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# Characterisation and antioxidant properties of aqueous extracts from capelin (*Mallotus villosus*)

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Skýrsluágrip Rannsóknastofnunar fiskiðnaðarins
Icelandic Fisheries Laboratories Report Summary

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| *Titill / Title* | **Characterisation and antioxidant properties of aqueous extracts from capelin (*Mallotus villosus*)** | | |
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| *Ágrip á íslensku:* | Möguleg nýting loðnu í verðmætar afurðir felst m.a. í áhugaverðum andoxunareiginleikum vatnsleysanlegra þátta í loðnu, sem sýnt hefur verið fram á að eru til staðar í loðnumjöli. Nokkrar aðferðir voru notaðar til að skilgreina andoxunareiginleika loðnu með því að meta hæfni til að binda DPPH radíkal, járnbindigetu og með súrefnis-radíkal gleypni (ORAC). Hrásafi eða pressuvökvi (vatnsútdrættir úr hráu sýni) sem unninn var úr heilli loðnu, svo og úr slægðri og hausaðri loðnu, var borinn saman við soð (vatnsútdrættir úr soðnu sýni), sem einnig var greint í þætti samkvæmt mólþunga. Þessi þrjú próf á andoxunarvirkni gáfu sambærilegar niðurstöður. Meiri andoxunarvirkni mældist í slægðri og hausaðri loðnu sem veidd var í 5. viku ársins en í heilli loðnu sem veidd var í 9. viku ársins. Breytileiki í próteininnihaldi og amínósýru/peptíð samsetningu sýnanna mælt með hárpípurafdrætti virtist ekki hafa áhrif á andoxunarvirkni eins og hún var mæld með þessum þremur prófum. Meiri þráahvatavirkni vegna hærra hemóglóbín innihalds í heilu loðnunni (frá 9. viku) gæti hafa dregið úr andoxunarvirkni allra vökvasýna sem unnin voru úr þessu sýni. Niðurstöðurnar sýna að breytilegir ytri þættir eins og árstíðasveiflur og meðhöndlun geta haft áhrif á andoxunarvirkni. Auk þess er áhugavert að skoða nánar mismunandi mólþætti til að skilgreina betur hugsanlega andoxunareiginleika mismunandi peptíða, amínósýra eða annarra vatnsleysanlegra þátta í loðnu með mögulega nýtingu afurða í huga. | | |
| *Lykilorð á íslensku:* | *Þráahindra próf, DPPH, ORAC, járnbindigeta, hárpípurafdráttur, peptíð, loðna, vökvafasi* | | |

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*Summary in English:*

The potential use of capelin into value added products is based on interesting antioxidant properties of this species that has been linked to components in the aqueous fraction of fishmeal from capelin. Several antioxidant assays were used to characterize antioxidative properties of capelin by assessing scavenging abilities of the DPPH radical, ferrous chelating capacity and by the oxygen radical absorbance capacity (ORAC) test. Aqueous extracts (raw press juice) from whole capelin vs. gutted and beheaded capelin were compared to broths from cooked samples, which were also separated according to molecular size by ultra-filtration. Results from the three tests for antioxidant activity agreed quite well. Higher antioxidant activities were observed in samples from gutted capelin harvested in week 5 compared to whole capelin from week 9. Variation in protein content and amino acid/peptide profiles obtained by capillary electrophoresis did not appear to affect the antioxidant activities of the samples as measured by these three tests. More prooxidant activity due to higher hemoglobin content in whole capelin (week 9) could most likely counteract the antioxidant activity of all aqueous samples derived from this sample. The results indicate that extrinsic factors like seasonal changes and handling influence the antioxidative properties. Further studies are needed to characterize antioxidant capacities of different aqueous fractions related to the content of peptides, amino acids or other components with antioxidant properties for possible further use into valuable products.

*English keywords:* *Antioxidant assays, DPPH, ORAC, chelating capacity, capillary electrophoresis, peptides, capelin, aqueous extracts*

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## 1. INTRODUCTION

Antioxidants in a food system normally refer to substances that can inhibit fatty acid autoxidation. The major antioxidants are metal chelators and chain-breaking antioxidants acting as hydrogen atom donors. Living organisms have developed a complex antioxidant network to protect their cells from reactive oxygen species and reviews on the natural compounds that participate in the antioxidative defence mechanism of fish have been published (Hultin 1992a, 1994; Undeland 1997, Bragadóttir 2001a). These include enzymes (catalase, peroxidase, glutathione and superoxide dismutase), carotenoids, peptides, amino acids and phenolic compounds (tocopherols, ubiquinones). Most amino acids have antioxidative properties, depending on pH and other compounds that may have synergistic effects with them. The most abundant free amino acids in capelin from winter season have been reported in order of decreasing amount as taurine, alanine, glutamic and lysine (Bragadóttir 2001b). Extracts from fish muscle contains sarcoplasmic proteins, enzymes, polypeptides, amino acids, blood components, amines (TMAO and biogenic amines), nucleotides and minerals, but many of those have antioxidative properties (Hultin 1992a, 1994; Undeland 1997a). Carnosine, anserine and ophidine are dipeptides and are among few found in fish extracts. They contain the amino acid histidine and some of them have shown antioxidant activity (Decker and Xu 1998). Glutathione is a tripeptide (γ-Glu-Cys-Gly) and a known antioxidant. Carnosine, anserine and glutathione are not seriously affected by heat treatment, but decrease upon storage in mackerel, bluefish and turkey (Decker og Xu 1998). The poly-amines putrescine, spermidine and spermine, that are found in almost all animal tissues prevent autoxidation and their activity increases with increased amount of amine groups (e.g. spermine>spermidine> putrescine). Ureic acid is an antioxidative compound that contains amine. The function of poly amines and ureic acid as antioxidants is unknown. Besides the antioxidative and/or prooxidant activity of amino acids and peptides, they can give both sweet and bitter taste.

The great number of different antioxidants in fish and other biological samples makes it difficult to measure each antioxidant separately. Therefore, several rapid methods have been developed and used to determine the total antioxidant capacities of various biological samples (Prior and Cao 1999, Prior and others 2005, Roginski and Lissi 2005).

None of these methods have been validated as a reliable measure of antioxidant capacity, because one-dimensional methods are problematic to use to evaluate multifunctional food and biological antioxidants (Huang and others 2005, Frankel and Meyer 2000). One of the most widespread used assays for antioxidant screening is the DPPH (2,2-diphenyl-1-dicrylhydrazyl) assay. This is a simple and rapid test based on the measurement of the reducing ability of antioxidants toward DPPH radical. The DPPH radical has a deep purple colour that fades upon reduction which can by monitored by a spectrophotometer. DPPH is one of a few stable organic radicals, but it has no similarity to the highly reactive and short-lived peroxyl radicals involved in lipid peroxidation (Prior and others 2005). The ORAC assay measures anti-peroxyl radical activity and thus reflects classical radical chain breaking antioxidant activity. An azo-compound, AAPH (2,2´-azobis (2-methylpropionamidine) dihydrochloride) is used as a generator of peroxyl radical which reacts with the fluorescent indicator, causing a decrease in fluorescence. In the presence of antioxidants the fluorescence decay is prevented. Recently, antioxidant capacity of more than 100 different kinds of foods was determined using the ORAC method (Wu and others 2004). The capacity to bind ferrous ion is also a useful indication of antioxidant activity. Ferrous ion is one of the most powerful prooxidants that can interact with hydrogen peroxide to produce hydroxyl free radical which can initiate lipid oxidation. Chelating capacity may be estimated by several methods. A simple and rapid method is to monitor the influence of the antioxidant on the decrease in maximal absorbance of iron(II)-ferrozine complex (Dinis and others 1994, Benjakul and others 2005).

Antioxidant activity of aqueous extracts from several fish species has been reported. Aqueous extracts from the white muscle of cod, haddock, dab and winter flounder were found to have antioxidant properties in haemoglobin-mediated oxidation of cod muscle membrane lipids (Undeland and others 2003). The same antioxidative properties were found after heating of the aqueous extracts in all cases except herring. Antioxidative properties were also found in water soluble components of fishmeal made from capelin, which undergoes heating during processing (Bragadóttir 2001b). Fishmeal from capelin does also contain lipid soluble antioxidants, although in much less amounts than the fresh capelin (Bragadóttir and others 2002, Bragadóttir and others 2004). Fresh capelin is an especially interesting fish to examine for antioxidative properties because, unlike other

similar pelagic fish species, it has an exceptionally long shelf life during frozen storage (Jangaard 1974, Shaw and Botta 1977, Botta and others 1983).

The purpose of this preliminary experiment was to investigate the influence of raw material handling and quality of capelin on antioxidative properties of its aqueous extracts. This was done by comparing whole capelin vs. gutted and beheaded capelin and by different extraction methods of the aqueous phases. The extraction was done on raw capelin, as well as cooked capelin, and the cooked extract was also separated according to molecular size by ultra-filtration. The aim was to study if inherent characteristics like protein, fat and water content and amino acid/peptide profiles obtained by capillary electrophoresis, as well as factors related to handling, like pH and the hemoglobin content of the different aqueous extracts from capelin could explain their antioxidative properties, Scavenging abilities of the DPPH radical, ferrous chelating capacity and the ORAC test were used for evaluating the antioxidative properties.

## 2. MATERIALS & METHODS

### Capelin

Two samples of capelin (*Mallotus villosus*) were obtained from the 5$^{th}$ and 9$^{th}$ week of the year, respectively. The former sample (5$^{th}$ week ) was caught in net East of Iceland (Reyðafjarðardjúp, catching zone 462) on the 5$^{th}$ of February 2006. The fish was cooled on board the fishing vessel in seawater tanks and landed the next day. The temperature during landing and transportation to the laboratory ranged form 0 to 5 °C. The latter sample (9$^{th}$ week ) was caught in net on February 21st South of Iceland (Reykjanesgrunn, catching zone 372). Temperature of the fish in the hold on board the ship was 7 °C and no cooling was applied. The sample was stored in an insulated plastic cooler and shipped by airfreight from Vestmannaeyjar on the following day and arrived to the laboratory in the morning of February 23$^{th}$. Preparation of aqueous extracts from capelin was done on day 3 from catch for both samples.

## Capelin aqueous extracts

The capelin obtained in week 5 was beheaded and gutted and rinsed in cold water, but the 9th week sample was utilized whole without rinsing. Both samples were coarsely minced in a mixer (Braun electronic) at medium speed. Aqueous extracts were made from raw and cooked capelin (**Table 1**). The raw press juice was made by centrifuging minced samples (180 g) in 275 mL centrifuge bottles at 27 500g for 120 min. The press juice was filtered through diaper cloth and stored frozen (-75 °C) until used (PJ-w5 and PJ-w9). The rest of the mince was cooked to make broth in microoven at highest power for approximately 6 min. The broth from the cooked samples were squeezed out using diaper cloth and stored frozen (-75 °C) until used (B-w5, and B-w9).

**Ultra-filtration**. The broth from week 5 (B-w5) was ultra-filtrated to make broth with particle size >300 Da and 150-300 Da, respectively. The ultra-filtration was done in NF-Lab-unit (ROP 970 Type Microlab 80) with PCI membranes, AFC 40 MWCO 150 – 300 Da (GEA-Filtration). The total membrane area was 0.094 $m^2$ and other operating parameters were 32 °C for the temperature, 35 bars pressure and the flux was 1.25 L/min for the retentate and 0.025 mL/min for the permeate. Samples were stored in freezer until analyzed (-75 °C).

## Proximate analyses of capelin

Water content was determined by drying in an oven at 102 to 104 °C for 4 h by ISO method 6496 (ISO 1999). Fat content was determined by the Soxhlet method Ba 3-38 (AOCS 1997) using petroleum ether (Bp. 30-40 °C) for extraction.

**Table 1. Overview of samples of aqueous phase prepared from raw and cooked capelin.**

| Sample description | Abbreviation |
|---|---|
| Raw press juice-week 5 | PJ-w5 |
| Broth-week 5 | B-w5 |
| Broth (w5) >300 Da | B-w5: >300 Da |
| Broth (w5): 150-300 Da | B-w5: 150-300 Da |
| Raw press juice-week 9 | PJ-w9 |
| Broth-week 9 | B-w9 |

Crude protein content was determined with the Kjeldahl method (E) 5983-2 (ISO 2005) and by multiplying the nitrogen content by 6.25.

**TVN - total volatile nitrogen**

Total volatile nitrogen (TVN) was determined on steam distillate of minced capelin (10 g) or broth (16 – 18 g) according to the official magnesium oxide method 920.03 (AOAC, 2000).

**Protein content of aqueous fractions**

Protein determination of the capelin aqueous phases was done according to the Biuret protein assay (Layne 1957, Torten and Whitake 1964). Protein content, expressed as mg/mL, was calculated using bovine serum albumin (BSA) as standard. Samples and standards (600 µl) were mixed with 2.5 mL Biuret solution in disposable plastic cuvettes. The samples were mixed and allowed to stand for 35 minutes. Afterwards the absorbance was measured at 540 nm.

**Chelating capacity**

The capacity of the samples for chelating ferrous iron was determined according to a modified method of Benjakul and others (2005). The samples were diluted 100 times and measured in the range of 500 nm to 800 nm with UV-Vis spectrometer. A volume of 0.05 mL $FeCl_2$ (0.2 mM) and 0.1 mL ferrozine (1 mM) were mixed in disposable cuvettes with 1 mL of the diluted sample. Thereafter the mixture was allowed to stand for 10 min before measuring the absorbance. Distilled water was used as control. The areas of the curves were calculated with the following equation:

$$b = \frac{Y_{end} - y_{start}}{x_{end} - y_{start}} \qquad a = y_{start} - \left( \frac{Y_{end} - y_{start}}{x_{end} - y_{start}} \right) x_{start}$$

$$\sum \left[ \left( \frac{(y_{i+1} + y_i)}{2} \right) - a - \frac{b}{2}(x_{i+1} + x_i) \right]$$

i= start wavelength of the curve

The chelating capacity was calculated according to the following equation:

$$Chelating\_capacity(\%) = \frac{area_{control} - area_{sample}}{area_{control}} * 100$$

**DPPH radical scavenging capacity**

The DPPH (2,2-diphenyl-1-dicrylhydrazyl) method was used to test the radical scavenging capacity of the capelin aqueous extracts. A variation of the method based on Yen and Wu (1999) was applied. The reduction of the DPPH radical was followed by measuring the absorbance of diluted samples (×100). One mL of the diluted sample was used to measure the turbidity between 400 nm and 800 nm. Subsequently 10 µl DPPH (10 mM) was mixed with the sample. The mixture was allowed to stand for 40 min before measuring the absorbance again between the two wavelengths. The area of the curves was calculated with the following equation:

$$b = \frac{Y_{650} - y_{454}}{x_{650} - y_{454}} \qquad a = y_{454} - \left( \frac{Y_{650} - y_{454}}{x_{650} - y_{454}} \right) x_{454}$$

$$i_1 = 454$$

$$\sum \left[ \left( \frac{(y_{i+1} + y_i)}{2} \right) - a - \frac{b}{2}(x_{i+1} + x_i) \right]$$

The DPPH scavenging effect was calculated with the following equation:

$$DPPH\_scavenging\_effect = \left( \frac{area_{control} - area_{sample}}{area_{control}} \right) * 100\%$$

## Oxygen Radical Absorbance Capacity (ORAC)

A modification of the method of Dávalos and others (2004) was used. Mx300 Real-Time PCR System (Stratagene) was used for the fluorescence measurements controlled by MxPro computer program. The capelin samples had a high radical scavenging capacity and therefore the concentration of the 2,2´-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and the 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were increased. All reagents were prepared in 75 mM sodium phosphate-buffer (pH 7). The samples were diluted 100 times and 20 µL were placed in the well (200 µL) of a micro-plate (Starstedt). Afterwards 120 mL fluorescein (70 nM) was added and the mixture was pre-incubated at 37 °C for 15 min. Subsequently, 120 µL AAPH (0.24 M) were added and placed immediately in the reader. The fluorescence (FAM) was recorded at 37 °C every minute for 80 min. A blank was prepared the same way as the sample, using distilled water instead of a sample. The first data measured after 6 seconds was used as t=0. The antioxidant curves (fluorescence versus time) were normalized. The data from the curves were multiplied by the factor:

$$\frac{fluorescence_{blank,t=0}}{fluorescence_{sample,t=0}}.$$

The area under the fluorescence decay curve (AUC) was calculated of the normalized curves with the following equation:

$$AUC = 1 + \sum_{i=0,4}^{i=80} \frac{f_i}{f_0}$$

$$f_i = fluorescence\_reading\_at\_time\_i$$

$$f_0 = initial\_fluorescence\_reading\_at\_0.2\_\min$$

The net AUC was calculated by subtracting the $AUC_{blank}$ from the $AUC_{sample}$.

## Peptide analysis by Capillary Electrophoresis (CE)

### Materials

Gly-Gly-Gly ($(Gly)_3$), anserine (β-ala-methyl-his), carnosine (β-ala-his), and amino acid standards were obtained from Sigma-Aldrich Inc. All other chemicals were of analytical grade.

**Preparation of the low-molecular-weight peptide and free amino acid fraction**

**Acetone extracts**. 2 mL (depending on the protein concentration, **Table 3**) of aqueous phase from capelin was suspended in 2 mL acetone and mixed with a Vortex for 20 s; pH of the suspension was adjusted to 4.6 with 4 M HCl. Precipitated protein was removed after 1 hour at room temperature by centrifugation at 4500 rpm for 30 min. The supernatant was filtered through a Whatman filter no. 1 and an internal standard $(Gly)_3$ added to a final concentration of 0.1 mg $(Gly)_3$ $ml^{-1}$ acetone extract. The extract was concentrated to 500 µL by evaporation of the acetone with nitrogen, and an equal amount of 10 mM phosphate buffer, pH 2.75, was added. The extract: buffer (1:1) was filtered through 0.45 µm filter prior to CE analysis.

**Capillary electrophoresis analysis (CE)**

All CE separations were performed on an Agilent CE system (Agilent, Germany). Separations were carried out on a standard 50 µm id capillary with an effective length 24.5 cm and an enlarged bubble cell in the detector window. A 100 mM phosphate buffer, pH 2.75, filtered through a 45m filter and degassed, was used as separation buffer. Prior to the first use and after extensive use the capillary was flushed with 1M NaOH (60min), 0.1 M NaOH (15 min), $H_2O$ (5 min), 100 mM phosphoric acid (10 min) and separation buffer (15 min). The capillary was thermostated to 25 °C. Samples (the concentrated ethanol extracts) were injected by pressure at 15 $kNsm^{-2}$ and separated at a constant voltage of 15kV. UV absorbance was monitored at 214 nm and 228 nm using Diode Array Detector. The separation time was 30 min between each run the capillary was preconditioned with 100 mM phosphoric acid for 5 min and with separation buffer for 8 min. Separation buffer was renewed each separation. The total corrected peak area was related to the corrected peak area of the internal standard $(Gly)_3$.

## 3. RESULTS

### Capelin

The chemical composition of the capelin used as raw material for the aqueous extracts varied somewhat and reflected mainly the seasonal changes in lipid content of capelin decreasing from 9.1% to 5.2% in samples from week 5 and week 9, respectively (**Table 2**). The higher TVN value of the week 9 sample was expected, because no cooling was applied aboard the fishing vessel, apparently resulting in higher activity of autolytic enzymes and more rapid microbial growth.

**Table 2**. Proximate composition and total volatile nitrogen (TVN) of capelin.

| Samples (harvesting time) | Water (%) | Protein (%) | Lipid (%) | TVN (mg N/100g) |
|---|---|---|---|---|
| Week 5 | 73.8 | 13.9 | 9.1 | 11.6 |
| Week 9 | 78.8 | 13.3 | 5.2 | 19.3 |

### Aqueous extracts

#### pH and sensory description

The press juice and broth from week 5 had a little lower pH than the respective extracts from capelin harvested in week 9 (**Table 3**). The highest pH of 7.07 was observed in the clear ultra-filtrated aqueous fraction; B-w5: 150-300 D.

The ultra-filtrated aqueous fractions from week 5 were tasted by three trained sensory judges. The clear broth fraction; B-w5: 150-300 Da had a shell-odour and the flavour was shell-like, with a bitter and astringent aftertaste. The very cloudy B-w5: >300 Da fraction had a milky appearance and a characteristic capelin like, dried-fish and shellfish-like net odour, but the flavour was described as sweet and creamy with a dried-fish aftertaste.

## Protein content of aqueous fractions from capelin

The protein content of the aqueous phases varied considerably (**Table 3**). The ultra-filtrated broth fraction B-w5: >300 Da had the highest protein content with almost eight times higher protein than its corresponding broth B-w5. The broth from B-w5 contained lower amounts of protein than its corresponding PJ, while the broth B-w9 contained almost twice as high as its corresponding press juice. The TVN content of the week 9 broth was more than 50% higher than the B-w5 sample, which indicated a more degraded raw material, reflected in a higher protein content.

**Table 3. Measurements of pH, protein and Hb content of capelin aqueous fractions.**

| Sample[a] | pH | Protein (mg/mL) | TVN (mg N/100g) | Hemoglobin (g/L) | Remarks[b] | Sensory description |
|---|---|---|---|---|---|---|
| PJ-w5 | 6.81 | 73.1 | | 3.0 | brownish | |
| B-w5 | 6.73 | 56.0 | 11.6 | | cloudy | |
| B-w5: 150-300 Da | 7.07 | 0.1 | | | clear | shell-odour and flavour, bitter and astringent aftertaste |
| B-w5: >300 Da | 6.89 | 426 | | | very cloudy, milky | capelin like, dried-fish and shellfish-like net odour, sweet and creamy flavour, dried-fish after taste |
| PJ-w9 | 6.86 | 51.1 | | 4.1 | reddish | |
| B-w9 | 6.86 | 96.2 | 17.5 | | cloudy | |

[a]For abbreviations see Table 1. [b]Remarks on the appearance of the solutions.

## Chelating capacity

The capacity of the samples for chelating ferrous iron was determined after some modifications of the method of Dinis and others (1994). Several attempts were made to measure the chelating capacity according to the original method by reading the absorbance at 562 nm. Various dilutions of the samples were tested, as well as concentrations of reagents, because of high turbidity of most of the samples (**Table 3**, remarks). The final results from these experiments were to decrease the amount of $FeCl_2$ and ferrozine by 50% and to measure the absorbance of the ferrozine complex in diluted samples (×100) at wavelength range of 500 nm to 800 nm.

An example of the absorbance measurements of a sample (B-w5) and control (water) is shown in **Figure 1**. The difference between the area of the control and the sample was calculated as the chelating activity of the sample. Accordingly, the press juice from capelin harvested in week 5 (PJ-w5) had the highest chelating activity of 95%, while the PJ from week 9 had 71% (**Figure 2**) The broth had somewhat lower chelating capacity of 65% and 47% for the B-w5 and B-w9, respectively. The ultra-filtrated fraction 150-300 Da from B-w5 had just 6% chelating capacity but the fraction with over 300 Da had approximately 53%.

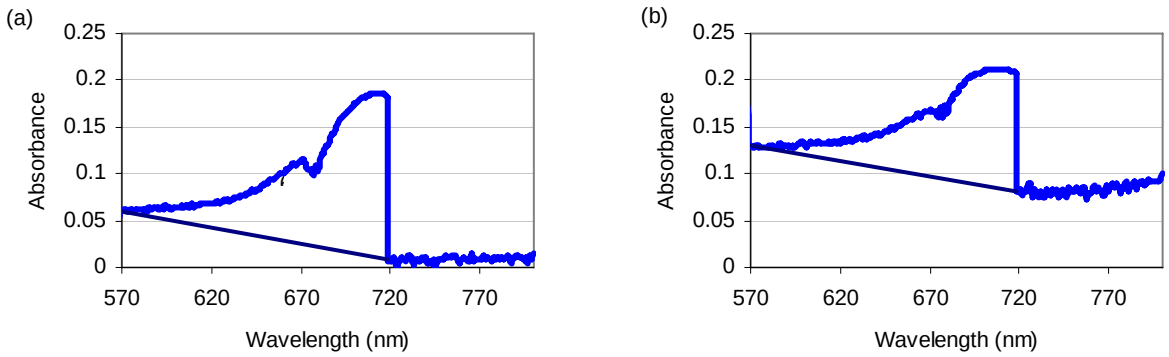


*Figure 1. Absorbance of (a) control (water) and (b) sample (B-5w, dilution ×100) after adding $FeCl_2$ and ferrozine in the chelating capacity test.*

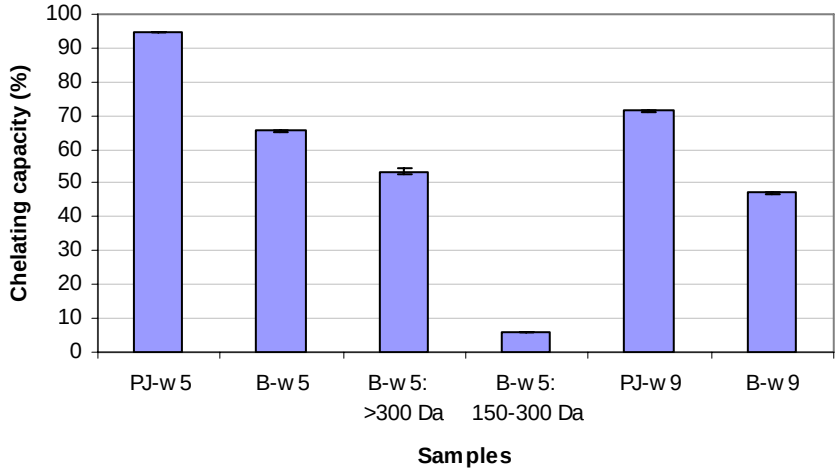


*Figure 2. Chelating capacity of capelin aqueous extracts. The bars represents a mean value (n = 2) ± SD of each sample. For abbreviations see Table 1.*

**DPPH radical scavenging capacity**

As with the previous methods the absorbance of the turbid samples interfered with this measurement using the original method of monitoring the loss of DPPH colour at 515 nm (Yen and Wu 1999). The capacity of the samples for scavenging DPPH (2,2-diphenyl-1-dicrylhydrazyl) radical was therefore determined after some modifications. The final results from these experiments were to measure the absorbance of the samples between 500 nm and 800 nm before and after addition of DPPH. An example of the absorbance measurements of a sample (B-w5: 150-300 Da) and control (water) is shown in **Figure 3**. The difference between the area of the control and the sample was calculated as the DPPH scavenging capacity.

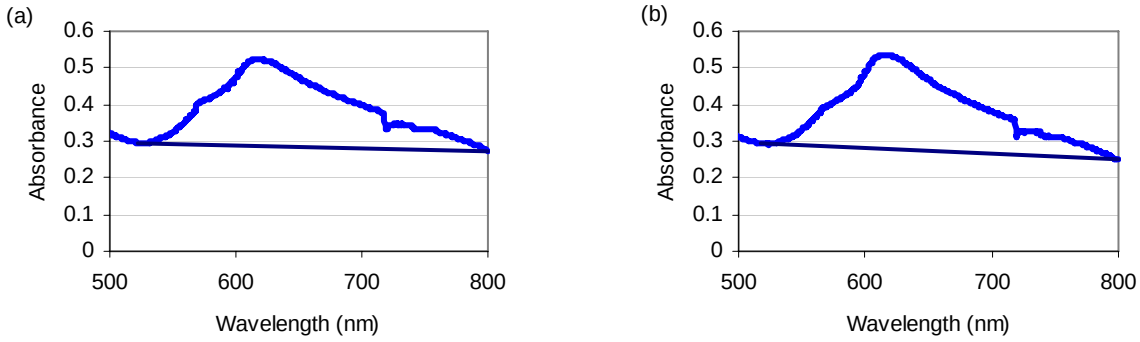


*Figure 3. Absorbance of (a) control (water) and (b) sample (B-w5: 150-300 Da, dilution ×100) after adding DPPH.*

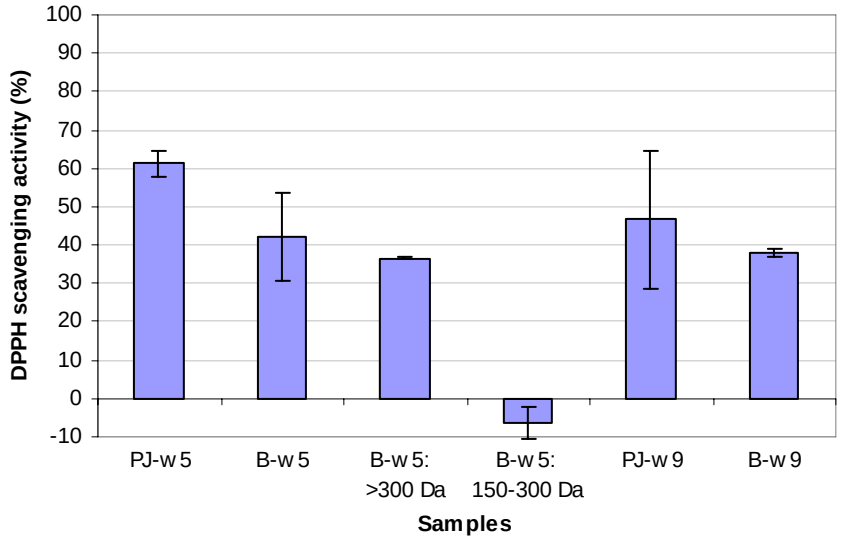


*Figure 4. DPPH (2,2-diphenyl-1-dicrylhydrazyl) scavenging capacity of capelin aqueous extracts. The bars represents a mean value (n = 2) ± SD of each sample. For abbreviations see Table 1.*

Accordingly, the press juices from capelin had the highest average DPPH scavenging activities of approximately 61 ± 3% and 47 ± 18% for samples PJ-w5 and PJ-w9, respectively (**Figure 4**). Both samples had on the average somewhat lower DPPH scavenging capacity of approximately 42 ± 11% and 38 ± 1% for samples B-w5 and B-w9, respectively. The ultra-filtrated fraction 150-300 Da from B-w5 had no DPPH scavenging capacity, or below zero, measured by this method, while the fraction with over 300 Da had approximately 37% DPPH scavenging capacity.

**Oxygen Radical Absorbance Capacity (ORAC)**

The course of the reaction of fluorescein with AAPH was similar for Trolox and the blank (results not shown). The antioxidant activity of Trolox should have delayed the fluorescein decay, but the concentration (4-16 µM) was probably not high enough, to have this effect. The calibration against Trolox could therefore not be used to express the data as Trolox equivalent. The samples did however show delayed decay curves for the reaction of fluorescein with AAPH, indicating oxygen radical absorbance capacity. An example of the fluorescence decay curves of blank and samples are shown in **Figure 5**.

The area under the curves (AUC) of samples, subtracted the AUC of blank are presented here as indication of ORAC activity (**Figure 6**).

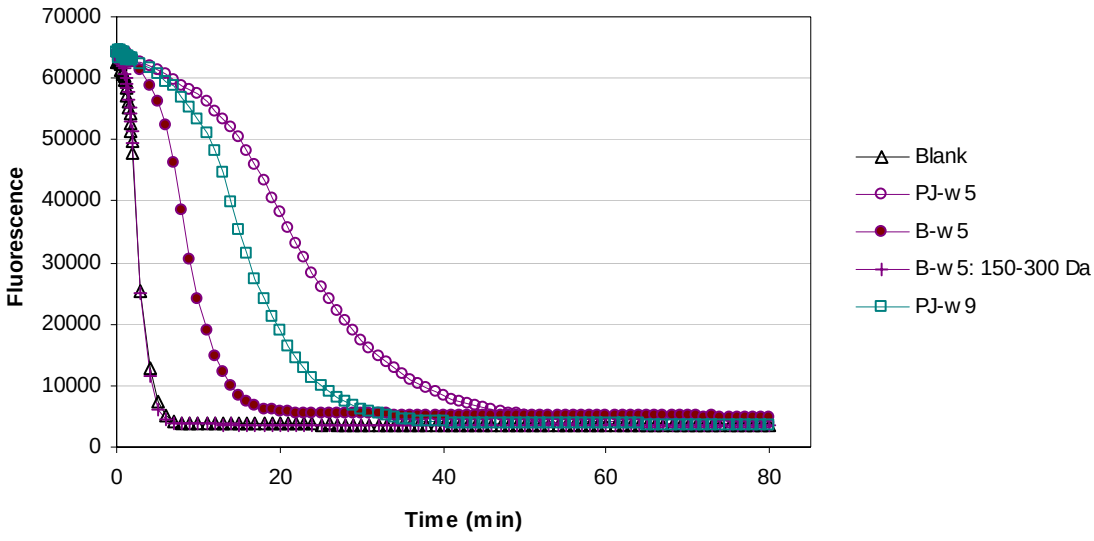


*Figure 5. Time course of the reaction of fluorescein with AAPH in the absence (Blank) and presence of different samples (PJ-w5, B-w5, B-w5: 150-300 Da and PJ-w9) in the ORAC test. For abbreviations see Table 1.*

Accordingly, the PJ samples had the highest average activity of 20 and 13, for Pj-w5 and PJ-w9, respectively. The broth samples resulted in similar values with an average of approximately 9 for both B-w5 and B-w9. The ultra-filtrated fractions from B-w5 had very low activity of approximately 4 for the >300 Da and zero for the 150-300 Da size.

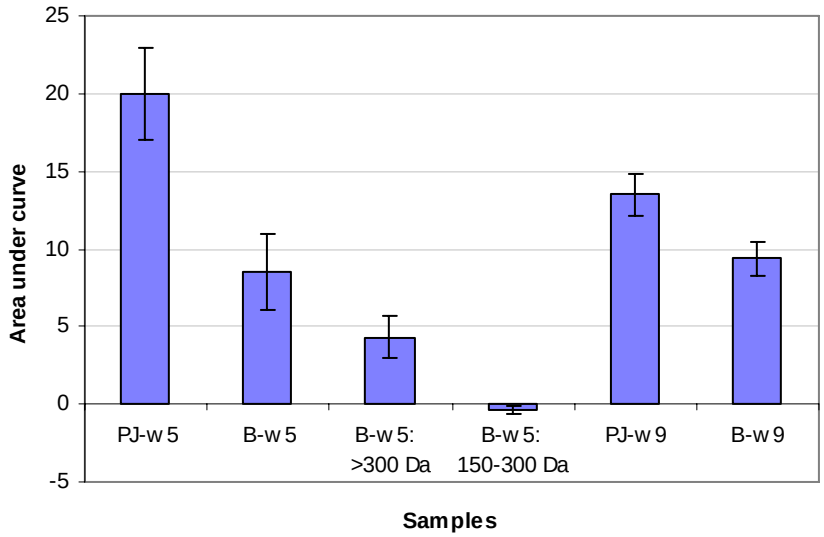


*Figure 6. ORAC scavenging capacity of capelin aqueous extracts. The bars represents a mean value (n = 2) ± SD of each sample. For abbreviations see Table 1.*

**Comparing results of the ORAC, Chelating capacity and DPPH**

Combining the results of the DPPH, chelating capacity and the ORAC tests together on a single figure, revealed a certain pattern in the antioxidant capacity of the samples (**Figure 7**). These three antioxidant activity tests gave highest results for the press juice samples, but considerably higher results for the week 5 sample (PJ-w5) than the sample from week 9 (PJ-9w). The broth samples had the same tendency to be higher for the week 5 sample (B-w5) than the week 9 sample (B-w9), especially for the chelating capacity. The ultra-filtrated samples gave clear messages that the lower molecular weight fraction (B-w5: 150-300 Da) had very little antioxidant activity, compared to the higher weight fraction (B-w5: >300 Da). Ranking the samples by these tests combined, gave the following order of decreasing antioxidant activity: PJ-w5 > PJ-w9 > B-w5 > B-w9 > B-w5: >300 Da > B-w5: 150-300 Da.

These tests showed quite good correlation, especially the DPPH and the chelating capacity ($r$ = 0.98), while the correlation between the ORAC test and the chelating capacity was $r$ = 0.92 and 0.89 with the DPPH capacity.

Other measures like the pH value of the samples showed negative correlation with the antioxidant activity of the samples. Samples with lower pH had a tendency to have higher antioxidant activity, while the protein content had very low correlation with the antioxidant tests.

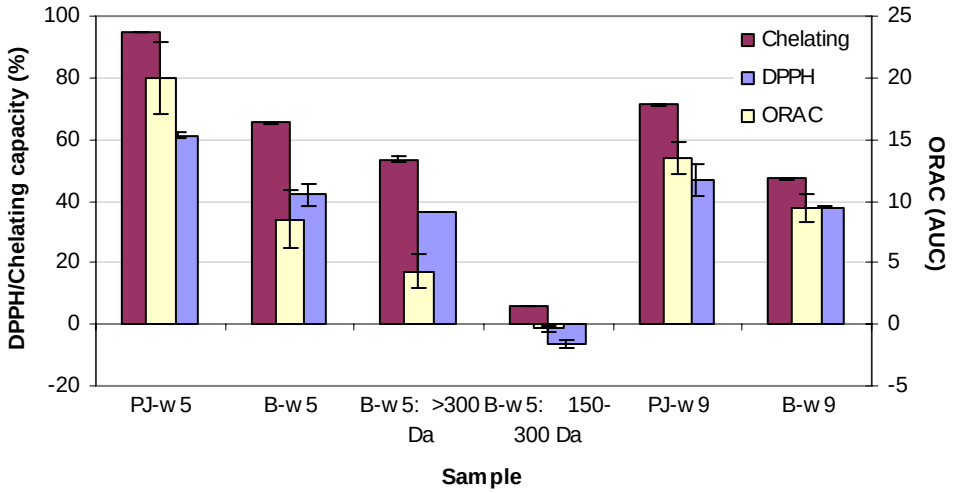


*Figure 7. Comparison of chelating capacity, DPPH scavenging capacity and ORAC (AUC) of capelin aqueous extracts. The bars represents a mean value (n = 2) ± SD of each sample. For abbreviations see Table 1.*

## Capillary electrophoresis analysis (CE)

The CE analysis of the acetone extracts was used to evaluate the differences between the different aqueous phases from capelin, i.e. press juice and broth from weeks 5 and 9. Amino acid/peptide profiles in the different samples absorbing at 214 nm are shown in Figure 8. A shift in migration time was noticed, that may be explained by the different matrix, but was corrected by calculating the relative migration time (in relation to the internal standard).

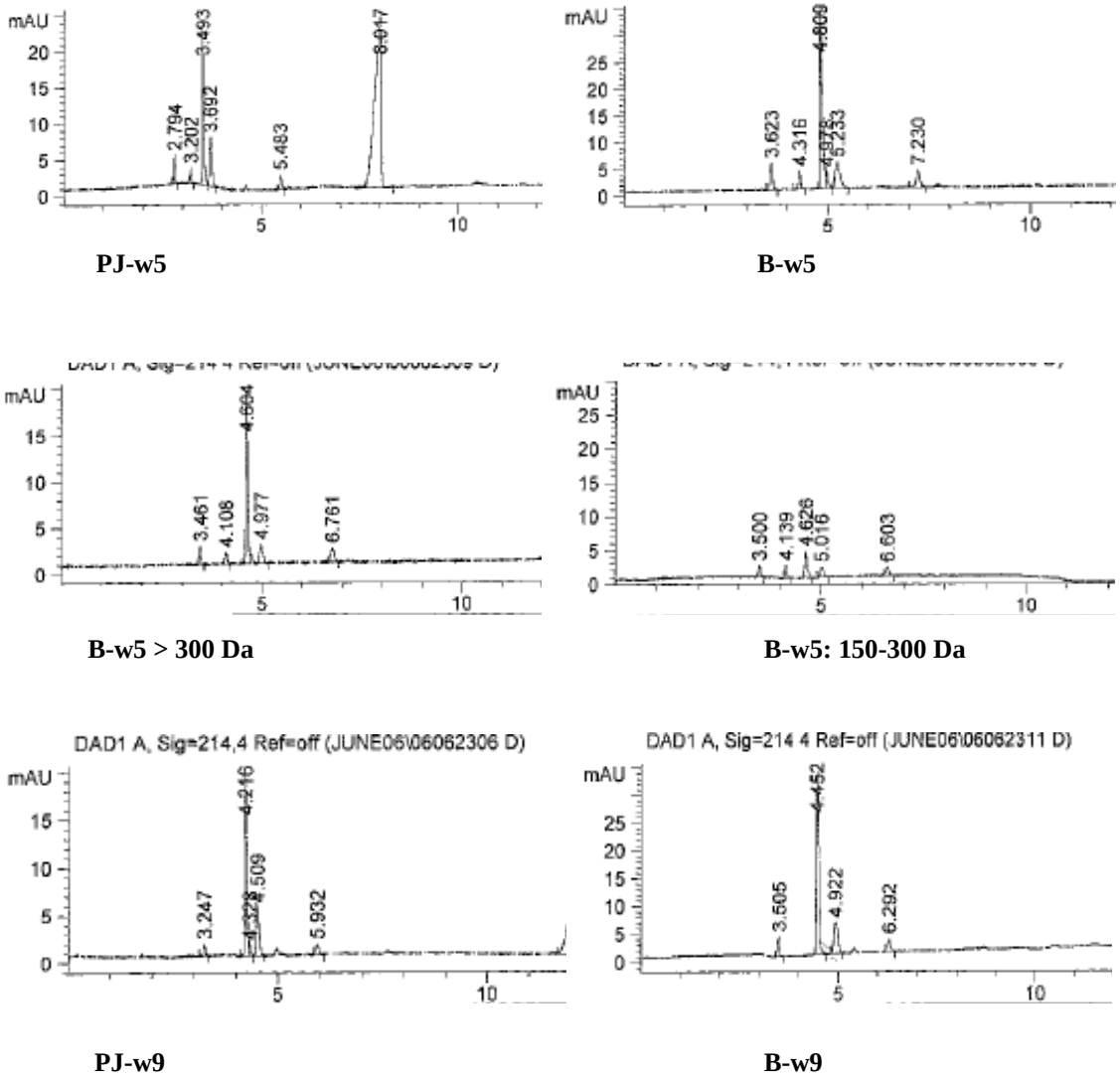


*Figure 8. Capillary electrophoresis electrograms of acetone extracts of different aqueous phases from capelin. For abbreviations see Table 1.*

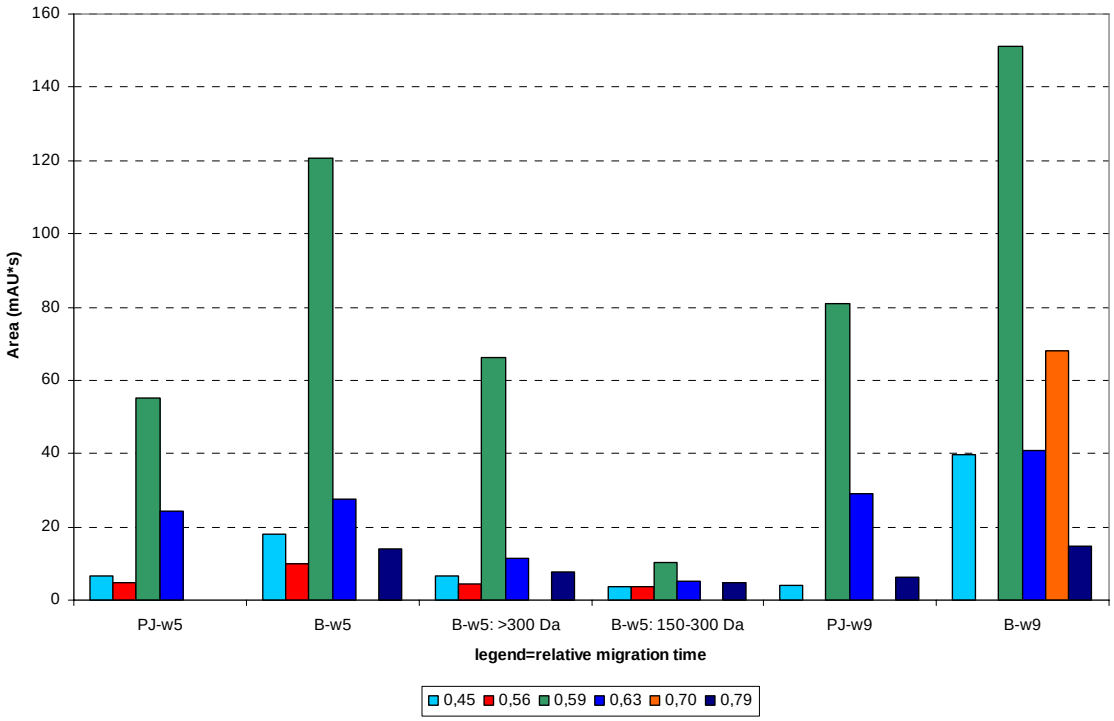


*Figure 9. Amount of compounds (area, AU*s) identified in different aqueous phases of capelin.*

By comparison with standards, e.g. the dipeptides carnosine and anserine, the compound with migration time ca 4.5 min (relative migration time = 0.59) is most likely anserine. The migration time for carnosine and anserine is very similar, with carnosine having slightly shorter migration time.

Lowest amount of compounds (area, AU*s) was seen in broth from week 5 (B-5w:150-300 Da) that was ultra-filtrated to particle size 150-300 Dalton (**Figure 8**). The profile however was similar to the other samples (**Figure 9**). The area of the possible anserine peak was much greater B-w5 >300 Da, than in B-w5:150-300 Da. The size of anserine is 299 Dalton and is therefore possibly identified in both fractions although in higher amount in fraction with particle size >300 Dalton (B-w5>300 Da). The content of anserine appears to be higher in the broth samples (B-w5 and B-w9) compared to the press juice samples (PJ-w5 and PJ-w9) and higher in week 9 than in week 5. Anserine has been identified in higher concentration than carnosine, in fish species like yellow fin tuna, skipjack, and swordfish (Ikeda, 1980).

Other compounds were difficult to identify but by comparison with amino acid standards it can be suggested that three of the peaks were alanine, lysine and glutamic acid. The

large front tailing peak was not identified. These amino acids were all identified earlier in high levels in capelin (Bragadóttir, 2001). Taurine is in the highest level in capelin but was not identified by this method.

## 4. DISCUSSION & CONCLUSIONS

The results from the three tests for antioxidant activity in capelin aqueous extracts conformed quite well. The high capacity of the extracts for chelating ferrous iron may indicate that the antioxidant activities as measured by DPPH radical scavenging capacity and oxygen radical absorbance capacity (ORAC) were explained by this property.

Higher antioxidant activities were observed in the samples from capelin harvested in week 5 compared to week 9. The protein content of the samples did not affect the antioxidant activities as measured by these three tests, although earlier studies have shown that amines, peptides and amino acids have antioxidant properties. The broth appeared to contain higher levels of suggested anserine and other compounds giving rise to higher expected antioxidant activity of the B-w9 sample. More prooxidant activity due to higher hemoglobin content in PJ-w9 could most likely counteract the antioxidant activity of all aqueous samples derived from the capelin harvested in week 9.

The protein content of PJ-w5 was higher than PJ-w9 but their respective broths, B-w5 and B-w9 had opposite connection. The reason for this disagreement in protein content between press juice and broth is not clear. It may be due to some difference in sample treatment or biological variation related to the change in fat and water content that changed drastically over the time interval of four weeks between sample treatments (Table 2). It can be speculated if this biological variation may have influenced the solubility of the proteins in the broth and therefore may have influenced the protein content. Also, the manual squeezing of the broth from the cooked capelin may need to be standardised. Another likely explanation may be related to higher degradation of the week 9 capelin sample, as reflected by higher TVN value of the raw material, as well as in the broth B-w9, compared to B-w5, which was made of beheaded and gutted capelin.

Parallel studies in the Oxifish project on the antioxidant capacity of the capelin extracts in washed cod model system gave contradictory results regarding the antioxidant properties. The broth from week 9 appeared to have antioxidant effect, while the press juice (9 week)

exhibited pro-oxidant activities when evaluated by TBARS, color, rancid odor and GC analysis of volatile compounds (Ólafsdóttir *et a*l., 2006). This can possibly be explained by higher protein content of the broth compared to the press juice and higher content of peptides and amino acids. These results indicate that it may be misleading to predict antioxidant effect in the muscle based on simple, rapid tests. However the overall characterisation obtained when using different test may eventually lead to better understanding of the role of pro- and antioxidants in oxidation in a complex matrix like the fish muscle. Furthermore, when using whole fish including viscera and blood containing high levels of hemoglobin the pro-oxidative effect of iron will result in rapid oxidation and formation of volatile compounds and off odours as was shown in washed cod muscle system with added hemoglobin (Jónsdóttir and others, submitted).

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