Lipid Oxidation in Herring (*Clupea harengus*) Light Muscle, Dark Muscle, and Skin, Stored Separately or as Intact Fillets

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ABSTRACT: Light muscle, dark muscle, and skin from herring (*Clupea harengus*) were stored separately or as intact fillets at –18°C. After 0, 2, 8, 12, and 18 wk, all tissues were analyzed for conjugated dienes (A_{234}) and lipid hydroperoxides. In tissues stored separately, total absorbance at 268 nm (A_{268}) and lipid-soluble fluorescent oxidation products (FP) were also monitored. Further, prior to storage these tissues were subjected to measurement of total lipids, lipid classes, fatty acid pattern, α-tocopherol, iron, copper, selenium, and total aqueous pro-oxidative activity. When light muscle, dark muscle, and skin were stored as intact fillets, the following ranking order was seen for A_{234} and levels of lipid hydroperoxides at the end of the storage period: skin > dark muscle > light muscle. The corresponding ranking order for tissues stored separately was: dark muscle > skin > light muscle, whereas for A_{268} and FP the orders were: dark muscle > light muscle > skin and light muscle > dark muscle > skin, respectively. The compositional data obtained indicate the highest level of pro-oxidants in dark muscle and the highest level of polar lipids in light muscle. These observations reveal that pro-oxidants, to a greater extent than lipid composition, influence the increase in A_{234}, hydroperoxides, and A_{268}, whereas the reverse seems to be true for the increase in FP. The results also point to the strong influence from oxygen contact and tissue interactions on the progress of lipid oxidation in herring during storage.

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A strong limiting factor in the processing and storage of herring (*Clupea harengus*) is the susceptibility of its lipids to oxidation. This susceptibility is caused by the powerful pro-oxidative systems present in fish and the highly unsaturated nature of the lipids (1). As a result, alterations in flavor, texture, nutritional value, and color may develop (2). Because both economic and nutritional factors call for an increase in the production of food from herring, there is a desire to understand, and thereby avoid, any changes in quality that result from lipid oxidation. Removal of specific unstable fractions from the fish is a possible way to achieve products with a longer shelf life (1). Against this background, several studies have been performed to compare the oxidative stability of light muscle, dark muscle, and skin from herring and other fatty fish (3–6). These types of tissue differ not only in their physiological roles but also in their content of lipids, pro-oxidants, and antioxidants (1).

Herring contains both enzymatic and nonenzymatic pro-oxidants (7). Lipoxygenases (8) and microsomal enzymes (9,10) belong to the former group, whereas hemoproteins, transition metals, and ascorbate are important examples of the latter (1,7). Among the potential antioxidants in herring are enzymes, such as coenzyme Q (11) and glutathione peroxidase (12), and nonenzymatic compounds, such as α-tocopherol (13) and ascorbate (14).

Light muscle is known to contain low levels of all mentioned pro-oxidants (15,16), whereas dark muscle contains large amounts of hemoproteins, low-molecular-weight (LMW) metals, and microsomal enzymes (1,7). Skin is also rich in LMW metals (17) and has been reported to be a possible source of lipoxygenases (8,18). From previous studies of the development of lipid oxidation in these three tissues, both skin and dark muscle have been reported as the least stable part (3–6). A possible explanation of this contradiction may be differences in storage conditions between the various studies (4–6). Apart from temperature (4), oxygen availability is a decisive factor (5,6,19). The latter is closely related to the degree of disintegration to which the fish has been subjected. Storing the fish whole or as fillets provides the most realistic conditions for studying the three parts in question. However, differences in their contact with air and other tissues make it difficult to draw conclusions regarding the influence of various pro- and antioxidants on their lipid stability. Storing dark muscle, light muscle, and skin separately, on the other hand, limits such differences and thus gives a truer picture of the prerequisites for each part to develop lipid oxidative changes.

This study investigated the influence of "external factors" (oxygen availability and tissue interactions) and compositional factors (lipids, pro-oxidants, and antioxidants) on the development of various oxidation products in light muscle, dark muscle, and skin from herring during frozen storage.

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MATERIALS AND METHODS

Herring samples. The fish used in this study was herring (*C. harengus*) caught in March and November 1995 off the west coast of Sweden. After landing in Göteborg, the fish was prepared into double fillets, i.e., headed, gutted, and deboned. Prior to further preparation, the fillets were stored on ice for one night.

The double fillets from herring caught in March [length: 147 ± 25 mm, weight: 46 ± 10 g, fat content: 109 ± 0.4 g/kg (mean ± SD, $n = 5$)] were divided into two single fillets (A and B). Each was packed in a 125-mL HDPE (high-density polyethylene) box, which was then covered with polyamide-laminated polyethylene film. Immediately after packaging, the fish were frozen at −40°C in a tunnel freezer. After freezing, all A fillets were stored at −70°C to be used as reference fillets. The B fillets were stored at −18°C for 2, 8, and 12 wk and then stored at −70°C until analyzed. Prior to dissection into light muscle, dark muscle, and skin, the fillets were thawed at room temperature for 15 min. On a weight basis, the three fractions accounted for 73.6 ± 2.3, 18.2 ± 2.0, and 8.8 ± 2.4%, respectively, of the fillet weight (mean value ± SD, $n = 5$).

The November-caught double fillets [length: 107 ± 8 mm, weight: 34 ± 3 g, fat content: 106 ± 0.2 g/kg (mean ± SD, $n = 5$)] were treated in the same way as the ones caught in March, except that dissection of the A and B fillets into light muscle, dark muscle, and skin was performed prior to freezing. Thus, the different types of tissue were packed and stored separately. A fourth storage point, 18 wk, was used in addition to 2, 8, and 12 wk. The distribution (w/w) among the three fractions was the following: light muscle: 70.4 ± 2.2%, dark muscle: 17.4 ± 2.2%, and skin: 12.2 ± 0.9%.

To compensate for the high degree of biological variability between individual fish, each B tissue was extracted for lipids and analyzed for oxidation products in parallel with its corresponding A tissue. The results from the analysis of the A tissues were then subtracted from those of the B tissues, yielding results that are expressed as Δ values. This method is generally referred to as the paired fillet technique (20) and was used for both batches of fish. All compositional analyses were only performed on the November-caught herring and only prior to storage.

Statistics of sampling and analyses. After each storage period, light muscle, dark muscle, and skin from n A fillets plus their corresponding B fillets were extracted for total lipids. Depending on the analytical procedure to be used, each lipid extract was then analyzed once, in duplicate, or in triplicate, thus $a = 1$, 2, or 3. The same was true for the extraction of aqueous pro- and antioxidants from each tissue. To establish the variation among the n samples concerning a certain attribute, mean values from the a analyses were used. The analytical error, i.e., the variation among the a analyses of a specific sample, is expressed as the relative standard deviation (RSD%).

Analysis of iron (Fe). Samples (~1.0 g) of light muscle, dark muscle, and skin were each ashed at 500°C (21). The ash was dissolved in 5 mL 6.0 M hydrochloric acid (Merck, Darmstadt, Germany) and diluted to 50 mL with double-deionized water. The concentration of iron was then measured with a flame atomic absorption spectrometer (Perkin-Elmer 5000; Norwalk, CT) with deuterium background correction (22) ($n = 2$, $a = 2$). The limit of detection was 1 mg/kg tissue, and the repeatability of the method was RSD% 5.0. For quality assurance purposes, internal or certified reference materials were analyzed together with the materials.

Analysis of copper (Cu). Samples (~1.0 g) of each of the three herring fractions were disintegrated with concentrated nitric acid (J.T. Baker, Philipsburg, NJ) in a microwave oven (2455 Hz, 650 W) for 75 min in Teflon destruction vessels. After this disintegration, the samples were diluted to 50 mL with double-deionized water. The copper content in each sample was determined with a Perkin-Elmer 5100 graphite furnace atomic absorption spectrometer with Zeeman background conditions (23) ($n = 2$, $a = 2$). The limit of detection was 0.1 mg/kg tissue, and the repeatability of the method was RSD% 6.0. For quality assurance purposes, internal or certified reference materials were analyzed together with the materials.

Analysis of selenium (Se). A ~1.0 g sample of each herring fraction was disintegrated with concentrated nitric acid (J.T. Baker) and hydrogen peroxide (Merck) in a microwave oven (2455 Hz, 650 W) for 75 min in Teflon destruction vessels. After disintegration, the selenium was reduced in the microwave oven by treatment with 37% hydrochloric acid (Merck). The reduced disintegrated sample was then diluted to 25 mL with double-deionized water. The content of selenium was analyzed by flow injection hydride atomic absorption spectrophotometry (Perkin-Elmer 5100) (24) ($n = 2$, $a = 2$). The limit of detection was 0.010 mg/kg tissue, and the repeatability of the method was RSD% 8.0. For quality assurance purposes, internal or certified reference materials were analyzed together with the materials.

Measurement of pro-oxidative effect of herring buffer extracts. Ten grams each of light muscle, dark muscle, and skin was homogenized on ice for 1 min in a Sorvall Omnimixer (Ivan Sorvall Inc., Norwalk, CT) at speed 5, together with 40 mL of potassium phosphate buffer (Merck), pH 7.4. The homogenates were centrifuged at 46,000 × g, 4°C for 15 min (Sorvall Superspeed RC2-B, Instrument AB Lambda, Stockholm, Sweden), and the supernatants were collected. The supernatants were then added, either crude or heat-treated (30 min at 55°C or 10 min at 100°C), to a 10-mM linoleic acid emulsion (pH 6.8), after which the oxygen consumption was measured at 25°C (25,26) ($n = 2$, $a = 2$). Results are expressed as the slope of the curve (k-value) where the maximum rate of oxygen consumption occurred.

Analysis of total lipid content. The total lipid content in light muscle, dark muscle, and skin was determined gravimetrically after extraction with a modified version of the Bligh and Dyer procedure (27) as described by Undeland *et al.* (19) ($n = 3$, $a = 1$). Results are expressed as g/kg of tissue.

Analysis of fatty acid pattern. The fatty acid pattern was measured in light muscle, dark muscle, and skin by using the lipid extracts prepared for total lipid determination. The fatty acids were converted into methyl esters and analyzed by gas chromatography according to Ekstrand *et al.* (28) ($n = 3$, $a = 2$). Results are expressed as g/kg of lipid.

Extraction of lipids for the analysis of lipid classes, α-tocopherol, and oxidation products. Total lipids were extracted basically according to the method of Burton *et al.* (29), but with the use of lower levels of SDS as described by Undeland *et al.* (30).

Analysis of lipid class distribution. The contents of neutral lipids (NL), phospholipids (PL), and free fatty acids (FFA) in the total lipids from each herring fraction were determined gravimetrically by solid phase extraction according to the method of Kaluzny *et al.* (31) ($n = 2$, $a = 2$). Results are expressed as g/kg of lipid.

Analysis of α-tocopherol. α-Tocopherol was determined by normal-phase high-performance liquid chromatography (HPLC) according to the method of Piironen *et al.* (32), with minor modifications as described by Undeland *et al.* (19) ($n = 2$, $a = 2$). The repeatability of the method for α-tocopherol analyses was RSD% 3.0 ($n = 1$, $a = 6$). The levels of α-tocopherol are expressed as g/kg of lipid.

Analysis of hydroperoxides by chemiluminescence (CL). Under alkaline conditions, in the presence of microperoxidase, lipid hydroperoxides are easily broken down into various radicals and active oxygen species. These reactive compounds can convert luminol into a luminol endoperoxide, which gives rise to chemiluminescence with an absorbance maximum around 430 nm (33,34). To analyze hydroperoxides on the basis of this reaction, total lipids were dissolved in isopropanol/*n*-heptane (7:3) (LiChrosolv, Merck) to a concentration of 4 μg/mL and then subjected to flow injection analysis (FIA) (35,36) by the following procedure ($n = 2$, $a = 2$): A sample solution (10 μL) was injected into an HPLC system (Hewlett-Packard 1090; Waldbronn, Germany) with methanol (LiChrosolv, Merck) as the mobile phase. In a mixing tee, the sample flow was allowed to mix with a reagent that was prepared in the following way: To 1000 mL of methanol was added 0.35 g luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma Chemical Co., St. Louis, MO) and to 1000 mL of water was added 38 g disodium tetraborate decadehydrate (Merck) plus 0.010 g microperoxidase (MP-11 sodium salt) (Sigma). The pH of the water phase was then adjusted to 10.0 with concentrated sodium hydroxide, after which it was mixed with the methanol phase. The flow rate of both the mobile phase and the reagent was set to 1 mL/min. After mixing with reagent, the sample flow was passed through a chemiluminescence detector (Jasco 825-CL; Hachioji, Tokyo, Japan) to record signals at 430 nm. Because no column was installed in the system, the signal was represented by one single peak, which arrived with the void volume. Its area represented the total chemiluminescence in the sample. To determine the linearity of the detector response, lipid samples from freshly caught herring were spiked with different amounts of 13-hydroperoxy-9,11-octadecadienoic acid [13(*S*)-HpODE; Cayman Chemical Co., Ann Arbor, MI] and analyzed as described above. The limit of detection was ~0.8 μg of 13(*S*)-HpODE. No quantitation was performed in relation to the analysis of 13(*S*)-HpODE because the response factors are believed to vary for different types of lipid hydroperoxides (37). Results are therefore expressed simply as area units/μg of lipid. The repeatability of the method for analyzing chemiluminescence was RSD% 6.2 ($n = 1$, $a = 9$).

Analysis of peroxide value (PV). Peroxide value (PV) was analyzed by the official ferric thiocyanate method of the International Dairy Federation (IDF) (38) as modified by Ueda *et al.* (39) ($n = 2$, $a = 2$). To decrease the toxicity of the method, total extracted lipids were dissolved in isohexane (HPLC grade, Fisons, Loughborough, England) instead of *n*-hexane as was described by Ueda *et al.* (39). This exchange did not influence the results. The repeatability of the method was RSD% 1.6 ($n = 1$, $a = 6$) and the results are expressed as meq peroxide/kg of lipid.

Analysis of absorbance at 234 (A_{234}) and 268 nm (A_{268}). Oxidized fatty acids that contain a conjugated diene system absorb ultraviolet light strongly at 234 nm. At 268 nm, conjugated trienes, and various bifunctional oxidation products, such as ethylenic diketones and oxodienes, show an absorption maximum (40,41). These properties have been used as the basis for the detection of oxidation products in biological samples by using simple spectrophotometry (42). In this study, absorption at 234 and 268 nm in fat from the three herring fractions was measured by FIA ($n = 2$, $a = 2$) as described by Undeland *et al.* (19). Results are expressed as area units/μg of lipid, and the repeatability of the method for analyzing A_{234} was RSD% 0.5 ($n = 1$, $a = 6$), and for analyzing A_{268}, RSD% = 4.5 ($n = 1$, $a = 6$).

Analysis of fluorescent lipid oxidation products (FP). FP are formed through the interaction of peroxidizing lipids, such as hydroperoxides and aldehydes, with cellular constituents that contain free amino groups (43). In this study, lipid-soluble fluorescence with an excitation maximum at 367 nm and an emission maximum at 420 nm was followed in the three types of herring tissue by FIA ($n = 2$, $a = 2$), as described by Undeland *et al.* (19). Results are expressed as area units/μg of lipid. The repeatability of the method was measured to be RSD% 4.7 ($n = 1$, $a = 6$).

RESULTS AND DISCUSSION

Compositional data. (i) Trace elements (Fe, Cu, Se). As shown in Table 1, the iron and copper content varied substantially among the three herring fractions. Dark muscle tissue contained almost eight times as much iron as white muscle and three times as much as skin. These results reflect the high levels of various hemoproteins (44,45) and LMW iron (46) in dark muscle. The relatively high level of skin-bound iron is, according to Ke and Ackman (17), mainly of a nonheme nature.

Concentrations of copper were five to eight times lower than iron concentrations in all types of tissue, something that

TABLE 1
Compositional Data of Light Muscle, Dark Muscle, and Skin from Herring (*Clupea harengus*) Caught in November 1995

	Light muscle	Dark muscle	Skin
Trace elements			
Fe (mg/kg wet sample)[a]	3.0 ± 0	22.5 ± 0.5	7.5 ± 0.5
Cu (mg/kg wet sample)[a]	0.62 ± 0.05	4.1 ± 0.2	1.1 ± 0.1
Se (mg/kg wet sample)[a]	0.25 ± 0	0.29 ± 0.02	0.26 ± 0
Lipid components			
Total lipids (g/kg wet sample)[b]	79 ± 1	220 ± 3	285 ± 3
Neutral lipids (g/kg lipid)[a]	880 ± 10	880 ± 10	890 ± 6
Phospholipids (g/kg lipid)[a]	97 ± 7	73 ± 5	43 ± 6
Free fatty acids (g/kg lipid)[a]	21 ± 5	24 ± 3	27 ± 5
α-Tocopherol (g/kg lipid)			
Indiv A[c]	0.090	0.098	0.042
Indiv B[c]	0.18	0.18	0.069
Indiv C[c]	0.060	0.018	0.0022

[a]Mean value (n = 2) ± (max − min value)/2. For each of the two samples, a = 2. Mean values from these two analyses were used to establish sample variation.
[b]Mean value (n = 3) ± SD. For each of the three samples, a = 1.
[c]a = 2.

has also been seen in striped bass (13), mackerel (17,46), and flounder (47). However, the tissue-related differences in copper content followed the same order as those of iron content, i.e., dark muscle > skin > light muscle. These results are partly in accordance with those of Decker and Hultin (46) and Ke and Ackman (17).

The selenium level was measured because it reflects, to some extent, the content of glutathione peroxidase (48–51). This antioxidative enzyme, which contains one mole of selenium in each of its four subunits (51), acts together with glutathione to reduce cellular peroxides to their corresponding alcohols without the formation of radical intermediates (48,49). As described by Jia *et al.* (12), glutathione peroxidase probably contributes to most of the non-LWM selenium that is present in mackerel (50–60% of total soluble selenium). As shown in Table 1, the present study showed only small differences in selenium content among the three types of tissue. As an indicator of glutathione peroxidase, these observations are therefore not in accordance with those of Nakano *et al.* (50), who found the glutathione peroxidase activity in skin sardine skin to be nearly three times as high as in light muscle (dark muscle was not measured). However, according to Stadtman (52), selenium-containing proteins other than glutathione peroxidase may also be present in fish, which would explain why the findings of Nakano *et al.* (50) were not reflected in the present data.

(ii) Aqueous pro-oxidative activity as measured by oxygen consumption. The net water-soluble pro-oxidative activity in light muscle, dark muscle, and skin, before and after heating, is shown in Table 2. Addition of dark muscle extracts to the linoleic acid emulsion gave an oxygen consumption which was approximately three times as high as when skin extracts were added and approximately four times as high as when light muscle extracts were added. These results, which may be explained by the high levels of metals in dark muscle (Table 1), are in accordance with those of Nagayama *et al.* (53) but contradict those of Cho *et al.* (54) and Fujimoto *et al.* (55). The latter indicated the following order of oxygen consumption upon addition of freeze-dried defatted sardine to an ethyl-eicosapentaenoic acid (ethyl-EPA) model system: skin > dark muscle ≈ light muscle. The authors claimed a pro-oxidative enzyme in the skin to be responsible for their findings. In the present study, the presence of pro-oxidative enzymes was studied by heating. This treatment lowered the pro-oxidative activity in all fractions but to a different extent (Table 2). Heating for 30 min at 55°C resulted in a lowering of the activity by 30% in light muscle, by 60% in dark muscle, and by 70% in skin. After 10 min heating at 100°C, 40% of the activity was lost in light muscle, 75% in dark muscle, and 97% in skin. Thus, compared to muscle, skin was more sensitive to high temperatures, and was the only type of tissue that was almost completely inactivated by heat treatment.

TABLE 2
Oxygen Consumption (k-value)[a] in a Linoleic Acid Emulsion (pH 6.8) upon Addition of Buffer[b] Extracts of Herring Light Muscle, Dark Muscle, and Skin

Treatment	Light muscle	Dark muscle	Skin
None	0.47 ± 0.06[c]	1.94 ± 0.2	0.58 ± 0.1
Heating 55°C, 30 min	0.32 ± 0.05	0.73 ± 0.09	0.19 ± 0.04
Heating 100°C, 10 min	0.27 ± 0.05	0.52 ± 0.09	0.019 ± 0.02

[a]k-Values represent the slope at the steepest part of the oxygen-consumption curve.
[b]Potassium phosphate buffer, pH 7.4. The relationship fish/buffer during homogenization was 1:4.
[c]Mean value ± (max − min value)/2. For each of the two samples a = 2. Mean values from these two analyses were used to establish sample variation.

Complete inhibition of the pro-oxidative activity in sardine skin was seen by Mohri *et al.* (18) after 30 min at 60°C or 10 min at 100°C, and by Fujimoto *et al*. (55) after 5 min at 100°C. These findings, together with our data, indicate a purer enzymatic nature of the pro-oxidative activity in skin than in muscle. Some of this activity may originate from lipoxygenases because these enzymes have been demonstrated in skin of sardine in at least three studies (18,54,55). The response of dark muscle extracts to heating is probably more complex and reflects several simultaneous reactions, e.g., denaturation of hemoproteins (56,57) and inactivation of microsomal enzymes (9,58). Concerning the former, Fujimoto *et al.* (55) actually found sardine dark muscle to yield a higher pro-oxidative activity after cooking, something which is most likely related to the exposure/liberation of heme groups (57). According to Undeland *et al.* (59), different hemoproteins responded in different ways to heating at 55 and 100°C; myoglobin increased its pro-oxidative activity, whereas the inverse was seen for hemoglobin. These data further stress the complexity of the ways in which muscle tissues respond to heating.

(iii) Total lipid content. According to Table 1, skin had a slightly, but not significantly, higher total lipid content than dark muscle. However, both dark muscle and skin contained significantly ($P < 0.05$) more total lipids than light muscle. The same ranking order was found in previous studies of sardine (6) and mackerel (4). The high total lipid content in skin is most likely due to its attachment to the subcutaneous fat layer (4).

(iv) Lipid classes. The data in Table 1 show that lipids from the three herring fractions differed mainly in their content of PL but also to a minor extent in their content of FFA. As is generally true when low- and high-fat animal samples are compared, light-muscle lipids contained somewhat more PL than lipids from dark muscle and about twice the PL amount of skin lipids. The same tendencies have been seen in white sucker (16), mackerel (45), and lake herring (60). The FFA levels indicated slight hydrolysis during handling and transport, somewhat more so in skin than in light and dark muscle.

(v) Fatty acid pattern. Analysis of the fatty acid patterns did not detect any significant ($P < 0.05$) differences between the three tissues (Table 3). The only notable difference was the slightly but nonsignificantly lower content of $C_{22:6}$ (docosahexaenoic acid, DHA) in skin, compared to muscle lipids. This observation was in accordance with previous studies of sardine (6), mackerel (4), and lake herring (60). As shown in Table 3, the identified fatty acids account for only ~55% of the total lipid content. Unidentified peaks in the chromatogram could account for at most another 10%, and the non-fatty acid portion of the NL and PL can be estimated to account for ~10% of the total lipid content. Thus, the total recovery was only ~75%, which may have originated from incomplete methylation. However, as recovery was similar between the various herring lipid samples, the data were considered valid for comparative purposes.

(vi) α-Tocopherol. α-Tocopherol content showed large individual variations, both in terms of the range of levels and the distribution between light muscle, dark muscle, and skin lipids (Table 1). Similar variations were seen by Hägg and Kumpulainen (61) and may be explained by genetic differences or unequal storage/transport conditions of the various fillets (e.g., access to air, temperature, blood contamination). However, one common feature among the three fillets was that the skin lipids contained the lowest levels of α-tocopherol. This is in accordance with the findings of Ke and Ackman (17), who compared α-tocopherol levels in lipids from mackerel skin and muscle (light and dark). As regards α-tocopherol in light- and dark-muscle lipids, the content was about the same in two of the fillets, whereas in one the light muscle

TABLE 3
Content of Fatty Acids (g/kg lipid)[a] in Total Lipids from Light Muscle, Dark Muscle, and Skin of Herring (*Clupea harengus*) Caught in November 1995

Fatty acid	Light muscle	Dark muscle	Skin
Saturates			
C:14:0	42.2 ± 7.4	41.4 ± 4.6	46.7 ± 9.0
C16:0	80.8 ± 7.1	74.5 ± 7.3	74.0 ± 10.6
C18:0	7.4 ± 0.6	7.4 ± 0.9	7.1 ± 0.6
Sum	130.4 ± 13.7	123.3 ± 11.5	127.7 ± 19.6
Monoenes			
C16:1	29.8 ± 6.8	29.8 ± 6.2	32.6 ± 9.3
C18:1	48.5 ± 6.7	53.1 ± 9.7	52.9 ± 12.8
C20:1	61.8 ± 12.8	62.3 ± 8.7	63.1 ± 15.0
C22:1	33.8 ± 4.2	33.1 ± 5.0	32.9 ± 6.9
Sum	173.9 ± 26.1	178.3 ± 23.1	181.4 ± 38.7
Polyunsaturates			
C18:2	12.0 ± 2.3	12.3 ± 1.7	13.2 ± 2.9
C18:3	8.4 ± 1.7	8.0 ± 1.1	9.2 ± 2.0
C18:4	16.4 ± 3.8	15.8 ± 2.4	18.6 ± 4.4
C20:5	127.9 ± 20	122.1 ± 13.9	121.3 ± 21.9
C22:6	77.5 ± 8.1	71.3 ± 9.6	53.4 ± 7.4
Sum	242.1 ± 25.2	229.4 ± 18.5	215.8 ± 30.3

[a]Mean value ± SD ($n = 3$, $a = 1$).

showed substantially higher values. In contrast to these findings, lipids from mackerel dark muscle have previously been found to contain three times as much α-tocopherol as the light-muscle lipids (11,44,45). The authors explained these results by the abundance of mitochondria in dark muscle.

Oxidative stability at –18°C. The oxidative stability of lipids in light muscle, dark muscle, and skin from fatty fish is known to differ widely. Skin and dark muscle are generally considered as being the least stable (3–6); however, contradictory results have been reported as to their internal ranking order. To elucidate the role of oxygen contact and tissue interactions for these contradictions, a smaller study, in which light muscle, dark muscle, and skin were stored frozen as intact herring fillets, was compared with a larger one, where these fractions were stored separately. In the latter, the relationship between oxidative stability and compositional parameters was also taken into account. To measure lipid oxidation, several different methods were used to get a more complete picture of the oxidation process. The development of conjugated dienes and lipid hydroperoxides was followed in both studies. The former were measured with absorbance at A_{234} and the latter with CL or PV. In the larger study, measurements of conjugated trienes/oxodienes/diketones at A_{268} and of lipid-soluble FP were also performed. All data on oxidation products are expressed on a lipid basis. This is important to keep in mind because the total lipid contents differed widely among the three herring fractions (Table 1). Furthermore, as the paired fillet technique (20) was used in the present study, all results are given as Δ values. No development of oxidation products was seen in the reference tissues (A tissues) throughout the entire storage period.

(i) Influence of oxygen contact and tissue interactions on oxidative stability. Comparison between the stability of herring light muscle, dark muscle, and skin, when stored as intact fillets vs. separately, is based on Figures 1A and 2A, which show the increase in conjugated dienes (A_{234}), and on Figures 1B and 2B, which show the increase in hydroperoxides as measured by CL and PV.

Figures 1A and 2A show that it is difficult to differentiate between samples on the basis of A_{234} measurements which can be explained by the high background absorbance from the native lipid at 234 nm (62). However, the trend in these curves points to a smaller oxidative deterioration of dark muscle when this fraction is stored within an intact fillet, as compared to when it is stored separated from skin and light muscle. In the former, the mean A_{234} values after 12 wk of storage ranked the three tissues in the following order: skin > dark muscle > light muscle, and in the latter: dark muscle > skin > light muscle.

Development of lipid hydroperoxides followed the same dynamics as the conjugated dienes but, as shown in Figures 1B and 2B, gave rise to a clearer differentiation between the three samples. Thus, again, but now in a more pronounced way, the skin fraction oxidized fastest when the herring was stored as intact fillets, whereas when the three types of tissues were separated prior to storage, the development of oxidation was fastest in dark muscle. Under these two sets of circumstances, light muscle proved to be the most stable fraction. The results from Figures 1 and 2 can be compared with earlier studies of thiobarbituric acid (TBA) values in sardine (6) and mackerel (5) light muscle, dark muscle, and skin, stored at –5°C, either within whole fish or as separate minces. The sardine study gave results similar to ours, i.e., after whole storage, skin showed the highest TBA values, whereas after storage as separate minces, dark muscle gave the highest values. In mackerel, on the other hand, skin oxidized fastest under both sets of storage conditions. High instability of skin lipids during storage of whole fatty fish was also shown by Vicetti and Palma (3) who observed the following ranking of TBA values after storage at –5°C: skin > dark muscle > light muscle. Together with the present results, these studies clearly point to the protective surroundings of dark muscle within an intact fillet. The outer tissues, namely, skin and white muscle, obviously consume a great deal of oxygen and thus prevent it from reaching the dark muscle. Even though dark muscle constitutes only about 18% (w/w) of the entire

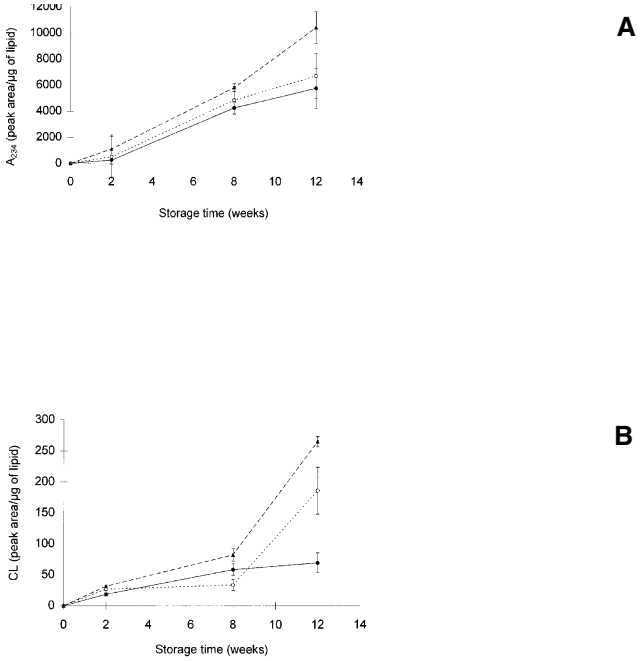
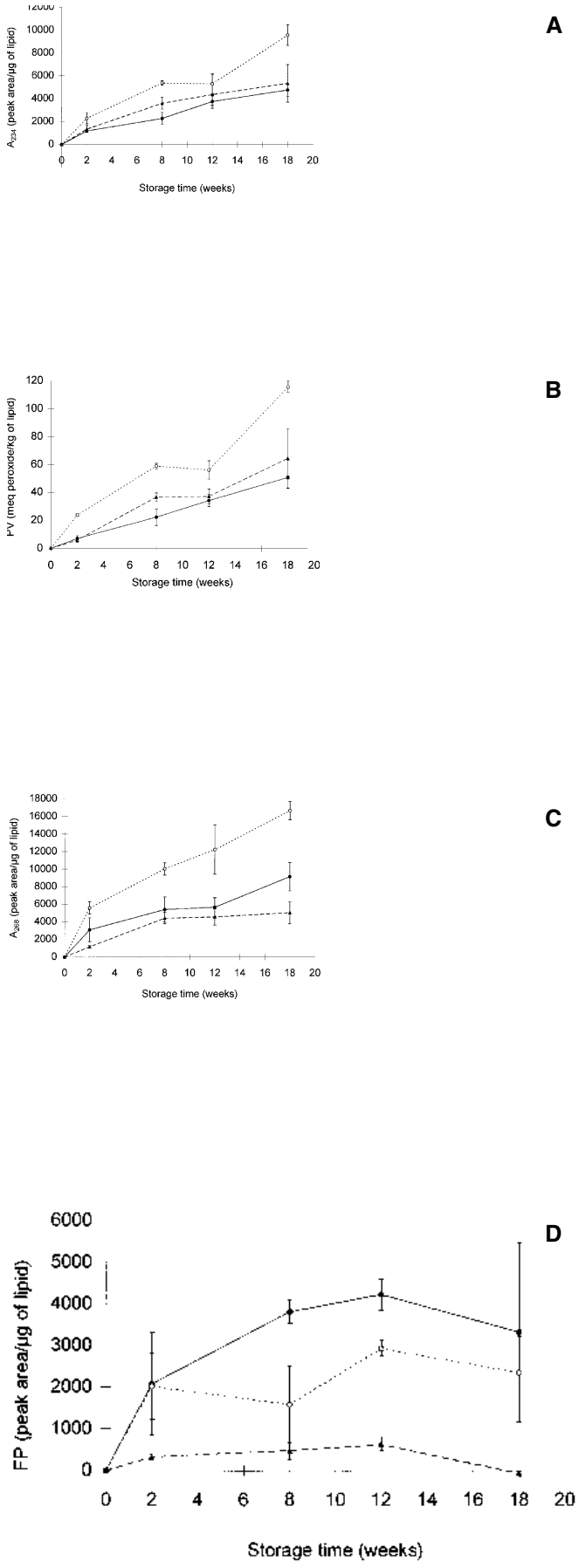


FIG. 1. The progress of (A) absorbance at 234 nm (A_{234}) and (B) chemiluminescence (CL) in light muscle (●), dark muscle (○), and skin (▲) when stored as intact fillets at –18°C for 0, 2, 8, and 12 wk. All points in the curves represent mean △ values from two stored fillets and their corresponding reference fillets (n = 2). Each sample was analyzed in duplicate (a = 2), and mean values from these two analyses were used to establish sample variation. The repeatability of the methods was RSD% 0.5 for A_{234} and RSD% 6.2 for CL (n = 1, a = 6). Bars indicate the span between maximum and minimum values.



fillet, a drastic decrease in the quality of this tissue will have a great impact on the entire flavor impression of the fillet. This points to the importance of storing fillets of fatty fish with the skin on for as long as possible. This conclusion is also strongly supported by previous studies on the protective role of skin on various layers of herring fillets (19).

(ii) Influence of composition on oxidative stability. The high instability of dark muscle when it is separated from the other types of tissue prior to storage points to a second conclusion to be drawn from the present results, namely, that dark muscle appears to have the least favorable composition, with respect to oxidative stability, when compared with light muscle and skin. Thus, it seems to have the worst balance between pro- and antioxidants and/or the least stable lipids. This conclusion could not have been drawn from storage stability studies of intact fillets, as dissimilarities in the contact with air and surrounding tissues constitute disturbing factors.

The development of primary oxidation products, i.e., conjugated dienes and lipid hydroperoxides, has already been discussed to some extent. As shown in Figures 2A and 2B, these products increased at the highest rate in dark muscle throughout the entire storage period. It was difficult to distinguish between light muscle and skin, but the trends in both figures indicate a slightly higher formation rate in skin. These results are well supported by the intensity of pro-oxidative activity as measured in a linoleic acid emulsion (Table 3) and can be explained in several ways. To begin with, dark muscle has the highest total content of iron (Table 1), most of which is bound to various hemoproteins or LMW compounds (1). In mackerel dark muscle, the concentration of LMW iron was reported to be four–five times higher than in light muscle (46). Several investigations in fish have revealed that this fraction of iron is a stronger pro-oxidant than heme-bound iron (17,63). Ferrous LMW iron is particularly critical because it can catalyze the production of various active oxygen species, such as the hydroxyl radical, ·OH (1,64). However, LMW-Fe^{2+} iron is also critical owing to its ability to participate in chain branching (65), a process that also can be brought about by heme iron. Iron bound to metmyoglobin may further be activated by low concentrations of hydrogen peroxide under the formation of a porphyrin cationic radical. This species has been shown to initiate lipid oxidation in poultry muscle (66) and in fish sarcoplasmic reticulum (67). The high copper content in dark muscle may be an additional

FIG. 2. The progress of (A) absorbance at 234 nm (A_{234}), (B) peroxide value (PV), (C) absorbance at 268 nm (A_{268}), and (D) lipid-soluble fluorescent oxidation products (FP) in light muscle (●), dark muscle (○), and skin (▲) when stored separately at –18°C for 0, 2, 8,12, and 18 wk. All points in the curves represent mean △ values from two stored fillets and their corresponding reference fillets (n = 2). Each sample was analyzed in duplicate (a = 2), and mean values from these two analyses were used to establish the sample variation. The repeatability of the methods was RSD% 0.5 for A_{234}, RSD% 1.6 for PV, RSD% 4.5 for A_{268}, and RSD% 4.7 for FP (n = 1, a = 6). Bars indicate the span between maximum and minimum values.

explanation for its high instability (Table 1). According to Decker and Hultin (46), 7–38% of the copper in mackerel is associated with the LMW fraction (<10 kD). Even if it is not as active as LMW iron (63), several studies in fish have pointed to the importance of LMW copper as a pro-oxidant (17,47,49,63). Copper-catalyzed reduction of ferric iron has been reported as one catalytic effect (68); however, participation in the production of active oxygen species and in the breakdown of lipid hydroperoxides have also been suggested (69). The relatively high formation rate of primary oxidation products also in skin may also be related to its low α-tocopherol level, its fairly high metal content, and its likely content of catalytic enzymes. However, in comparison with dark muscle, its somewhat lower content of PL and $C_{22:6}$ may explain its more stable nature.

In addition to Figures 2A and 2B, Figure 2C, which shows the increase in A_{268}, supports the conclusion about unfavorable composition of the dark muscle. Instability of the dark muscle lipids was actually more pronounced based on this measure, compared to the measurements of dienes and hydroperoxides. One explanation may be that secondary oxidation products are also taken into account when analyzing A_{268}. According to the literature, conjugated trienes, ethylenic diketones, and oxodienes all absorb at 268 nm (40,41). As previously discussed, iron and copper effectively decompose primary oxidation products into secondary ones. The substantial rate at which dark muscle increased in A_{268} may therefore be partly related to its high content of these metals. Comparison between light muscle and skin shows that the latter had a higher content of iron and copper but still increased, with a slightly lower rate, in absorbance at 268 nm. This may possibly be due to differences in the physical distribution of lipids. Skin lipids are bound in membranes to a smaller extent than muscle lipids and may therefore have less contact with the metal-containing aqueous phase. Further, because fatty acids need three or more double bonds to form a conjugated triene system (70), the somewhat smaller amount of $C_{22:6}$ (Table 2) in skin may be an additional explanation.

Figure 2D shows the increase in fluorescence intensity (excitation/emission: 367/420 nm) in lipids from pre-separated light muscle, dark muscle, and skin. This fluorescence is believed to originate from various Schiff's bases, formed in the reaction between hydroperoxides and aldehydes with PL that contain free amino groups (43,71). The formation rate of these so-called tertiary oxidation products was highest in light muscle, followed by dark muscle and then skin. Thus, the three tissue types were ranked in an order that markedly differed from the orders seen in relation to primary and secondary oxidation products. According to Tables 1–3, these results are probably better explained by variations in lipid composition among the tissues than by the content of various pro-oxidants. The PL content (Table 1), which ranked the three tissues in the same order as did the development of FP, is likely the most important factor here (43,71). Regarding individual fatty acids, Fletcher *et al.* (71) described fluorescence with excitation/emission wavelengths of 360–380/440–470 nm as characteristic for the oxidation of lipids with 4–6 double bonds. Thus, the somewhat lower content of $C_{22:6}$ in skin compared to muscle lipids may in part explain its low production of fluorescence. Fujimoto and co-workers (54,55) found skin to give the highest fluorescence (excitation/emission: 380/440 nm), followed by dark and then light muscle, during storage of freeze-dried defatted sardine tissues in ethyl-EPA. The use of different lipid substrates probably explains the lack of agreement with our observations. As shown in Figure 2D, a decrease in fluorescence was observed in all samples at the end of the storage period. Similar observations were made by Fujimoto *et al.* (55) and Cho *et al.* (54), and one explanation offered was polymerization between Schiff's bases, which caused the formation of yellow-brownish discoloration (72). Copolymerization with proteins to form lipid-insoluble fluorescence has also been suggested as an explanation of this phenomenon (73).

(iii) Significance of the various methods for measuring lipid oxidation. As previously discussed, several different methods were chosen to follow the progress of lipid oxidation in the three fractions of herring. There were two main reasons for this approach; firstly, the desire to measure products that are formed at various stages in the lipid oxidation chain, thus possibly at various stages of the storage period, and secondly, because light muscle, dark muscle, and skin, owing to compositional differences, vary substantially in their ability to form individual lipid oxidation products.

The first reason, which was based on the belief that the four methods should be of different importance at various stages during the storage period, was not confirmed. The development of the different lipid oxidation products was not at all as strongly separated in time as has sometimes been suggested in the literature (74). The so-called primary, secondary and tertiary products all developed more or less simultaneously (Figs. 1,2), and the first group of products that started to decrease was actually the tertiary group. However, with respect to the second reason, the results presented here reveal that the use of several methods was highly profitable. Depending on the method used, the ranking order among the three types of tissue differed (Fig. 2), and this gave us the opportunity to discuss some of the individual mechanisms that are involved in the oxidation of lipids in light muscle, dark muscle, and skin.

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