	50	70	250	410
arginine	nd	0	50	340	130	180	170	640
taurine	30	40	30	50	40	60	30	50

[a] Analyses were performed on pooled samples for all samples. nd: not detected.

acids with known antioxidant activity (*25*−*27*). The content of these amino acids increased from the start of the digestion to after 75 min in both saithe and shrimp (**Table 2**). Shrimp contained higher amounts of amino acids such as proline, glycine and arginine, compared to saithe. This is in accordance with Lie et al. (*28*). Many of the amino acids known to inhibit oxidation, such as alanine, proline, leucine and glycine, are in higher abundance in saithe compared with herring (*28*), and this could also explain why the PJ of saithe had higher AOC than the PJ of herring. Species differences in proteins, e.g., content of amino acids and exposure patterns of them, may explain why the rise in AOC during the GI digestion was not proportional between the species.

Degree of Hydrolysis. In **Figure 1** the increase in the amount of free amino groups during the simulated GI digestion is illustrated. At the start of digestion, shrimp muscle showed a higher DH than saithe muscle, which is probably due to a higher amount of FAA, such as glycine, in shrimp muscle (**Table 2**). The analyzed samples of both saithe and shrimp showed a general increase in DH from the start to the end of GI digestion.

Oxygen Radical Absorbance Capacity (ORAC). *Press Juice*. After 75 min of digestion, the reaction mixture may be regarded to reflect a mixture entering the small intestine, where peptides and FAA may be absorbed. At this point the ORAC values of the PJ samples had increased significantly compared to the start of digestion. The samples displayed ORAC values of approximately 38 mmol TE/L PJ of autumn saithe and approximately 30 mmol TE/L PJ of winter saithe and shrimp, compared to 4 mmol TE/L PJ of autumn and winter saithe and 16 mmol TE/L PJ of shrimp at the start of digestion. Again the high amount of FAA in shrimp may explain the differences at the start of digestion (**Table 2**). Throughout the subsequent digestion, the ORAC values remained fairly stable and did not change significantly. The ORAC

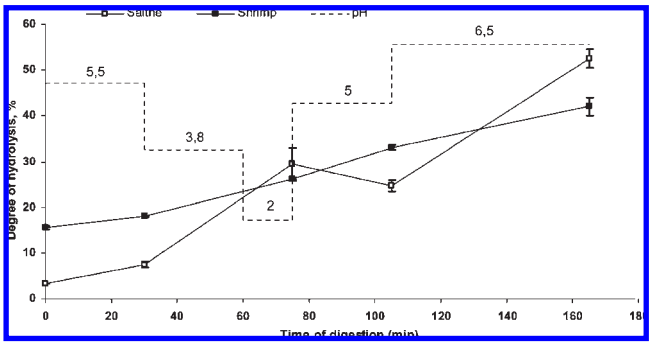

Figure 1. Degree of hydrolysis of autumn saithe and shrimp during an *in vitro* gastrointestinal digestion of 1 g of muscle.

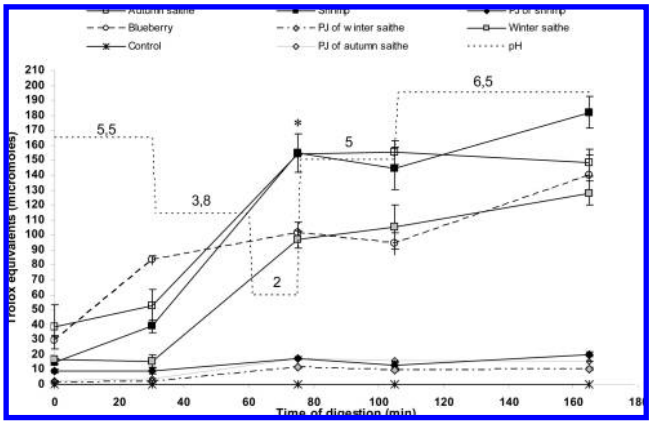

Figure 2. Antioxidative capacity (ORAC) of 1 g of muscle, the corresponding amount of press juice (PJ) and 1 g of blueberry during an *in vitro* gastrointestinal digestion. The results are presented as the mean ± standard deviation of three parallels of digestion. *: significant difference from samples earlier in the digestion at $p < 0.05$.

values obtained during GI digestion of PJ of autumn saithe correlated positively ($r = 0.844$) with the protein concentration (table **1**). The ORAC values of PJ of shrimp and the protein concentration was poorly correlated ($r = 0.583$). After 165 min of digestion the ORAC values had increased 6- and 2-fold compared to the start of digestion for PJ of autumn saithe and shrimp respectively. The protein concentration had increased 2.0- and 1.4-fold respectively, at this time interval, thus the AOC is not solely correlated to the amount of proteins, peptides and amino acids. Sannaveerappa et al. (*17*) reported that the highest ORAC value during the digestion of 15 mL of PJ obtained from 100 g of herring was observed after 75 min of digestion and was approximately 25 mmol TE/L PJ. In this study, the ORAC values after 75 min of digestion exceeded 30 mmol TE/L PJ of both autumn and winter saithe as well as for PJ of shrimp. This indicates that PJ from less than 100 g of saithe and shrimp muscle exhibited elevated AOC compared to PJ from 100 g of herring.

Whole Muscle. In **Figure 2** the ORAC values of 1 g of muscle together with the corresponding ORAC values of the PJ samples are shown. One gram of muscle corresponded to 0.23 mL of PJ of autumn saithe, 0.20 mL of PJ of winter saithe and 0.40 mL of PJ of shrimp. The patterns of changes were similar. After 75 min, when the saithe and shrimp samples had been digested until the stage simulating the small intestine, the increase was significant ($p < 0.05$) compared to the start of digestion for all the muscle samples. At this point the ORAC value of the muscle of autumn saithe was approximately 150 μmol TE/g. The ORAC value of the muscle of winter saithe, however, was slightly lower;

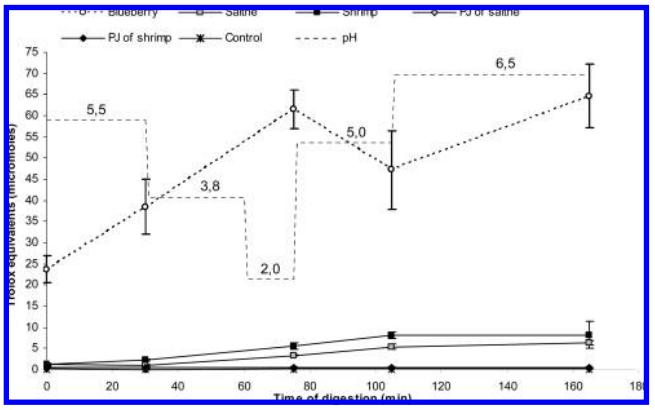

Figure 3. Antioxidative capacity (FRAP) of 1 g of muscle, the corresponding amount of press juice (PJ) and 1 g of blueberry during an *in vitro* gastrointestinal digestion. The results are presented as the mean ± standard deviations of three parallels of digestion.

approximately 100 μmol TE/g. Shrimp exhibited an ORAC value of approximately 160 μmol TE/g after 75 min of digestion. The general development in the ORAC values of the muscle samples were well correlated with those of the protein concentration measured in the samples during the GI digestion of autumn saithe and shrimp (r = 0.813 and r = 0.903, respectively). A correlation was also made for the ORAC values and the DH, and for autumn saithe and shrimp it was 0.903 and 0.904 respectively. The difference between the autumn saithe and the winter saithe could be due to seasonal variations (*29*). The increase in AOC of muscles as opposed to PJs was observed throughout digestion, although not significant after the phase simulating the small intestine. After 165 min of digestion the ORAC values had increased approximately 5-fold for the autumn saithe and 10-fold for winter saithe and shrimp

Blueberry. Blueberry exhibited generally lower ORAC values than both autumn saithe and shrimp, with approximately 100 μmol TE/g after 75 min of digestion (**Figure 2**). The increase in the AOC of the blueberry samples during digestion was not significant. After 165 min of digestion the ORAC values had increased approximately 5-fold. AOC in 100 g of undigested blueberry measured by ORAC has been reported to vary between 3 and 7 mmol TE/100 g (*30*, *31*).

Ferric Reducing Antioxidant Power. *Press Juice*. The FRAP values obtained from the digested PJs were lower, but showed a similar pattern as the ORAC values. The seasonal difference in saithe were not seen in the FRAP assay, and consequently, the results of the saithe are presented generally. Initially the samples exhibited FRAP values between 0.3 and 0.5 mmol TE/L PJ of saithe and shrimp, respectively. After 75 min of digestion, an increase was observed and the samples exhibited FRAP values between 1.2 mmol TE/L PJ of saithe and 0.6 mmol TE/L PJ of shrimp. The increase was, however, not significant.

Whole Muscle. In **Figure 3** the FRAP values of 1 g of muscle along with the FRAP values of the PJ samples, corresponding to 1 g of muscle (0.23 mL PJ of saithe and 0.4 mL PJ of shrimp), are presented. The digested muscle samples of saithe and shrimp exhibited FRAP values of approximately 1 and 2 μmol TE/L PJ, respectively, at the beginning of the digestion. After 75 min of digestion the FRAP values had increased three times compared to the start of the digestion. The FRAP assay is performed at pH 3.6. Under such acidic conditions the carboxyl groups of the amino acids are protonated. Additionally the FRAP assay does not detect thiols because the reduction potential of thiols is generally lower than that of the Fe^{3+}/Fe^{2+} half-reaction (*32*). Thus the FRAP assay may not be optimal in quantification of AOC in protein sources.

Table 3. Comparison of the Antioxidative Capacity (AOC) Measured by ORAC and FRAP Assay of Digested Press Juice (PJ) Corresponding to 100 g of Seafood and 100 g Seafood or Blueberry

	AOC (mmol TE/100 g)	
raw material	ORAC	FRAP
PJ of autumn saithe	1.9 ± 0.2	0.02 ± 0.0
PJ of winter saithe	1.1 ± 0.1	
PJ of shrimp	2.3 ± 0.1	0.05 ± 0.0
PJ of herring[a]	0.75	
autumn saithe	15.8 ± 1.2	0.3 ± 0.0
winter saithe	9.7 ± 0.6	
shrimp	15.5 ± 1.3	0.6 ± 0.1
blueberry	10.0 ± 0.7	6.2 ± 0.5

[a] Adopted from Sannaveerappa et al. (*17*).

Blueberry. Contradictory to the results obtained from the ORAC method, the results obtained from the FRAP method showed that blueberry exhibited 10 times the AOC of shrimp and saithe after 75 min of digestion (60 μmol TE/g) (**Figure 3**). The FRAP value of blueberry has previously been reported to be 4 and 8 mmol/100 g of cultivated and wild blueberry respectively (*32*).

Comparison of Press Juice and Muscle. In order to more accurately resemble the AOC one would get from a diet, the digested muscles were compared with the digested PJs. The AOC, measured by ORAC and FRAP, of 100 g of muscle and the amount of PJ possible to "extract" from 100 g of muscle is presented in **Table 3**.

Further Work. Enhancement of *in vivo* AOC in the human plasma by digested seafood proteins has recently been discussed by Parra et al. (*33*). In an energy-restricted diet to treat obesity, and thus a diet expected to enhance fatty acid metabolism resulting in a higher oxidative stress, they reported that a cod-based diet was the most effective strategy to reduce oxidative stress. The results from Parra et al. (*33*) indicate that antioxidative compounds, like the ones found in this study, which could withstand the GI digestion, may have a physiological impact. The most accurate method to study changes in AOC of foods in humans would be corresponding clinical trials. This is expensive and time-consuming and therefore not applicable at a screening level. *In vitro* models are, in comparison, inexpensive and rapid and may serve as efficient tools for screening AOC of foods. A cellular based antioxidative assay would be more accurate in assessing the uptake and AOC of peptides and possible protective effects. Such studies are foreseen.

ABBREVIATIONS USED

AOC, antioxidative capacity; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; PJ, press juice.

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