Lipid Oxidation in Fillets of Herring (*Clupea harengus*) during Ice Storage

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The influence of ice storage on lipid oxidation, odor, antioxidants, water-soluble catalysts, and microorganisms was investigated in fillets of herring (*Clupea harengus*) during 15 days. Based on linear regression analyses of the data, significant rises ($p \leq 0.05$) in lipid oxidation products were seen after 2−3 days and in "rancid" odor after 2.5 days. Peroxide value (PV), fluorescent product (FP), and ascorbic acid analyses were the chemical measures most strongly correlated to "rancid" odor ($r = 0.97$). Antioxidants decreased in the following order: α-tocopherol > ascorbic acid > glutathione peroxidase (GSH-px); however, GSH-px correlated best to the development of lipid oxidation products ($r_{mean} = -0.96$). The activity of aqueous pro-oxidants, which were enzymatic in nature to a great extent, had decreased by 75% at day 15. No significant increase in total bacteria was seen until after 7 days. There were major local differences in both composition and stability throughout the fillet. Oxidation proceeded most rapidly in the tissue right under the skin, probably explained by its high initial pro-oxidative activity.

Keywords: *Antioxidants; Clupea harengus; herring; ice storage; lipid oxidation*

INTRODUCTION

Herring (*Clupea harengus*) is a fish with many advantages as a raw material for food production: abundance, low price, and a high content of omega-3 fatty acids. Unfortunately, the development of lipid oxidation often limits the possibilities for storage and processing of this species. This is due to close contact between the highly unsaturated herring lipids and strong catalytic systems. However, a certain control of the lipid−catalyst interactions is provided for by the presence of natural antioxidants in the tissue. Immediately after slaughter, the antioxidant level is generally high, and most oxidative attacks are inhibited. However, with time post mortem, an array of changes takes place in the tissue that disturbs the delicate balance that initially exists between catalysts and antioxidants. Among these changes are a decrease in reducing capacity (Hultin, 1994), an increase in free iron (Decker and Hultin, 1990), activation of hemoproteins (Kanner et al., 1987), and membrane disintegration (Huang et al., 1993).

Freezing and refining of herring is most often preceded by a storage period on ice. Under these conditions, a number of studies have pointed to a slow progress of lipid oxidation (Bilinski et al., 1978; Karsti and Blockhus, 1966; Sigurdsen, 1947; Smith et al., 1980) and instead pointed to microbial growth as the main spoilage process taking place (Aubourg et al., 1995; Harris and Tall, 1989). However, in several other species, large losses of antioxidants have been reported during ice storage (Bandarra et al., 1997; Petillo and Hultin, 1995; Watanabe et al., 1996) as well as substantial increases in both primary (Botta and Shaw, 1976; Sen and Bhandary, 1978), secondary (Aubourg, 1997; Kolakowska et al., 1992; Ramanathan and Das, 1992; Wood and Hintz, 1971), and tertiary (Aubourg et al., 1995, 1997) lipid oxidation products. Thus, contemporaneous with microbial changes, lipid oxidation, or at least the conditions for lipid oxidation to take place, appear to be greatly favored by preprocessing storage on ice. As a result, this storage period may have a severe influence on the stability of the final product and should therefore be controlled. To do so, the dynamics of changes in lipid oxidation products, antioxidants, and catalysts during ice storage need to be well understood. This would clarify which compounds are the most relevant as indicators of lipid oxidation and also what kind of protective strategy should be applied.

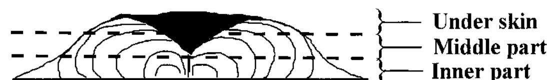

Figure 1. Cross-section of a herring (*Clupea harengus*) fillet showing the three layers investigated: under skin, middle part, and inner part.

The aim of the present study was to monitor chemical measures of lipid oxidation, sensory changes, antioxidant loss, total water-soluble catalytic activity, and bacterial growth in herring fillets stored for 0−15 days on ice. To discover local differences within the fillet, the analyses were also to be carried out at certain intervals on fillets sliced into three horizontal layers: under skin, middle part, and inner part (Figure 1).

MATERIALS AND METHODS

Equipment. Slicing of herring fillets was performed with a slicing machine (EMIDE, Type AS 220 W, Germany). Protein was measured with a Perkin-Elmer nitrogen analyzer (PE 2410 series II, Germany). Extraction of lipids and aqueous pro-oxidants from the samples was performed in a Sorvall omnimixer (Ivan Sorvall Inc., Northwalk, CT), and cold centrifugation was performed in a Sorvall Superspeed RC2-B (Ivan Sorvall Inc., Norwalk, CT). Computers used during the sensory analysis were obtained from PSA (Version 2.07a, Oliemans

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Punter & Partners, The Netherlands). The statistical software Systat (Version 6.0, SPSS Inc., Chicago, IL) was used for regresssion analyses. Selenium-based glutathione peroxidase activity (GSH-px) was analyzed in an enzyme-linked immunosorbent assay (ELISA) reader from IEMS (Labsystems DY, Finland). Color was measured with a spectrophotometer (Macbeth Color-eye 2180, New Windsor, NY).

Samples. Herring (*Clupea harengus*) caught off the west coast of Sweden in October 1997 was stored in refrigerated seawater (RSW) tanks for 24 h before it was mechanically headed, gutted, and deboned with commercial equipment. The double fillets obtained, which had a length of 10.2 ± 1.3 cm and a weight of 30.8 ± 7.4 g, were packed in paper boxes (5 kg of herring/box) and stored surrounded by plastic bags filled with ice in a refrigerated room (2 °C) for up to 15 days. The mean temperature of the fish during this period was −0.4 °C. After 0, 1, 2, 3, 4, 5, 6, 8, 9, 12, and 15 days, the fillets were removed from the boxes, packed individually in polyethylene film, and frozen at −40 °C in a tunnel freezer. After being frozen, they were stored at −70 °C. On the day of analysis, the polyethylene film was removed from seven fillets from the same storage point, and these were placed in a polyethylene bag and thawed for 11 min in cold running tap water. Following thawing, the skin was removed manually, and the seven fillets were homogenized together for 1 min in a food processor. From this pooled herring mince, n samples were removed for the different analyses which were repeated a times.

For the study of different horizontal layers, fillets from storage points 0, 6, and 12 days were treated in a similar way as described above. However, the thawing was shortened to 4 min, and after removal of the skin, the fillets were sliced into three horizontal layers, under skin, middle part, and inner part (Figure 1), using a slicing machine (Undeland et al., 1998a). Twenty-one slices of each type were then homogenized together as previously described.

Analysis of Protein. Protein was measured at time 0 in whole and sliced fillets as nitrogen × 6.25 according to the Association of Official Analytical Chemists (1990) using a nitrogen analyzer ($n = 2$, $a = 2$). Results were expressed as grams of protein/kilogram of tissue.

Analysis of Total Lipid Content. Total lipids were extracted with petroleum ether from 0 day whole and sliced fillets in a Soxhlet extractor as described by Pearson (1973) (for the whole fillets, $n = 5$, $a = 1$; for the slices, $n = 3$, $a = 1$). Results were expressed as grams of lipid/kilogram of tissue.

Analyses of Lipid Oxidation Products. For analysis of oxidation products, lipids were extracted with water, sodium dodecyl sulfate (SDS), ethanol, and heptane as described by Undeland et al. (1998b).

Analysis of Peroxide Value (*PV*). PV was analyzed using the ferric thiocyanate method as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). The repeatability of the method, described as relative standard deviation (% RSD), was 1.6 ($n = 1$, $a = 6$), and the results were expressed as milliequivalents of peroxide/kilogram of lipid.

Analysis of Absorbance at 268 nm (A_{268}). A_{268} of the lipids extracted from whole and sliced fillets was measured using flow injection analysis (FIA) as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). Results were expressed as $\log_{10}$ area units/microgram of lipid, and the repeatability of the method was 4.5% RSD ($n = 1$, $a = 6$).

Analysis of Total Lipid-Soluble Fluorescent Lipid Oxidation Products (*FP*). FP having an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in lipids from whole and sliced fillets with FIA as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). Results were expressed as $\log_{10}$ area units/microgram of lipid. The repeatability of the method was measured to be 4.7% RSD ($n = 1$, $a = 6$).

Analysis of Yellow Pigments (*YP*). Color of the dorsal and ventral side of the whole fillets were measured as CIE $L^*a^*b^*$ using a spectrophotometer. Yellowness was indicated in this coordinate system by b^* and ranged from −60 (blueness) to +60 (yellowness). The results were expressed as Δb^* values with the 0 day samples as reference ($n = 5$, $a = 9$).

Sensory Analysis. The odor of the pooled samples of whole herring fillets was assessed by descriptive sensory analysis as described by Andersson and Lingnert (1997) ($n = 7$, $a = 2$). Twenty grams of the samples was placed in 180-mL balloon glasses covered by watch glasses. Prior to serving, the samples were equilibrated for 2 h at ambient temperature. Seven panel members were used, and four training sessions were performed prior to the evaluation of the intensity of the following descriptors: total odor, rancid, sharp/acrid, old fish, fresh fish, and shellfish. To evaluate the data, the unipolar line scale used during the assessments was converted to intensity values ranging from 0 to 100.

Analysis of Antioxidants. *Analysis of α-Tocopherol.* Following extraction with water, SDS, ethanol, and heptane (Undeland et al., 1998b), α-tocopherol was determined in lipids from whole and sliced fillets by normal-phase high-performance liquid chromatography (HPLC), as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). The repeatability of the method for α-tocopherol analyses was measured to be 3.0% RSD ($n = 1$, $a = 6$), and the levels of α-tocopherol were expressed as grams/kilogram of lipid.

Analysis of Ascorbic Acid. Ascorbic acid was acidically extracted from whole and sliced fillets and analyzed with reversed-phase HPLC coupled to an electrochemical detector set at 0.6 V according to the method of Meeland and Waagbø (1998) ($n = 2$, $a = 1$). Results were expressed as milligrams of ascorbic acid/kilogram of tissue.

Analysis of Selenium-Based GSH-px Activity. The selenium-based GSH-px activity was monitored in whole and sliced fillets at 340 nm as the consumption of NADPH over time ($n = 1$, $a = 8$). This method was originally described by Bell et al. (1985) but was scaled down by Lygren et al. (1999) to be carried out using ELISA plates and an ELISA reader. Results were expressed as units [micromoles of NADPH oxidized minute^{-1} (gram of protein)$^{-1}$].

Analysis of Total Water-Soluble Pro-oxidative Activity. Buffer extracts from whole and sliced herring fillet samples were prepared, heated (12 min at 55 or 100 °C), and analyzed for total aqueous pro-oxidative activity, as described by Undeland et al. (1998c) ($n = 2$, $a = 2$). Results were expressed as the slope of the curve (k value) where the maximum rate of oxygen consumption occurred.

Total Bacterial Count. Total bacterial count in whole fillet samples was measured as spread plate counts on seawater agar after 7 days incubation at 15 °C (Dalgaard et al., 1997). Results were expressed as $\log_{10}$ colony forming units (cfu)/gram of tissue.

Statistical Evaluation. Data from all measures were subjected to regression analyses in order to fit a model describing their most likely changes with storage time. This technique also provided the possibility to compare the rates at which oxidation products changed during storage in different samples. Both linear ($y = kt + m$) and logarithmic ($\log_{10}y = kt + m$) models were tested, and based on calculations of R^2, the one describing the greatest variance in the data was selected. Confidence intervals were constructed around the lines in order to estimate significant differences between individual storage points (Harper, 1971a). A significant difference between two storage points was obtained when their confidence intervals did not overlap. For elucidation of whether lipid oxidation developed at significantly different rates in the three layers of herring fillets, a significance test as described by Undeland et al. (1998a) was applied to the results obtained from each of the measures. Pearson's correlation coefficient (r) (Harper, 1971b) was calculated in order to study the correlation between various measures. The level of significance for all tests was set at $p = 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the regression models that were fitted to all the data obtained from chemical, sensory, and

Table 1. Statistics Describing the Outcome of a Regression Analysis of the Data[a]

measure	whole fillet model[b]	whole fillet R^2 (lin/log)[c]	under skin model[b]	under skin R^2 (lin/log)[c]	middle part model[b]	middle part R^2 (lin/log)[c]	inner part model[b]	inner part R^2 (lin/log)[c]
PV	PV = 1.4t − 0.07	**0.94**/0.87	PV = 2.0t − 1.6	**0.94**/0.89	PV = 0.5t + 0.9	**0.90**/0.80	PV = 0.7t + 2.7	**0.96**/0.42
A_{268}	log A_{268} = 0.03t + 3.7	0.87/**0.88**	log A_{268} = 0.03t + 3.7	0.90/**0.94**	log A_{268} = 0.01t + 3.7	0.93/**0.94**	log A_{268} = 0.007t + 3.9	0.76/**0.86**
FP	log FP = 0.06t + 2.7	0.81/**0.91**	log FP = 0.09t + 2.3	0.97/**0.91**	log FP = 0.04t + 2.5	0.94/**0.98**	log FP = −0.006t + 3.2	0.13/**0.09**
TO	TO = 1.3t + 41.2	**0.81**/0.79						
R	R = 2.8t + 4.1	**0.91**/0.74						
S/A	S/A = 3.0t + 8.0	**0.91**/0.78						
O	O = 2.3t + 9.1	**0.90**/0.73						
F	F = −1.2t + 37.2	**0.87**/0.87						
S	S = −1.2t + 26.4	**0.87**/0.87						
α-T	α-T = −0.01t + 0.13	**0.87**/0.80	α-T = −0.01t + 0.11	**0.81**/0.88	α-T = −0.006t + 0.16	**0.91**/0.93	α-T = −0.004t + 0.17	**0.69**/0.67
G	G = −5.7t + 238	**0.72**/0.69	G = −7t + 240	**0.71**/0.73	G = −1.4t + 174	**0.07**/0.05	G = −1.6t + 170	**0.11**/0.13
Asc	Asc = −0.3t + 5.8	**0.89**/0.72	Asc = −0.37t + 5.8	**0.92**/0.97	Asc = −0.3t + 5.8	**0.99**/0.97	Asc = −0.3t + 6.0	**0.96**/0.95
Po	Po = −0.05t + 1.1	**0.90**/0.88	Po = −0.12t + 1.8	**0.95**/0.96	Po = −0.02t + 0.8	**0.22**/0.16	Po = 0.02t + 0.4	**0.26**/0.24
TB	log TB = 0.17t + 4.7	0.67/**0.83**						

[a] Abbreviations: TO, total odor; R, rancid; S/A, sharp/acrid; O, old; F, fresh; S, shellfish; α-T, α-tocopherol; G, GSH-px; Asc, ascorbate; Po, aqueous pro-oxidative activity; TB, total bacteria; t, storage time in ice (days). [b] Linear or logarithmic model depending on which gave the highest R^2 (shown in bold text) for the whole fillets. [c] lin, R^2 obtained for a linear model; log, R^2 obtained for a logarithmic ($\log_{10}$) model. R^2 for the model described is indicated in bold text.

bacterial analyses of whole fillets as well as inner, middle, and under skin layers. R^2 values express how much of the variance in the data that could be explained by the models.

Development of PV, A_{268}, FP, and YP. High R^2 values of the models created with untransformed PV data indicate an almost linear development of these primary oxidation products, at a rate of 1.4 mequiv of peroxide (kg of lipid)$^{-1}$ day^{-1}, in the whole fillets. On the basis of the confidence intervals shown in Figure 2A, a significant increase in PV was already obtained after 2 days in these samples. The dotted and broken lines in Figure 2A specify how the PV changed in the three different layers of the fillets (Figure 1). The under skin layer, which contained the largest amount of fat, 190 g/kg of tissue (Table 2), increased by the highest rate followed by the inner and then the middle part. The two latter contained only 47 and 18 g of fat/kg of tissue, respectively, which explains why most of the change in the whole fillets was described by changes in the under skin layer. In accordance with the present data, 3 days of storage at 1−3 °C has previously been shown to be critical for the PV development in minced oil sardine (Sen and Bhandary, 1978). In contrast, no significant changes in PV were seen in herring over a 3-day (Karsti and Blockhus, 1966), 4-day (Bilinski et al., 1978), 5-day (Sigurdsen, 1947) or 10-day period on ice (Smith et al., 1980).

Although A_{268} is a measure of both primary (conjugated triene hydroperoxides) and secondary (ethylenic diketones and oxodienes) lipid oxidation products (IUPAC, 1979; Brown and Snyder, 1982), the correlation between the $\log_{10}$ transformed data from this measure and PV was as high as $r = 0.92$ (Table 3). A significant increase was seen after 3 days in the whole fillets, and as shown in Figure 2B, the under skin layer lipids again contributed to most of the total rise in A_{268}. The highest 0 day absorbance was attained by the inner part lipids. However, as no significant increase occurred in this layer during the 12 days of storage, it was believed to be a phenomenon related to absorbance of the native lipids. A completely different pattern of development during ice storage of fish has been reported for the thiobarbituric reactive substances test (TBARS), another measure of secondary products. Here it has been shown that an immediate sharp increase is followed by a rapid fall during the second week of storage (Aubourgh et al.,1995). According to Aubourgh et al. (1997), the reason is that carbonyls are unstable and react easily with other compounds.

The development of lipid-soluble FP, a measure of carbonyls linked to phospholipids through a conjugated Shiff's base (Erickson, 1993), is shown in Figure 2C. Apart from in the under skin layer, an exponential increase was once again obtained (Table 1), and a significant increase in these tertiary oxidation products could be seen already after 2 days in the whole fillets. Among the three layers, the highest rate of increase was seen in the under skin layer; however, due to rather high sample variation, the increase here did not become significant until after 5.5 days. In the middle part, the rate of increase was somewhat lower, but already at day 3, a significant increase was detected. Although the inner part again gave very high 0 day values, no significant change was seen during the entire storage period. The latter may explain the very poor models created for the inner part (Table 1) and also indicates that analyzing FP is of limited value when monitoring lipid oxidation in white herring muscle during ice storage. In agreement with our data, Aubourgh et al. (1997) found a steady increase in the ratio between FP with ex/em maxima of 393/463 and 327/415 nm, respectively, in sardines during 16 days on ice. However, in contrast to us, they found FP to correlate better with microbial growth than with lipid oxidation. Presently r_{FP-TB} was only 0.88, while r_{FP-PV} and $r_{FP-A268}$ were 0.93 and 0.94, respectively (Table 3).

As YP has been found to form as a result of lipid oxidation (Fujimoto, 1970; Pokorny et al., 1974), these products were measured as b^* values on the ventral and dorsal side of intact, skinned fillets throughout storage. The 0 time b^* value was significantly higher on the ventral side than the dorsal side, but whereas the former did not change throughout the storage period, the latter had increased significantly after 12 days. To some extent, these results resembled those obtained for A_{268} and FP, supporting the theory given for the formation of YP, i.e., polymerization of fluorescent Shiff's bases (Fujimoto, 1970; Pokorny et al, 1974). When YP data from the ventral side were correlated with the $\log_{10}$ treated A_{268} and FP data from the inner part, r values of −0.63 and −0.72, respectively, were obtained. The corresponding correlation between the dorsal side and under skin layer data gave $r = 0.91$ and 1.0, respectively. The change in color observed under the skin was in accordance with earlier findings of

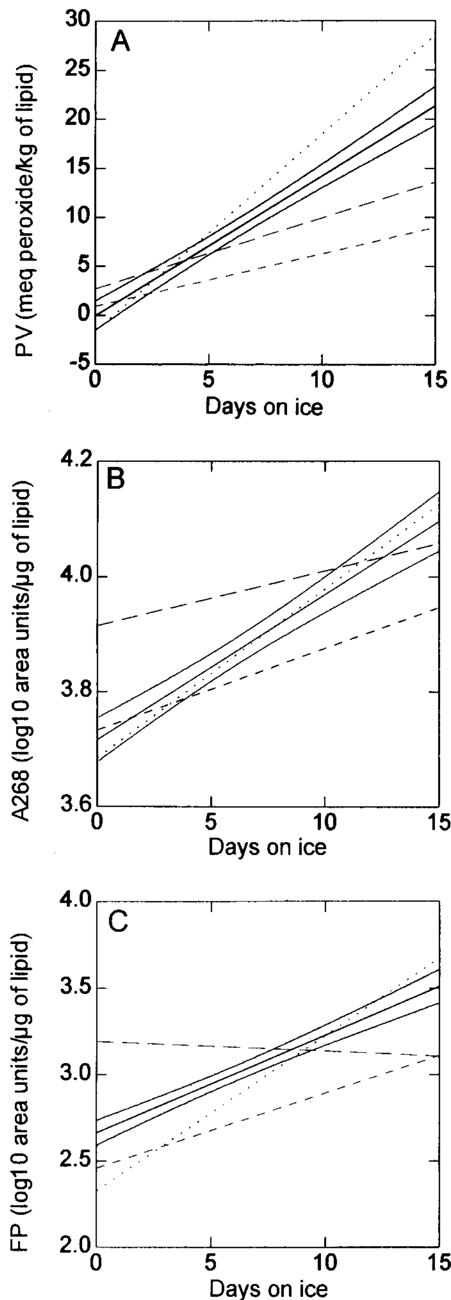

Figure 2. Linear regression lines illustrating the progress of (A) peroxide value (PV), (B) absorbance at 268 nm (A_{268}), and (C) lipid-soluble fluorescent oxidation products (FP) in herring fillets stored for 0–15 days in ice. (—), whole fillets; (···), under skin layer; (- - -), middle part; and (— —), inner part. Models describing these lines are shown in Table 1. The confidence bands shown for the whole fillet regression lines are based on $p = 0.05$.

Amano et al. (1967) and Buttkus (1975). Large fish-to-fish variation in the levels of YP made the models fitted to these data very poor. The best model, which was obtained for the dorsal data, explained only 15% of the variance.

Sensory Changes. The intensities of all six descriptors used to assess odor changed almost linearly throughout the storage period, either increasing or decreasing (Table 1). In Figure 3, total odor, rancid, and fresh fish are shown as examples. Rancid and sharp/acrid were strongly related ($r = 0.99$) and although the intensity of the latter increased at the highest rate, the first significant increase in rancid odor was observed after 2.5 days as compared to after 3 days for sharp/acrid. Old was ranked third with respect to development rate, and total odor was the last. Fresh fish and shellfish, which were also strongly correlated ($r = 0.98$), had both decreased significantly after 3.5 days. Of the three chemical tests used to monitor lipid oxidation, log FP in general correlated most strongly to the changes in odor ($r_{mean} = 0.96$) (Table 3). Rancid showed the highest correlation with PV and log FP ($r = 0.97$), which was a bit surprising as none of these measures directly respond to volatile oxidation products. However, Botta and Shaw (1976) also found a high correlation between PV and the onset of rancidity. Rancidity was previously detected in minced oil sardine after 2 days at 1–3 °C (Sen and Bhandary, 1978), in Baltic herring after 6 days on ice (Kolakowska et al., 1992), and in mackerel after 4–6 days on ice (Petillo and Hultin, 1995). Smith et al. (1980), on the other hand, reported no rancidity development in herring after 10 days of ice storage. According to Kolakowska et al. (1992), the fishing season has a strong influence on the level of oxidation products required to make the rancid smell evident. In lean spawning herring (~4% fat), these authors found rancidity to develop at a PV of 21, whereas in fattier autumn herring (~11% fat), a PV of 40 had to be reached. In the present study, where the fillets contained in average 8.6% fat (Table 2), rancidity was first significantly detected at a PV of ~4.

The correlation coefficients relating total microbial count to the sensory changes ranged between 0.65 and 0.87, which was increased to 0.77–0.93 following a logarithmic teatment of the microbial data (Table 3). It should be kept in mind, however, that the microbial analyses were made on frozen samples, which may have influenced the total bacterial count and thereby the correlation to sensory changes. It has previously been shown by Magnússon and Martinsdóttir (1995) that frozen storage of cod fillets at −25 °C for >14 weeks significantly decreased the total bacterial population. However, for fillets of ocean perch, no decrease was found, indicating that freezing-induced suppression of bacterial growth is highly species dependent.

Antioxidant Losses. The α-tocopherol consumption during storage is illustrated in Figure 4A. As shown in Table 1, the linear model created for the whole fillets explained 87% of the variation in the data, whereas for the various layers, R^2 ranged between 0.69 and 0.91. For the middle and under skin layers, slightly better models were obtained after a $\log_{10}$ transformation, indicating a more exponential change. In the whole fillets, a significant lowering in α-tocopherol was obtained after 3 days, and at day 13, the entire α-tocopherol depot was depleted. The under skin layer contributed most to this decrease, whereas the middle layer still had 55% left after 12 days. In the inner part, no significant change was seen. To some extent, these data support those of Petillo and Hultin (1995), observing a faster loss of α-tocopherol in dark than light mackerel muscle. However, while these authors found the dark muscle to have 3.3 times higher initial values than the light muscle, the present data showed that the under skin lipids initially had 30% lower α-tocopherol concentration than the lipids from the other parts of the fillet.

The activity of selenium-bound GSH-px, an antioxidative enzyme reducing peroxides to their corresponding alcohols (Kadiiska et al., 1993), changed according to Figure 4B. As can be seen in Table 1, poor models were

Table 2. Compositional Data for Unstored Whole Skinned Herring Fillets and for the Three Slices of These Fillets: Under Skin, Middle Part, and Inner Part

	whole fillets	under skin	middle part	inner part
protein[a](g/kg of tissue)	181 ± 3.3	177 ± 3.2	183 ± 0.8	189 ± 2.1
dry matter[a](g/kg of tissue)	281 ± 0.2	356 ± 0.5	248 ± 0.5	227 ± 0.3
ascorbic acid[a](mg/kg of tissue)	6.2 ± 0.8	6.2 ± 0.06	5.8 ± 0.3	5.9 ± 0.6
GSH-px[b](units/g of protein)	235 ± 14	241 ± 31	184 ± 13	163 ± 12
α-tocopherol[c](g/kg of lipid)	0.15 ± 0.01	0.13 ± 0.005	0.17 ± 0.004	0.18 ± 0.01
total lipids[d](g/kg of tissue)	85.6 ± 0.2	190.0 ± 3.0	47.1 ± 0.7	18.0 ± 0.8

[a] $n = 2$, $a = 1$, results given as mean ± (max-min)/2. [b] $n = 1$, $a = 8$, results given as mean ± SD. [c] $n = 3$, $a = 2$, results given as mean ± SD. [d] For the whole fillets, $n = 5$, $a = 1$; for the slices, $n = 3$, $a = 1$.

Table 3. Correlation As Described by Pearsons Correlation Coefficient (*r*) between the Different Measures Used To Follow Quality Changes during Ice Storage of Herring Fillets[a]

measure	PV	log A_{268}	log FP	TO	R	S/A	O	F	S	α-T	G	Asc	Po	log TB
PV		0.92[b]	0.93[b]	0.91[c]	0.97[c]	0.96[c]	0.94[c]	−0.97[c]	−0.96[c]	−0.85[b]	−0.96[c]	−0.93[c]	−0.97[c]	0.94[c]
log A_{268}			0.94[b]	0.95[c]	0.95[c]	0.96[c]	0.95[c]	−0.93[c]	−0.97[c]	−0.88[b]	−0.96[c]	−0.94[c]	−0.95[c]	0.86[c]
log FP				0.94[c]	0.97[c]	0.96[c]	0.97[c]	−0.96[c]	−0.97[c]	−0.90[b]	−0.95[c]	−0.95[c]	−0.97[c]	0.88[c]
TO					0.93[c]	0.97[c]	0.96[c]	−0.89[c]	−0.95[c]	−0.90[c]	−0.88[c]	−0.94[c]	−0.89[c]	0.77[c]
R						0.99[c]	0.97[c]	−0.98[c]	−0.99[c]	−0.92[c]	−0.96[c]	−0.97[c]	−0.95[c]	0.91[c]
S/A							0.98[c]	−0.96[c]	−0.99[c]	−0.94[c]	−0.95[c]	−0.98[c]	−0.95[c]	0.88[c]
O								−0.96[c]	−0.99[c]	−0.96[c]	−0.94[c]	−0.99[c]	−0.96[c]	0.85[c]
F									0.98[c]	0.91[c]	0.96[c]	0.96[c]	0.98[c]	−0.93[c]
S										0.96[c]	0.97[c]	0.99[c]	0.97[cc]	−0.88[c]
α-T											0.93[c]	0.98[c]	0.94[c]	−0.80[c]
G												0.96[cc]	0.97[c]	−0.93[c]
Asc													0.96[c]	−0.85[c]
Po														−0.93[c]
log TB														

[a] Abbreviations as explained in Table 1. [b] $n = 30$. [c] $n = 10$.

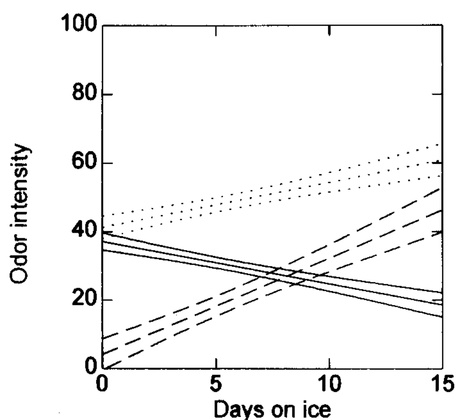

Figure 3. Linear regression lines illustrating the change in (···) total odor, (– –) rancid odor, and (—) fresh fish odor. Models describing these lines are shown in Table 1. The confidence bands are based on $p = 0.05$.

obtained for the inner and middle parts, whereas 72 and 71%, respectively, of the GSH-px variation in whole fillets and under skin layers could be described. In the whole fillets, a significant decrease was seen after 3 days, and at the end of the storage, the activity was lowered by 35%. As further shown in Figure 4B, the under skin layer had about 1.3 times the initial activity of the middle part and 1.5 times that of the inner part. Similar data were previously found in carp, where dark muscle had ~3 times the activity of light muscle (Nakano et al., 1992). During storage, the inner and middle parts retained their GSH-px activity, whereas the under skin layer lost ~35%. A faster decrease than the present one was observed in Japanese jack mackerel and skipjack tuna stored at 4 °C (Watanabe et al., 1996). Here, 83−92% of the initial activity was lost after 5 days, something that was ascribed to enzyme hydrolysis by intracellular proteinases or to enzyme denaturation. In contrast, Nakano et al. (1992) observed a significant increase (~1.5 times) in GSH-px activity in salmon fillets during 8 days of storage at 4 °C. Membrane disruption and/or alteration in enzyme configuration were given as explanations.

In Figure 4C, it can be seen that the content of ascorbic acid in whole herring fillets decreased by ~80% throughout the storage period. This decrease, which was significant after 3 days, was of a linear character; the model fitted could explain 89% of the variation in the data (Table 1).The lack of significant local differences within the fillet points to a lower ability of ascorbic acid to differentiate between samples in terms of their stability to lipid oxidation in comparison with GSH-px activity and α-tocopherol. Similarly, Erickson (1993) found ascorbic acid to be a less sensitive measure than α-tocopherol for differentiation between the oxidative stability of striped bass and hybrid striped bass. These findings may be related to the multifunctional role of ascorbic acid in the tissue (St. Angelo, 1996) and thus to its rather unspecific role as an antioxidant (Srinivasan and Hultin, 1995). The latter is most strongly coupled to the ability of ascorbic acid to regenerate α-tocopherol (Lambelet et al., 1985), a reaction that is efficient only when the ascorbic acid to α-tocopherol ratio is over 10:1−15:1 (Kunert and Ederer, 1985). In the present herring fillets, this ratio was initially only 1.3:1, which probaly explains why α-tocopherol was not retained for more than 13 days. In contrast to our data, Petillo and Hultin (1995) found a faster loss of ascorbic acid in dark than in light mackerel muscle during storage on ice. In the former, 80% was lost in 4 days, whereas in the latter, the corresponding figure was 60%. An extremely quick consumption of ascorbate during ice storage was detected by Decker and Hultin (1990). These authors found 50% of the initial ascorbate in previously frozen thawed mackerel fillets to be lost after 1 day, whereas after 7 days no ascorbate could be detected.

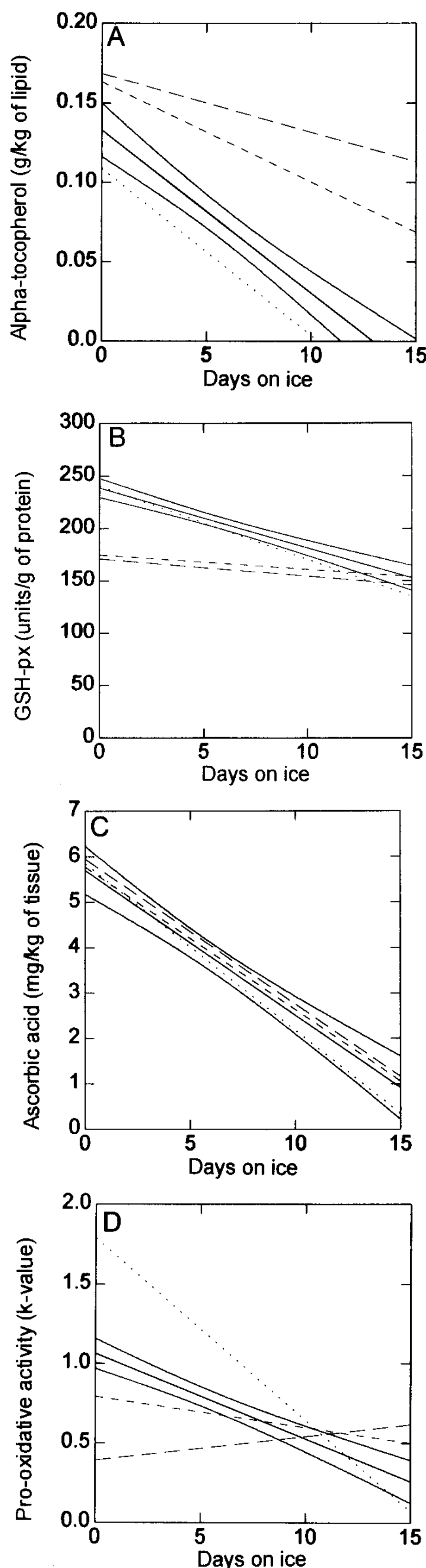

Figure 4. Linear regression lines illustrating the change in (A) α-tocopherol, (B) glutathione peroxidase (GSH-px), (C) ascorbic acid, and (D) aqueous pro-oxidative activity in herring fillets stored for 0−15 days in ice. (—) whole fillets, (···) under skin layer, (- - -) middle part, and (— —) = inner part. Models describing these lines are shown in Table 1. The confidence bands shown for the whole fillet regression lines are based on $p = 0.05$.

Taking into consideration all the data presented in Figure 4A−C, it can be seen that the relative order of antioxidant loss in whole herring fillets was the following: α-tocopherol > ascorbic acid > GSH-px activity. This order contradicts the so-called "pecking order" (Buettner, 1993) which, based on the antioxidant's reducing capacity, predicts that ascorbic acid would decompose before α-tocopherol. This order has previously been confirmed in channel catfish (Brannan and Erickson, 1996) and in mackerel (Petillo and Hultin, 1995). However, in the present study, the order of antioxidant loss was largely dependent on which layer of the fillet was considered. In the inner part, the antioxidants decreased according to ascorbic acid > α-tocopherol ≈ GSH-px activity; in the middle part, it was ascorbic acid > α-tocopherol > GSH px activity; and in the under skin, it was α-tocopherol > ascorbic acid > GSH-px activity. These data, in combination with those presented in Figure 3A−C, point to large differences in the microenvironment throughout the fillet. A few such differences have previously been reported by us (Undeland et al., 1998a,d), such as a decrease in fat content, triglycerides, eicosapentaenoic acid (EPA), iron, and copper when moving from the under skin layer toward the inner part. This distribution of metals is probably an important explanation of the instability of the under skin layer.

As concerns the relation between lipid oxidation and antioxidant consumption, the depletion of α-tocopherol at day 13 did not result in any dramatic rise in oxidation products, indicating that the presently found levels of α-tocopherol, 0−0.18 g/kg of tissue, were not within the range where α-tocopherol efficiently prevents lipid oxidation in ice-stored herring. This was further stressed by the fact that the correlation between oxidation products and α-tocopherol was poorer ($r = -0.85$ to -0.90) than the correlation between oxidation products and the aqueous antioxidants ($r = -0.93$ to -0.96) (Table 3). Among the latter, GSH-px activity gave the highest correlation coefficients, $r = -0.95$ to -0.96, which was in accordance with the findings of Watanabe et al. (1996). Although the r values provided for by ascorbate was only slightly lower, -0.93 to -0.95, and although a very strong correlation was found between ascorbate and the development of rancidity in the whole fillets ($r = -0.97$), the role of this compound in lipid oxidation was doubtful. As shown in Figure 3C, ascorbate was the only measure of lipid oxidation that could not differentiate between the under skin, the middle part, and the inner part of the fillets, indicating that other processes than lipid oxidation were the primary ascorbic acid consumers during storage. These findings were also supported by those of Erickson (1993).

Pro-oxidants. The first significant change in total aqueous pro-oxidative activity was seen after 3 days, and at day 15, the whole fillets had lost 75% of their initial activity. Figure 4D shows that the pro-oxidative activity initially was very unevenly distributed throughout the fillets. The under skin layer had more than 4 times the activity of the inner part and twice that of the middle part. During storage, the under skin layer continuously decreased down to 3% of its initial activity whereas the other parts did not decrease significantly. This probably explains the low R^2 values obtained for the middle and inner part models (Table 1). The present data stress two features characterizing lipid oxidation in herring fillets. First, as oxidation products continued

to develop under the skin, despite a severe decrease in pro-oxidative activiy, the latter seemed to be most important during the intitial phase of lipid oxidation. This finding is in agreement with the autocatalytic nature of the lipid oxidation process (Belitz and Grosch, 1987). Second, the data point to limited oxygen availability in the interior of the fillets during storage (Flink and Goodhart, 1978; Undeland et al., 1998a,c,d). Although the initial pro-oxidative activity ranked the three layers as follows, under skin > middle part > inner part, a limited diffusion of oxygen trough the tissue appeared to supress the significance of pro-oxidants for hydroperoxide formation in the middle layer. This is shown by the order of PV development in Figure 3A: under skin > inner part > middle part. There is no straightforward explanation of the fact that the under skin layer lost so much of its pro-oxidative activity. Masking from antioxidants formed during the continuous microbial growth could be one reason (Erickson, 1992); storage-induced protein denaturation could be another. The contribution from enzymes to the pro-oxidative activity was investigated from heating of the buffer extracts to 55 and 100 °C. This test revealed that enzymes contributed to a great deal of the aqueous pro-oxidative activity measured also at late stages of storage. In extracts from whole fillets, heating to 55 °C for 12 min decreased the activity by 67% on average, and 100 °C for 12 min inhibited the activity totally. There was a tendency toward heating exerting a relatively greater effect at the end of the storage period, something that contradicts several earlier findings. According to German et al. (1992), 2-lipoxygenase in carp and trout had a half-life of <3 h at 0 °C, and as reported by Decker and Hultin (1990), elevated concentrations of nonenzymatic pro-oxidants (low molecular weight iron and copper) were found in mackerel during storage on ice. It should not be excluded, however, that the high thermal instability observed for the pro-oxidants late during storage is a misleading result associated with the use of a linoleic acid emulsion to simulate the situation in fish muscle. According to Kühn et al. (1981), hemoglobin at low concentrations (0.01−1 μM) have a quasi-lipoxygenase activity that is specific only to linoleic acid, inhibited by heating, and inactive toward biological membranes. Concerning the effect of heating on extracts from the various parts of the fillets, 55 °C produced almost no differences, whereas 100 °C initially had the strongest effect on the inner part extracts followed by the middle part and then the under skin extracts. These results may indicate a relatively higher level of thermostable enzymes in the inner part than in the under skin layer. However, it may also be an illustration of the complex way in which hemoprotein-rich tissues, such as the one right under the skin, respond to heating (Undeland et al., 1998d).

Microbiology. The lag phase of total measured microorganismes lasted for ~6 days, after which an exponential increase started. The limit of acceptability, 10^6 cfu/g of tissue, as recommended by the International Commission on Microbiological Specifications for Foods (ICMSF) (1974) was reached between day 9 and day 12. This was somewhat later than in the study of Bennour et al. (1991), where iced whole mackerel reached a bacterial load of 10^6 between 6 and 9 days. The logarithmic model fitted to the data could explain 83% of the variance and showed that the increase was significant after 7 days. According to the early detection of lipid oxidation (Figures 2 and 4), these data indicate that microbial changes were slower than chemical changes under the present storage conditions. This contradicts the findings of Aubourg et al. (1995) and Harris and Tall (1989), but as was discussed in the Sensory section might be due to a suppression of the bacterial growth caused by the freezing of the samples prior to analysis.

It has been described that microbial growth can both inhibit (Erickson, 1992) and enhance (Vercellotti et al., 1992) lipid oxidation. In this study, there were no dramatic changes in the development of oxidation products at the time when the bacterial lag phase ended. In accordance with the findings of Lee and Toledo (1977), this indicates that the influence from bacteria and/or their products on the oxidation process was limited. The completely different kinetic patterns of lipid oxidation product formation and bacterial growth were also reflected in relatively low correlations between these processes, $r = 0.82-0.91$. These figures were however raised to 0.86−0.94 (Table 3) after a $\log_{10}$ transformation of the microbial data.

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ABBREVIATIONS USED

a, number of analyses of each sample preparation; A_{268}, absorption at 268 nm; cfu, colony forming units; em, emission; EPA, eicosapentaenoic acid; ex, exitation; ELISA, enzyme-linked immunosorbent assay; FIA, flow injection analysis; FP, lipid-soluble fluorescent lipid oxidation products; GSH-px, glutathione peroxidase; HPLC, high-performance liquid chromatography; ICMSF, International Commission on Microbiological Specifications for Foods; *k*, slope of a curve; *m*, curve intercept; *n*, number of sample preparations; PV, peroxide value; *r*, Pearson's correlation coefficient; R^2, fraction of the response variation explained by the model; % RSD, relative standard deviation; RSW, refrigerated seawater; SDS, sodium dodecyl sulfate; *t*, storage time (days); TBARS, thiobarbituric reactive substances test; YP, yellow pigments.

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