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Hemoglobin-Mediated Lipid Oxidation and Compositional Characteristics of Washed Fish Mince Model Systems Made from Cod (*Gadus morhua*), Herring (*Clupea harengus*), and Salmon (*Salmo salar*) Muscle

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The use of washed cod light muscle minces in mechanistic studies of hemoglobin (Hb)-mediated fish lipid oxidation has largely increased in the past 5 years. Although cod light muscle has a low level of intrinsic lipid oxidation catalysts, a prerequisite for a good oxidation model system, we believe it cannot fully mimic the oxidation kinetics taking place in other fish species being more susceptible to lipid oxidation. The aim of this study was to systematically investigate whether washed mince model systems useful in Hb-mediated oxidation studies could be prepared also from herring (*Clupea harengus*) and salmon (*Salmo salar)* light muscles. The kinetics of oxidation in the washed models was measured during ice storage (+/−Hb), and the results were related to compositional differences. Minces from cod, herring, and salmon light muscles were washed 3 times with 3 volumes of water and buffer. A 20 μM portion of Hb and 200 ppm streptomycin was then added, followed by adjustment of pH and moisture to 6.3 and 86%, respectively. Samples with or without Hb were then stored on ice, and oxidation was followed as peroxide value (PV), rancid odor, redness (*a**) loss and yellowness (*b**). Prior to storage, all minces and models were also analyzed for total lipids, fatty acids, α-tocopherol, proteins, Hb, Fe, Cu, and Zn. Hb-mediated lipid oxidation appeared within 2 days on ice in all models. Small differences in the oxidation rates ranked the models as herring > cod > salmon. These differences were ascribed to more preformed peroxides and trace elements in the herring model, and more antioxidants in the salmon model. Controls, without Hb, stayed stable in all cases except herring, where a very slight oxidation appeared, especially if the herring raw material had been prefrozen. In conclusion, fattier fish like dark muscle species and salmonoids are useful for making washed mince model systems and would be a better choice than cod if there is an interest in the oxidation kinetics of such species.

KEYWORDS: Fish; lipid oxidation; washed mince; hemoglobin; cod; salmon; herring

INTRODUCTION

Development of model systems for use in pro- and antioxidant research is a compromise between reality and simplicity. The oxidation substrate, surrounding matrix, and storage conditions should resemble a real system as much as possible. Yet, the system should be simplified enough in order to draw mechanistic conclusions. Within marine research, examples of models that have been used to study the oxidation process in fish tissue is bulk fish oils, emulsions, micellar systems (e.g., from linoleic acid), isolated bilayer structures (fish microsomes, marine phospholipid liposomes), and fish minces (washed and unwashed). In recent years, washed fish mince (usually treated by three washes with three volumes of water and/or a buffer pH 6.2–6.5) has emerged as a particularly useful tool in oxidation research. This is because washed fish mince provides a matrix that has the structure of muscle, i.e., with myofibrillar proteins and membranes, but has a reduced content of endogenous triacylglycerols and aqueous pro- and antioxidants. Controlled physiological levels of oxidation catalysts can then be added and studied in relation to lipid oxidation under various conditions of pH, moisture etc. By adding an antibacterial agent (usually 200 ppm streptomycin), the time frame during which oxidation can be studied during ice storage is extended, normally up to 8–14 days.

To date, most trials with washed fish mince as a model have been done using light muscle from cod (*Gadus morhua*) as this species naturally is very low in total lipids and catalysts (*1*–*9*).

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To start up oxidation, endogenous levels of fish hemoglobin (Hb) (5.8–20 μM), have almost exclusively been used (*1–5*, *9*). However, a few recent studies also exist where marine (sperm whale, trout) myoglobins (Mb) have been added (*6–8*). Heme proteins have in the past 10 years emerged as main mediators of oxidation in post mortem fish muscle. When running trials at the slightly acidic pH values (6.2–6.5) commonly found in post mortem fish, Hb-mediated oxidation in washed cod is usually measurable within 1–5 days during storage on ice. Studies in the washed cod mince model system have contributed to knowledge about the role of Hb deoxygenation, autoxidation, hemin loss, Fe loss, relative pro-oxidative activity of Hb vs Mb, and also of different forms of Hb (e.g., anodic/cathodic and Hb from different fish species) (*4*, *5*, *8*, *9*).

Since lipid oxidation generally is more of a problem in other fish species than cod, we feel that it is not completely accurate to use cod muscle for mimicking such fish in lipid oxidation research. It would be more realistic if lipid oxidation studies could be conducted in models made from the specific fish species in question. In this study, the overall aim was therefore to systematically investigate what the possibilities are to prepare and use washed minces from more sensitive fish like herring and salmon. These species differ from cod particularly regarding their content of total fat, individual fatty acids, carotenoids, mitochondria, and heme proteins; all of which could affect the oxidation kinetics. This investigation was carried out by comparing the kinetics of Hb-mediated oxidation in washed muscle minces made from fresh and prefrozen cod, herring, and salmon during ice storage. Compositional differences between the minces, both before and after washing, were also to be compared and linked to the oxidative stability.

MATERIALS AND METHODS

Fish Supply. In the spring and fall of 2004, fresh cod (*Gadus morhua*) and salmon (*Salmo salar*) were obtained from Leröy Allt i Fisk (Göteborg, Sweden) and herring (*Clupea harengus*) was obtained from Paul Mattsson AB (Ellös, Sweden). The post mortem ages of the fish when arriving in laboratory were between 1 and 2 days for herring, and 2–3 days for cod and salmon.

Bleeding of Trout. Fish blood was obtained from trout (*Oncorhynchus mykiss*) kept at the Department of Zoology, Göteborg University (Sweden) (*10*). The trout was anaesthetized for ~2 min in 3-aminobenzoic acid ethyl ester (0.1 g/L H_2O plus 0.2 g sodium bicarbonate/L H_2O to neutralize). Blood was drawn from the caudal vein using evacuated blood collection tubes pretreated with lithium heparin (BD Vacutainer, Plymouth, UK) and put on ice until preparation.

Hemolysate Preparation and Quantification. Hemolysate was prepared according to the method of Fyhn et al. (*11*) and colorimetric Hb quantification was done according to Brown (*12*). Both methods were modified as described by Richards and Hultin (*13*). Bovine Hb was used to make a standard curve.

Washed Mince Model System. Fish mince model systems were prepared from both fresh and prefrozen fillets from the three different species. While the model system preparation from fresh fish were done using fish from two different seasons (spring and fall), the freezing effect was only studied using the spring-caught fish. To avoid batch-to-batch variations when comparing model systems from the fresh and prefrozen fillets, one fillet of each fish was frozen at −80 °C, while the other was used directly for making a model system. Thawing of the prefrozen fish fillets was done in a sealed plastic bag under running cold water. From both fresh and prefrozen fillets, skin and dark (i.e., red) muscle were manually removed, and the light (i.e., white) muscle was ground in a kitchen grinder using a hole plate with a hole diameter of 5 mm (Ultra Power, Model KSM90, Kitchen Aid, St Joseph, MI). A 600 g portion of fish mince was then washed once in 3 volumes (w/w) of distilled, deionized water, and twice in 3 volumes of 50 mM phosphate buffer (pH 6.6). In the two first washes, mince and solution were stirred for 2 min, after which they was allowed to leach 15 min on ice. Between the washes, water was removed using a kitchen sieve. In the third wash, mince and solution were homogenized for 1 min and 15 s using an Ultra Turrax homogenizer (speed 3, Ultra Turrax model T18 basic, IKA Works, Wilmington, NC). The washing solution was then removed by centrifugation (15 000*g*, 25 min, 4 °C, Sorvall Superspeed RC-5C Plus, Kendro Laboratory Products, Stockholm, Sweden). In each wash, the pH was checked and if necessary adjusted to pH 6.6. The pH and moisture of the final model systems were pH 6.40–6.72 and 81.3–85.2%, respectively (see the section Analysis of Moisture Content and pH for details). The model systems were frozen at −80 °C until use.

Preparation of the Oxidation System. Frozen washed model systems from cod, herring, and salmon were thawed in sealed plastic bags under running cold water. An appropriate volume of 50 mM phosphate buffer, pH 6.3, was then added to adjust the moisture of all samples to 86%. A 200 ppm portion of streptomycin was added to prevent bacterial growth, and pH was adjusted to 6.3 with HCl. Each fish model system was then split into two portions with trout hemolysate added to one of the portions (final Hb concentration 20 μM), and the same volume of Tris/saline buffer added to the other (control). Each sample (20 g) was finally flattened out on the bottom of a 250 mL screw-capped Erlenmeyer flask, giving a thickness of ~5 mm, and stored on ice in darkness for up to 15 days. The storage was ended at the point in time when a spoiled odor became sensorically evident in the Erlenmeyer flasks. Typical spoiled odors were "sour" and "putrid".

Sampling. To follow the Hb-mediated lipid oxidation, chemical, sensory, and colorimetric analyses were performed regularly during the ice storage period. Samples for chemical analysis (~1 g "plugs") were taken out from the Erlenmeyer flask using a hollow cylinder to obtain a constant surface-to-volume ratio between different samplings. The plug was wrapped in aluminum foil and kept at −80 °C until analysis.

Sensory Analysis. A small panel (2–3 panelists) regularly smelled the headspace above the samples by uncapping the Erlenmeyer flasks. The sensory analysis was focused on rancid odor which was quantified using a scale ranging from 0 to 100, with 100 representing the strongest intensity. The lag phase for rancid odor in a sample was set to the time when the intensity passed 10 on the sensory scale. This intensity was defined as "slightly rancid". The relative standard deviation (RSD%) among the panelists regarding sensory analysis of oxidized samples was 18%. For nonoxidized samples, the RSD% was always lower.

Colour Measurement. During ice storage of the samples, redness (a^*) and yellowness (b^*) in the CIE Laboratory colour scale were regularly monitored with a colorimeter (Minolta Chroma Meter CR-300, Minolta Corp., Ramsey, NJ). Calibration of the instrument was done using a white Minolta calibration plate with a D_{65}illuminant and 2° observer. For analyzing samples, the probe was held against the bottom of the Erlenmeyer flask and five different spots were analyzed (thus, $a = 5$). An average value was used in further calculations. The RSD% for the a^*-value and b^*-value analyses was 4.9% and 3.2%, respectively ($a = 6$).

Total Lipid Content. Total lipid fractions were extracted from 2 g of each mince and model system with chloroform and methanol according to Lee et al. (*14*). For samples with high fat content (>6%), chloroform:methanol was used in a ratio of 2:1. For medium fat samples (2–6%), a 1:1 ratio was used, and for lean samples (<2%), a 1:2 ratio was used. The fat content was determined gravimetrically by evaporating 1–2 aliquots (~3 mL) of the chloroform phase under nitrogen at ambient temperature. Lipid extractions were performed on duplicate or triplicate samples ($n = 2-3$), and lipid content was expressed as a percent of the sample wet weight.

Peroxide Value (PV). Total lipids were extracted from 1-g samples using chloroform:methanol (*14*). To determine the peroxide value (PV), the ferrithiocyanate method was used as described by Undeland et al. (*15*). The chloroform extracts from salmon samples naturally had an intrinsic red colour. To handle this, the absorbance of extracts *without* reagents added was subtracted from samples *with* reagents added. Quantification was done using a standard curve made from cumene

hydroperoxide. PV was determined on duplicate samples ($n = 2$). In a few cases when the sample amount was limited, $n = 1$. Results were expressed as micromoles of peroxide per kilogram of sample (wet weight (ww)). The RSD% of the PV analysis method was 1.1% ($a = 6$).

Fatty Acid Pattern Analyses. The fatty acid pattern of minces and washed model systems was determined in duplicate on aliquots of the chloroform lipid extracts obtained by the total lipid extraction (*14*). The method of Lepage and Roy (*16*) as modified by Lindqvist et al. (*17*) was used, which is based on methylation of the fatty acids. C:17 was added to all samples as an internal standard. The fatty acid methyl esters (FAME) were analyzed using a capillary gas chromatograph equipped with an auto-injector coupled to a flame ionization detector (Hewlet Packard 5890, Waldbronn, Germany). The separation of FAME was done on a DBwax column (30 m × 0.25 mm) (J&W scientific, Folsom, CA) using hydrogen as a carrier gas. The column temperature started at 180 °C and increased at 5 °C/min until 250 °C. The injection temperature was 300 °C, and the detection temperature was 325 °C. The chromatogram was evaluated using Borwin evaluation chromatography software (Le Fontanil, France). Fatty acids were identified by comparison of retention times with a mixture of 11 standards. Results are expressed both as milligrams per kilogram of sample and percent of total integrated peak area.

α-Tocopherol Determination. α-Tocopherol was extracted from minces and model systems by vortexing two 1-g samples ($n = 2$) with 5 mL methanol for 1 min, followed by incubation at 80 °C for 30 min. Samples were then vortexed for 1 min, sonicated for 1 min, and vortexed again for 1 min. Centrifugation was performed at ambient temperature for 5 min at 1600*g*, and the supernatant was used for α-tocopherol determination. Tocopherols were separated by high performance liquid chromatography (HPLC) on a Kromasil C18 column (150 mm × 2.1 mm, 5 μm) (Eka Chemicals, Bohus, Sweden). The mobile phase consisted of 98% methanol (HPLC, Laboratory-Scan LtD, Dublin, Ireland) in water, and the flow rate was 0.4 mL/min. Peaks were detected with a Shimadzu RF-551 spectrofluorometric detector (Kyoto, Japan) using an excitation wavelength of 295 nm and emission wavelength of 330 nm. α-Tocopherol peaks were quantified against a DL-α-tocopherol standard (Calbiochem, an affiliate of Merck KGaA, Darmstadt, Germany). Results were expressed as milligrams α-tocopherol per kilogram of sample (ww). The relative standard deviation (RSD%) of the α-tocopherol analysis method was 1.8% ($a = 6$).

Protein Determination. Total protein content in minces and model systems was determined in duplicate or triplicate ($n = 2-3$) by homogenizing a 1-g sample with 10 mL 1 M NaOH for 50 s at speed 3 using an Ultra Turrax homogenizer (Ultra Turrax model T18 basic, IKA Works, Wilmington, NC). The homogenate was let to stand over night at 4 °C to allow complete dissolving. Protein concentration was determined twice on each homogenate ($a = 2$) according to the method of Lowry et al. (*18*), modified by Markwell et al. (*19*). Results are expressed as percent protein per sample wet weight.

Moisture Content and pH. Moisture content was measured on 2.5-g samples of each mince and model system using an HA300 moisture balance (Precisa HA 300, Zurich, Switzerland). Moisture content was determined in duplicate or triplicate ($n = 2-3$), and results are expressed as percent of wet weight. The pH of both solutions and solid matrixes was recorded by a Hamilton double pore electrode (Hamilton Double Pore, Bonaduz, Switzerland) in conjunction with a pH-meter (Radiometer analytical PHM210, Villeurbanne, France).

Quantification of Hemoglobin. Heme protein was extracted from three samples of each mince and model system ($n = 3$) by homogenizing a 4-g sample in 50 mL of buffer (50 mM Tris, 80 mM KCl, 1 mM EDTA, pH 8) for 1 min (speed 4.5) using an Ultra Turrax homogenizer (Ultra Turrax model T18 basic, IKA Works, Wilmington, NC). The homogenate was centrifuged at 37 000*g* for 40 min at 4 °C, and the supernatant was filtered through a Munktell 00H filter. Quantification of heme protein was done in duplicate on each sample extract ($a = 2$) according to the method of Brown (*20*) as modified by Richards and Hultin (*21*). Bovine Hb was used to prepare a standard curve, and results are expressed as micromoles of Hb per kilogram of sample (ww).

Trace Mineral Content. Fe, Zn, and Cu content were determined on freeze-dried minces and model systems by ion chromatography following the work of Fredriksson et al. (*22*). A microwave digestion (Milestone microwave laboratory system EthosPlus, Sorisole, Italy) was done by mixing at the most 0.4 g sample, 0.75 mL concentrated HNO_3, 0.15 mL HCl, and 3 mL H_2O in a teflon vial. The sample was digested to a transparent solution by a temperature program reaching 180 °C in 15 min and this temperature was then kept for 20 min. After cooling, the sample was decanted into a test tube and diluted to a final volume of 10 mL. A 0.1 mL portion of ascorbic acid (20 mg/mL) was added to 0.9 mL of the digested sample before analysis. The ion chromatography method is based on the formation of mineral complexes by pyridine-2,6-dicarboxylic acid in the mobile phase. The complexes are then postcolumn derivatized with 4-(2-pyridylazo)resorcinol (PAR), resulting in mineral–PAR complexes that are detected by UV–vis absorption at 500 nm. Trace elements were analyzed in duplicate or triplicate samples ($n = 2-3$). Results were expressed as milligrams per kilogram of sample (ww).

Statistics. During the lipid oxidation storage trials, the use of sensory analysis limited the possible number of replicate sample flasks to one in each experiment. However, to confirm the data found in the first trial, the whole experiment was instead repeated a second time, using model systems that were made from fish caught during another season (fall). Similar results from two completely different sample batches are thought to create stronger evidence than repeated trials on the same material. In order to not hide valuable information on species differences, results from the two experiments are not merged into average values, but instead interpreted separately. In order to illustrate sample variation, the span between maximum and minimum values is used when there are only two replicate samples and standard deviation (SD) is used when there are three replicates. In order to significantly differentiate between oxidation data obtained from different storage points, or from different sample types, data were compared with a *t* test. Differences among mean values obtained from the compositional analyses of model systems were tested by ANOVA where significance levels were obtained with Tukey's HSD multiple range test. The software used was SPSS (version 14.0 for windows). Differences are regarded as significant when $p \leq 0.05$.

RESULTS

Fish Species Differences in Hb-Mediated Lipid Oxidation. Hb-mediated lipid oxidation was studied in nine different washed fish minces. These were made from fresh and prefrozen cod, herring, and salmon light muscles from spring-caught fish, as well as from fresh cod, herring, and salmon light muscles from fall-caught fish. Herring and salmon light muscle had not been used before for preparing this kind of model system, the same being true for prefrozen raw materials. Below, data are given on the oxidative stability and composition of these new models in combination with data from the more well-established fresh cod light muscle model. **Figure 1a–d** shows changes in PV, rancid odor, redness (*a**), and yellowness (*b**), respectively, in washed minces made from fresh fall fish. **Figure 2** shows rancid odor and *a**-value data in models from fresh and prefrozen spring-caught fish.

The data in **Figure 1a** shows that after 1 day, the order of Hb-mediated PV development was herring > cod > salmon. However, at day 2, salmon had reached higher PV's than the cod, and the order was changed to herring > salmon > cod. After 2.9 days, the PV's peaked for all three species. The patterns obtained for the development of PV in models from spring fish were similar (data not shown). However, already after 2 days, the PV's of herring and salmon samples peaked; around 1300 and 1800 μmol peroxide/kg, respectively. Thus, differently from in models from fall fish, herring samples made from spring fish obtained slightly lower values than salmon at this "peak" sampling point.

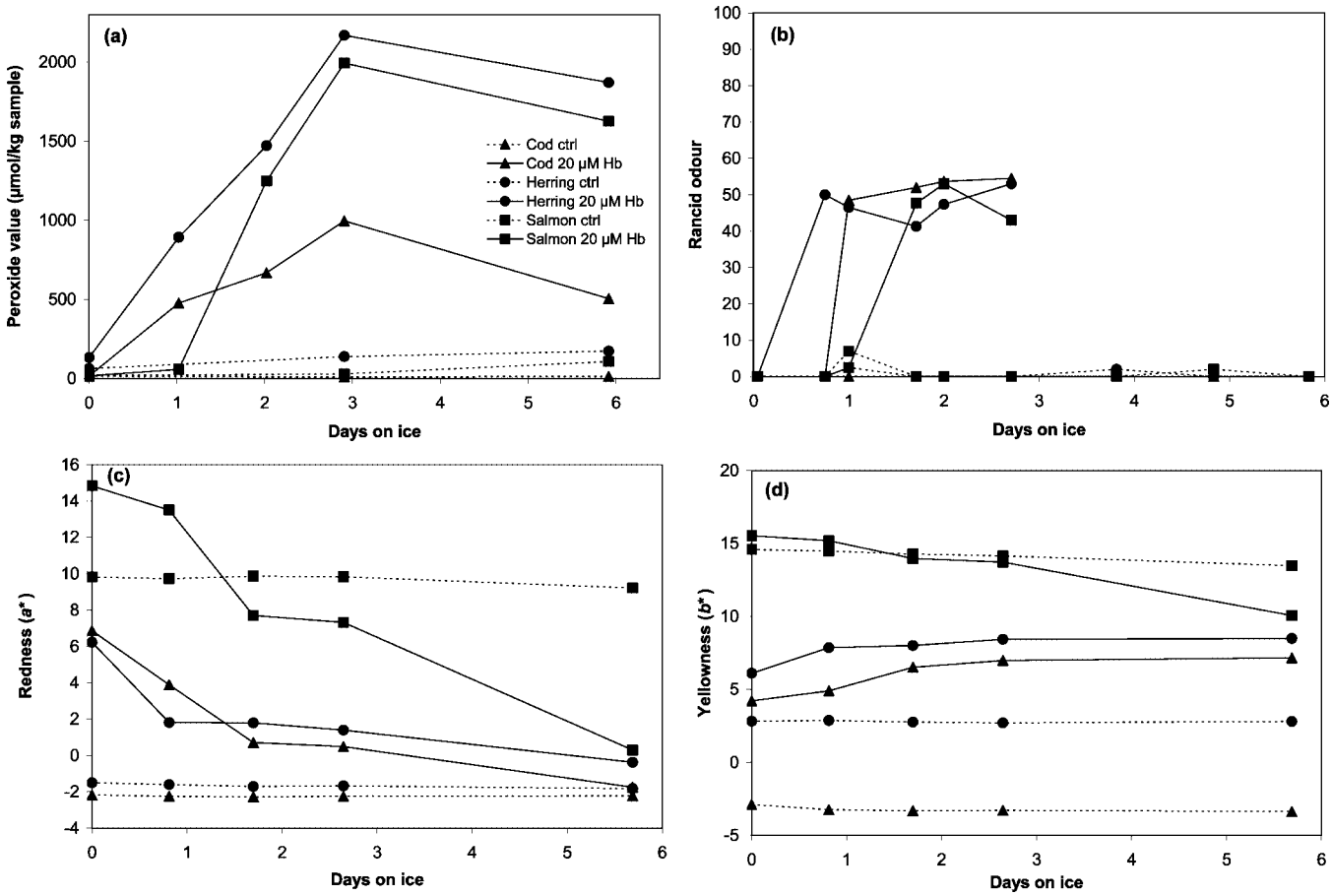

Figure 1. Changes in (a) PV, (b) rancid odor, (c) redness, a^*, and (d) yellowness, b^*, during ice storage of washed fish mince model systems from cod, herring, and salmon caught in the fall, with and without the addition of 20 μM trout Hb. All samples were adjusted to pH 6.3 and 86% moisture. A 200 ppm portion of streptomycin was added to slow down bacterial growth. The model system was flattened out in screw-capped glass Erlenmeyer flasks to a thickness of ~5 mm. Details on the number of sample replicates and the analytical variations are given in the Materials and Methods section.

Regarding sensory analysis (**Figures 1b** and **2a**), rancid odor developed significantly faster in the herring model than in the cod and salmon models. In both fall and spring herring, rancid odor was detected already after 0.7 days. Thus, no lag phase could be determined. In the cod and salmon models, rancid odor had developed first at day 1 and day 1.9, respectively. All samples then stabilized at a rancid odor intensity of around 50–60.

The PV and sensory data were confirmed by the changes in redness (a^*); i.e., met-Hb formation (**Figures 1c** and **2b**). The relative decrease in red colour was the fastest in the herring samples. Between 0 and 0.7 days, the drop in a^*-values was from 6/6.8 to 2/1.5 in fall/spring herring. Then, it changed only slightly up to 6 days. In cod, the period before stabilization of a^*-values was longer: 1.7 days and 1 day in fall and spring fish, respectively. In salmon, this period was 1.7 days in both batches of fish. From 1.7 to 6 days, salmon then continued to loose some redness, but at a slower rate. The large initial differences in a^* among samples were caused by the presence of carotenoids (cod, herring vs salmon) and the added hemoglobin (controls vs oxidizing samples).

In **Figure 1d**, a significant increase in yellowness (b^*) can be seen in herring and cod models from fall fish. In salmon models, which had much higher initial b^*-values due to their carotenoids, a significant decrease in b^*-values was seen over the entire storage period.

On the basis of all the methods used in this trial to follow lipid oxidation (**Figures 1a–d** and **2a** and **b**), the Hb-free control samples made from fresh fish were stable during the ice storage period of 9 days. Only some very slight PV increase was seen in models from fall herring (**Figure 1a**), which was absent in models from spring herring (not shown).

Prefreezing of Fillets Prior to Model System Preparation. To improve the usefulness of the washed fish mince model systems, the possibility of using frozen fillets was also evaluated. Fish caught in spring were used for testing whether freezing of the raw material at −80 °C before making a washed fish mince model system had any influence on the kinetics for Hb-mediated lipid oxidation. However, in this evaluation, only sensory and redness changes were followed.

As can be seen in **Figure 2a**,**b**, prefreezing of the raw material only had minor, nonsignificant effects on the kinetics of Hb-mediated oxidation in the washed models. Rancid odor development and redness loss was slightly delayed for model systems made from prefrozen cod when compared to the model made from fresh cod. In contrast, the model systems based on prefrozen salmon were slightly more prone to oxidation. In the herring models, no differences at all could be recorded between models from fresh and prefrozen fish. When it comes to control samples originating from prefrozen raw materials (**Figure 2a**,**b**), cod and salmon models showed the same good stability that was found for models made from fresh fish. For the control made from prefrozen herring, however, rancid odor started developing at day 3, and between day 4.7 and 5.8, it became significantly higher than in the control made from fresh raw material. An interesting observation was that bacterial spoilage

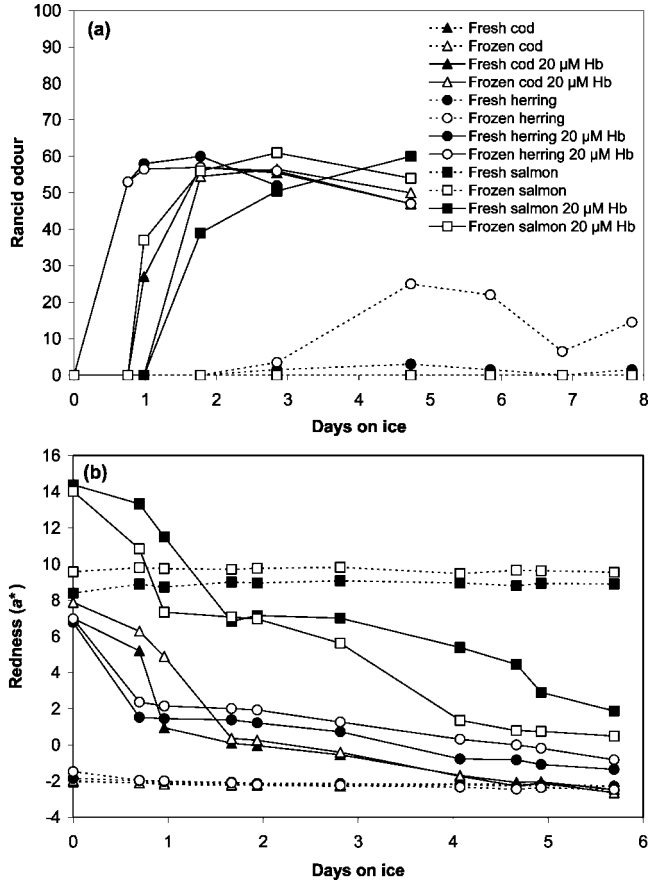

Figure 2. Changes in (a) rancid odor and (b) redness, *a**, during ice storage of washed fish mince model systems prepared from fresh and frozen fillets of fish caught in the spring, with and without the addition of 20 μM trout hemoglobin. All samples were adjusted to pH 6.3 and 86 % moisture. A 200 ppm portion of streptomycin was added to slow down bacterial growth. The model system was flattened out in screw-capped glass Erlenmeyer flasks to a thickness of ~5 mm. Details on the number of sample replicates and the analytical variations are given in the Materials and Methods section.

of the ice-stored samples became sensorially apparent a few days later when using models made from prefrozen raw material as compared to when using fresh raw material.

Composition of Minces and Model Systems. Data on moisture, total lipids, total protein, heme protein, lipid hydroperoxides, trace elements, and α-tocopherol for minces and models are shown in **Table 1** . Note that the data for the models are shown after adjusting the moisture to the running conditions (86%), enabling the composition to be related to oxidation kinetics.

Table 1 illustrates the large seasonal variations in fat and moisture content that is a characteristic for herring. It was however interesting to notice that the salmon used in this study did not suffer from such seasonal variations, probably a result of its aquacultured origin. **Table 1** also illustrates that the fattier the starting material, the larger the relative removal of lipids during the washing. Comparing the lipid data on a dry weight (dw) basis (numbers not shown), there was essentially no differences in lipid content between minces and models made from cod and spring herring, while models made from the fattier fall herring and spring/fall salmon had 19% and 27–31% less fat than the minces, respectively. However, despite a larger lipid removal from high-fat fish minces, the lipid content of their corresponding models was still significantly higher than that of the other models; 2.96–3.52% (ww basis) vs 0.56–0.97% (ww

Table 1. Composition of Minces and Model Systems from Cod, Herring, and Salmon Light Muscle Minces and Model Systems Caught and Prepared in the Spring and Fall of 2004[a]

	cod mince		cod model		herring mince		herring model		salmon mince		salmon model	
	spring	fall	spring	fall	spring	fall	spring	fall	spring	fall	spring	fall
moisture %	80.7 ± 0.62	81.1 ± 0.28	86	86	77.8 ± 1.33	70.8 ± 1.27	86	86	61.0 ± 2.33	63.6 ± 1.51	86	86
lipid %	0.91 ± 0.04	0.72 ± 0.04	0.69 ± 0.00^{a,b}	0.56 ± 0.10^{a}	1.46 ± 0.06	7.53 ± 0.28	0.97 ± 0.08^{b}	2.96 ± 0.1^{c}	13.41 ± 0.38	12.17 ± 0.39	3.52 ± 0.38^{d}	3.41 ± 0.11^{d}
protein %	18.2 ± 0.96	17.9 ± 0.75	15.7 ± 0.17^{a}	15.0 ± 0.65^{a,b}	16.6 ± 0.36	19.3 ± 0.37	13.5 ± 0.11^{b,c}	12.0 ± 0.05^{c}	19.3 ± 0.44	22.1 ± 0.19	11.8 ± 0.11^{c}	13.2 ± 0.29^{c}
Hb (μmol/kg)	0.23 ± 0.02	0.03 ± 0.03	nd	nd	2.89 ± 0.08	3.18 ± 0.15	nd	nd	0.93 ± 0.10	2.08 ± 0.08	nd	nd
peroxides (μmol/kg)	5.2 ± 4.7	8.6 ± 0.3	3.9 ± 0.8^{a}	14.3 ± 0.9^{b}	14.6 ± 12.2	17.9 ± 2.1	33.0 ± 2.3^{c}	62.6 ± 2.6^{d}	14.1 ± 5.4	8.3 ± 2.4	10.6 ± 0.2^{a,b}	17.8 ± 0.8^{b}
Fe (mg/kg)	0.44 ± 0.10	0.23 ± 0.02	0.44 ± 0.05^{a}	0.32 ± 0.04^{a}	0.94 ± 0.15	2.63 ± 0.08	0.71 ± 0.10^{b}	1.07 ± 0.01^{c}	1.01 ± 0.26	1.64 ± 0.04	0.31 ± 0.02^{a}	0.69 ± 0.02^{b}
Cu (mg/kg)	0.09 ± 0.01	0.10 ± 0.01	0.07 ± 0.01^{a}	0.11 ± 0.02^{a}	0.25 ± 0.03	0.33 ± 0.01	0.41 ± 0.05^{b}	0.39 ± 0.01^{b}	0.10 ± 0.03	0.18 ± 0.02	0.11 ± 0.01^{a}	0.17 ± 0.03^{a}
Zn (mg/kg)	2.85 ± 0.05	2.75 ± 0.03	2.01 ± 0.06^{a}	2.02 ± 0.05^{a}	3.12 ± 0.36	4.15 ± 0.31	2.40 ± 0.04^{b}	2.45 ± 0.01^{b}	2.69 ± 0.20	2.72 ± 0.15	1.32 ± 0.01^{c}	1.79 ± 0.06^{d}
α-tocopherol (mg/kg)	5.30 ± 0.02	3.05 ± 0.04	3.46 ± 0.08^{a}	2.15 ± 0.00^{b}	2.61 ± 0.17	2.28 ± 0.30	0.25 ± 0.01^{c}	0.86 ± 0.04^{d}	2.34 ± 0.05	2.84 ± 0.35	1.89 ± 0.17^{b}	2.24 ± 0.15^{b}

[a] Significance testing was done only to establish the differences between models, which were adjusted with respect to pH (6.3) and moisture (86%). Figures in the same row followed by different letters are significantly different ($p \leq 0.05$). All data are expressed on a wet weight (ww) basis. Here, nd means not detectable.

Table 2. Content of Fatty Acids in Lipids from Cod Light Muscle Mince and Model Systems Prepared in the Spring and Fall of 2004[a]

fatty acid	cod mince spring (mg/kg sample %)		cod model spring (mg/kg sample %)		cod mince fall (mg/kg sample %)		cod model fall (mg/kg sample %)	
				Saturates				
C14:0	33.1 ± 1.2	0.7	22.2 ± 1.5	0.6	37.8 ± 0.0	1.0	30.3 ± 1.1	0.9
C16:0	776 ± 22	16.3	574 ± 11	16.8	692 ± 7.5	17.6	577 ± 0.6	17.3
C18:0	201 ± 3.8	4.2	144 ± 3.6	4.2	176 ± 1.9	4.5	146 ± 1.7	4.4
sum	1010 ± 27	21.3	740 ± 16	21.6	905 ± 9.4	23.0	753 ± 0.0	22.6
				Monoenes				
C16:1	72.4 ± 2.4	1.5	49.1 ± 2.4	1.4	44.8 ± 4.6	1.1	39.4 ± 0.1	1.2
C18:1	539 ± 13	11.3	376 ± 1.6	11.0	445 ± 10.7	11.3	352 ± 2.6	10.6
C20:1	34.8 ± 0.7	0.7	22.6 ± 2.3	0.7	62.2 ± 8.7	1.6	49.9 ± 0.2	1.5
C22:1	11.5 ± 0.5	0.2	3.0 ± 0.4	0.1	19.5 ± 1.3	0.5	10.3 ± 0.8	0.3
sum	658 ± 16	13.9	451 ± 6.7	13.2	571 ± 13.5	14.5	452 ± 3.7	13.6
				Polyunsaturates				
C18:2(n-6)	43.9 ± 1.6	0.9	33.4 ± 1.5	1.0	28.8 ± 1.0	0.7	23.6 ± 0.4	0.7
C18:3(n-3)	11.4 ± 0.5	0.2	8.1 ± 0.7	0.2	8.5 ± 0.0	0.2	6.6 ± 0.7	0.2
C18:4(n-3)	10.6 ± 0.4	0.2	7.4 ± 0.5	0.2	16.1 ± 0.1	0.4	12.8 ± 0.0	0.4
C20:4 (n-6)	264 ± 4	5.6	191 ± 3.5	5.6	141 ± 1.1	3.6	119 ± 0.5	3.6
C20:5(n-3)	805 ± 14	17.0	599 ± 5.9	17.5	569 ± 3.6	14.5	482 ± 1.4	14.5
C22:5(n-3)	136 ± 3	2.9	95.3 ± 0.4	2.8	73.1 ± 0.8	1.9	60.0 ± 0.1	1.8
C22:6(n-3)	1423 ± 44	30.0	1066 ± 0.4	31.2	1369 ± 15	34.8	1152 ± 0.2	34.6
sum	2693 ± 64	56.7	2000 ± 11	58.5	2206 ± 20	56.1	1855 ± 1.9	55.7
total sum	4360 ± 106	91.8	3191 ± 34	93.3	3682 ± 43	93.7	3059 ± 5.5	91.9

[a] Results are expressed both as milligrams per kilogram of sample and percent of total integrated peak area. Note that the moisture content differs between minces and model systems according to the information given in **Table 1**.

basis). Altogether, the models were ranked as follows based on average total lipid: salmon > herring > cod.

Regarding total proteins, it is well established that most sarcoplasmic proteins are removed in the washing, and thus, the proteins of the models consist mainly of myofibrillar proteins. A somewhat higher total protein content was found in the cod models than in models from herring and salmon. The difference between cod and salmon was significant. Hemoglobin could not be detected in any of the models after washing.

The content of lipid hydroperoxides was slightly higher in minces from herring and salmon than from cod. During model making, the PV's largely increased, especially when using herring. As a result, the herring models had final PV's that were significantly higher than those in cod and salmon models (33–62.6 vs 3.9–17.8 μmol/kg). Prefreezing the herring (in this case the spring herring) gave a model with even higher PV (154 μmol/kg).

According to the result in **Table 1**, it appears as if the trace metals were quite strongly attached to the muscle matrix and thus, difficult to remove in the washes. On a dw basis (results not shown), the content of most trace elements had either increased or remained unchanged after washing. The content of Fe in the models ranked them as follows: herring (significant only for fall herring) > cod ~ salmon. The content of Zn ranked the models as follows: herring > cod > salmon. And, Cu ranked the models as follows: herring > salmon ~ cod.

Initially, cod mince had the highest α-tocopherol level. During washing, the largest decrease in α-tocopherol was seen for herring mince, followed by cod and then salmon minces. This decrease could be a result both of actual wash-out and oxidative degradation. The residual levels of α-tocopherol ranked the models as follows: cod (significant only for spring cod) > salmon > herring.

The results from the fatty acid pattern analysis are shown in **Tables 2–4**; both as absolute values on a ww basis and as percent of total peak areas obtained in the chromatograms. In general, there were less fatty acids in the models than in minces, which is in accordance with the loss of fat and moisture uptake during washing and preparation. However, the relative fatty acid compositions were very similar between minces and models. Some slight relative increases in DHA (22:6) were notified in herring and salmon fat, probably reflecting a relative increase in membrane lipids after washing.

Relative PUFA-levels were highest in fat from cod models (55.7–58.5% of total peak area) (**Table 2**), followed by herring models (28.9–43.2%) (**Table 3**) and then salmon models (30–32.8%) (**Table 4**). The large variation seen for the two batches of herring was due to the high differences in the total fat content between spring and fall herring (1.5 vs 7.5%, ww-basis). Absolute amounts of PUFA were highest in the salmon models, followed by herring and then cod.

Relative levels of monoenes ranked the models as follows: fall herring > salmon > spring herring > cod. Absolute levels of monoenes ranked the models as follows: salmon ~ fall herring > spring herring > cod. Even though, there was a big variation in absolute quantity of saturated fatty acids, the relative amounts were very similar (20–24 %) in minces and model systems from all species.

DISCUSSION

Useful models in fish muscle lipid oxidation studies are minces, unwashed or washed. Regarding oxidation stability studies on unwashed fish minces, with and without extra additions of oxidation reactants, plenty of information is available in the literature, (*23–28*). The advantage with whole minces is that realistic information, e.g. on the effect of anti and pro-oxidants can be given, as most endogenous substances are present. The disadvantage of whole minces is the difficulty to draw any mechanistic conclusions on how a certain anti or pro-oxidant will act, as it is more or less impossible to have full control over the content of all such components. In the past 5–10 years, this has lead to a fairly wide-spread use of ice-stored washed cod light muscle minces in fish lipid oxidation research. Cod light muscle is very low in lipids and endogenous oxidants but not perfect for mimicking other types of fish where

Table 3. Content of Fatty Acids in Lipids from Herring Light Muscle Mince and Model Systems Prepared in the Spring and Fall of 2004[a]

fatty acid	herring mince spring (mg/kg sample %)		herring model spring (mg/kg sample %)		herring mince fall (mg/kg sample %)		herring model fall (mg/kg sample %)	
			Saturates					
C14:0	366 ± 0.5	3.9	233 ± 14	3.6	4053 ± 175	6.4	1503 ± 100	6.0
C16:0	1647 ± 20	17.4	1172 ± 26	18.0	8178 ± 409	12.9	3387 ± 139	13.6
C18:0	196 ± 2.7	2.1	144 ± 2.0	2.2	732 ± 142	1.1	299 ± 2.5	1.2
sum	2209 ± 23	23.3	1549 ± 42	23.8	12962 ± 725	20.4	5190 ± 242	20.8
			Monoenes					
C16:1	209 ± 3.2	2.2	138 ± 16	2.1	2826 ± 225	4.4	975 ± 43	3.9
C18:1	730 ± 4.6	7.7	527 ± 32	8.1	5216 ± 282	8.2	1920 ± 90	7.7
C20:1	612 ± 17	6.5	368 ± 19	5.7	6670 ± 199	10.5	2572 ± 141	10.3
C22:1	1316 ± 35	13.9	760 ± 40	11.7	12962 ± 320	20.4	5091 ± 262	20.4
sum	2867 ± 53	30.3	1794 ± 107	27.5	27674 ± 1025	43.5	10559 ± 535	42.4
			Polyunsaturates					
C18:2(n-6)	102 ± 1.4	1.1	75.0 ± 4.0	1.1	959 ± 25	1.5	376 ± 17	1.5
C18:3(n-3)	53.0 ± 0.6	0.6	38.0 ± 1.9	0.6	814 ± 37	1.3	316 ± 21	1.3
C18:4(n-3)	59.0 ± 0.1	0.6	40.9 ± 2.6	0.6	1692 ± 81	2.7	667 ± 40	2.7
C20:4 (n-6)	99.6 ± 2.9	1.1	72.0 ± 0.5	1.1	232 ± 8.9	0.4	124 ± 4.5	0.5
C20:5(n-3)	805 ± 8.4	8.5	572 ± 9.5	8.8	4076 ± 145	6.4	1649 ± 62	6.6
C22:5(n-3)	57.3 ± 0.6	0.6	40.4 ± 1.6	0.6	430 ± 27	0.7	171 ± 9.4	0.7
C22:6(n-3)	2707 ± 34	28.6	1972 ± 13	30.3	8005 ± 226	12.6	3887 ± 99	15.6
sum	3883 ± 48	41.0	2810 ± 33	43.2	16208 ± 531	25.5	7189 ± 253	28.9
total sum	8959 ± 123	94.6	6152 ± 182	94.5	56844 ± 2281	89.4	22937 ± 1030	92.0

[a] Results are expressed both as milligrams per kilogram of sample and percent of total integrated peak area. Note that the moisture content differs between minces and model systems according to the information given in **Table 1**.

Table 4. Content of Fatty Acids in Lipids from Salmon Light Muscle Mince and Model Systems Prepared in the Spring and Fall of 2004[a]

fatty acid	salmon mince spring (mg/kg sample %)		salmon model spring (mg/kg sample %)		salmon mince fall (mg/kg sample %)		salmon model fall (mg/kg sample %)	
			Saturates					
C14:0	4564 ± 16	4.0	1369 ± 22	4.6	4208 ± 181	4.3	1098 ± 23	3.8
C16:0	16788 ± 444	14.5	4294 ± 110	14.5	15329 ± 582	15.6	4709 ± 79	16.5
C18:0	3318 ± 92	2.9	884 ± 23	3.0	2794 ± 79	2.8	862 ± 22	3.0
sum	24670 ± 520	21.4	6546 ± 155	22.1	22328 ± 843	22.7	6669 ± 124	23.3
			Monoenes					
C16:1	7324 ± 142	6.3	1938 ± 68	6.5	5667 ± 66	5.8	1364 ± 54	4.8
C18:1	23858 ± 652	20.7	4814 ± 117	16.2	20049 ± 800	20.4	5568 ± 152	19.5
C20:1	5843 ± 144	5.1	1909 ± 52	6.4	6631 ± 208	6.8	1762 ± 48	6.2
C22:1	5933 ± 141	5.1	1656 ± 30	5.6	6983 ± 234	7.1	1838 ± 4.2	6.4
sum	42958 ± 1079	37.2	10317 ± 267	34.8	39329 ± 1308	40.1	10532 ± 251	36.9
			Polyunsaturates					
C18:2(n-6)	5675 ± 123	4.9	1017 ± 21	3.4	4846 ± 223	4.9	1364 ± 48	4.8
C18:3(n-3)	1610 ± 61	1.4	293 ± 1.2	1.0	1223 ± 42	1.2	348 ± 10	1.2
C18:4(n-3)	1575 ± 40	1.4	372 ± 11	1.3	1276 ± 33	1.3	321 ± 12	1.1
C20:4 (n-6)	808 ± 16	0.7	231 ± 5.8	0.8	536 ± 12	0.5	176 ± 8.0	0.6
C20:5(n-3)	8166 ± 250	7.1	2288 ± 53	7.7	6158 ± 167	6.3	1830 ± 53	6.4
C22:5(n-3)	4220 ± 143	3.7	1187 ± 30	4.0	3168 ± 89	3.2	883 ± 23	3.1
C22:6(n-3)	14355 ± 433	12.4	4349 ± 82	14.7	10577 ± 224	10.8	3652 ± 96	12.8
sum	36409 ± 1064	31.5	9737 ± 204	32.8	27783 ± 792	28.3	8573 ± 250	30.0
total sum	104037 ± 2663	90.2	26600 ± 627	89.7	89440 ± 2943	91.1	25774 ± 625	90.2

[a] Results are expressed both as milligrams per kilogram of sample and percent of total integrated peak area. Note that the moisture content differs between minces and model systems according to the information given in **Table 1**.

lipid oxidation might be more of a problem. This study was undertaken to systematically investigate the possibilities of using darker muscle fish (herring) and salmonoids (salmon) in preparing washed fish mince models for use in lipid oxidation studies. The challenges with these two species lie in their high content of lipids (both species), carotenoids (salmon), and heme proteins (herring). Important questions to answer in this investigation were how much of the endogenous oxidation reactants could be washed out, whether the washed models would stay stable in the absence of an added pro-oxidant (especially the herring models), and how fast oxidation would proceed in pro-oxidant-fortified samples (here, 20 μM Hb). It is important that the exponential oxidation phase occur well before microbial spoilage sets in, which is usually after 6–10 days on ice in the presence of 200 ppm streptomycin. If the model gets spoiled before it gets rancid, the full efficacy of an antioxidant cannot be studied. Finally, since it is not always possible to get fresh raw material, it was also of great interest to study whether prefrozen raw materials could be used for model preparation.

From our ice-storage studies, it was seen that controls from fresh salmon and cod stayed completely stable during the 6–12 days on ice passing before microbial spoilage became sensorially apparent (**Figures 1** and **2**). In the controls made from fresh fall herring, some very slight oxidation was seen (**Figure 1**), which escalated if prefrozen herring was used (**Figure 2**). The latter could be due, e.g., to slight peroxide formation or heme activation during the freezing process. Prefrozen herring is

therefore not recommended when making these kinds of models. The herring models were also the most prone to oxidation with Hb added (**Figures 1** and **2**). In models from both spring and fall fish, Hb immediately induced rancid odor in the herring model (no lag phase), followed by the cod (0.8 day lag phase) and then the salmon model (1 day lag phase) (**Figures 1b** and **2a**). The same order of rates was also found for PV (**Figure 1a**) and was supported by decreases in *a**-value (i.e., met-Hb formation) (**Figures 1c** and **2b**) as well as increases in *b**-value (i.e., yellow pigment formation) (**Figure 1d**). The detected differences in the length of the oxidation lag phases between models from the three species were probably not linked to the total lipid content (salmon model 3.4–3.5% ww > herring model 1–3% > cod model 0.6–0.7%) (**Table 1**), the total n-3 PUFA content (salmon > herring > cod) (**Tables 2–4**), or the *relative* amount of n-3 PUFA in the fat (cod > herring ~ salmon). The lack of impact from fat content was further supported by the fact that spring and fall herring models showed very similar oxidation lag phases despite their different fat content (1% vs 3%). The fact that an increase in total fat or the ratio of unsaturated fatty acids does not increase Hb-mediated oxidation of washed fish mince was recently also found by Undeland et al. (*15*) and Richards et al. (*29*), respectively. Rather, the small oxidation differences we detected appear to be linked to residual levels of the antioxidant α-tocopherol in the washed models (cod > salmon > herring) (**Table 1**). Less α-tocopherol would make particularly the membranes more susceptible to oxidation. Visual inspection and colour analyses (**Figures 1c** and **d** and **2b**) also revealed a much higher level of carotenoids in the salmon model compared to the other two models. The contribution from carotenoids to both *a**- and *b**-values is established (*30*). Both α-tocopherol and carotenoids therefore probably contributed to the longer oxidation lag phase seen in washed salmon mince. The oxidation differences could also be linked to the small residues of trace elements in the three models (**Table 1**). Total Fe and Cu ranked the models as follows: herring > cod ~ salmon, And, Zn ranked them as follows: herring > cod > salmon. The trace metals could, e.g. via Fenton chemistry, be responsible for the preformed peroxides present in the models. Upon addition of the trout Hb, these peroxides could then quickly break down to free radicals. Copper has also been shown to accelerate met-heme protein formation (*31*), which would further accelerate the Hb-mediated peroxide breakdown. From PV analyses of the models at time 0 (**Table 1**), it was seen that, in all minces, peroxides were formed during the actual washing process, but in herring, this formation was higher than for the other species. Thus, the herring models had significantly higher 0-time PV's than the other models, especially the one made from fall herring (62.6 μmol/kg). The fact that there was essentially no oxidation-increase in this model during ice storage *without* Hb (i.e., in the control) indicates that the residual levels of trace metals of this model per se could not induce any significant breakdown of the 62.6 μmol/kg peroxides present. However, in the model made from prefrozen herring, where the level of preformed peroxides was more than doubled, some oxidation was actually induced even in the absence of Hb during ice storage, i.e. by the residual trace elements alone (**Figure 2**).

While maximum rancidity scores were about the same in all three systems (**Figures 1b** and **2a**), higher maximum PV levels were reached in the salmon model, followed by herring and then cod (**Figure 1a**). The latter order was the same as that found regarding the residual lipid levels in the models (see above). These results agree with those of Sánchez-Alonso et al. (*32*), where maximum PV levels became higher in washed cod mince after adding 10% cold pressed herring oil, although the oxidation lag phase length was unaffected. We therefore believe that the higher lipid substrate level prolonged the time span for PV development and thus yielded higher maximum levels of peroxides.

It can be observed in **Figure 1** that, in Hb-containing samples, both primary (PV) and secondary (rancid odor) oxidation products increased more or less simultaneously, which contradicts the classic sequential development of primary and secondary products which is often suggested from bulk oil studies (*33*). The fact that the measured oxidation products leveled out after a few days, and sometimes even declined, indicates fast reactions of secondary products, e.g. with proteins of the models forming so called Schiff's bases (*34*). This behaviour has repeatedly been seen before in washed cod mince models with added Hb (*3, 5, 15*). The latter was confirmed by the slight increases in yellowness (*b**) (Schiff's bases polymerization) (*35*) in the cod and herring models in the same time span as odor and PV changed (**Figure 1d**). Salmon behaved somewhat differently in this aspect in that yellowness rather decreased, probably due to carotenoid bleaching resulting from co-oxidation of lipids (*36*).

In conclusion, light muscle from other species than cod (salmon and herring) were suitable for making washed fish mince oxidation model systems. Without Hb, the models were stable, except for when prefrozen herring was used. With 20 μM Hb, oxidation developed well before microbial growth became sensorially evident in all models. The small species differences noted in the rate of Hb-mediated oxidation (herring > cod > salmon) were ascribed residual levels of antioxidants, trace elements, and preformed hydroperoxides rather than lipid content or fatty acid pattern. Although these models work well for mechanistic studies, it is always suggested that hypotheses on anti or pro-oxidants developed from washed fish mince studies are confirmed in unwashed muscle before making recommendations (e.g., to industry).

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