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Loss of Redness (*a**) as a Tool To Follow Hemoglobin-Mediated Lipid Oxidation in Washed Cod Mince

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Instrumental measurement of redness loss (decrease in *a** value) was evaluated as a tool to follow hemoglobin (Hb)-mediated lipid oxidation in fish muscle. Two washed cod mince model systems were used (prepared at pH 6.5 and 5.5), both fortified with 15 μmol/kg of trout Hb and adjusted to pH 6.5 and 81% moisture. The rate of oxidation was varied through pH alterations (pH 6.1 and 6.9) and addition of an antioxidative cod muscle press juice. During ice storage, TBARS, painty odor, and *a** values were followed. In all "oxidizing" samples, *a** values correlated well with TBARS and painty odor development; $r = -0.95$ and -0.77, respectively. In press juice containing samples, the correlation was lower (0.55 for *a** vs TBARS) because there was a slight *a** value decrease even in the absence of measurable lipid oxidation. *a** values distinguished between "oxidizing" and stable samples within 1 day, *before* any lipid oxidation products could be chemically detected. It was confirmed in an aqueous phosphate buffer model system that the redness loss corresponded to a buildup of brownish met-Hb at the expense of oxy- and deoxy-Hb. The *a** value data were best used as a lipid oxidation index by calculating the rate of decrease (*k* value) in the "initial phase" of the redness loss (before accumulation of lipid oxidation products) or in the "differentiation phase" (during the exponential raise in TBARS/painty odor). Calibration to lipid oxidation products must, however, be made for each specific sample type. Washing method, pH, Hb-type, etc., all affected both *k* values and absolute *a** readings. Small yellowness (*b**) increases also occurred along with *a** value losses, possibly the result of polymerized Schiff bases.

KEYWORDS: Redness; *a; lipid oxidation; cod; hemoglobin; TBARS; Hb**

INTRODUCTION

Today the world faces the problem of the marine supply being exhausted by extensive fishing. Among the threatened species are cod and tuna. Herring (*Clupea harengus*) and other pelagic species are, however, still available in large quantities, but less than half of the catch is used for food production. One of the reasons for this is the high susceptibility of the lipids and proteins of these fish to oxidation, which quickly post-mortem affects taste, smell, color, and nutritional value in a negative way. To be able to control the oxidation reaction within the fish-processing sector, there is an urgent need for fast and reliable methodologies for rancidity/freshness assessment that can replace time-consuming sensory and chemical methods. Near-infrared reflectance (NIR) (*1*), hybrid gas-sensor array systems (*2*), and fluorescence spectroscopy (*3*, *4*) have been evaluated for these purposes with fairly good results. However, costly equipment is needed, and the interpretation of data is not straightforward. In addition, the actual compound that is measured (oxidation product or others) is often unknown, making it difficult to understand the development mechanism and, thus, prevent the oxidation reaction.

Heme proteins, especially the oxidized met-forms, have been identified as highly critical for the onset of oxidation in muscle (*5*), particularly in fish from cold waters (*6*). In studies of lipid oxidation in minced herring (*7*), minced/sliced tuna (*8*), and washed cod mince model systems containing beef, chicken, and fish hemoglobins (Hbs) (*9*−*11*), it was measured instrumentally and visually that there was a dramatic loss of red color in parallel with hydroperoxide and thiobarbituric acid reactive substances (TBARS) development during cold storage. Lee et al. (*8*) concluded that the decrease in *a**- value of tuna was due to conversion of the bright red oxy-myoglobin (Mb) to the brownish met-Mb form. Met-forms of Hb have been confirmed during ice storage of phosphate buffer model systems containing mackerel, menhaden, flounder, and pollock hemolysates (*6*). In the work by Richards et al. (*9*), *a** value loss occurred more quickly in washed cod mince containing Hb from trout than from warm-blooded animals such as chickens and cows. It was also seen (*11*) that washed cod mince containing anodic trout Hb lost red color more rapidly than cathodic trout Hb. The former also mediated TBARS development more rapidly than cathodic trout Hb. According to Lee (*8*), tuna oxy-Mb oxidizes

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more quickly than mammalian Mbs, which was ascribed to differences in the amino acid composition. These findings indicate that although the high instability and pro-oxidative activity of oxidized fish heme proteins certainly cause problems, their brownish color can be taken advantage of as a basis for detecting the oxidative changes they give rise to. Thus, we believe there is the possibility to follow the development of lipid oxidation in heme-containing fish products using instrumental analyses of redness (*a** value) loss. Color measurements are fast and nondestructive and allow the evaluation of other oxidation-derived color parameters in parallel. Lipid oxidation in fish can, for example, also cause yellow pigment formation (*13*) and bleach carotenoid pigments (*14*). To date, instrumental color analysis within the muscle foods sector has primarily been applied for meat, and more as a classification tool than as a direct/indirect measure of biochemical degradations affecting other sensory attributes. For example, it has been used to sort beef carcasses with respect to initial muscle color. Wulf and Wise (*15*) studied how bloom time, post-mortem storage time, pH, cut surface area, and moisture affected instrumental color readings of beef. Brewer et al. (*16*) studied factors affecting color (bloom time) of muscles from pork carcasses of various ultimate pH values. Wiegand et al. (*17*) used color readings to study the effect of feeding conjugated linoleic acid (CLA) on the quality of loin chops from pork (*17*). In their study, only *b** values were affected by storage time. Within the fish sector, instrumental measures of whiteness/lightness (*L**) of surimi (*18*) and fish mince (*19*) as well as measures of redness/yellowness (*a**, *b**) of farmed salmon muscle have been the most common applications of color analyses (*20*). Recently, data on redness (*a**) of spring-spawning herring fillets as a function of season were reported (*21*).

The ultimate aim of this study was to evaluate loss of redness (*a**) as an indirect tool to follow Hb-mediated lipid oxidation in fish muscle during ice storage. The evaluation was done by comparing redness loss with TBARS and painty odor development in washed cod mince model systems designed to develop lipid oxidation at different rates. The latter was achieved by adding an antioxidative cod muscle press juice and by varying the pH of the model system. Both of these factors are believed to affect the reaction between the Hb molecule and membrane lipids. To confirm the involvement of met-Hb in the color changes, spectrophotometric analysis of a buffer-based model system at different pH values was done in parallel with colorimetric analysis of redness (*a**). Because the color of fish muscle is known to be affected, for example, by microbial spoilage, cut surface area, moisture content, and raw material characteristics (diet, catching season, etc.), microbial growth was suppressed, and moisture content, surface area, and fish raw material source for the samples within each experiment were standardized.

MATERIALS AND METHODS

Fish and Fish Blood Supply. Fresh cod (*Gadhus morhua*) was obtained from Leröy Allt i Fisk AB (Göteborg, Sweden). The white muscle was manually removed and trimmed so that all of the dark muscle, cartilage, and vein remains were removed. The purified, white cod muscle was ground using a kitchen grinder (Ultra Power, model KSM90, KitchenAid, St. Joseph, MI). Fish blood was obtained from rainbow trout (*Onchorhynchus mykiss*) kept at the Department of Zoology (Göteborg University, Sweden).

Washed Minced Cod Muscle Model System. Two washed cod muscle model systems were prepared, one with a moisture level resembling physiological moisture content (~81%), the other with lower moisture (~70%). The latter was done to allow addition of large amounts of antioxidative cod muscle press juice, without exceeding the physiological moisture content. For the first model system, 600−700 g of minced cod muscle was washed once with 3 volumes of Milli-Q water and twice with 3 volumes of 50 mM sodium phosphate buffer, pH 6.5. For the low-moisture model system, 50 mM NaCl was used instead of 50 mM sodium phosphate buffer and the pH was lowered to 5.5. In the first two washes, the mince was stirred for 1 min with washing solution and was then allowed to settle for 15 min on ice before the washed mince was collected in a sieve. In the third wash, the mince and wash solution were homogenized for 1 min at speed 3 using an Ultra Turrax (model T 18 basic, IKA Works). The homogenate was then centrifuged for 15 min (4 °C at 15000*g*) in a Sorval Superspeed centrifuge (model RC-2B, Instrument AB Lambda, Stockholm, Sweden) using an SLA-1500 rotor. The supernatant was removed, and the washed mince was frozen in plastic bags at −80 °C until use.

Bleeding of Fish, Preparation of Hemolysate, and Quantification of Hemoglobin. Rainbow trout were anesthetized for 3 min in neutralized 3-aminobenzoic acid ethyl ester solution (0.5 g/L H_2O). One milliliter of blood was drawn from the caudal vein using a 5 mL syringe equipped with a 27 gauge needle and preloaded with 1 mL of sodium heparin (120 units/mL 150 mM NaCl). Preparation of the hemolysates was done according to the method of Fyhn et al. (*22*) and quantification of the Hb levels according to the method of Brown (*23*); both methods were modified as described by Richards and Hultin (*24*).

Preparation of Cod Muscle Press Juices. Pure white minced muscle from cod was packed in 200 mL polypropylene centrifuge bottles (200 g in each) and centrifuged at 22000*g* for 2 h at 4 °C. The resulting supernatant (press juice) was filtered through a Munktells filter paper (no. 1010) and stored at −80 °C until use.

Preparation of Oxidation System. The "physiological" moisture washed cod mince was used to study hemoglobin from trout at different pH values. The "low-moisture" washed cod mince was used to study the inhibition of Hb-mediated oxidation by fish muscle press juice. Both model systems were thawed under cold running water and analyzed for moisture content. The regular-moisture mince was adjusted to pH 6.1, 6.5, or 6.9 using 0.1−1 M NaOH and/or 0.1−1 M HCl. Streptomycin and hemolysate were then added to the system to reach final levels in the moisture fraction of 200 ppm and 15 μM Hb, respectively. Both streptomycin and hemolysate were stirred in manually for 2 min each using a stainless steel spatula. The samples (final weight of ~20 g) were then flattened out with an L-shaped stainless steel spatula in the bottom of a 250 mL screw-capped glass Erlenmeyer flask, resulting in a sample thickness of ~5−6 mm. The thawed low-moisture washed cod mince was subjected to manual squeezing in a cotton towel to remove as much excess/unbound water as possible. The "dried" mince was then chopped for 2 min in a small precooled mixer (Hugin, model MC-851). After analysis of the moisture content, cod muscle press juice or, for the controls, 50 mM phosphate buffer, pH 6.5, was added to raise the moisture to 81%. The addition of press juice brought about a 1.8-fold dilution of the same into the moisture of the washed cod. The pH was measured and, if needed, adjusted to 6.5. As described above, streptomycin and hemolysate were then added to 20 g of the "moisturized" system, and the samples were flattened out in the bottom of Erlenmeyer flasks. Samples were stored on ice in darkness for up to 14 days.

Color Measurement. During storage of the Erlenmeyer flasks, changes in redness (*a**), lightness (*L**), and yellowness (*b**) of the cod mince samples were measured using a colorimeter (Minolta Chroma Meter CR-300, Minolta Corp., Ramsey, NJ) using the CIE Lab color scale. Standardization of the instrument was done using a white Minolta calibration plate with a D_{65} illuminant and 2° observer. The colorimeter was connected to a personal computer, and SpectraMatch software (Minolta Corp.) was used to handle the measurements and to store the data. Measurements were done by pressing the probe (diameter = 0.5 cm) against the bottoms of the Erlenmeyer flasks. Three replicate measurements were done at different locations of the bottom, and an average value was used in further calculations.

Analysis of Moisture Content and pH. The moisture content of minces was measured using an HA300 moisture balance (Precisa balance 310M). pH was recorded with an Orion PerpHect Sure-Flow electrode (Termometer fabriken Orion Research Inc., Beverly, MA) in conjunction with a pH-meter (model MA235, Mettler Toledo, New York, NY). In muscle minces, the pH was measured after manual stirring of 1 part of mince with 9 parts of Milli-Q water.

TBARS. During storage of the samples, ~1 g sample "plugs" were taken at regular intervals for analysis of TBARS. The samples were picked from the bottom of the Erlenmeyer flasks using a hollow cylinder with a diameter of ~1 cm so that a constant surface-to-volume ratio was achieved. Samples were wrapped individually in aluminum foil and stored at −80 °C until analysis. TBARS analysis was performed according to the method of Lemon et al. (*25*), after extraction of the 1 g sample with 6 mL of trichloroacetic acid solution (7.5% TCA, 0.1% propyl gallate, and 0.1% EDTA in Milli-Q water).

Sensory Analysis. During storage of the Erlenmeyer flasks, a small internal panel (two or three trained people) performed a sensory screening for painty odor by sniffing the headspace above the samples (*6*). The intensity of the odor was judged according to a scale from 0 to 10, with 0 indicating no smell and 10 the "maximum". Average values from the panelists are reported in the figures.

Trout Hb Oxygenation and Oxidation Status. An aqueous model system was used to spectrally follow conformational changes in the hemoglobin molecule during storage. This system was constructed to resemble the washed cod mince control samples in size, composition, and the way it was stored. Sixteen milliliters of phosphate buffer (50 mM, pH 6.5) was mixed with 200 ppm of streptomycin sulfate and 15 μM trout Hb. A blank was constructed by excluding the addition of trout Hb. Twenty milliliters of the samples was stored on ice in 250 mL Erlenmeyer flasks for 24 days. At regular intervals, 1 mL samples were scanned between 650 and 450 nm. The peak values around 576 and 630 nm as well as the valley value at 560 nm were recorded for all of the samples. Calculations of the oxy-, deoxy-, and met-Hb were done according to the equations described by Benesch et al. (*26*). To evaluate in what pH range the most dramatic deoxygenation and oxidation reactions of the Hb molecule took place, we made an identical aqueous model system as described above, but adjusted it to six different pH values in the range of 8.6−5.8. The samples were immediately scanned without any storage, and the oxy-, deoxy-, and met-Hb values were calculated as above.

Statistics. One to three sample replicates (n) were used in each experiment (the exact n is given in each figure caption). The number of analytical replicates (a) were three for analyses of color and Hb, two for TBARS, and one for sensory analysis. For each sample (n) and sampling point, an average of the analytical data (a) was used for further calculations. In cases of $n = 3$, sample variations are shown in the figures as standard deviations (SD). One-way analysis of variance (ANOVA) combined with pairwise comparisons with Tukey's test was used to determine at what storage time point TBARS, painty odor, redness, yellowness, lightness, met-Hb, oxy-Hb, or deoxy-Hb formation had changed significantly from the starting values. It was also used to test significant differences between curves within the same figure. The level of significance is shown as the p value. Pearson's correlation coefficients (r) were calculated to estimate relationships between redness and TBARS, painty odor, met-Hb, oxy-Hb, or deoxy-Hb formation. Each experiment was repeated two or three times, with results from one of these experiments illustrated in the figures. The reason for not merging data from different experiments was that different batches of raw material had been used and, also, there were small differences in the exact sampling time points. Variations caused by these factors could then hide valuable information on changes caused by the variables we wished to study (pH, antioxidants, and time). The experiment presented in **Figure 6** was done only once, but with each sample as triplicates ($n = 3$).

RESULTS

Hb-mediated changes in TBARS and redness were studied in two different model systems previously used in oxidation studies (*6*, *27*): a "physiological moisture washed cod mince" (81%) and a "low-moisture washed cod mince" (70%). In the latter, the moisture was raised to 81% with phosphate buffer or cod muscle press juice before storage.

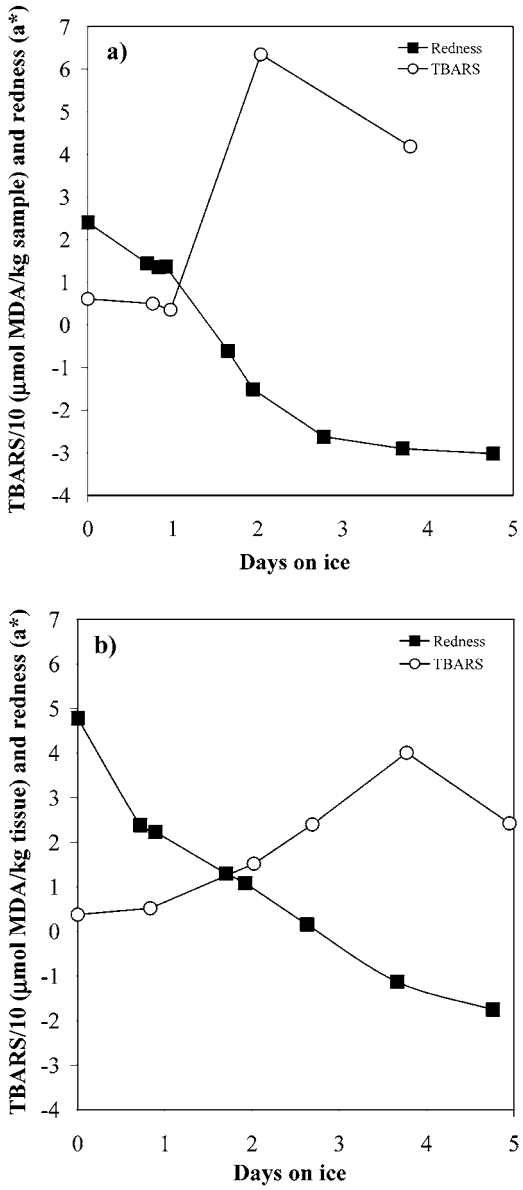

Figure 1. Hb-mediated changes in TBARS and redness (a^* value) during ice storage of (**a**) washed cod mince prepared at physiological moisture (81%) ($n = 2$) and (**b**) washed cod mince prepared at low moisture (70%) and then adjusted with phosphate buffer (50 mM, pH 6.5) to 81% moisture ($n = 2$). Oxidation was initiated with 15 μM trout Hb, and 200 ppm of streptomycin was added to prevent bacterial growth. The final pH of both model systems was 6.5.

When the physiological moisture washed cod mince was stored on ice at pH 6.5, a significant increase in TBARS was detected after 1 day ($p = 0.01$), with a maximum value of ~63 μmol of malondialdehyde (MDA)/kg reached after 2 days (**Figure 1a**). During the same time period redness (a^*) decreased significantly ($p < 0.001$), from ~2.5 to −1.5. After ~4−5 days, redness leveled off at −3. In low-moisture washed cod mince with 50 mM phosphate buffer, pH 6.5, added to bring the moisture to physiological level (81%), TBARS had increased significantly after 2 days ($p = 0.05$) with a maximum value of ~40 μmol of MDA/kg detected after 4 days. During this time period, redness dropped significantly ($p = 0.01$), from ~5 to

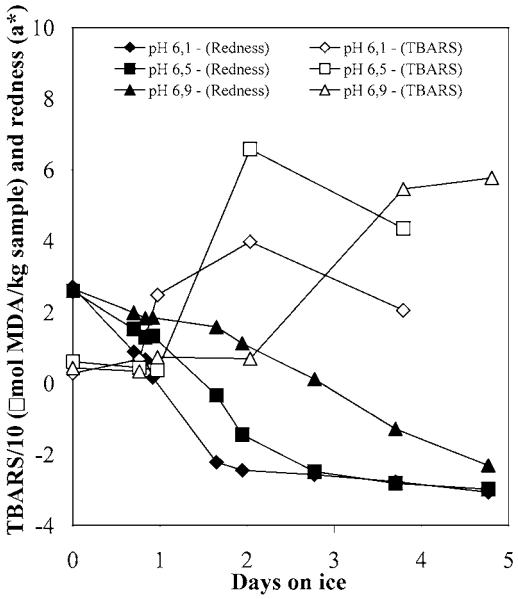

Figure 2. Hb-mediated changes in TBARS (open symbols) and redness (*a** value) (solid symbols) in washed cod mince prepared at physiological moisture (81%) and adjusted to pH 6.1 ($n = 1$), 6.5 ($n = 2$), or 6.9 ($n = 1$). Oxidation was initiated with 15 μM trout Hb, and 200 ppm of streptomycin was added to prevent bacterial growth.

−1 (**Figure 1b**). After that, *a** values started to level off at ∼ −2.

To see if changes in redness could reflect different rates of Hb-mediated lipid oxidation, model systems with slightly different pH values were used. When physiological moisture washed mince at pH 6.1, 6.55, and 6.9 was stored on ice (**Figure 2**), it was seen that TBARS had increased significantly from the starting value after 1, 2, and 3.8 days, respectively, in the pH 6.1, 6.5, and 6.9 samples ($p \leq 0.05$). Maximum TBARS occurred at the same time (2 days) in the pH 6.1 and 6.5 samples. The pH 6.9 samples still increased in TBARS after 4.8 days. The order of TBARS development was again reflected in changes in redness of the samples. The pH 6.1 sample declined first, followed by the pH 6.5 and 6.9 samples, the latter being significantly slower ($p \leq 0.05$) than the two former. It was interesting that sample differences in redness, but not TBARS, could be seen within 1 day. After 4.8 days, all three samples approached the same redness value (∼ −3).

It was tested if an antioxidative cod muscle press juice could prevent redness loss in addition to lipid oxidation (**Figure 3**). In control samples, containing 50 mM phosphate buffer, pH 6.5, instead of press juice, TBARS had increased significantly after 1.8 days ($p < 0.001$) and was still increasing after 5 days on ice. In the presence of press juice, no changes in TBARS occurred, and the sample was significantly differentiated from the control during the whole storage period ($p < 0.05$). Painty odor had increased significantly in the control after 2.8 days ($p < 0.05$), whereas no significant sensory changes occurred in the sample with press juice added. The addition of press juice gave a significantly lower drop in redness ($p < 0.05$). Whereas the control changed by 6.5 units (from ∼5 to −1.5), the sample with press juice added changed by only 3 units (from 4.5 to 1.5). In **Figure 3**, an attempt is made to distinguish the redness changes in the oxidizing control sample and the nonoxidizing press juice containing sample into three different phases: "initial phase", "differentiation phase", and "stationary phase". The differentiation phase is the phase in which the oxidation and color responses changed the most.

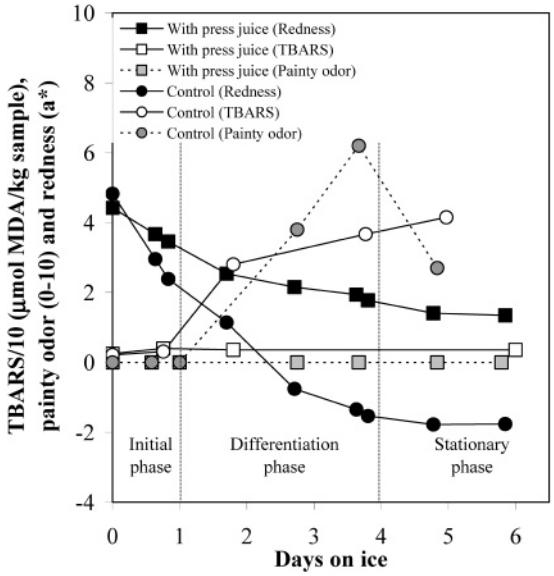

Figure 3. Hb-mediated changes in TBARS, painty odor, and redness (*a** value) in washed cod mince prepared at low moisture (70%) and then adjusted with phosphate buffer (50 mM, pH 6.5) ($n = 2$) or cod muscle press juice ($n = 2$) to 81% moisture. Upon addition to the model system, the press juice became diluted 1.8 times into the moisture fraction. Oxidation was initiated with 15 μM trout Hb, and 200 ppm of streptomycin was added to prevent bacterial growth. The final pH of the model systems was 6.5. The graph is divided into three phases, "initial phase", "differentiation phase", and "stationary phase", describing the different stages of oxidation/redness loss.

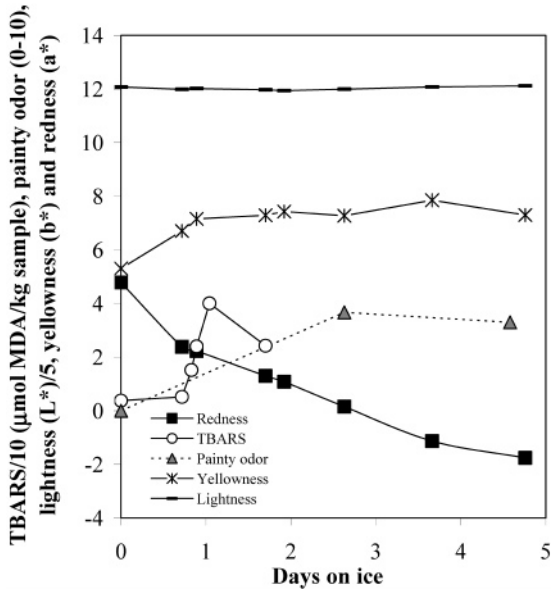

Figure 4. Hb-mediated changes in TBARS, painty odor, redness (*a** value), yellowness (*b** value), and lightness (*L** value) during ice storage of washed cod mince prepared at low moisture (70%) and then adjusted with phosphate buffer (50 mM, pH 6.5) to 81% moisture ($n = 2$). Oxidation was initiated with 15 μM trout Hb, and 200 ppm of streptomycin was added to prevent bacterial growth. The final pH of the model systems was 6.5.

In one experiment, lightness (*L**) and yellowness (*b**) values were also recorded along with the *a** values. **Figure 4** shows the changes in TBARS, painty odor, the *L**, *a**, and *b** values in low-moisture washed cod muscle mince brought to 81% moisture with 50 mM phosphate buffer, pH 6.5. Again, TBARS and painty odor increased [changes were significant ($p = 0.05$)

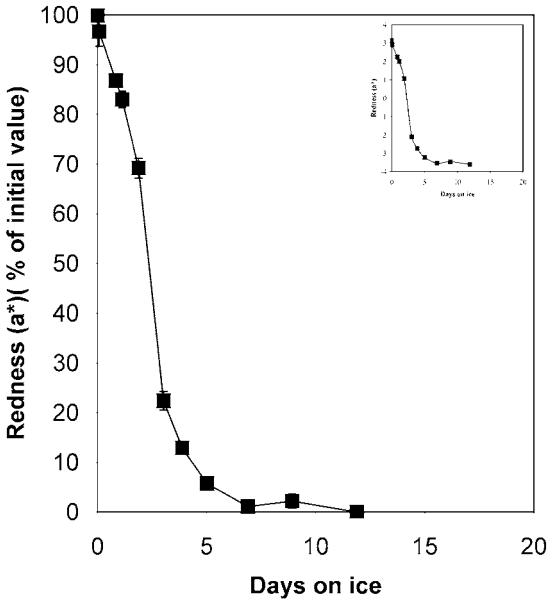

Figure 5. Relative (percent) changes in redness (*a**) during ice storage of washed cod mince prepared at physiological moisture (81%), pH 6.5, with 15 μM trout Hb and 200 ppm of streptomycin added ($n = 3$). Error bars show standard deviations (SD). (Inset) Absolute redness (*a** value) data.

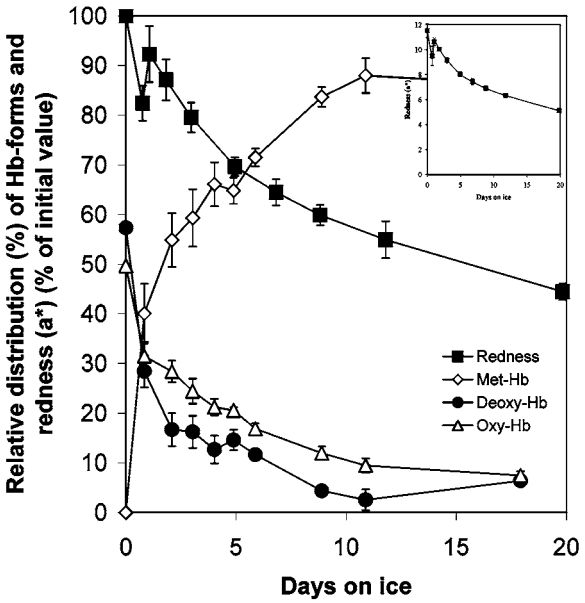

Figure 6. Relative (percent) changes in redness (*a** values), oxy-, deoxy-, and met-forms of trout Hb during ice storage of an aqueous model system consisting of 50 mM phosphate buffer (pH 6.5), 15 μM trout Hb, and 200 ppm of streptomycin ($n = 3$). Error bars show standard deviations (SD). (Inset) Absolute redness (*a** value) data.

after 0.8 and 2.6 days, respectively], whereas redness had decreased significantly ($p < 0.05$) after 3.6 days. Lightness (L*) did not change, but yellowness (b*) increased significantly ($p < 0.001$) within the first day (from 5.2 to 7.2). This was in the same period as TBARS reached its maximum value. In stable samples protected by cod muscle press juice, no such early raise in b* was seen (data not shown).

Because the change in redness was thought to be due to Hb autoxidation, an aqueous model system was set up to confirm the formation of met-Hb. Using 15 μM Hb from the same batch of hemolysate, redness losses were followed simultaneously in the washed cod mince system (**Figure 5**) and in the aqueous model system (**Figure 6**), both adjusted to pH 6.5. In the aqueous system, deoxygenation and autoxidation of the Hb molecule were also followed spectrophotometrically.

In the mince system (**Figure 5**), the redness was significantly reduced after 0.8 days ($p < 0.001$), whereas in the aqueous system it was not significantly reduced until after 1.8 days ($p < 0.001$) (**Figure 6**). As seen in the insets, the *a** values of the mince systems decreased ~8 units within 6−7 days, whereas this decrease took 24 days for the aqueous system. Significant alterations in the relative levels of oxy-, deoxy-, and met-Hb were seen within 0.8 day ($p < 0.001$). The rapid increase in met-Hb formation slowed somewhat after day 5 (**Figure 6**). After 10 days, the formation leveled off at ~85%. Deoxy-Hb and oxy-Hb levels decreased from 60 and 50% to 28 and 32%, respectively, during the first 0.8 day. Thereafter, the decrease continued but more slowly. After 10 days, the changes leveled off at just below 10% for both deoxy-Hb and oxy-Hb.

To evaluate if the presence of relatively large levels deoxy-Hb (60% of the total) at the start of the experiment was a result of a pronounced Bohr effect at the pH used (6.5), scans were taken after the trout hemolysate had been diluted in buffers ranging from the initial pH of the hemolysate (8.6) to pH 5.9. As shown in **Figure 7**, the most dramatic deoxygenation took place from pH 7.6 to 6.6. The relative amount of oxy-Hb was significantly lower, and the amount of deoxy-Hb significantly higher ($p < 0.05$), below than above pH 7. Met-Hb formation

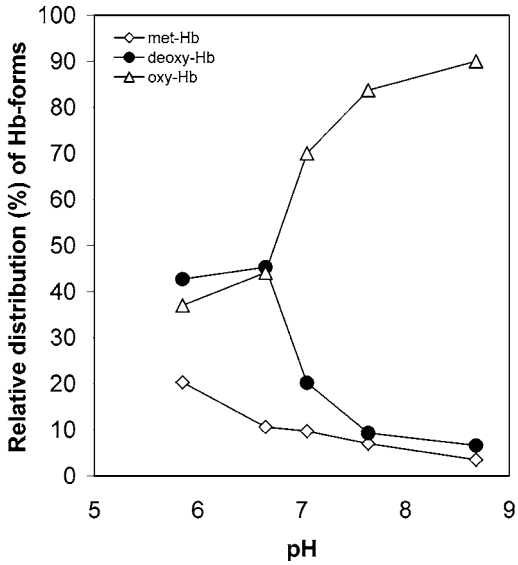

Figure 7. Relative distribution of oxy-, deoxy-, and met-forms of trout Hb in an ice-cold aqueous model system consisting of 50 mM phosphate buffer adjusted to pH 5.85, 6.65, 7.05, 7.5, or 8.7 ($n = 2$). The Hb level was 15 μM trout Hb.

became particularly evident below pH 6.6, but the only value significantly higher than the others was that at pH 5.85 ($p < 0.05$).

DISCUSSION

Post-mortem, Hb can easily become mixed with lipids and muscle proteins via natural or process-induced bursting of blood capillaries and lysis of erythrocytes. Met-Hb has been found to be a strong catalyst of lipid oxidation (*5*, *6*). We wished to study whether the changes in color (*a** value) caused by autoxidation of Hb could be used as an index to follow lipid oxidation in fish muscle. To minimize the interference from other reactions (enzymic and/or bacterial), washed minced cod muscle with

added antimicrobial agent was used as a model system. This system has the structure of muscle, that is, with intact myofibrillar proteins and membranes, but is virtually free of endogenous triacylglycerols, pro- and antioxidants. Hb could then be added and studied under controlled conditions.

Lipid Oxidation. TBARS (**Figures 1−4**) and painty odor analyses (**Figures 3** and **4**) showed that 15 μM trout Hb effectively mediated lipid oxidation in the washed cod mince prepared according to two different procedures. In accordance with previous studies (*6*, *27*), TBARS and painty odor increased and then often decreased again during ice storage. Well-known reactions of aldehydic secondary oxidation products in muscle are those with free amino groups, for example, in membrane lipids, proteins, and peptides to form Schiff bases (*28*) and/or with active methylene compounds such as in histidine to form Michael addition products (*29*, *30*). The Schiff bases can further polymerize into yellow pigments (*13*).

TBARS developed more rapidly in the model system prepared at physiological moisture (81%) than in the one prepared at low moisture (70%) (**Figure 1**). This was despite the fact that oxidation in both of the systems was evaluated at 81% moisture. A reason could be the exposure of the cod mince to low pH (pH ~5.5) during the washing in order to achieve efficient dewatering. Such treatment irreversibly reduced the solubility of cod myofibrillar proteins (*31*) and could also affect the membrane conformation. The presence of free −SH groups and the exposure of the phospholipids would both be critical for the manifestation of lipid oxidation in muscle.

The rate of TBARS development was increased by reducing the pH to 6.1 and decreased by raising it to 6.9. Acidification accelerates Hb deoxygenation (the Bohr effect) and increases the oxidation of oxy-Hb and/or deoxy-Hb to met-Hb (*6*). These acid-induced alterations of trout Hb were also confirmed here (**Figure 6**). Deoxy-Hb, met-Hb, and the hypervalent ferryl-Hb that form in the reaction between H_2O_2 and met-Hb are thought to promote lipid oxidation more than oxy-Hb (*11*, *32*). In the presence of cod muscle press juice diluted 1.8 times, TBARS and painty odor were completely prevented. The antioxidative effect of cod muscle press juice is believed to be due to heat-resistant, low molecular weight antioxidative compounds (*27*).

Redness. Our study showed that the factors controlling TBARS and painty odor development also controlled redness loss in a similar way. Compared to the control sample prepared at physiological moisture and pH 6.5, redness changed more slowly in the model system prepared at low moisture, in the model with pH raised to 6.9, and in samples containing cod muscle press juice (**Figures 1−4**). In contrast, redness disappeared more quickly in the model with pH reduced to 6.1 (**Figure 2**).

However, it was interesting to see that while TBARS were completely prevented in the presence of cod muscle press juice (**Figure 3**), there was still some loss of redness in these samples. Compared to the oxidizing control samples, the kinetics of redness loss in "stable" samples were, however, more linear. Also, the a^* readings never reached values as low as in the oxidizing samples, the difference being ~4 units. Because bacterial growth was prevented, the a^* value change in the "stable" samples could be due to some spontaneous conversion of oxy-/deoxy-Hb to met-Hb and the superoxide anion radical over time, but obviously not enough to initiate oxidation. It is also possible that the myofibrillar proteins denatured to some degree over time, that their interaction with Hb changed (*33*), and/or that there was a slight drying of the surface. It is known that physical properties of tissue systems (e.g., particle size, moisture content, and thickness) can change the reflection of light and, thereby, L^*, a^*, and b^* values (*18*). This is why redness data must be calibrated against TBARS or other chemical oxidation data for each specific system before potentially being used.

The need for calibration is also supported by the different initial a^* values given when 15 μM Hb was mixed with the two model systems prepared with different washing methodologies, but adjusted to the same moisture (81%) and pH (6.5) (**Figure 1**). In the model system prepared at physiological moisture, 15 μM trout Hb gave on average a^* values of 3.4 ± 0.26 ($n = 6$, SD). In the model system prepared at low moisture, the average a^* value was 4.8 ± 0.02 ($n = 2$, SE). Initial a^* values were also affected by the pH of the model system. In the physiological moisture system, 15 μM trout Hb gave initial a^* values of 2.8, 3, and 3.2 at pH 6.1, 6.5, and 6.9, respectively (**Figure 2**), most likely reflecting immediate met-Hb formation and suggesting that data should be normalized if the kinetics of different types of samples are compared. The acid-induced increase in levels of the met-form of trout Hb is confirmed in **Figure 6**. It was shown by Richards et al. (*9*) that the species of the Hb also affects initial a^* values when added into a washed cod mince system (88% moisture, pH 6.3). At 6 μM, in cow Hb gave highest redness at time 0 ($a^* = 1.2$), followed by chicken Hb ($a^* = 0.7$) and then trout Hb ($a^* = -0.9$). The capacity of these Hbs to catalyze peroxide value (PV) and TBARS ranked them in the opposite order: trout Hb > chicken Hb > beef Hb. These results further stress the need for calibration of redness changes in each specific case.

Roughly, the kinetics of redness during development of lipid oxidation could be separated into three distinct phases (**Figure 4**): "initial phase", "differentiation phase", and "stationary phase". The initial phase usually occurred within 1 day, and despite the lack of development of TBARS, there were some loss of redness in all samples (**Figures 1−4**). This early decrease was, however, larger in samples that later started to oxidize, indicating that redness loss was a very early indicator of differences in lipid oxidation. In the "differentiation phase", an exponential increase in lipid oxidation products (TBARS, painty odor) took place in control samples, and the differences in a^* values between "stable" and "oxidizing" samples became more evident. The stationary phase was the final phase, during which small changes in redness were detected over time but during which absolute differences in a^* values were largest.

Obviously, there are several different aspects of the redness data that can be related to data on lipid oxidation. Examples are the rate of change in the initial phase and/or differentiation phase, the final a^* value reached in the stationary phase, or the total number of a^* units lost from start to the stationary phase. Because there were quite large differences in initial a^* values and absolute a^* value losses between different types of model systems and with different types of Hb added (*9*), the rate of change (k) in one of the two first phases seems to be the most useful. Using 15 μM trout Hb, the k value in the initial phase was on average 2.9 a^* value units/day in control samples made from the low-moisture washed cod (**Figures 1b** and **3**). In the sample protected from oxidation by press juice, k was 1.2 a^* value units/day (**Figure 3**). In the differentiation phase, k values of 1.25 and 0.5 a^* value units/day, respectively, were obtained. In the control samples made from physiological moisture washed cod and 15 μM Hb (pH 6.5) (**Figures 1a** and **2**), the k value was lower in the initial than in the differentiation phase, 1 versus 2.2 a^* value units/day, respectively. Thus, as for absolute a^* values, the k values that characterize oxidizing and stable

samples must be calibrated for each specific system, even when they contain the same Hb level. In agreement with this, Richards et al. (*9*) showed that chicken Hb and trout Hb gave the same *k* values for redness loss in the differentiation phase, although the TBARS and PV development was much faster in washed cod mince with trout Hb.

To determine how the TBARS and sensory methods correlated to redness measurements, Pearson's correlation coefficients were calculated for the data presented in **Figures 2** and **3**. For all oxidizing samples (i.e., controls), high negative correlations were achieved. For TBARS against a^*, $r = -0.95$, and for paintiness against a^*, $r = -0.77$. For the sample protected by cod muscle press juice, the correlation (r) between a^* values and TBARS was only -0.55. The latter was due to the above-mentioned a^* value changes also in samples without TBARS increases.

Our hypothesis was that the observed loss of redness occurring simultaneously as trout Hb-catalyzed lipid oxidation was due to oxidation of heme iron from the ferrous to the ferric state under production of a brownish gray color (*34*). Studies of trout hemolysate in a phosphate buffer model system confirmed that Hb oxidation took place under the applied conditions of pH, Hb concentration, storage temperature, and addition of antimicrobial agent (**Figure 6**). The correlation of redness loss with met-Hb formation and oxy-Hb loss, respectively, was -0.82 and 0.88 in the aqueous system. It is assumed that this Hb oxidation also occurred in the fish system containing myofibrillar proteins and membranes. However, the buffer system was chosen over the muscle model to study this reaction because of the difficulty in extracting Hb from muscle, particularly after some storage. Lee et al. (*8*) detected accumulation of met-Mb and TBARS on the surface of 2.5 cm thick yellowfin tuna steaks and tuna patties during ice storage. Along with these oxidation reactions, there were significant decreases in a^* value and sensory scores.

From the spectral Hb analyses, it could be seen that the met-Hb formation was accompanied by loss of the reduced Hb forms, oxy-Hb and deoxy-Hb (**Figure 6**). In the most common reaction pathway explained for met-Hb formation from oxy-Hb (*35*), Hb deoxygenation proceeds Met-Hb formation. In our studies, loss of deoxy-Hb during ice storage was somewhat larger than the loss of oxy-Hb. However, it cannot be seen from our data whether all initially present oxy-Hb was deoxygenated before oxidation. A reaction pathway is also described whereby oxy-Hb is directly converted to Met-Hb (*36*).

Already at time 0, 50% of the Hb was in the form of deoxy-Hb (**Figure 6**). The presence of deoxy-Hb has been shown to accelerate met-Hb formation compared to fully oxygenated Hb (*35*). This indicated that deoxygenation of trout Hb started momentarily after the pH of the hemolysate was lowered from its original pH 8.6 to 6.5, which was used for the phosphate buffer. To clarify the exact location of the Bohr range for trout Hb, we evaluated the relative distribution of the three Hb forms in 50 mM phosphate buffer between pH 8.6 and 5.85 (**Figure 7**). This experiment confirmed that significant deoxygenation starts at pH 7.6 and increases down to pH 6.65, after which the relative level of deoxy-Hb leveled off at 45%. Richards et al. (*9*) found a dramatic deoxygenation of trout Hb from pH 7.25 to 6.75, after which no further changes occurred. Met-Hb increased from 5 to 20% between pH 8.6 and 5.8. It is seen in **Figure 6** that there was still some small redness loss in the buffer model system after the formation of met-Hb had ceased at ~87%. The reason for this is not known, but as discussed previously, other physical and chemical changes in the model system after long-term storage might affect color readings.

There was a more rapid redness loss in the cod muscle model system than in the buffer model system. This could indicate that lipid and/or protein oxidation products accelerated Hb autoxidation. To study the role of lipid oxidation products in Mb oxidation, Lee et al. (*8*) incubated oxy-Mb in the presence of 0.1−1 mM hexanal, hexenal, and 4-hydroxynonenal (4-HNE) for 4 h at 37 °C at pH 7.2. They found that all aldehydes increased oxidation of oxy-Mb in a concentration-dependent manner relative to controls free of aldehydes.

The use of L^* and b^* values as indicators of lipid oxidation in washed cod mince was also tested (**Figure 4**). The increase in yellowness (b^* value) in the same region as TBARS increased could originate in the polymerization of Schiff bases into yellow pigments (*13*). Schiff bases, also called tertiary oxidation products, form from the reactions, for example, between aldehydes and free amino groups of phospholipids and proteins (*27*). Yellowness could also be a result of changes in only the Hb molecule, such as met-Hb formation (**Figure 6**), or other reactions (e.g., heme detachment, ferryl formation). The latter is supported by the slight yellowness increase (from 10.7 to 12.9) seen also in the muscle-free aqueous model system during the first 6 days on ice (data not shown). In trials with decoloration of slaughterhouse blood by oxidation with H_2O_2 (*37*), a yellowish proteinaceous material was obtained.

After further evaluations on the origin of yellow pigmentation, it may be possible to measure lipid oxidation in Hb-containing fish muscle according to two different principles with instrumental color analyses: indirectly (heme protein oxidation) and directly (accumulation of tertiary oxidation products). The former could become a very early and sensitive tool, but, as stressed repeatedly, the relationship between a^* values and the buildup of oxidation products must be carefully controlled for each specific system.

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