Lipid Oxidation in Fillets of Herring (*Clupea harengus*) during Frozen Storage. Influence of Prefreezing Storage

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Fillets of herring (*Clupea harengus*) were kept on ice for 0, 3, 6, and 9 days prior to storage at −18 °C for 0, 21, 42, 63, and 84 days. At each storage point, peroxide value (PV), absorbance at 268 nm (A_{268}), fluorescent products (FP), α-tocopherol, glutathione peroxidase (GSH-px) activity, and ascorbic acid were measured. As shown by regression analyses, samples held for 6 days on ice formed oxidation products at the highest rate during frozen storage, followed by, for PV and FP, the 9-day samples. These data indicate that severe changes that negatively affect the oxidation process took place in the herring muscle between 3 and 6 days after catch. Both the initial antioxidant levels and the rate of antioxidant loss at −18 °C decreased with increased prefreezing holding time, the latter being most obvious for GSH-px activity and ascorbic acid. α-Tocopherol showed the largest losses and had disappeared entirely from the 6- and 9-day samples at the end of the frozen storage. Partial least-squares regression analysis of the data showed that ice storage had a greater effect than frozen storage on changes in PV, A_{268}, FP, α-tocopherol, and ascorbic acid. For GSH-px activity, frozen storage had the greatest effect.

INTRODUCTION

An increased use of herring (*Clupea harengus*) for food production is desirable for many reasons. However, processing and storage of this species is difficult due to the susceptibility of the herring lipids to oxidation. This reaction, which quickly gives rise to rancidity (Pokorny, 1987), is believed to be particularly important during frozen storage. This is due to the inhibition of microbial growth (Harris and Tall, 1989), the mechanical destruction of membranes (Nilsson, 1994), and the concentration of pro-oxidative solutes (Apgar and Hultin, 1982), which take place below 0 °C. As there is increasing interest nowadays in using frozen herring for further processing, for example, for salting, it is important to understand how different ways of handling herring affect lipid oxidation during frozen storage. Factors that have previously been studied are the conditions and length of prefreezing storage (Kolakowska, 1981; Bilinski et al., 1981), the degree of tissue disruption (Smith et al., 1980; Kolakowska, 1981), oxygen accessibility (Santos and Regenstein, 1990; Undeland et al., 1998a), freezing methods (Vyncke, 1978; Jhaveri and Constantinides, 1981), and storage temperature (Ke et al., 1977). The conclusion has been that prefreezing storage in refrigerated seawater (RSW) followed by minimal tissue disruption, removal of oxygen, fast freezing, and low freezer storage temperatures reduces rancidity and gives the longest shelf life. However, the effect of the duration of prefreezing storage on oxidation is still not fully understood. Most studies have pointed to a faster increase in oxidation products when prefreezing storage is extended (Kolakowska, 1981; Bilinski et al., 1978; Hiltz et al., 1976; Hardy and Smith, 1976; Ke et al., 1976), although some interesting findings have indi-

cated that processing and storage of very fresh fish increase the susceptibility toward oxidation during subsequent frozen storage (Kolakowska, 1981; Deng, 1978). Antioxidative effects of compounds formed during proteolysis, lipid–protein interactions, and lipid hydrolysis have been cited as possible explanations. However, it should also be noted that contamination with blood during butchering of the fish decreases with time post-mortem, and blood can be a strong pro-oxidant (Richards et al., 1998).

Consequently, for fish that are to be skinned, filleted, or minced prior to freezing, there seem to be two critical prefreezing periods: the one prior to butchering and the one prior to actual freezing. Our aim was to investigate how the length of the latter influences the change in different oxidation products and antioxidants in skin-on herring fillets during subsequent storage at −18 °C.

MATERIALS AND METHODS

Equipment. The fillets were packed in polyethylene film from Plus Pack (article no. 571.303, Båstad, Sweden). Freezing was carried out in a tunnel freezer from Wollgrens kyl AB (Göteborg, Sweden). Homogenization of the fillets was done in a household food processor from Philips (type HR 2373, Klagenfurt, Austria). Protein was measured with a Perkin-Elmer nitrogen analyzer (PE 2410 series II, Norwalk, CT). Extraction of lipids was performed in a Sorvall Omnimixer (Ivan Sorvall Inc., Norwalk, CT) and cold centrifugation in a Sorvall Superspeed RC2-B (Ivan Sorvall Inc.). Selenium-based glutathione peroxidase activity (GSH-px) was analyzed in an enzyme-linked immunosorbent assay (ELISA) reader from IEMS (Labsystems DY, Helsinki, Finland). The statistical software Systat (version 6.0, SPSS Inc., Chicago, IL) was used for linear regression analyses, Excel (IMS Akademidata AB, Göteborg, Sweden) for the difference tests, and Modde (Umetri AB, Umeà, Sweden) for the partial least-squares regression analysis (PLS).

Reagents. β-Nicotinamide adenine dinucleotide phosphate (NADPH) in reduced form, which was used in the analyses of

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Se-based GSH-px activity, was from Sigma (Heidenreich AS, Oslo, Norway). For the ascorbic acid analyses metaphosphoric acid and ethylenediaminetetraacetic acid (EDTA) were obtained from Merck (Phil AS, Ågotnes, Norway), and DL-dithiothreitol was from Sigma (Heidenreich AS).

Samples. Herring (*Clupea harengus*) caught off the west coast of Sweden in October 1997 was stored in RSW tanks (0 to −1 °C) for 24 h before it was headed, gutted, and deboned using commercial equipment. The double fillets obtained, which had a length of 10.2 ± 1.3 cm [mean ± standard deviation (SD), $n = 10$], a weight of 30.8 ± 7.4 g ($n = 10$), and a fat content of 85.6 ± 0.2 g/kg of tissue ($n = 3$, $a = 1$) were packed in waxed cardboard boxes (5 kg/box) and stored, surrounded by plastic bags filled with ice, in a refrigerated room (2 °C) for up to 9 days. The mean temperature of the fish during this period was −0.4 °C. After 0, 3, 6, and 9 days, fillets were removed from the boxes, packed individually in polyethylene film, and frozen at −40 °C in a tunnel freezer set at 36 m/s. After freezing, they were moved to an −18 °C chamber, where they were stored for 0, 21, 42, 63, and 84 days. Following storage, they were stored at −70 °C until analysis. On the day of analysis, the polyethylene film was removed from seven fillets from each treatment, and these were placed in a polyethylene bag and thawed for 11 min in cold running tap water. After thawing, the skin was manually removed, and the seven fillets were homogenized together for 1 min in a food processor. From this pooled herring mince were then removed n samples for the different analyses, which were repeated a times.

Analysis of Protein. Prior to storage, protein was measured as N × 6.25 according to an AOAC (1990) method using a nitrogen analyzer ($n = 2$, $a = 2$). Results were expressed as grams of protein per kilogram of tissue.

Analysis of Total Lipid Content. Prior to storage, total lipids were extracted with petroleum ether in a Soxhlet extractor as described by Pearson (1973) ($n = 3$, $a = 1$). Results were expressed as grams of lipid per 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).

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 the relative standard deviation (RSD%), was 1.6 ($n = 1$, $a = 6$), and the results were expressed as milliequivalents of peroxide per kilogram of lipid.

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

Total Lipid Soluble Fluorescent Lipid Oxidation Products (FP). FP with an excitation (ex) maximum at 367 nm and an emission (em) maximum at 420 nm were measured in the extracted lipids using FIA as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). Results were expressed as peak area units per microgram of lipid. The RSD% was 4.7 ($n = 1$, $a = 6$).

Analysis of Antioxidants. α-*Tocopherol.* Following extraction with water, SDS, ethanol, and heptane (Undeland et al., 1998b), α-tocopherol was determined by normal-phase high-performance liquid chromatography (HPLC), as described by Undeland et al. (1998a) ($n = 3$, $a = 2$). The RSD% was 3.0 ($n = 1$, $a = 6$), and α-tocopherol was expressed as grams per kilogram of lipid.

Se-Based GSH-px Activity. The Se-based GSH-px activity was monitored 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 micromoles of NADPH oxidized per minute per gram of protein.

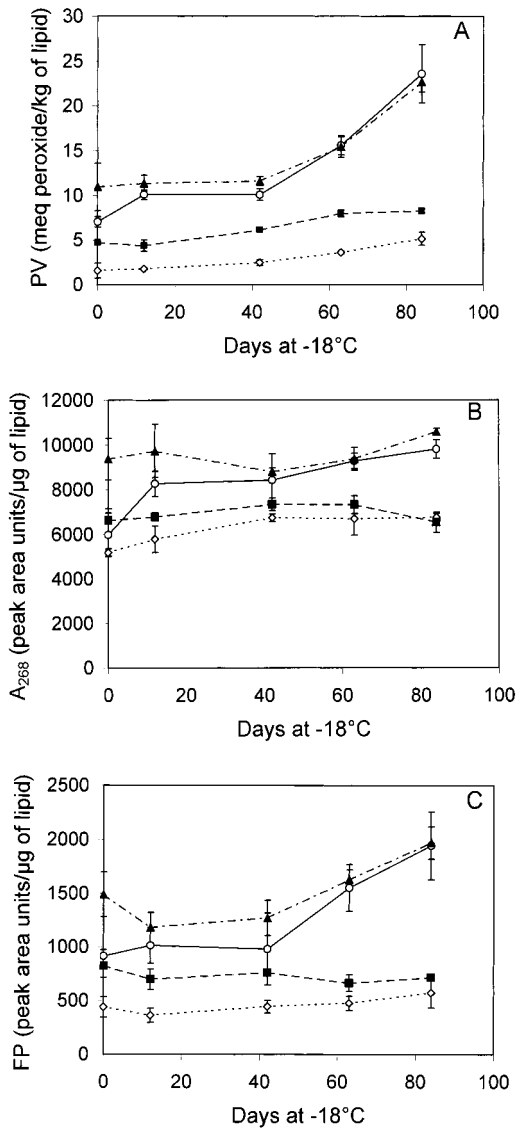*Ascorbic Acid.* Ascorbic acid was extracted from the fish with 5% metaphosphoric acid containing 0.1% dithiothreitol and 0.54 mM EDTA. Analysis was carried out with reversed phase HPLC coupled to an electrochemical detector set at 0.6 V according to the method of Meeland and Waagbø. (1998) ($n = 2$, $a = 1$). Results were expressed as milligrams of ascorbic acid per kilogram of tissue.

Statistical Evaluation. Data from all measurements were subjected to regression analyses to fit a model describing their most likely changes with frozen storage time. Both linear ($y = kt + m$) and logarithmic (log $y = kt + m$) models were tested; however, because a logarithmic treatment of the data increased the explainability of the model in only 4 of the 24 cases, for PV in the 6- and 9-day fillets as well as for ascorbic acid in the 3- and 6-day fillets, only linear models are described for A_{268}, FP, α-tocopherol, and GSH-px. To estimate whether the models, that is, the slopes and intercepts, were significantly different, a multiple comparison significance test based on the creation of confidence intervals around the means (Box et al., 1978) was carried out. To obtain a significance level of $p = 0.05$, despite the multiple comparisons done (six for each measure), the Bonferroni correction (Bland, 1995) was used. This correction was made by using a significance level of 0.01 in the calculations, which is based on the fact that 0.99^6 gives a confidence level of ∼95%. The same test was used to evaluate whether the 0-, 3-, 6-, and 9-day fillets were significantly different at time 0. To estimate when the levels of oxidation products and antioxidants were first significantly different from the 0-day data, confidence intervals were constructed around the regression lines (Harper, 1971a). A significant difference ($p = 0.05$) was obtained when these intervals did not overlap. Pearson's correlation coefficient (r) (Harper, 1971b) was calculated to study the correlation between various measures. The significance of these coefficients was calculated according to the method of Davies and Goldsmith (1976) using a significance level of $p = 0.05$. To evaluate whether ice (Ic) or frozen storage (Fr) had the greater effect on the oxidative changes, all of the data were subjected to a PLS analysis which, for each measure, gave rise to the following model: $Y = \beta_0 + \beta_1 Ic + \beta_2 Fr + \beta_{12} Ic \times Fr$. In this equation, β_0 is the intercept and the sizes of β_1, β_2, and β_{12} are equal to the effect of ice storage, frozen storage, and the interaction between these variables, respectively. The level of significance in this test was set at $p = 0.05$.

RESULTS AND DISCUSSION

Figures 1 and 2 illustrate how herring fillets that had been stored for 0, 3, 6, and 9 days on ice changed with regard to oxidation products and antioxidants, respectively, during storage for up to 84 days at −18 °C. In Table 1, the regression models are presented. In this table, R^2 indicates how much of the variance in the data could be explained by the model.
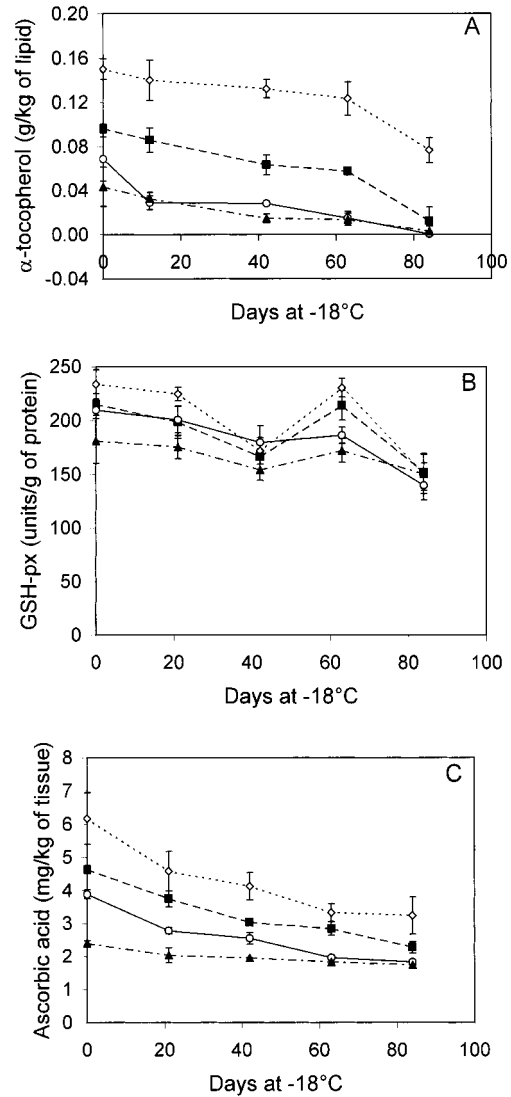
Development of Oxidation Products. Figure 1A illustrates how the PV changed in the four groups of fillets during frozen storage. At freezing, mean PV was highest for 9 days > 6 days > 3 days > 0 days; however, due to the limited number of samples analyzed ($n = 3$), no significant differences were observed. The latter was true also for the 0-day A_{268} and FP data. For the 6- and 9-day fillets, slightly better regression models were obtained after a logarithmic treatment of the data. However, on the basis of the generally high R^2 values (0.72−0.91), both the linear and logarithmic models were considered as reliable for all of the samples (Lindgren, 1995). The rate of PV increase was significantly higher in the 6- and 9-day-fillets than in fillets stored for 0 and 3 days on ice. The highest mean rate, 0.16 mequiv of peroxide/kg of lipid/day at −18 °C, was seen for the 6-day fillets, which also were the samples that first had changed significantly from time 0 (after 28 days). It is therefore reasonable to assume that critical changes increasing the susceptibility toward

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Figure 1. Progress of (A) the peroxide value (PV), (B) absorbance at 268 nm (A_{268}), and (C) lipid soluble fluorescent oxidation products (FP) during storage at $-18\,°C$ of herring fillets held on ice for 0 (◇), 3 (■), 6 (○), and 9 days (▲) prior to freezing. For each measure, $n = 3$, $a = 2$. Error bars indicate the standard deviation at each storage point.

Figure 2. Changes in (A) α-tocopherol, (B) GSH-px activity, and (C) ascorbic acid during storage at $-18\,°C$ of herring fillets held on ice for 0 (◇), 3 (■), 6 (○), and 9 days (▲) prior to freezing. For α-tocopherol, $n = 3$, $a = 2$, for GSH-px, $n = 1$, $a = 8$, and for ascorbic acid, $n = 2$, $a = 2$. Error bars indicate the standard deviation at each storage point.

lipid oxidation took place in the fillets between 3 and 6 days on ice.

Figure 1B shows how the absorbance at 268 nm changed in the herring lipids during storage at $-18\,°C$. This measure represents an increase in, for example, conjugated trienes, diketones, and oxodienes (IUPAC, 1979; Brown and Snyder, 1982). On the basis of mean A_{268} values, the four samples ranked at freezing 9 days > 3 days > 6 days > 0 days. According to Table 1, reliable regression models were only obtained for the 0- and 6-day fillets, which were the only ones in which significant changes had taken place. Although the rates of change in these two samples were not significantly different, the 6-day fillets had the highest mean rate and changed significantly more quickly than the 3- and 9-day fillets. For the two latter sample groups, the models turned out to be very poor, with only 12 and 28%, respectively, of the data variance explained. The problem was due to the small storage-induced increase in relation to the rather high sample variation. As both the native and oxidized lipids absorb light at 268 nm,

variations in lipid composition among samples may further enhance the sample-to-sample variation.

Figure 1C shows how lipid soluble FP changed. These *tertiary* lipid oxidation products form as a result of interactions between carbonyls and phospholipids (Dillard and Tappel, 1973) and have previously been shown to correlate strongly to changes, for example, in TBARS (Aubourg et al., 1997) and rancid odor (Undeland et al., 1999). Initially, the mean levels of FP ranked similar to PV. However, throughout storage, the 0- and 3-day samples, as well as the 6- and 9-day samples, approached each other and ended up with similar values. Due to the relatively small storage-induced changes and the fairly high analytical variation, the regression model were not reliable. As shown in Table 1, only the 6-day model gave an acceptable R^2 value, 0.67. The FP of fillets stored for 6 days increased at the highest rate throughout the storage, followed by the fillets stored for 9, 0, and 3 days. However, the 6- and 9-day fillets did not differ significantly.

The data presented in Figures 1 support, to some extent, the conclusions from previous studies, which

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Table 1. Regression Models[a] for the Oxidation Products and Antioxidants Measured

measure	0 days on ice model	R²	3 days on ice model	R²	6 days on ice model	R²	9 days on ice model	R²
PV	$PV = 0.043^a t + 1.1^a$	0.83	$PV = 0.051^a t + 4.2^b$	0.86	$PV = 0.16^b t + 6.0^{bc}$	0.84	$PV = 0.13^b t + 8.9^c$	0.72
	$\log PV = 0.0067^{ab} t + 0.13^a$	0.83	$\log PV = 0.0036^a t + 0.63^b$	0.83	$\log PV = 0.0059^{ab} t + 0.84^c$	0.91	$\log PV = 0.0037^{bc} + 0.98^d$	0.76
A_{268}	$A_{268} = 19^{abc} t + 5409^a$	0.63	$A_{268} = 1.7^d t + 6853^b$	0.012	$A_{268} = 41^{ab} t + 6623^{ab}$	0.71	$A_{268} = 13^{cd} t + 8904^c$	0.28
FP	$FP = 1.7^{ab} t + 397^a$	0.27	$FP = -1.1^a t + 782^{bd}$	0.18	$FP = 12^{cd} t + 763^{ade}$	0.67	$FP = 6.6^{bde} t + 1228^{ce}$	0.42
α-T[b]	$\alpha\text{-}T = -0.00080^a t + 0.16^a$	0.70	$\alpha\text{-}T = -0.00079^a t + 0.1^b$	0.90	$\alpha\text{-}T = -0.00061^a t + 0.05^{cd}$	0.79	$\alpha\text{-}T = -0.00056^a t + 0.05^{de}$	0.84
G[c]	$G = -0.76^{ab} t + 235^a$	0.40	$G = -0.50^{ab} t + 211^a$	0.25	$G = -0.74^a t + 215^a$	0.67	$G = -0.30^b t + 180^b$	0.25
Asc[d]	$Asc = -0.034^a t + 5.5^a$	0.90	$Asc = -0.027^a t + 4.4^b$	0.93	$Asc = -0.023^a t + 3.6^c$	0.89	$Asc = -0.008^b t + 2.4^d$	0.95
	$\log Asc = -0.0034^a t + 0.77^a$	0.83	$\log Asc = -0.0035^a t + 0.65^b$	0.95	$\log Asc = -0.0038^a t + 0.56^c$	0.94	$\log Asc = -0.0015^b t + 0.36^d$	0.82

[a] Slopes and intercepts, respectively, in the same row bearing the same following letter are not significantly different ($p \leq 0.05$). t in the equation is equal to storage time in days. [b] α-T, α-tocopherol. [c] G, GSH-px. [d] Asc, ascorbic acid.

indicate that prolonged prefreezing holding increases the rate of development of oxidation products during frozen storage (Kolakowska, 1981; Bilinski et al., 1978; Hiltz et al., 1976; Hardy and Smith, 1976; Ke et al., 1976). However, for PV, which gave the most reliable models, this conclusion was valid only up to 6 days of holding on ice, after which time a slight reduction in the PV rate was observed. Similarly, Kolakowska (1981) observed that mince produced from 2-day Baltic herring had a faster increase in oxidation products during subsequent frozen storage than herring stored for a period longer or shorter than 2 days. The fact that these authors found a maximum rate 4 days earlier than we did is most likely related to the mincing process, which is known to accelerate lipid oxidation (Harris and Tall, 1989). There are also observations that show that the length of prefreezing storage is not critical to the stability during subsequent frozen storage. Deng (1978) found no difference in the rate of TBARS production at −18 °C between mullet skin-on fillets prepared from fish held in the round for 1 or 7 days on ice. However, as no storage times between 1 and 7 days were analyzed, a maximum or minimum may have been passed. Also, an inverse relationship between the length of prefreezing storage and lipid oxidation has been reported. Kolakowska (1981) found that increased prefreezing holding of Baltic herring mince decreased the PV development after 2 months of frozen storage. This author also reported that the frozen storage stability of minced and round Baltic herring was lower in samples frozen in a superfresh state than in samples frozen after 4 and 2 days on ice, respectively. Antioxidative effects of proteolysis and lipid−protein interactions were given as the explanations in that study. Similarly, Deng (1978) found that the TBARS development rate at −18 °C in skin-off mullet fillets was lower in fillets prepared from fish held in the round for 7 days on ice than in fillets prepared from 1-day fish. Although the author discussed elevated lipid hydrolysis as an explanation, it should not be excluded that the fillets prepared from the 1-day fish became more contaminated with blood during filleting than the fillets prepared from the 7-day fish (Richards et al., 1998).

To explain our findings, the oxidation stage of the 6-day samples at the moment of freezing has to be considered. Most likely, the oxidation process in the 0- and 3-day samples was still in the so-called "induction phase" (Erickson, 1993; Brannan and Erickson, 1996), whereas between 3 and 6 days, it approached the "propagation phase", leading to a rapid buildup of hydroperoxides during subsequent frozen storage. When the ice-holding period was further extended up to 9 days, it could be postulated that the rates of hydroperoxide cleavage and hydroperoxide reactions exceeded the hydroperoxide formation rate. However, the latter would imply that A_{268} and FP would form more rapidly in the 9-day than in the 6-day samples, which was not the case. For this reason, it cannot be excluded that compounds arising from protein changes or lipid hydrolysis had reached levels at this stage at which they might possess an inhibiting effect. The rate of degradation processes of this kind—protein alterations in particular—probably have a large impact on lipid oxidation dynamics, both positively and negatively. It has been suggested that the production of protein radicals is crucial to the initiation of lipid oxidation (Srinivasan and Hultin, 1995) and that proteolysis brings lipids and

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Table 2. PLS Models[a] Describing the Influence of Days on Ice (Ic) and Days of Frozen Storage at −18 °C (Fr) as well as Days on Ice and at −18 °C in Combination (Ic × Fr) on Changes in the Oxidation Products and Antioxidants Measured

measure	PLS model	R^2
PV	$PV = 9.2 + 5.0*^{a}Ic + 2.5*^{b}Fr + 0.15Ic \times Fr$	0.80
A_{268}	$A_{268} = 7770 + 1236*^{a}Ic + 536*^{b}Fr + 77Ic \times Fr$	0.70
FP	$FP = 964 + 373*^{a}Ic + 116*^{b}Fr + 46Ic \times Fr$	0.66
α-T[b]	$\alpha\text{-}T = 0.058 - 0.039*^{a}Ic - 0.022*^{b}Fr + 0.00038Ic \times Fr$	0.82
G[c]	$G = 175 - 8.78*^{a}Ic - 22.2*^{b}Fr + 8.64*^{c}Ic \times Fr$	0.81
Asc[d]	$Asc = 3.0 - 0.82*^{a}Ic - 0.75*^{a}Fr + 0.15*^{b}Ic \times Fr$	0.90

[a] Coefficients bearing an asterisk are significant on the level $p = 0.05$. Significant coefficients bearing a different following letter are significantly different ($p = 0.05$). [b] α-T, α-tocopherol. [c] G, GSH-px. [d] Asc, ascorbic acid.

catalysts, which are normally separated, in close contact with each other (Harris and Tall, 1989). Thus, by varying the length of prefreezing holding, the conditions for oxidation to take place during subsequent frozen storage may change dramatically. However, in the present study, which was designed to study the extent of lipid oxidation within the frozen storage period recommended for fillets of fatty fish by Codex Alimentarius (1978), the impact of the changed biochemical conditions was illustrated to only a limited extent. Beyond 3 months at −18 °C, the four groups would probably be more clearly differentiated.

To investigate whether ice or frozen storage within the studied experimental domain had the greatest effect on lipid oxidation, all of the data were evaluated using PLS analysis. In this evaluation, the whole experiment was considered as a full factorial design (Undeland et al., 1998c) with two variables: ice and frozen storage, set at four and five levels, respectively. Table 2 shows the models and, thus, the β-coefficients, or effects, of each storage type for the development of PV, A_{268}, and FP. R^2 for the models describing these effects ranged between 0.66 and 0.80 and were, therefore, considered as reliable. Both ice storage and frozen storage significantly promoted all of the oxidation products measured. However, the larger effect arose from storage on ice, which stresses the importance of limiting the holding on ice of herring prior to frozen storage. None of the oxidation products were significantly affected by the interaction between ice and frozen storage.

The development of rancid odor further supports a minimization of the ice storage period. In our previous study (Undeland et al., 1999), we reported that rancidity in herring fillets could be detected sensorially after 2.5 days on ice. At this point, the PV was only ∼4. Thus, not only from a chemical point of view, but also on the basis of sensory data, fillets of herring should preferably be frozen sooner than 3 days after catching. According to the linear PV model in Table 1, fillets frozen immediately could be stored for up to 64 days before a PV of 4 was reached. As reported by Hardy and Smith (1976), 0−2 days of prefreezing storage of whole and gutted mackerel led to a discernible rancid taste after 22 weeks at −14 °C, after 35 weeks at −21 °C, and after 44 weeks at −29 °C. After prefreezing storage for 3−4 days, the corresponding figures were 22, 24, and 44 weeks, respectively. In that study, the development of rancid flavor during frozen storage obviously had a weak relationship with the time on ice.

Consumption of Antioxidants. As can be seen in Figure 2A, large losses of α-tocopherol had already

occurred prior to freezing in the 3-, 6-, and 9-day samples. On the basis of the mean values, 36% of the initial 0.15 g of α-tocopherol/kg of lipid had been lost after 3 days at −18 °C, 54% after 6 days, and 71% after 9 days. However, as previously mentioned, the limited number of replicates ($n = 3$) made significant differences between the sample groups difficult to detect. The linear regression models describing further losses during frozen storage are described in Table 1. These models could explain 70−90% of the variation in the data and were, thus, all reliable. Although the main conclusion from the present models was that α-tocopherol was consumed at similar rates in the four sample groups, there was a slight tendency toward lower rates with increased holding on ice. However, according to the equations described in Table 1, the 6- and 9-day samples ran out of α-tocopherol first, something that occurred after 82 and 89 days, respectively. At this point, the 0- and 3-day samples still had 0.094 and 0.035 g of α-tocopherol/kg of lipid left, respectively, and, as described by the models, did not reach a 0 level until after ∼200 and ∼130 days, respectively.

Figure 2B shows the loss of GSH-px activity during storage at −18 °C. On the basis of mean GSH-px activity values, the initial rank order was similar to that of α-tocopherol, that is, 0 days > 3 days > 6 days > 9 days. However, the 0- and 3-day as well as the 3- and 6-day fillets did not differ significantly. The losses that had taken place during the ice storage were 8, 10, and 23%, respectively, in the 3-, 6-, and 9-day samples. Throughout the storage at −18 °C, all of the samples, particularly those held for 0, 3, and 9 days on ice, displayed a dip in activity after 42 days. However, it is currently unknown whether this dip was due to analytical errors or if there is a biochemical explanation. Nevertheless, the complex way GSH-px activity changed made modeling difficult, and the only model giving an acceptable R^2 value, 0.67, was the 6-day model. As can be seen in Table 1, the only fillets differing significantly in terms of rates of losses were the 6- and 9-day fillets, with the latter changing at the lowest rate.

Figure 2C illustrates the loss of ascorbic acid in the four sample groups during storage at −18 °C. Although the mean initial values decreased with storage on ice, no significant differences could be detected. Throughout the frozen storage period, the differences between the sample groups decreased, and the initial range of mean ascorbic acid levels, 2.4−6.2 mg/kg of tissue, decreased to 1.8−3.2 mg/kg of tissue by 84 days at −18 °C. The linear models describing the loss of ascorbic acid gave rise to high R^2 values, 0.89−0.93. However, for the 3- and 6-day fillets, the logarithmic models could explain slightly more of the data variance. As shown by the linear models, the mean rate of breakdown decreased with increasing prefreezing storage, similar to what was observed for α-tocopherol. However, the rates of the 0-, 3-, and 6-day fillets did not differ significantly. The present data were comparable to the data reported earlier by Brannan and Erickson (1996). These authors observed a linear loss ($R^2 = 0.85$) of ascorbic acid at a rate of 0.075 mg/kg of tissue per day in channel catfish stored at −6 °C. In the present study, the rate of loss varied between 0.008 and 0.034 mg/kg of tissue per day.

To explain the reduced rate of ascorbate loss when ice storage was extended, it should be mentioned that we recently found a significant lowering in the aqueous pro-oxidative activity, and thus, in the need for anti-

oxidants, during storage of herring fillets on ice (Undeland et al., 1999). In addition, it should not be excluded that a reduction, for example, in the activity of various proteases, slowed the loss of GSH-px activity at −18 °C. For ascorbic acid, a relationship with the reduced levels of α-tocopherol is also possible; the correlation between these two antioxidants was significant ($r = 0.89$). The role of ascorbic acid in the regeneration of α-tocopherol is well-known (Lambelet et al., 1985).

PLS analyses were also made with the antioxidants to evaluate whether ice storage or frozen storage had the greater effect on changes in these substances. The PLS models, which are described in Table 2, had R^2 values between 0.81 and 0.90 and showed that both types of storage conditions had significant effects on all three antioxidants measured. In addition, an interaction between ice and frozen storage significantly prevented the loss of ascorbic acid and GSH-px activity. With respect to the main effects, ice storage had the largest effect on α-tocopherol and ascorbic acid, whereas for the GSH-px activity (Figure 2B), frozen storage was of greater significance. Denaturation/damage to the GSH-px protein during the freezing process probably destabilized its enzyme activity (Richardson and Hyslop, 1985) and made it more sensitive than α-tocopherol and ascorbic acid to storage at −18 °C.

Relationship between Antioxidants and Changes in Lipid Oxidation. To interpret how the reduced rates of antioxidant consumption in the aged samples affected the formation of oxidation products at −18 °C is not a completely straightforward process, and possibly, the absolute antioxidant levels at the moment of freezing were of greater importance. Thus, although the 9-day samples, according to the models described in Table 1, retained their aqueous antioxidants the longest, it is possible that the ranges of antioxidant levels in these samples at the moment of freezing were already far below the levels at which either α-tocopherol, GSH-px, or ascorbic acid effectively inhibit lipid oxidation. It has been postulated that inhibition takes place only during the "induction phase" of the oxidation process when the antioxidant concentration is above a "critical concentration". According to Figure 1A,C, a clear change in the rate of PV and FP formation occurred between 42 and 63 days of frozen storage with the 6- and 9-day samples, which might indicate a breakpoint between the "induction" and "propagation" phases. Even though it is difficult to pinpoint critical antioxidant levels, this observation may indicate that between days 42 and 63, one or more antioxidants in the herring tissue had reached a critical level. Recalculation of the data obtained from the 6- and 9-day models (Table 1) showed that α-tocopherol was the antioxidant available at the lowest levels during this period; 17−9 and 15−7 nmol/g of dry weight tissue in the 6- and 9-day samples, respectively. The corresponding figures for GSH-px in these samples were 119−108 and 107−104 µmol of NADPH oxidized/min/g of dry weight tissue and for ascorbic acid 53−45 and 42−38 µmol/g of dry weight tissue, respectively. Erickson (1993b) described how α-tocopherol plays a vital role in protecting membrane lipids against oxidation during the initial stages of storage. She found α-tocopherol at a dry weight basis of 35−44 nmol/g to be the critical level needed to protect frozen minced channel catfish under fluctuating frozen storage (between −6 and −18 °C).

That there is a strong relationship between α-tocopherol consumption and the development of oxidation products during frozen storage is further supported by the significant correlation coefficients (r) obtained between α-tocopherol and PV, A_{268}, and FP: −0.82, −0.75, and −0.76, respectively. Ascorbic acid gave slightly lower, or similar, r values (−0.80, −0.75, and −0.65) and GSH-px the lowest values (−0.62, −0.48, and −0.48). These findings contrasted with our previous study of ice-stored herring (Undeland et al., 1999), in which $r_{PV, A268, FP}$ ranked the three antioxidants as follows: GSH-px > ascorbic acid > α-tocopherol. Thus, as was earlier described by Brannan and Erickson (1996), freezing of the muscle drastically changes the prerequisites for chemical and biochemical interactions between and among antioxidants, enzymes, and other cellular constituents to take place. As a result, components serving as good indicators of oxidative changes in fatty fish are not necessarily the same before as after freezing of the tissue.

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