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# Protein and Lipid Oxidation during Frozen Storage of Rainbow Trout (*Oncorhynchus mykiss*)

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This study aimed at investigating protein and lipid oxidation during frozen storage of rainbow trout. Rainbow trout fillets were stored for 13 months at −20, −30, or −80 °C, and samples were analyzed at regular intervals for lipid and protein oxidation markers. Lipid oxidation was followed by measuring lipid hydroperoxides (PV), as well as secondary oxidation products (volatiles) using dynamic headspace GC-MS. Free fatty acids (FFA) were measured as an estimation of lipolysis. Protein oxidation was followed using the spectrophotometric determination of protein carbonyls and immunoblotting. Significant oxidation was observed in samples stored at −20 °C, and at this temperature lipid and protein oxidation seemed to develop simultaneously. FFA, PV, and carbonyls increased significantly for the fish stored at −20 °C, whereas the fish stored at −30 and −80 °C did not show any increase in oxidation during the entire storage period when these methods were used. In contrast, the more sensitive GC-MS method used for measurement of the volatiles showed that the fish stored at −30 °C oxidized more quickly than those stored at −80 °C. Detection of protein oxidation using immunoblotting revealed that high molecular weight proteins were oxidized already at $t = 0$ and that no new protein oxidized during storage irrespective of the storage time and temperature. The results emphasize the need for the development of more sensitive and reliable methods to study protein oxidation in order to gain more explicit knowledge about the significance of protein oxidation for food quality and, especially, to correlate protein oxidation with physical and functional properties of foods.

**KEYWORDS: Protein oxidation; protein carbonyls; lipid hydroperoxides; trout; frozen storage**

## INTRODUCTION

Fatty fish such as salmon and trout contain a high level of omega-3 polyunsaturated fatty acids (PUFA), and due to the beneficial health effects of omega-3 PUFA (*1, 2*) their consumption is highly encouraged. Prolonged frozen storage of fatty fish can lead to oxidation of omega-3 PUFA, which can result in the development of unpleasant off-flavors (*3–5*). However, some of the quality changes observed in fish during frozen storage such as toughness and loss of protein functional properties cannot be attributed solely to lipid oxidation (*6, 7*).

Earlier investigations have reported that oxidation of protein could result in severe loss of protein functionality (*8*), and this is believed to explain some of the changes associated with quality loss during frozen storage of fish (*9–11*). The impact of protein oxidation on myofibrillar protein functionality and on muscle food quality has recently received more attention (*12, 13*), and protein oxidation has been shown to affect protein solubility, decrease gel elasticity, and affect water distribution in muscle foods (*14–16*).

In the muscle, reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radical, are generated in situ by metals or enzymes. These reactive oxygen species can catalyze oxidative reactions that may have detrimental effects on fish quality, affecting both lipids and proteins. It is now recognized that protein oxidation is an important parameter, which may explain some changes associated with quality loss both during processing and storage of muscle foods (*14–17*). A previous proteomic and 2D-immunoblotting study of protein oxidation in rainbow trout showed that some proteins are more susceptible to oxidation than others (*18*). However, little is known about the kinetics of protein oxidation, its importance in muscle foods, and, especially, the interaction between protein and lipid oxidation. It is unclear if protein and lipid oxidation are concomitant processes or if one event precedes the other. Lipid oxidation products such as malonaldehyde and 4-hydroxy-nonenal (4-HNE) can interact with proteins, resulting in protein damage (*19, 20*). On the other hand, proteins are also able to bear free radicals and to transfer them to other macromolecules such as lipids, DNA, starch, or other proteins (*21–23*). It has also been suggested that depending on their ability to scavenge free radicals or chelate metals, proteins can be either prooxidative or antioxidative (*24*). In biological systems it is now fully

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**Table 1.** Fatty Acid Composition[a] of Rainbow Trout Muscle Fed Fish Oil and Astaxanthin (±SD)

| fatty acid | % |
|---|---|
| C14:0 | 4.13 (±0.18) |
| C16:0 | 14.95 (±0.32) |
| C18:0 | 3.08 (±0.10) |
| Σ saturated | 22.16 (±0.38) |
| C16:1(n-7) | 5.57 (±0.26) |
| C18:1(n-7) | 2.79 (±0.13) |
| C18:1(n-9) | 13.76 (±0.24) |
| C20:1(n-9) | 5.23 (±0.17) |
| C22:1(n-11) | 4.82 (±0.11) |
| Σ MUFA | 32.17 (±0.39) |
| C18:2(n-6) | 5.69 (±0.80) |
| C18:3(n-3) | 1.20 (±0.09) |
| C18:4(n-3) | 1.48 (±0.10) |
| C20:4(n-3) | 1.24 (±0.04) |
| C20:5(n-3) | 6.12 (±0.24) |
| C21:5(n-3) | 0.40 (±0.02) |
| C22:5(n-3) | 2.28 (±0.16) |
| C22:6(n-3) | 17.27 (±1.36) |
| Σ PUFA | 35.68 (±1.95) |
| others[b] | 9.99 (±1.31) |

[a] The fatty acid composition is expressed as percent of total fatty acids and is determined using an internal standard C:23. [b] Others are fatty acids that could not be identified.

recognized that proteins are a primary target for free radical attacks, but little is known about the mechanisms responsible for their prooxidative or antioxidative activity (*25*). In muscle-based foods, knowledge about the kinetics of protein oxidation processes is lacking, and systematic investigations of both lipid and protein oxidation processes are required to obtain a better understanding of changes in quality during storage.

The objective of this investigation was to reveal if prolonged frozen storage of rainbow trout induces oxidative changes in the protein fraction correlating with the changes observed in the lipid fraction. Protein oxidation was measured using both spectrophotometric determination of protein carbonyls and immunoblots to detect protein carbonyls, whereas lipid oxidation was evaluated using traditional analytical methods, that is, determination of lipid hydroperoxides and secondary volatile oxidation products.

## MATERIALS AND METHODS

**Materials.** All chemicals and reagents used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). Bovine serum albumin dinitrophenyl conjugated standard was from Molecular Probes (Eugene, OR). Protease inhibitor Complete was from Roche (Mannheim, Germany). Tocopherol standards were purchased from Calbiochem (San Diego, CA).

**Raw Material.** For the pre and postrigor study a total of nine rainbow trout (*Oncorhynchus mykiss*) were obtained alive from Asnjs Fiskeopdrjt (Kalundborg, Denmark) and killed in the laboratory. For the prolonged storage experiment, fish fed for 64 days with a feed containing 24.4% fish oil, 110 ppm of astaxanthin, and 18.2, 2.1, 1.3, and 0.7 ppm of α-, β-, γ-, δ-tocopherol, respectively, were obtained from Biomar A/S (Brande, Denmark). The fatty acid composition of fish used in the frozen storage experiment is presented in **Table 1**. and calculated as relative concentration in percent. The fish were not bled but slaughtered by electrocution when they reached approximately 350 g, filleted as butterfly fillets using an industrial filleting machine, frozen at −30 °C on an industrial steel belt freezer with a freezing time of 20 min, and stored at −30 °C for 1 week ($t = 0$) before they were transferred to temperatures of −20, −30, or −80 °C. Fish were packed individually in standard polyethylene bags with a thickness of 70 μm (Tobiplast A/S, Greve, Denmark) and stored frozen for up to 13 months. Reference samples (−80 °C) were vacuum-packed in plastic bags made with laminar film with low gas permeability (NEM 40/LLDE 75, Amcor Flexible A/S, Horsens, Denmark) (thickness = 116 μm; water vapor transmission = 1 g/m$^2$/24 h; and oxygen transmission = 6 cm$^3$/m$^2$/24 h/1 atm). All analyses were conducted on a minimum of three fish for each storage condition, and measurements were performed on individual fillets, in triplicate. The part of the fillet from the gill to the dorsal fin was used for determination of the oil content, fatty acid composition, free fatty acid, antioxidants, and protein analysis. In addition, for the determination of protein carbonyls and immunoblot a small part of the white muscle was used. The tail part of the individual fillet was used for the analysis of secondary oxidation volatiles products.

**Oil Content.** Fish muscle samples of 50 g were ground, and 10 g in duplicate was used for determination of the oil content, which was determined gravimetrically using chloroform and methanol according to the protocol of Bligh and Dyer, but using a reduced amount of solvent (*26*).

**Fatty Acid Composition.** Fatty acids in the Bligh and Dyer extract were transesterified to methyl ester using a base-catalyzed transesterification followed by a boron trifluoride catalyzed esterification according to the AOCS method (*27*). The methyl esters were dissolved in *n*-heptane to a concentration of approximately 20 mg/mL. A HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector was used for the separation of the fatty acid methyl esters. The column was an Omegawax 320 fused silica capillary (0.32 mm × 30 m × 0.25 μm) (Supelco, Bellefonte, PA). The injection volume was 0.2 μL injected in the split mode with a split ratio of 1:50. The injection and detection temperatures were 250 and 240 °C, respectively. The initial oven temperature was 160 °C, immediately raised by 3 °C/min to 200 °C, held for 1 min, further raised by 3 °C/min to 220 °C, and held for 12 min The helium carrier gas flow was 21 cm/s. An internal standard, methyl tricosanoate (C:23), was used to calculate the relative percentage of the different fatty acids in the lipid fraction.

**Free Fatty Acid (FFA).** FFA content was determined by acidometric titration of the Bligh and Dyer extract using NaOH (0.1 M). The FFA content was calculated as oleic acid according to the AOCS method (*28*).

**Antioxidants.** Tocopherol and astaxanthin contents were determined on the Bligh and Dyer extract using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA), equipped with a fluorescence detector or a UV diode array detector respectively. A fraction of the lipid extract was evaporated under nitrogen and redissolved in 2 mL of *n*-heptane before injection. For tocopherol analysis an aliquot (40 μL) of the *n*-heptane fraction was injected on a Spherisorb s5w column (250 mm × 4.6 mm) (Phase Separation Ltd., Deeside, U.K.) and eluted with an isocratic mixture of *n*-heptane/2-propanol (100:0.4, v/v) at a flow of 1 mL/min. Detection was performed using a fluorescence detector with excitation at 290 nm and emission at 330 nm and according to the AOCS method (*29*). Astaxanthin content was determined after injection of an aliquot (50 μL) of the *n*-heptane fraction onto a LiChrosorb S160 column (100 mm × 3 mm, 5 μm) equipped with a Crompsep silica (S2) guard column (Chrompack, Middleburg, The Netherlands) and eluted with a flow of 1.2 mL/min using *n*-heptane/acetone (86:14, v/v) and detection at 470 nm.

**Lipid Oxidation.** *Peroxide Values.* Peroxide values were measured on the lipid extract (Bligh and Dyer) by colorimetric determination using the ferric thiocyanate assay as described by Decker and Shantha (*30*). Results are expressed in milliequivalents of peroxide per kilogram of oil.

*Volatiles.* Volatiles were collected from the rainbow trout muscle by dynamic headspace sampling. The samples were prepared according to the protocol described by Refsgaard et al. (*31*). Briefly, 50 g of fish was frozen in liquid nitrogen and comminuted into a fine powder. The powder (10 g in triplicate) was slurried in water, and the sample was purged with nitrogen at 340 mL/min for 10 min at 37 °C. The volatiles were trapped on a Tenax-GR trap column (Varian Chrompack

International, Bergen op Zoom, The Netherlands), and volatiles were released from the column by thermal desorption (ATD-400, Perkin-Elmer, Boston, MA) and analyzed by GC-MS on a 30-m DB 1701 capillary column (J&W Scientific, Folsom, CA). The temperature program used was 40 °C for 10 min, ramping at 3 °C/min to 140 °C, at 5 °C/min to 170 °C, and at 10 °C/min to a 240 °C final temperature. The ionization energy was set to 70 eV in the IE and the scan range from 30 to 250 atomic mass units with a repetition rate at 3.4 scans/s. For quantification, calibration curves were made by injecting standards directly to the Tenax-GR trap column and analyzed as described above for the samples.

**Protein Oxidation.** *UV Spectroscopy.* Protein carbonyls were measured as described by Levine et al. (*32*, *33*). A fish sample (0.5 g) was homogenized in 10 mL of Tris buffer (pH 7.4, 50 mM, 1 mM EDTA) containing 0.01% (w/w) BHT. For spectroscopic determination of carbonyl groups 100 $\mu$L of the homogenate was precipitated with 50 $\mu$L of TCA (100%, w/v). After centrifugation (12600*g*, 3 min), the pellet was incubated with 1 mL of dinitrophenylhydrazine (DNPH) in 2 M HCl, in the dark for 1 h. For each sample a blank incubated in 2 M HCl and without DNPH was run in parallel. The samples were precipitated with 50 $\mu$L of TCA (100%, w/v), and the pellets were washed three times with 1 mL of ethanol/ethyl acetate (1:1, v/v). The pellet was redissolved in 6 M guanidine chloride in 20 mM $KH_2PO_4$. The carbonyl content was calculated using the absorbance measurements at 370 and 280 nm.

*SDS-PAGE and Immunoblotting.* For immunoblotting the DNPH reaction was performed directly on the protein homogenate or on the sarcoplasmic protein fraction (low salt soluble protein) obtained by centrifugation of the protein homogenate at 12600*g* for 3 min The supernatant contained the sarcoplasmic proteins and the pellet the myofibrillar proteins. Prior to DNPH reaction the protein concentration was adjusted to 5 mg/mL using the BCA kit (Pierce, Rockford, IL). The sample (30 $\mu$L) was mixed 1:1 with 12% SDS and further diluted with 60 $\mu$L of DNPH in 10% trifluoroacetic acid. The samples were incubated for 1 h in the dark at room temperature. The reaction was stopped by adding 60 $\mu$L of neutralizing solution (1.85 M Trizma-base, 28% glycerol, and 0.1 M DTT). The samples were centrifuged for 3 min at 12600*g* and loaded on the gel (10% Bis-tris gels or 12% Tris glycine gels; Invitrogen A/S, Taastrup, Denmark). After the run, one gel was stained with Coommasie Brilliant Blue G-250, and one gel was used for immunoblotting. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane 0.2 $\mu$m (Millipore, Billerica, MA) using a Hoefer TE-77 semidry transfer unit (Amersham Health AS, Hillerød, Denmark) or a Mini Cell SureLock, equipped with an XCell II blot module (Invitrogen A/S, Taastrup, Denmark) for approximately 60–70 min After transfer, the membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) buffer (0.137 M NaCl and 20 mM Tris-HCl, pH 8.0) and incubated with a 1:15000 dilution of rabbit anti-DNP (DAKO Denmark A/S, Glostrup, Denmark) in 1% skim milk, in TBS for 1 h. The membranes were washed in TBS and incubated in a 1:7500 dilution of the secondary antibody, peroxidase-conjugated swine antirabbit (DAKO Denmark A/S). After washing in TBS, the blot was either developed using the ECL ± kit (Invitrogen A/S) or incubated for 1 min in 2.7 mM $H_2O_2$, 0.07 mM *p*-coumaric acid, and 1.25 mM luminol (3-aminophthalhydrazide) in 0.1 M Tris-HCl, pH 8.6. Chemiluminescence was detected on hyper-film ECL (Amersham Health A/S, Hillerød, Denmark).

Image analysis of gels and blots were performed using the software Quantity One (Bio-Rad, Herlev, Denmark).

**Statistical Analysis.** The experiments were carried out on three fish unless otherwise stated. The results are given as mean values of triplicates with indication of the standard deviation. Evaluation of statistical significance of differences was performed using Student's *t* test.

## RESULTS

**Protein Oxidation.** Investigation of the development of protein oxidation in trout pre and postrigor mortis was measured on a total of nine rainbow trout. The results (**Table 2**) showed a great variation in protein carbonyl level between individuals at the time of slaughter ranging from 1.8 to 3.9 nmol of carbonyl/mg of protein. Postrigor (i.e., after 48 h on ice) the protein oxidation level was re-evaluated for each individual, and the results showed no changes in the protein carbonyl after 48 h on ice with levels ranging from 2.1 to 4.2 nmol/mg of protein. The mean value for the six individuals did not reveal any significant difference for pre and postrigor and reached 2.8 and 2.9 nmol/mg, respectively, which are in agreement with data reported for healthy biological tissue samples (*34*, *35*).

**Table 2.** Protein Oxidation in Nine Individual Rainbow Trout Expressed at Killing and after 48 h on Ice (±SD)

| fish | at killing (nmol of carbonyl/ mg of protein) | 48 h on ice (nmol of carbonyl/ mg of protein) |
|---|---|---|
| 1 | 3.9 (±0.4) | 4.2 (±1.1) |
| 2 | 3.5 (±0.4) | 3.4 (±0.7) |
| 3 | 2.9 (±0.7) | 2.9 (±0.5) |
| 4 | 2.3 (±0.7) | nd[a] |
| 5 | 1.8 (±0.5) | nd |
| 6 | 2.2 (±0.3) | nd |
| 7 | nd | 2.3 (±0.5) |
| 8 | nd | 2.1 (±1.0) |
| 9 | nd | 2.3 (±0.2) |
| mean | 2.8 (±0.8) | 2.9 (±0.8) |

[a] Not determined.

During frozen storage of rainbow trout for up to 13 months at −80 °C protein oxidation measurements revealed no significant changes in the level of protein carbonyls (**Figure 1**), indicating that protein did not oxidize in these samples. Fish stored at −30 °C showed the same tendency, and no significant increase in the level of protein carbonyls was observed even after 13 months of frozen storage. The protein carbonyl levels measured in fresh fish and in fish stored at −80 or −30 °C for up to 13 months were in the same range, and not statistically different, indicating that protein oxidation was minimal under these conditions, irrespective of the storage time and the storage temperature. In contrast, frozen storage of fish at −20 °C gave a significant increase in protein carbonyls already after 8 months with protein carbonyls, reaching 7.7 nmol/mg of protein after 13 months, revealing protein oxidation.

Fish proteins were separated by SDS-PAGE, and overall no major changes in any of the protein bands were observed during storage as shown for fish stored at −20 °C (**Figure 2A**). Immunoblot of protein carbonyl groups in the protein homogenate showed that high molecular weight proteins (above 200 kDa) and protein with size aound 200 and 38 kDa were heavily oxidized (**Figure 2B**). These latter bands were most likely myosin and actin, respectively, as already reported in our previous investigation using 2D immunoblotting (*18*). Samples stored for 8 and 13 months revealed slightly more intense bands in the 35–38 kDa area of the blot when compared to the initial sample ($t = 0$). However, no new protein seemed to be oxidized, as seen from **Figure 2B** representing a blot for fish samples stored at − 20 °C over time. Immunoblotting of sarcoplasmic proteins revealed thick bands around 320, 230, and 160–140 kDa, but no bands were detected at lower molecular weight (**Figure 3**). The identity of the oxidized protein corresponding to the protein bands observed in the sarcoplasmic fraction is unclear. After 13 months of frozen storage, only small differences in protein oxidation patterns and levels were observed for the different storage temperatures. However, the samples stored at −20 °C seemed to be slightly more oxidized compared to the other two storage temperatures. The results from the total

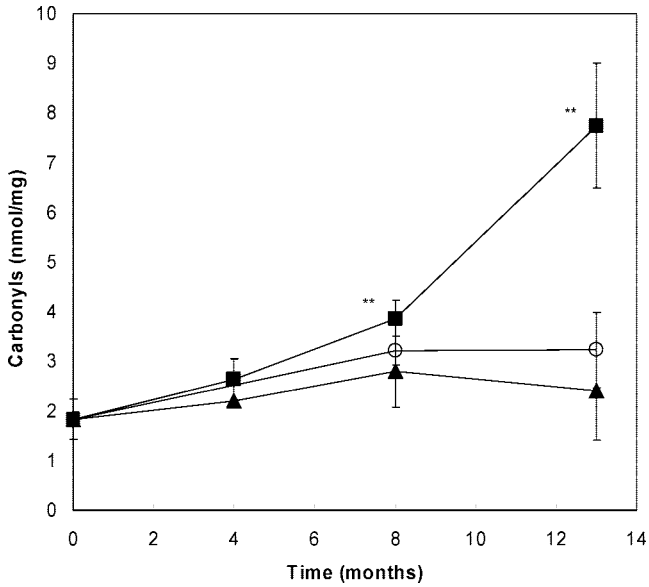


**Figure 1.** Formation of protein carbonyl groups during frozen storage of rainbow trout at −20 °C (■), −30 °C (▲), and −80 °C (○) for up to 13 months, with ∗∗ ($p < 0.01$) against fresh frozen sample ($t = 0$).

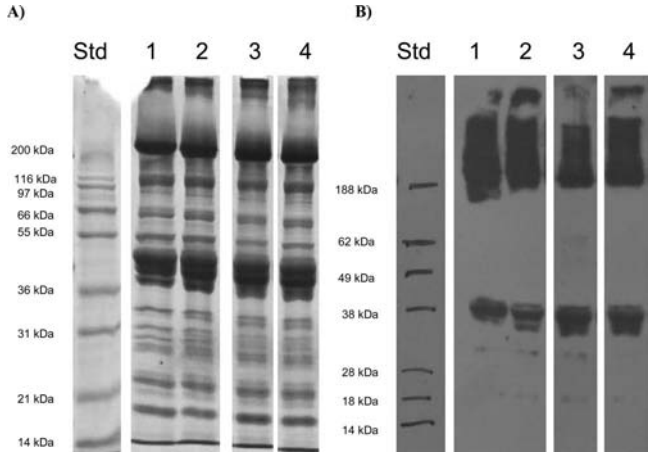


**Figure 2.** Rainbow trout muscle homogenate (1) at $t = 0$ or after frozen storage at −20 °C for (2) 4 months, (3) 8 months, or (4) 13 months, with (**A**) SDS-polyacrylamide gel electrophoresis and (**B**) immunoblotting against protein carbonyl groups. Std, molecular weight marker.

homogenate and the sarcoplasmic protein fraction are in agreement, meaning that no new proteins are oxidized and that the main difference observed between the samples might be a slight increased intensity (which is difficult to quantity) of the bands on the blot for −20 °C samples with prolonged storage time.

**Lipid Oxidation.** Measurement of the development of primary lipid oxidation products such as lipid hydroperoxides in the different fish samples was performed (**Figure 4**). There was a significant increase in the level of lipid hydroperoxides after 8 months of frozen storage for fish stored at −20 °C, which was even more pronounced after 13 months, reaching 6.6 mequiv/kg of oil, indicating ongoing oxidation. In contrast, samples stored at −80 and −30 °C did not show any significant increases in peroxides during the entire storage period (with $p = 0.26$ and $p = 0.07$, respectively). Free fatty acid levels (**Figure 5**) indicating lipid hydrolysis showed the same pattern and reached the highest level for samples stored at −20 °C, possibly revealing some enzymatic activity. Measurement of secondary oxidation products was followed for 13 months, and the development of hexanal, an oxidation product of linoleic

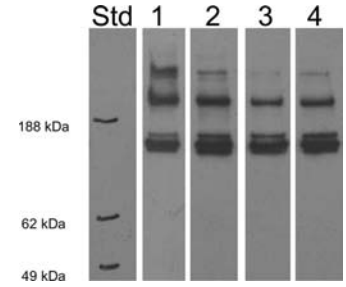


**Figure 3.** Western blot against protein carbonyl groups in the low salt soluble protein fraction of rainbow trout (1) at $t = 0$ or after $t = 13$ months of frozen storage at (2) −20 °C, (3) −30 °C, and (4) −80 °C. Std, molecular weight marker.

acid, and 1-penten-3-one and *t,t*-2,4-heptadienal, both oxidation products of omega-3 fatty acids, is presented in **Figure 6**. Other volatiles were also measured during storage (1-penten-3-ol, heptanal, 1-octen-3-ol, *t*-2-octenal, nonanal, *t,c*-2,6-nonadienal, decanal), and their development was generally in agreement with what is reported here for hexanal, 1-penten-3-one, and *t,t*-2,4-heptadienal. The development of hexanal matched the observed pattern for oxidation revealed by FFA, PV, and protein carbonyls, indicating that fish stored at −20 °C was the most oxidized and that little difference was observed between −80 and −30 °C. In contrast, volatiles from omega-3 PUFA increased significantly in fish stored at −30 °C for 13 months when compared to −80 °C. The volatile 1-penten-3-one reached the same concentration in fish stored at −20 and −30 °C after 8 and 13 months, whereas *t,t*-2,4-heptadienal followed the same pattern for the first 8 months but reached lower concentration for fish stored at −30 °C compared to −20 °C after 13 months. On the basis of these observations the ranking order −20 °C > −30 °C > −80 °C was obtained for the development of oxidation in fish stored at freezing temperatures. In addition, these results indicated that measurement of volatiles is a very sensitive method for measuring the development of lipid oxidation. Samples stored at −80 °C did not show any significant development of volatiles characteristic of lipid oxidation compared to the control sample ($t = 0$). This confirms the data obtained with the peroxides and the FFA (**Figures 3** and **4**), indicating that no lipid oxidation and no hydrolysis were detected in samples stored at −80 °C.

**Antioxidants.** Measurement of the antioxidant status in the fish muscle was performed after 13 months of frozen storage for the different temperatures (**Table 3**). Only samples stored at −20 °C showed a significant decrease in tocopherol content after 13 months, whereas astaxanthin levels present in the muscle were unaffected irrespective of the storage temperature and oxidative status.

## DISCUSSION

The aim of this study was to evaluate how frozen storage of rainbow trout at −20, −30, and −80 °C for up to 13 months affected the level of lipid and protein oxidation. Lipid and protein oxidations were followed simultaneously to obtain optimal information on the overall oxidative status and to gain new insights into the correlation between lipid and protein oxidation in fish.

In the results reported here a great variation in the protein carbonyl results could be ascribed to biological variation between individuals (**Table 2**). The relative standard deviation calculated from the data obtained with the six different individuals indicated a relatively high deviation of around 20%. The

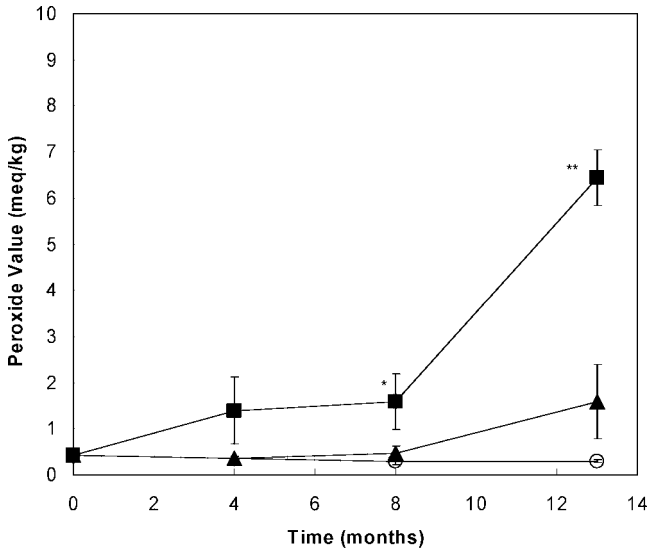


**Figure 4.** Development of lipid hydroperoxides during frozen storage of rainbow trout at −20 °C (■), −30 °C (▲), and −80 °C (○) for up to 13 months, with * ($p < 0.05$) and ** ($p < 0.01$) against fresh frozen sample ($t = 0$).

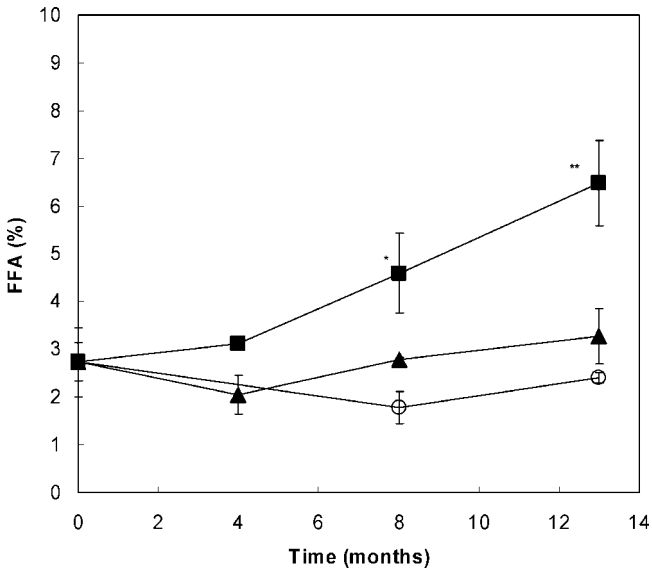


**Figure 5.** Formation of free fatty acids (FFA in percent oleic acid) during frozen storage of rainbow trout at −20 °C (■), −30 °C (▲), and −80 °C (○) for up to 13 months, with * ($p < 0.05$) and ** ($p < 0.01$) against initial level ($t = 0$).

biological variation in carbonyl content at the individual level was expected, and similar biological variation between individuals from the same family for fat, tocopherol, and astaxanthin contents has also been reported in farmed salmon (*38*). Measurement of protein carbonyl groups using the DNPH assay is widely used when protein oxidative status and damage in cells and in tissues are investigated (*39*, *40*). However, the assay itself-has been questioned for its reproducibility and sensitivity (*41*, *42*). Early investigations estimated its reproducibility level at around 5% (*34*).

Interestingly, the levels of oxidized protein were identical pre or postrigor, indicating that the onset of rigor mortis did not induce further free radical attack on the protein or that at this stage enzymatic system and antioxidants present in the fish muscle were able to cope with free radical attack. This might be due to the fact that protein oxidation is involved in cell signaling and apoptosis and that the cell is capable of tolerating

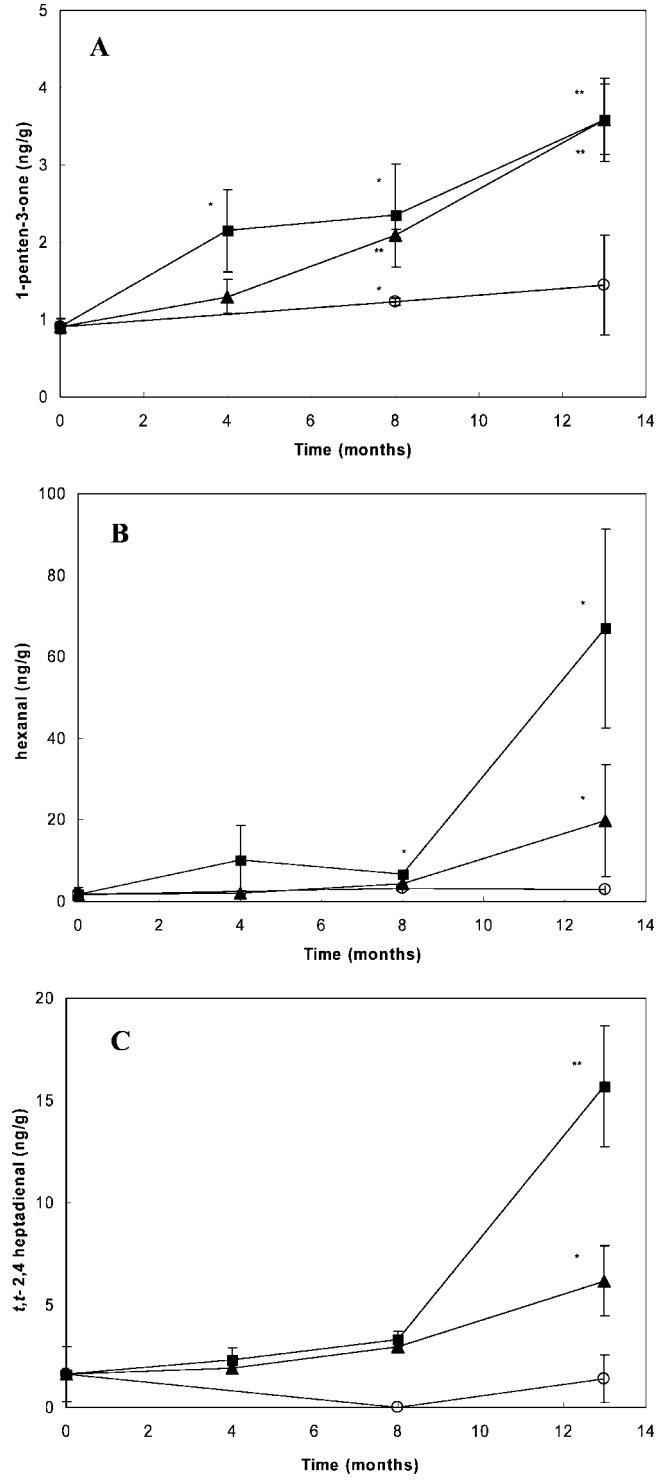


**Figure 6.** Volatiles formation during frozen storage of rainbow trout at −20 °C (■), −30 °C (▲), and −80 °C (○) for up to 13 months with (**A**) 1-penten-3-one, (**B**) hexanal, and (**C**) *t*,*t*-2,4-heptadienal, with * ($p < 0.05$) and ** ($p < 0.01$) against initial level ($t = 0$).

some free radical injury (*43*, *44*). On the basis of the calculation reported by Reznick and Packer (*34*) and assuming that the protein fraction has an average molecular mass of 50 kDa, for a sample containing 2.7–2.8 nmol of carbonyl/mg of protein, the percentage of carbonyl groups relative to the protein on a molar basis could represent at most around 14% of the total protein fraction. Indeed, the average molecular weight of muscle protein is expected to be much higher with contribution of myosin by 50% and other large proteins such as titnin and

**Table 3.** Consumption of Antioxidant, Astaxanthin, and α-Tocoperol in Rainbow Trout Muscle during Storage for 13 Months at −30, −20, or −80 °C (±SD)

| storage temp (°C) | time (months) | tocopherol (mg/kg) | astaxanthin (mg/kg) |
|---|---|---|---|
| 0 | 0 | 20.9 (±2.2) | 9.8 (±3.0) |
| −20 | 13 | 14.5* (±1.0) | 7.7 (±2.7) |
| −30 | 13 | 17.5 (±4.7) | 8.2 (±3.2) |
| −80 | 13 | 16.2 (±6.5) | 7.7 (±3.0) |

* $p < 0.05$ against fresh frozen sample ($t = 0$).

nebulin. The level obtained of around 2–3 nmol/mg of protein was very similar for different storage times and temperatures. Thus, even after 13 months of storage at −80 or −30 °C, little protein oxidation was detected, with carbonyl levels of 3.2 and 2.4 nmol/mg of protein, respectively. This indicates that if there were any small changes in oxidative modification at the protein level during frozen storage, they could not be detected using the DNPH assay. After 13 months of storage at −20 °C, an increase to 7.7 nmol/mg of protein was observed. High levels of protein carbonyls are usually reported in the pathological conditions of many diseases and in aging (*45–47*), but other studies have also reported such high levels for muscle foods (*12, 48*). In fish, previous studies have indicated an increase in carbonyl groups during ice storage (*8, 49*). Additionally, prolonged frozen storage of rainbow trout under the same conditions as performed in the present study revealed that the protein carbonyl group reached 5.2 nmol of carbonyl/mg of protein after 2 years of storage at −20 °C. This decrease in protein carbonyl groups with prolonged storage might be explained by further reactions of the protein carbonyls with other cellular constituents such as carbohydrates, lipids, and nucleophilic compounds and also polymerization of highly oxidized material, which in turn becomes insoluble (*50, 51*). A recent investigation also reported a decrease in protein carbonyl groups over time after inducing severe oxidation in pork myofibrils (*52*). From our data and from previous results in the literature (*34, 41, 42*) it is clear that the DNPH assay is not suitable for the detection of small modifications at the protein level and that only severe protein modifications can be detected. However, the increase in protein carbonyls observed after prolonged storage at −20 °C compared to the other temperatures indicated that the highest frozen storage temperature gave the largest increase in protein and lipid oxidation products. It has previously been reported that decreasing the storage temperature had a positive impact on fish protein quality during storage (*53*). From this it can be concluded that a storage temperature of −20 °C is not appropriate for prolonged storage of fatty fish.

Immunoblotting of oxidized protein after DNPH derivatization indicated that high molecular weight proteins were oxidized already at the beginning of the storage period. The two muscle structural proteins actin and myosin were primary targets for oxidation as already reported (*18, 54, 55*). Oxidation of actin and myosin has been reported not only to affect polymerization but also to induce cross-linking and formation of aggregates (*50, 52*). Park et al. (*52*) reported that oxidation of myosin led to destabilization of myofribrillar proteins, especially myosin in its rod domain. In meat, others have reported that myosin and actin were the proteins most susceptible to oxidative damage (*12, 55*). From our results it is observed that in the sarcoplasmic protein fraction only high molecular mass proteins with sizes above 140 kDa were oxidized. Cross-linking via disulfide bond formation and aggregation may be responsible for these high molecular weight protein bands; however, reducing agent was present in the samples. From the blots it was not possible to observe a significant effect of the storage time and storage conditions on protein oxidation, but there was no appearance of newly oxidized proteins during frozen storage, confirming our previous results observed by 2D immunoblot analyses (*18*). It is noteworthy that the significant increase in the protein carbonyl levels observed after 13 months of storage at −20 °C measured by spectroscopy was not clearly visible from the immunoblotting. In addition, it is obvious that some protein oxidation did occur during frozen storage of rainbow trout, but it is not possible with the present methods, that is, spectroscopy and immunoblotting of protein carbonyls groups, to make a very sensitive determination of the extent of protein oxidation in fish frozen.

Our investigation of lipid oxidation in frozen fish indicated that low storage temperatures were optimal for preserving fish from oxidative deterioration, and this conclusion was in agreement with previous findings. Similar increases in lipid oxidation have been previously reported in the development of rancidity in salmonoids during frozen storage (*3, 5*), and storage at higher frozen temperatures has been reported to result in increased rancidity and release of free fatty acids (*3, 37*). Surprisingly, the consumption of natural antioxidants during storage was only significant for tocopherol after 13 months for samples stored at −20 °C. Astaxanthin was not significantly consumed after 13 months at any of the temperatures. This finding illustrates the role of tocopherol in protecting the carotenoids. Some studies have reported that astaxanthin concentrations in salmonoids do not change during frozen storage, whereas others have reported a decrease in astaxanthin concentrations during frozen storage (*3–5, 37*). The different results might be due to different initial levels of astaxanthin and tocopherol present in situ in the fish muscle and perhaps also to different levels of prooxidant.

From a comparison of the data obtained from lipid oxidation and protein oxidation it appeared that in the −20 °C stored sample lipid and protein oxidation followed the same pattern; development of protein carbonyls was similar to the development of lipid hydroperoxides and seemed to indicate that protein and lipid oxidations were simultaneous. However, evaluation of lipid oxidation using dynamic headspace GC-MS, which is a powerful technique for the identification and quantification of secondary volatile oxidation products, revealed that lipid oxidation proceeded differently in fish stored at −30 °C compared to fish stored at −80 °C. Thus, it was possible to detect significant increases in volatile oxidation products in fish stored at −30 °C after 8 months. Similar increases in protein carbonyls could not be detected at this temperature. As expected, dynamic headspace GC-MS also revealed that oxidation of volatiles originating from the more unstable omega-3 PUFA was dominating.

With respect to oxidative changes both in the protein and in the lipid fraction, the findings suggested that the colorimetric methods are not so sensitive and that other analytical methods need to be developed and standardized to gain further knowledge about protein oxidative processes and about the formation of lipid hydroperoxides. Degradation of amino acid by Strecker reaction has been extensively studied by Hidalgo et al. (*56*), and they reported that Strecker aldehydes, 3-methylbutanal and 3-methylpropanal, not only originate from the interaction between amino acids and sugar but also could be the results of the interaction between oxidized lipids and protein. The volatile 3-methylbutanal was found in our investigation (not shown), but it is possible that it originated from the interaction between lipid and leucine during oxidation. However, no systematic investigations about its origin and significance for fish quality have been performed, and this topic deserves more attention.

The lack of sensitive methods makes it difficult to gain more knowledge about protein oxidation even if there is increasing evidence that protein oxidation can affect, for example, muscle food texture, protein functionality, or water-holding capacity. Indeed, more systematic investigations of changes in protein functionality relating it to modification of specific muscle proteins ought to be performed. The need for the development of sensitive methods to study protein modification is central if more knowledge about its implications for fish quality and food in general is required.

In summary, this study indicated that with the available methods it seemed that lipid and protein oxidations developed simultaneously in fish stored at −20 °C. Interestingly, the FFA, PV, and carbonyls increased for the fish stored at −20 °C, whereas the fish stored at −30 and −80 °C did not show any increase in oxidation using these methods. In contrast, the more sensitive GC-MS method used for the measurement of the volatiles showed that the fish stored at −30 °C were more oxidized than those stored at −80 °C. It was demonstrated that −80 °C was the best storage temperature and hindered deterioration of the lipids compared to −30 °C. This study highlights that the methods currently available to study protein oxidation and interactions between protein and lipid oxidation are unsatisfactory if more knowledge about the significance of protein oxidation for food quality is to be obtained.

## LITERATURE CITED


(1) Simopoulos, A. P. Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* **1991**, *5*, 438–463.

(2) Shekelle, R. B.; Stamler, J. Fish and coronary heart disease: the epidemiologic evidence. *Nutr. Metab. Cardiovasc. Dis.* **1993**, *3*, 46–51.

(3) Refsgaard, H. H. F.; Brockhoff, P. B.; Jensen, B. Sensory and chemical changes in farmed Atlantic salmon (*Salmo salar*) during frozen storage. *J. Agric. Food Chem.* **1998**, *46*, 3473–3479.

(4) Bjerkeng, B.; Johnsen, G. Frozen storage quality of rainbow trout (*Oncorhynchus mykiss*) as affected by oxygenilluminationand fillet pigment. *J. Food Sci.* **1995**, *2*, 284–288.

(5) Andersen, H. J.; Bertelsen, G.; Christophersen, A. G.; Ohlen, A.; Skibsted, L. H. Development of rancidity in salmonoid steaks during retail display. *Z. Lebensm. Unters. Forsch.* **1990**, *191*, 119–122.

(6) Saeed, S.; Howell, N. K. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomer scombrus*). *J. Sci. Food Agric.* **2002**, *82*, 579–586.

(7) Mackie, I. M. The effect of freezing on flesh proteins. *Food Rev. Int.* **1993**, *9*, 575–610.

(8) Srinivasan, S.; Hultin, H. O. Hydroxyl radical modification of fish muscle proteins. *J. Food Biochem.* **1995**, *18*, 405–425.

(9) Herrero, A. M.; Carmona, P.; Careche, M. Raman spectroscopic study of structural changes in hake (*Merluccius merluccius*) muscle proteins during frozen storage. *J. Agric. Food Chem.* **2004**, *52*, 2147–2153.

(10) Badii, F.; Howell, N. K. Changes in the texture and structure of cod haddock fillets during frozen storage. *Food Hydrocolloids* **2002**, *16*, 313–319.

(11) Careche, M.; del Mazo, M. L.; Fernandez-Martin, F. Extractability and thermal stability of frozen hake (*Merluccius merluccius*) fillets stored at −10 and −30 °C. *J. Sci. Food Agric.* **2002**, *82*, 1791–1799.

(12) Martinaud, A.; Mercier, Y.; Marinova, P.; Tassy, C.; Gatellier, P.; Renerre, M. Comparison of oxidative processes in myofibrillar proteins from beef during maturation and by different model oxidation system. *J. Agric. Food Chem.* **1997**, *45*, 2481–2487.

(13) Saeed, S.; Fawthrop, S. A.; Howell, N. K. Electron spin resonance (ESR) study on free radical transfer in fish lipid-protein interaction. *J. Sci. Food Agric.* **1999**, *79*, 1809–1816.

(14) Ooizumi, T.; Xiong, Y. L. Biochemical susceptibility of myosin in chicken myofibrils subjected to hydroxyl radical oxidizing systems. *J. Agric. Food Chem.* **2004**, *52*, 4303–4307.

(15) Bertram, H. C.; Kristensen, M.; Østdal, H.; Baron, C. P.; Feveile Young, J.; Andersen, H. J. Does oxidation affect the water functionality of myofibrillar proteins. *J. Agric. Food Chem.* **2007**, *55*, 2342–2348.

(16) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Influence of early postmortem protein oxidation on beef quality. *J. Anim. Sci.* **2004**, *82*, 785–793.

(17) Lund, M. N.; Hviid, M. S.; Skibsted, L. H. The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. *Meat Sci.* **2007**, in press.

(18) Kjjrsgård, I. V. H.; Nørrelykke, M. R.; Baron, C. P.; Jessen, F. Identification of carbonylated protein in frozen rainbow trout (*Oncorhynchus mykiss*) fillets and development of protein oxidation during frozen storage. *J. Agric. Food Chem.* **2006**, *54*, 9437–9446.

(19) Tironi, V. A.; Tomas, M. C.; Anon, M. C. Structural and functional changes in myofibrillar proteins of sea salmon (*Pseudopercis semifasciata*) by interaction with malonaldehyde (RI). *J. Food Sci.* **2002**, *67*, 930–935.

(20) Liu, Z.; Minkler, P. E.; Sayre, L. M. Mass spectroscopic characterization of protein modification by 4-hydroxy-2-(*E*)-nonenal and 4-oxo-2-(*E*)-nonenal. *Chem. Res. Toxicol.* **2003**, *16*, 901–911.

(21) Østdal, H.; Davies, M. J.; Andersen, H. J. Reaction between protein radicals and other biomolecules. *Free Radical Biol. Med.* **2002**, *33*, 201–209.

(22) Hakwins, C. L.; Davies, M. J. Generation and propagation of radical reactions on proteins. *Biochim. Biophys. Acta* **2001**, *1504*, 196–219.

(23) Veitch, N. C. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* **2004**, *65*, 249–259.

(24) Davies, M. J.; Dean, R. T. *Radical-Mediated Protein Oxidation*; Oxford University Press: New York, 1997.

(25) Davies, M. The oxidative environment and protein damage. *Biochim. Biophys. Acta* **2005**, *1703*, 93–109.

(26) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

(27) AOCS official method Ce 2-66. Preparation of methyl esters of long-chain fatty acids. *Official Methods of the AOCS*; AOCS: Champaign, IL, 1998.

(28) AOCS official method Ca 5a-40. Free fatty acids. *Official Methods of the AOCS*; AOCS: Champaign, IL, 1994.

(29) AOCS official method Ce 8-89. Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. *Official Methods of the AOCS*; AOCS: Champaign, IL, 1994.

(30) Shantha, N. C.; Decker, E. A. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *J. Assoc. Off. Anal. Chem.* **1994**, *77*, 421–424.

(31) Refsgaard, H. H. F.; Haahr, A. M.; Jensen, B. Isolation and quantification of volatiles by dynamic headspace sampling and mass spectrometry. *J. Agric. Food Chem.* **1999**, *47*, 1114–1118.

(32) Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **1994**, *233*, 346–357.

(33) Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **1990**, *186*, 464–478.

(34) Reznick, A. Z.; Packer, L. Oxidative damage to proteins—spectrophotometric method for carbonyl assay. *Methods Enzymol.* **1994**, *233*, 357–363.

(35) Starke-Reed, P. E.; Oliver, C. N. Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* **1989**, *275*, 559–567.

(36) Milo, C.; Grosch, W. Changes in the odorants of boiled trout (*Salmo fario*) as affected by the storage of the raw material. *J. Agric. Food Chem.* **1993**, *41*, 2076–2081.

(37) Jensen, C.; Birk, E.; Jokumsen, A.; Skibsted, L. H.; Bertelsen, G. Effect of dietary levels of fattocopherol and astaxanthin on colour and lipid oxidation during storage of frozen rainbow trout (*Oncorhynchus mykiss*) and during chill storage of smoked trout. *Z. Lebensm. Unters. Forsch. A* **1998**, *207*, 189–196.

(38) Refsgaard, H. H. F.; Brockhoff, P. B.; Jensen, B. Biological variation of lipid constituents and distribution of tocopherols and astaxanthin in farmed Atlantic salmon (*Salmo salar*). *J. Agric. Food Chem.* **1998**, *46*, 808–812.

(39) Chevion, M.; Berenshtein, E.; Stadtman, E. R. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radical Res.* **2000**, *33*, S99–S108.

(40) Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R. Protein carbonyls groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **2003**, *329*, 23–38.

(41) Fagan, J. M.; Sleczka, B. G.; Sohar, I. Quantification of oxidative damage to tissue proteins. *Int. J. Biochem. Cell Biol.* **1999**, *31*, 751–757.

(42) Cao, G.; Cutler, R. G. Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Arch. Biochem. Biophys.* **1995**, *320*, 106–114.

(43) Wiese, A. G.; Pacifici, R. E.; Davies, K. J. Transient adaptation of oxidative stress in mammalian cells. *Arch. Biochem. Biophys.* **1995**, *318*, 231–240.

(44) Kagan, V. E.; Fabisiak, J. P.; Shvedova, A. A.; Tyurina, Y. Y.; Tyurin, V. A.; Schor, N. F.; Kawai, K. Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Lett.* **2000**, *477*, 1–7.

(45) Chaudhuri, A. R.de; Waal, E. M.; Pierce, A.Van; Remmen, H.; Ward, W. F.; Richardson, A. Detection of protein carbonyls in aging liver tissue: a fluorescence-based proteomic approach. *Mech. Ageing Dev.* **2006**, *127*, 849–861.

(46) Smith, C. D.; Carney, J. M.; Starkereed, P. E.; Oliver, C. N.; Stadtman, E. R.; Floyd, R. A.; Markesbery, W. R. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer-disease. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10540–10543.

(47) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab. Rev.* **1998**, *30*, 225–243.

(48) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Influence of early postmortem protein oxidation on beef quality. *J. Anim. Sci.* **2004**, *82*, 785–793.

(49) Passi, S.; Cataudella, S.; Tiano, L.; Littarru, G. P. Dynamics of lipid oxidation and antioxidant depletion in Mediterranean fish stored at different temperatures. *BioFactors* **2005**, *25*, 241–254.

(50) Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Gagliano, N.; Lusini, L.; Milzani, A.; Di Simplicio, P.; Colombo, R. Actin carbonyl-hdation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radical Biol. Med.* **2001**, *31*, 1075–1083.

(51) Bader, N.; Grune, T. Protein oxidation and proteolysis. *Biol. Chem.* **2006**, *387*, 1351–1355.

(52) Park, D.; Xiong, Y. L.; Alderton, A. L. Concentration effect of hydroxyl radical oxidizing systems on biochemical properties of porcine muscle myofibrillar protein. *Food Chem.* **2006**, *101*, 1239–1246.

(53) Saeed, S.; Howell, N. K. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). *J. Sci. Food Agric.* **2002**, *82*, 579–586.

(54) McDonagh, B.; Tyther, R.; Sheehan, D. Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and Western blotting: actin as a target for oxidative stress. *Aquat. Toxicol.* **2005**, *73*, 315–326.

(55) Stagsted, J.; Bendixen, E.; Andersen, H. J. Identification of specific oxidatively modified proteins in chicken muscles using a combined immunologic and proteomic approach. *J. Agric. Food Chem.* **2004**, *52*, 3967–3974.

(56) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino-carbonyl reactions. *J. Agric. Food Chem.* **2000**, *48*, 3152–3158.

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