Lipid Oxidation in Minced Herring (*Clupea harengus*) during Frozen Storage. Effect of Washing and Precooking

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A 2^3 factorial experimental design was used to investigate the effect of washing in combination with precooking on the oxidative stability of lipids in minced herring (*Clupea harengus*) at −18 °C. The following variables were studied: washing (no, yes), cooking time (38, 54 min), and cooking temperature (55, 100 °C). The responses monitored were peroxide value (PV), absorbance at 234 nm (A_{234}), absorbance at 268 nm (A_{268}), and lipid soluble fluorescent products (FP). A partial least-squares regression analysis (PLS) revealed that the best lipid stability was obtained at minimum cooking time and at lower temperature. On the basis of compositional analyses and in vitro experiments, this finding was proposed to be due to heat inactivation of catalytic enzymes, without simultaneous activation, for example, of hemoproteins. Washing reduced these benefits from precooking by removal of pro-oxidative enzymes and also through a reduction in the amount of antioxidants as well as a relative increase in phospholipids and free fatty acids in the fat.

Keywords: *Clupea harengus; herring; lipid oxidation; washing; precooking*

INTRODUCTION

Herring (*Clupea harengus*) has the potential to be utilized for food production to a much greater extent than is the case today. Both economic and nutritional factors call for an increase in the use of this abundant, cheap, and nutritious species of fish. In recent years, one approach to bring about such an increase has been the development of herring mince products. In comparison with conventional refining, mincing offers several advantages, such as a multitude of possibilities for further processing (Kiesvaara and Granroth, 1985; Venugopal and Shahidi, 1995). Mincing, however, destabilizes the fish due to a high level of incorporated oxygen and cellular disruption, making the lipids susceptible to oxidation. Most methods for fish mince stabilization are based upon one or several of the following three strategies: **removal** of pro-oxidants, oxygen, or components susceptible to oxidation; **alteration** of pro-oxidants, antioxidants, or other components influencing the oxidation; or **addition** of components that can protect fish mince lipids against oxidation.

Washing of fish mince helps removal of various components that influence the oxidative stability, for example, aqueous pro-oxidants, pigments, and fat (Hultin, 1988). However, results have been reported pointing to both an increased (Lee and Toledo, 1977) and a decreased (Ekstrand et al., 1993a; Spencer and Bligh, 1988) storage stability of washed fish mince. These contradictions could be attributed to the simultaneous removal of antioxidative compounds and the relative increase in both polarity and unsaturation in the remaining lipid fraction (Ekstrand et al., 1993a; Hultin, 1988).

Heating has been reported to influence the oxidative stability of fish lipids (Bosund and Ganrot, 1970; Ke et

al., 1976), for example, from the alteration of pro-oxidative enzymes such as lipoxygenases (Wang et al., 1991), "lipoxidases" (Khayat and Schwall, 1983), and microsomal enzymes (Slabyj and Hultin, 1982). Furthermore, changes in the pro-oxidative properties of myoglobin and other hemoproteins due to heat-induced exposure of heme (Cho et al., 1989) and release of heme-iron (Mielche and Bertelsen, 1993) have been reported. In relation to these proteins, more specific changes, such as decreased quasi-lipoxygenase activity of hemoglobin (Kühn et al., 1981), conversion of metmyoglobin into a porphyrin cationic radical (Harel and Kanner, 1985), and conversion of the inactive red pigment ferrous nitric oxide hemochromogen into a gray ferric pro-oxidative form (Younathan and Watts, 1959), have also been observed. Heating may also enhance the production of aqueous (Wang et al., 1991) and lipid soluble (Fujita et al., 1994) antioxidants. Also, conversion of α-tocopherol into a more active dimer (Ishikawa and Yuki, 1975), as well as inactivation of superoxide dismutase (SOD) (Mei et al., 1994), catalase (Mei et al., 1994; Harel and Kanner, 1985), and glutathion peroxidase (GSH-px) (Mei et al., 1994), has been reported. Physical changes from heating include membrane disruption (Wang et al., 1991) and aggregation of lipids (Bosund and Ganrot, 1970). Both increased (Bosund and Ganrot,1970; Fujita et al., 1994; Ishikawa and Yuki, 1975; Hsieh et al., 1988; Wang et al., 1991; Khayat and Schwall, 1983; Sen and Bhandary, 1978; Cho et al., 1989) and decreased (Zipser and Watts, 1961; Wang et al., 1991; Cho et al., 1989) storage stability of fish lipids following heating have previously been observed. Such conflicting results could be due to the use of different precooking conditions, such as heating method and time−temperature relationship (Mielche and Bertelsen, 1993).

The aim of this study was to follow the extent of lipid oxidation in herring mince subjected to different combinations of washing and precooking. A two-level factorial design (Carlsson, 1992) was used with three

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Table 1. Details of the 2^3 Factorial Design

expt	washing[a]	precooking temp[b]	precooking time[c]
1	+1	+1	+1
2	−1	+1	+1
3	+1	−1	+1
4	−1	−1	+1
5	+1	+1	−1
6	−1	+1	−1
7	+1	−1	−1
8	−1	−1	−1
9	+1	no	no
10	−1	no	no

[a] +1 = with washing, −1 = without washing. [b] +1 = 100 °C, −1 = 55 °C. [c] +1 = 54 min, −1 = 38 min.

variables, namely, washing (wa), cooking temperature (te), and cooking time (ti). To examine the stability of the lipids, the levels of oxidation products were measured after storage of the treated mince at −18 °C for 0, 6, 20, and 40 weeks.

MATERIALS AND METHODS

Equipment. The meat−bone separator used for preparing the mince had a drum hole diameter of 2 mm and was supplied by Baader (Model 694, Allan Brattsröm o/c AB, Stockholm, Sweden). The centrifuge used during washing was delivered by Krauss-Maffei AG (Munchen, Germany), and the PC-temperature logger, which was equipped with software AAC-2 PC Soft (Version 8.08), was supplied by INTAB Interference Teknik AB (Stenkullen, Sweden). Extraction of lipids and aqueous pro-oxidants from the mince was performed in a Sorvall Omnimixer (Ivan Sorvall Inc., Northwalk, CT) and cold centrifugation in a Sorvall Superspeed RC2-B (Instrument AB Lambda, Stockholm, Sweden). The atomic absorption spectrophotometers (AAS) were supplied by Perkin-Elmer (Norwalk, CT): for Fe analysis, Model 5000; and for Cu and Se analyses, Model 5001.

Reagents. For trace element analyses, hydrochloric acid and hydrogen peroxide were of pro analysi (p.a.) grade and obtained from Merck (Darmstadt, Germany). Nitric acid of suprapure quality was supplied by J. T. Baker (Philipsburg, NJ). For the analyses of pro-oxidative activity, p.a. quality potassium phosphate buffer (Merck), >99% linoleic acid (Nu-Chek-Prep, MN), and 95 and 99% ethanol (Kemetyl, Haninge, Sweden) were used. Myoglobin and hemoglobin were supplied by Sigma (St. Louis, MO). The isohexane for peroxide value determinations was of HPLC grade (Fisher, Loughborough, England).

Multivariate Experimental Design. A 2^3 factorial design (Carlsson, 1992) was used in the present study. This means that the three variables, or factors, as given in Table 1, were investigated at two levels, +1 and −1. The results were then analyzed statistically with partial least-squares regression analysis (PLS) using the software MODDE (Umetri, Umeà, Sweden). The PLS analysis resulted in a mathematical model that correlated the three variables to the four responses peroxide value (PV), absorbance at 234 nm (A_{234}), absorbance at 268 nm (A_{268}), and lipid soluble fluorescent products (FP). This model could be expressed as follows: $Y_{(PV,A234,A268,logFP)} = \beta_0 + \beta_1 wa + \beta_2 te + \beta_3 ti + \beta_{12} wa \times te + \beta_{13} wa \times ti + \beta_{23} te \times ti$. In this equation, β_0 is a constant and the sizes of β_1, β_2, β_3, β_{12}, β_{13}, and β_{23} are equal to the size of the effect of each variable (wa, te, and ti) or two variables in combination (wa × te, wa × ti, and te × ti) on the response. The former is referred to as the *main effect*, and the latter as the *interaction effect*. The reliability of the model was evaluated from calculations of R^2 and Q^2. R^2 is the fraction of the response variation that is explained by the model, and Q^2 the part of this variation that can be predicted by the model (Umetri AB, 1992). Q^2 should be above 0.5 if conclusions are to be drawn from the model (Lindgren, 1995). It should be noted that the response matrices obtained after each of the

four storage periods were evaluated separately; thus, storage time was not included as a variable in the design.

Samples. Herring (*Clupea harengus*) was caught off the west coast of Sweden in November 1995. The fish was headed, gutted, and deboned on the day of landing. The average length and weight of the fillets obtained were 107 ± 8 mm and 26 ± 3 g, respectively [mean ± standard deviation (SD), $n = 6$]. After one night on ice, the fillets were minced in a meat−bone separator. Half of the mince was then washed using the following procedure: 50 L of filtered (Millipore, 20 μm) water (4 °C) containing 0.05 M NaCl was added to 10 kg of the mince. The slurry was manually stirred for 30 min and then centrifuged at 1600g for 10 min, after which time the mince was collected. Both the crude and washed minces were then divided into two batchess; one batch was packed into 120 mL high-density polyethylene (HDPE) boxes (93 × 93 × 22 mm) and the other into 210 mL boxes (93 × 93 × 38 mm). After filling, the boxes were covered with polyamide-laminated polyethylene film and subjected to heat treatment in water baths maintained at 55 and 100 °C. In both the 120 and 210 mL boxes, the precooking was allowed to continue until the center of the boxes had reached the temperature (±3 °C) of the surrounding water. After removal from the water bath, the boxes were held for 10 min under aluminum foil at room temperature. Details about the final length of the heating procedures are given in Table 1. After equilibration, the boxes were cooled at 5 °C for 30 min, after which time they were frozen at −40 °C in a tunnel freezer. From each of the precooked experiment groups (1−8 in Table 1), two boxes were then put directly into a −70 °C freezer and were used as 0-week samples. The remaining ones were stored at −18 °C for 6, 20, and 40 weeks, respectively. After completed storage at −18 °C, the boxes were transferred to −70 °C, at which temperature they were stored until analysis. At the day of analysis, two boxes ($n = 2$) from each storage point and each sample group were thawed for 15 min at room temperature and one 5 g sample (35 × 35 × 4 mm) was then taken from the top surface and one from the bottom surface of the boxes. Together these two samples were referred to as "surface sample". From the center of the boxes, a 10 g "center sample" was taken (23 × 23 × 17 mm). That the total thickness of the surface and center samples exceeded the thickness of the small box was due to expansion of the mince during freezing. Each surface and center sample was then extracted for total lipids, which were analyzed in duplicate for oxidation products ($a = 2$).

Washed and crude *uncooked* mince (experiment groups 9 and 10 in Table 1) was also packed, stored, and analyzed in the same way. These experiments were used as controls and were not included in the multivariate data analysis.

Compositional Analyses of Crude and Washed Mince. *Analysis of Selenium (Se).* About 1.0 g each of crude and washed mince was digested with 5 mL of concentrated nitric acid and 5 mL of hydrogen peroxide in a microwave oven (2455 Hz, 650 W) for 75 min using Teflon digestion vessels. Following this treatment, the selenium was reduced in the microwave oven by treatment with 37% hydrochloric acid. The reduced destruate was then diluted to 25 mL with double-deionized water. The content of selenium was analyzed by flow injection hydride atomic absorption spectrophotometry (AAS) (Perkin-Elmer, 1994) ($n = 2$, $a = 2$). The limit of detection was 0.010 mg/kg of mince, and the repeatability of the method was relative standard deviation (RSD%) 8.0. Internal or certified reference materials were analyzed together with the herring mince.

Analysis of Iron (Fe). About 1.0 g each of crude and washed mince was ashed at 500 °C (Isaak and Johnsson, 1975). The ash was dissolved in 5 mL of 6.0 M hydrochloric acid and diluted to 50 mL with double-deionized water. The concentration of iron was then measured with flame AAS using deuterium background correction (Perkin-Elmer, 1976) ($n = 2$, $a = 2$). The limit of detection was 1 mg/kg of mince, and the repeatability of the method was RSD% 5.0. Internal or certified reference materials were analyzed together with the herring mince.

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Analysis of Copper (Cu). About 1.0 g each of crude and washed mince was digested with 10 mL of concentrated nitric acid in a microwave oven (2455 Hz, 650 W) for 75 min using Teflon destruction vessels. After digestion, the samples were diluted to 50 mL with double-deionized water. The copper content in each sample was determined with graphite furnace AAS using Zeeman background conditions (Perkin-Elmer, 1992) ($n = 2$, $a = 2$). The limit of detection was 0.1 mg/kg of mince, and the repeatability of the method was RSD% 6.0. Internal or certified reference materials were analyzed together with the herring mince.

Analysis of Pro-oxidative Activity of Herring Mince Buffer Extracts, Hemoglobin, and Myoglobin. Ten grams each of crude and washed herring mince was homogenized on ice for 1 min together with 40 mL of 50 mM potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 46000g at 4 °C for 15 min, and the supernatants were collected. A portion of each supernatant (100 μL) was then added, either immediately or after heat treatment (30 min at 55 °C or 10 min at 100 °C), to 1.3 mL of 10 mM linoleic acid emulsion (LAE) in 0.1 M potassium phosphate buffer (pH 6.8), and the oxygen consumption was measured with a Clark electrode maintained at 25 °C (Axelrod et al., 1981) ($n = 2$, $a = 2$). For comparison, the pro-oxidative effects of hemoglobin and myoglobin were similarly measured by adding 100 μL of 50 mM potassium phosphate buffer (pH 7.4) containing pure myoglobin or pure hemoglobin at 5.12 and 1.4 μM, respectively, to the LAE (final concentration = 0.39 and 0.11 μM). The hemoprotein concentrations were selected to give the same level of heme groups. Results were expressed as the slope of the curve (k value) where the maximum rate of oxygen consumption occurred.

Analysis of Pro- and Antioxidative Effect of the Washing Water. After washing of the herring mince, the wash water was analyzed for content of water soluble and lipid soluble pro- and antioxidants. This was performed by adding either 50 μL of the aqueous phase or 0.7 mg of the lipid phase (floating on top of the aqueous phase) of the washing water to 2 mL of a 10 mM LAE (pH 6.8). The LAE mixtures were then stored for up to 4 weeks at 20 °C in the dark, and at periodic intervals 50 μL samples were withdrawn and analyzed for absorbance at 234 nm ($n = 2$, $a = 2$) after addition of 4 mL of 70% ethanol. Results were expressed as absorbance units at 234 nm.

Analysis of Total Lipid Content. The total lipid content in crude and washed herring mince was determined gravimetrically after extraction with a modified version of the Bligh and Dyer (1959) procedure as described by Undeland et al. (1998) ($n = 3$, $a = 1$). Results were expressed as grams of lipid per kilogram of mince.

Analysis of Fatty Acid Profiles. The fatty acid profiles of lipids in crude and washed herring mince were determined after extraction according to the modified Bligh and Dyer procedure. The lipids were converted into methyl esters and analyzed by gas chromatography (Ekstrand et al.., 1993b) ($n = 3$, $a = 2$). Results were expressed as grams per kilogram of lipid.

Extraction of Lipids for Analysis of Lipid Classes, α-Tocopherol, and Oxidation Products. Total lipids were extracted according to the method of Burton et al. (1985), with few modifications according to Undeland et al. (1998).

Analysis of Lipid Class Distribution. The contents of neutral lipids (NL), phospholipids (PL), and free fatty acids (FFA) in the total lipids from crude and washed herring mince were determined gravimetrically using solid-phase extraction (SPE) according to the method of Kaluzny et al. (1985) ($n = 2$, $a = 2$). Results were expressed as grams per kilogram of lipid.

Analysis of α-Tocopherol. α-Tocopherol was determined in the crude and washed mince lipids by normal-phase high-performance liquid chromatography (HPLC) according to the method of Piironen et al. (1984), with minor modifications ($n = 2$, $a = 2$). The repeatability of the method for α-tocopherol analyses was measured to be RSD% 3.0 ($n = 1$, $a = 6$). The levels of α-tocopherol were expressed as grams per kilogram of lipid.

Analyses of Lipid Oxidation Products. *Analysis of PV.* PV was analyzed using the official ferric thiocyanate method of the International Dairy Federation (IDF) (1991), as modified by Ueda et al. (1986) ($n = 2$, $a = 2$). However, for toxicity reasons, total extracted lipids were dissolved in isohexane instead of in *n*-hexane. The repeatability of the method was RSD% 1.6 ($n = 1$, $a = 6$), and the results were expressed as milliequivalents (mequiv) of peroxide per kilogram of lipid.

Analysis of Absorbance at 234 nm (A_{234}) and 268 nm (A_{268}). Absorption at 234 and 268 nm of the extracted lipids was measured using flow injection analysis (FIA) ($n = 2$, $a = 2$). Results were expressed as area units per microgram 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). Total lipid soluble fluorescence having an excitation maximum at 367 nm and an emission maximum at 420 nm was measured with FIA ($n = 2$, $a = 2$). Results were expressed as log area units per microgram of lipid. The repeatability of the method was measured to be RSD% 4.7 ($n = 1$, $a = 6$).

RESULTS AND DISCUSSION

Oxidative Stability at -18 °C. Table 1 shows the two-level factorial design that was used to elucidate how washing and precooking in combination affect the oxidative stability of herring mince lipids during storage at -18 °C. Experiment groups 9 and 10 in this table, which consisted of crude and washed mince in an uncooked state, were used only to determine the isolated influence of cooking. Thus, these two groups were not included in the multivariate evaluation.

Oxidative changes in the herring mince lipids were analyzed after 0, 6, 20, and 40 weeks using the following four parameters: PV, A_{234}, A_{268}, and FP. It may be mentioned that the results were expressed on a *lipid basis*. Thus, discussions and conclusions are solely dealing with the oxidative status of the herring mince lipids. Furthermore, to compensate for differences in the responses not originating from oxidative changes, results were given as Δ values. These values were obtained by subtraction of the 0-week sample results from the stored-sample results. The reason for this treatment of the data was the high background absorbance and background fluorescence, which were obtained in washed samples prior to storage. It was concluded that these differences were not due to oxidative changes, as no such washing-related difference was seen in the PV results. This phenomenon thus seemed to originate in interference from the native lipid (Situnayake et al., 1990).

Figures 1 and 2 show the storage-induced changes in PV and A_{268} in the surface (Figures 1A and 2A) and center samples (Figures 1B and 2B) from each of the 10 experiment groups. From comparisons between the A and B panels, it is obvious that throughout the 40 weeks of storage, lipid oxidation was almost completely inhibited in the center parts, which in the thin boxes were situated only 4 mm in from the surface. This indicates that oxygen incorporated during the mincing process very quickly was consumed and that slow diffusion then prevented further oxygen supply. Since the packaging box provided almost no barrier to oxygen, it was concluded that diffusion of oxygen was limited by the mince itself. According to Flink and Goodhart (1978), oxygen diffusion trough frozen fish mince is retarded due to plugging of the oxygen transport pathways and therefore is highly dependent upon sample thickness. Virtually no oxygen diffusion could
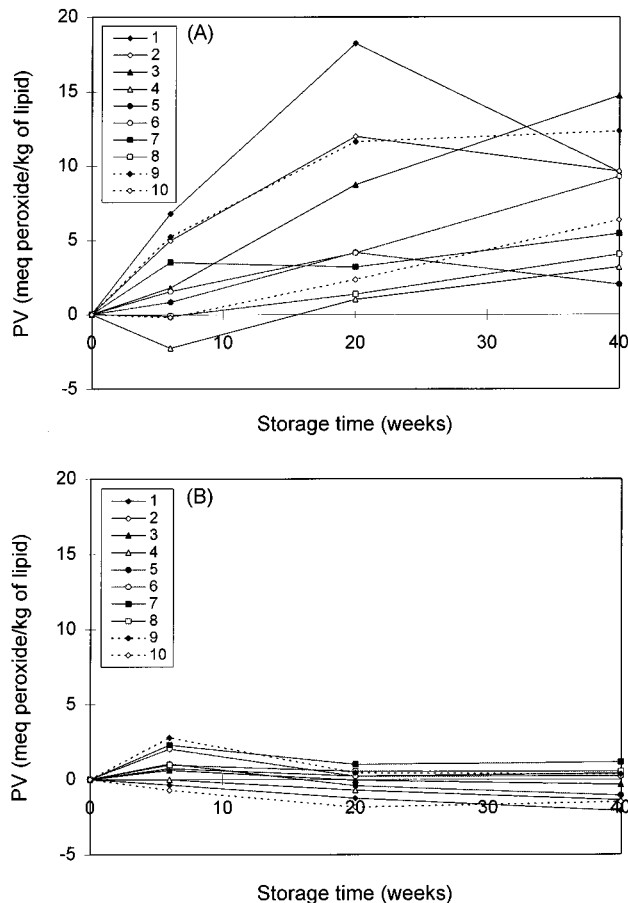
Figure 1. Progress of PV in (A) surface samples and (B) center samples taken from the 10 experiment groups after storage at −18 °C for 0, 6, 20, and 40 weeks ($n = 2$, $a = 2$). For each of the 10 groups, the results obtained at 0 weeks were subtracted from those obtained after 6, 20, and 40 weeks, respectively. Thus, each point on the curves represents a mean Δ value. The repeatability of the PV method was RSD% 1.6 ($n = 1$, $a = 6$).

be measured in frozen fish mince samples thicker than 5 mm, supporting the present observations. Similar findings were also presented by Lawrie (1974), showing that oxygen diffuses 1−4 mm into the surface of muscle tissue, the absolute distance depending on mitochondrial content and degree of tissue disruption. The intention was to use the center samples for studying the influence of heating rate on the oxidative stability. Since packaging boxes of two different sizes were used, two different rates of heating were obtained at each temperature. However, according to the high stability in the center, the multivariate evaluation could be based only on the changes taking place in the surface samples, explaining why the heating rate variable had to be excluded.

A clear color gradient was seen in the uncooked samples when moving from the surface into the center, which could be attributed to decreasing O_2 availability in the interior parts of the mince blocks. The more oxidized surface samples were grayish, whereas the stable samples from the center were reddish-pink. This indicates a possible relationship between the disappearance of red pigment and the development of lipid oxidation as previously reported in mullet (*Mugil cephalus*) (Zipser and Watts, 1961) and ham (Watts, 1957).

From Figures 1A and 2A it can be seen that some samples reached maximum PV and A_{268} values after 20
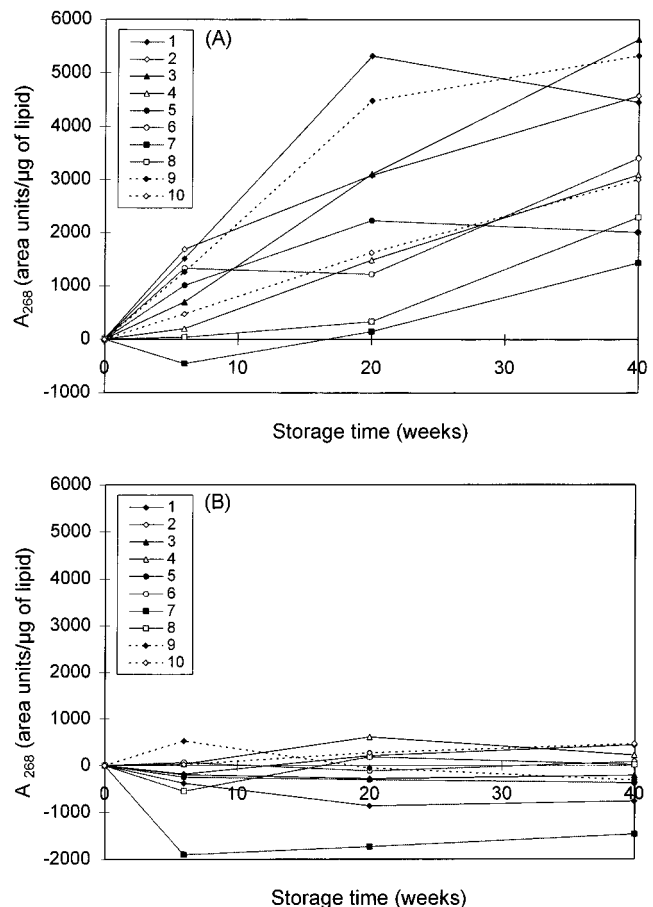

Figure 2. Progress of A_{268} in (A) surface samples and (B) center samples taken from the 10 experiment groups after storage at −18 °C for 0, 6, 20, and 40 weeks ($n = 2$, $a = 2$). For each of the 10 groups, the results obtained at 0 weeks were subtracted from those obtained after 6, 20, and 40 weeks, respectively. Thus, each point on the curves represents a mean Δ value. The repeatability of the A_{268} method was RSD% 4.5 ($n = 1$, $\alpha = 6$).

Table 2. Reliability of the PLS Model at Each Storage Point As Expressed by R^2 and Q^2

	PV		A_{234}		A_{268}		FP	
	R^2	Q^2	R^2	Q^2	R^2	Q^2	R^2	Q^2
6 weeks	0.90	0.78	0.74	0.45	0.89	0.71	0.83	0.60
20 weeks	0.95	0.80	0.96	0.81	0.87	0.68	0.97	0.89
40 weeks	0.93	0.75	0.87	0.72	0.93	0.84	0.89	0.72

weeks of storage and then decreased. This was most obvious in samples 1 and 5, the former having high settings for all variables and the latter for two of three (Table 1). The same was true for A_{234} and FP, although not shown here. These results indicate the possible involvement of lipid oxidation products in secondary reactions when 20 weeks of storage is exceeded. Therefore, 20 weeks of storage was used in the further evaluations in this study. Furthermore, when oxidative stability is studied, the influence of the three variables on early changes should preferably be evaluated. An additional reason for focusing on the 20-week results is given in Table 2, which shows the reliability (expressed as R^2 and Q^2) of the mathematical models obtained after each storage period. It can be seen that in three of four cases, the R^2 and Q^2 values were highest after 20 weeks.

Figures 1A and 2A also indicate that the extent of lipid oxidation in uncooked samples (dotted lines) was within the range of values obtained for cooked samples.

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Thus, some cooked samples had lower levels of lipid oxidation than uncooked ones. This was true for both crude and washed minces and indicates that certain time−temperature combinations during the precooking step were successful at improving the storage stability of these minces. A general trend was that groups which had been treated at low settings for the three variables were the most stable. To evaluate in greater detail the regions of the experimental domain which gave rise to the best stability, multivariate statistics was used. This tool also provided information on the influence of individual variables, or two variables in combination, on each of the responses.

Influence of the Three Processing Variables on PV, A_{234}, A_{268}, and FP. Figure 3 shows the β-coefficient plots for each of the four responses analyzed. The size of the β-coefficients (also referred to as effects) illustrates how large the influence from a specific variable, or combination of variables, is on the response in question. It should be noted that *positive* values on these coefficients imply that the variable has a *promoting* effect, whereas *negative* values are equal to a *retarding* effect. Error bars not crossing the *X*-axis indicate that the effect in question is significant. All error bars are based on a 95% confidence interval; thus, $P < 0.05$.

It can be seen that the three main variables, washing (wa), cooking temperature (te), and cooking time (ti), all had a significant promoting effect on the four responses; that is, PV (Figure 3A), A_{234} (Figure 3B), A_{268} (Figure 3C), and FP (Figure 3d). In all cases, the strongest effect was derived from cooking time, whereas washing and cooking temperature alternated in being ranked second, the former for A_{234} and FP and the latter for PV and A_{268}. With regard to interaction effects, wash × cooking time had a significantly promoting influence on all responses except for on A_{268}, whereas wash × cooking temperature proved to be insignificant. The effect of time × cooking temperature was different with respect to the four responses. For PV, this interaction was promoting, whereas in relation to FP it gave rise to a retarding effect. A_{234} and A_{268} were not significantly affected at all by this interaction.

Thus, as a whole, changes in the settings of the three variables had a similar influence on the four responses. The slight inconsistencies obtained, for instance, among the interaction effects, are probably related to the fact that the responses used, PV and FP in particular, monitor products formed at different stages in the lipid oxidation chain. The former, together with A_{234}, measures lipid hydroperoxides, or *primary* lipid oxidation products, whereas the latter monitors fluorescent Schiff bases, or *tertiary* products. These arise from interactions of peroxidized lipids, such as hydroperoxides and aldehydes, with cellular constituents containing free amino groups (Dillard and Tappel, 1973). A_{268} values monitor, to some extent, a stage between the two stages above, as it responds both to conjugated triene hydroperoxides and to various *secondary* products, such as ethylenic diketones and oxodienes (IUPAC, 1979; Brown and Snyder, 1982). Which combinations of the variables that gave rise to the lowest development of oxidation products are indicated more clearly in the response surface plots given in Figures 4 (A_{234}) and 5 (FP). These plots show the contour of the response surface, thus, which values for A_{234} and FP, respectively, that are obtained without washing (A panels) and with washing
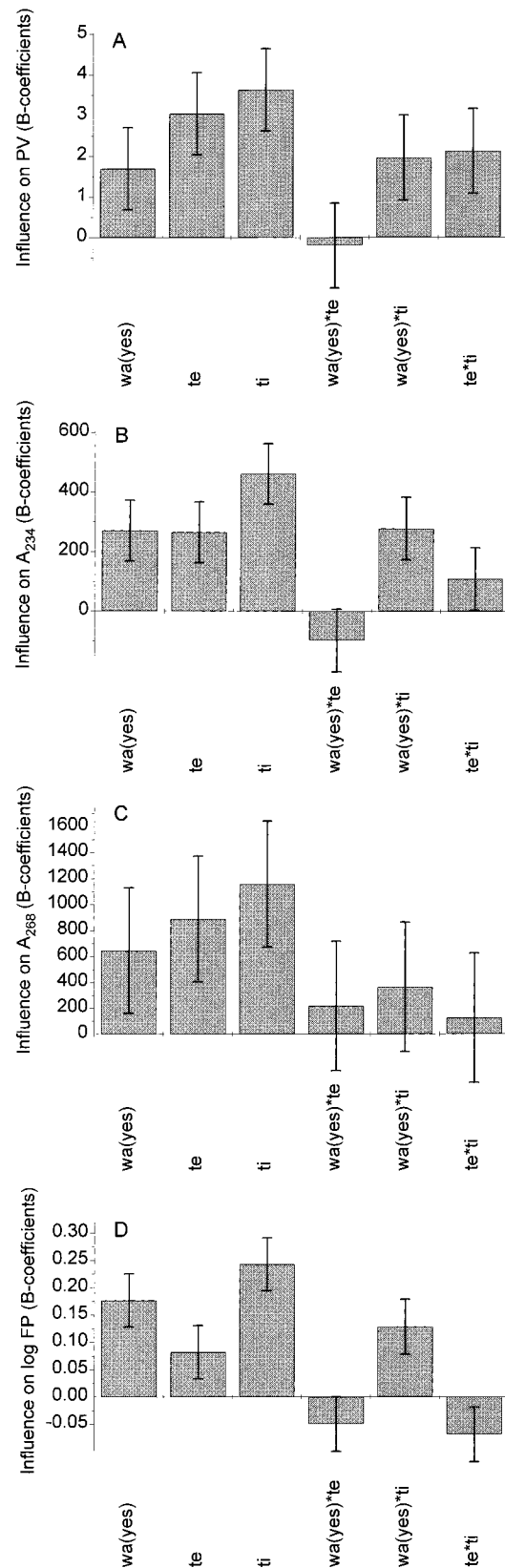

Figure 3. Main and interaction effects of the three variables wa, te, and ti on (A) PV, (B) A_{234}, (C) A_{268}, and (D) FP in fat from minced herring. The effects are described as β-coefficients, originating from a PLS analysis of the results obtained after 20 weeks of storage at −18 °C. For each of the four responses, the estimated PLS model can be expressed as follows: $Y_{(\text{PV},A234,A268,\log\text{FP})} = \beta_0 + \beta_1\text{wa} + \beta_2\text{te} + \beta_3\text{ti} + \beta_{12}\text{wa} \times \text{te} + \beta_{13}\text{wa} \times \text{ti} + \beta_{23}\text{te} \times \text{ti}$. Error bars are based on a 95% confidence interval.

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Table 3. Compositional Data for Crude and Washed Mince Made from Herring (*C. harengus*) Caught in November 1995

	crude mince	washed mince
trace elements		
Fe[a] (mg/kg of wet sample)	7 ± 0	4 ± 0
Cu[a] (mg/kg of wet sample)	1.1 ± 0	0.68 ± 0.01
Se[a] (mg/kg of wet sample)	0.21 ± 0.02	0.14 ± 0.01
lipid components		
total lipids[b] (g/kg of wet sample)	105 ± 8	33 ± 2
neutral lipids[a] (g/kg of lipid)	837 ± 11	744 ± 12
phospholipids[a] (g/kg of lipid)	99 ± 13	181 ± 15
free fatty acids[a] (g/kg of lipid)	16 ± 8	34 ± 3
α-tocopherol[a] (g/kg of lipid)	0.13 ± 0.005	0.22 ± 0.01

[a] Mean value $\pm$ (max$-$min value)/2, ($n = 2$, $a = 2$). Mean values from the two analyses were used to establish sample variation. [b] Mean value $\pm$ SD, ($n = 3$, $a = 1$).

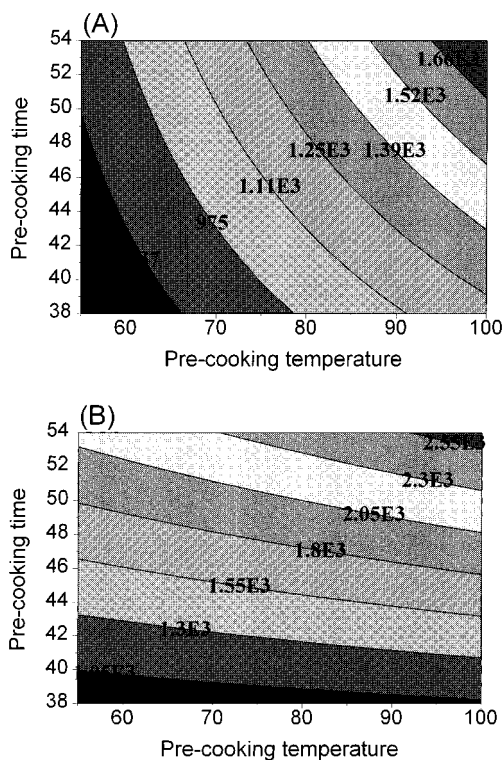**Figure 4.** Response surface plots from a PLS analysis of A_{234} obtained in fat from minced herring after 20 weeks of storage at -18 °C. The plots illustrate where in the experimental domain the lowest A_{234} values are obtained (A) without washing and (B) with washing.

Figure 5. Response surface plots from a PLS analysis of the levels of FP obtained in fat from minced herring after 20 weeks of storage at -18 °C. The plots illustrate where in the experimental domain the lowest FP values are obtained (A) without washing and (B) with washing.

(B panels) when different precooking conditions are used. It can be seen that the best stability is obtained when low settings are used both for cooking time and for cooking temperature, that is, 38 min and 55 °C, and furthermore, as can be seen in the B panels, when washing is avoided. The same was also true for PV and A_{268}. It was interesting to note that for unwashed mince, the storage stability was strongly dependent upon cooking temperature, whereas when washing was applied, the stability instead became strongly coupled to cooking time. This was particularly pronounced with regard to the development of primary products.

Various aspects of both washing and precooking will be discussed below to explain the present observations.

Washing. From the results shown in Figure 3, it was obvious that washing under the present conditions influenced the lipid stability in a negative way. This observation contradicted the results of Lee and Toledo (1977) but supported those of Ekstrand et al. (1993a) and Spencer and Bligh (1988). To explain the disadvantageous effects of washing, changes in the composi-
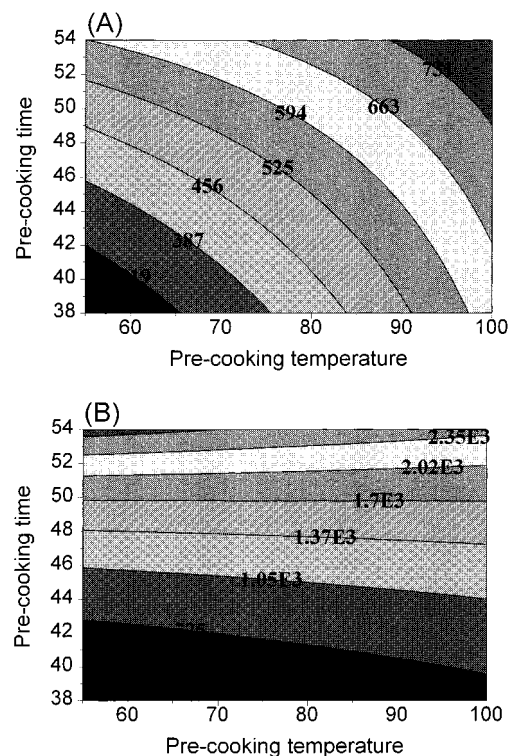
tion of antioxidants, pro-oxidants, and lipids were determined in crude and washed mince.

The content of Se, an index of glutathione peroxidase, which is an antioxidative enzyme (Kadiiska et al., 1993), decreased by 34% upon washing (Table 3). This enzyme reduces cellular peroxides to their corresponding alcohols without the formation of radical intermediates (Kadiiska et al., 1993). The α-tocopherol-to-lipid ratio increased by 40% upon washing. However, as the lipid class composition also changed during washing, there was a slight decrease in the α-tocopherol/phospholipid (PL) ratio. This may be of greater significance for the stability than the α-tocopherol/total lipid ratio, since α-tocopherol primarily is a constituent of the cell membranes (Fryer, 1993). The observation that a substantial amount of antioxidants was removed during washing was further confirmed by in vitro analyses of the washing water (Figure 6). After 10 days at 20 °C, a 50% lower level of conjugated dienes was seen in a linoleic acid emulsion to which a sample of the aqueous phase of the washing water had been added. In addition

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Table 4. Oxygen Consumption (k Value)[a] in an LAE (10 μM, pH 6.8) upon Addition of Crude/Washed Herring Mince Buffer Extracts,[b] Hemoglobin,[c] and Myoglobin[c]

treatment	crude mince	washed mince	hemoglobin	myoglobin
none	0.83 ± 0.07^c	0.21 ± 0.02	1.67 ± 0	0.09 ± 0.007
heating at 55 °C, 30 min	0.31 ± 0.09	0.22 ± 0.04	1.04 ± 0.04	0.14 ± 0.005
heating at 100 °C, 10 min	0.33 ± 0.02	0.10 ± 0.03	0.60 ± 0.05	0.16 ± 0.005

[a] K values represent the slope at the steepest part of the oxygen consumption curve (mean value $\pm$ (max−min value)/2, ($n = 2$, $a = 2$). Mean values from the two analyses were used to establish sample variation. [b] Potassium phosphate buffer, 50 mM (pH 7.4). The relation fish/buffer during homogenization was 1:4. [c] 5.12 μM myoglobin and 1.4 μM hemoglobin in 50 mM potassium phosphate buffer (0.39 and 0.11 μM final concentrations). These experiments were performed in the same way as those including herring mince buffer extracts.
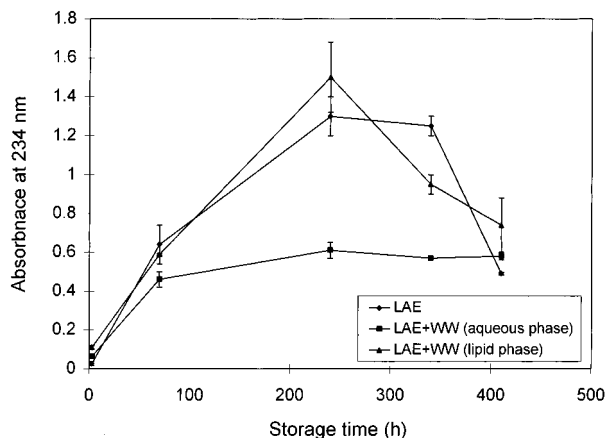

Figure 6. A_{234} in a 10 mM LAE (pH 6.8) upon addition of samples from the aqueous and lipid phases of the water used during washing of minced herring (WW) ($n = 2$, $a = 2$). The aqueous and lipid phases were added at concentrations of 25 μL/mL and 350 μg/mL, respectively, and the mixtures were stored in the dark for 2.75, 70, 240, 340, and 410 h at 20 °C. Error bars indicate the span between maximum and minimum values.

Table 5. Fatty Acid Pattern in Crude and Washed Mince from Herring (*C. harengus*) Caught in November 1995

fatty acid	crude mince[a] (g/kg of lipid)	washed mince[a] (g/kg of lipid)
saturates		
C14:0	47.4 ± 9.4	43.9 ± 6.0
C16:0	93.6 ± 12.4	98.2 ± 5.1
C18:0	9.6 ± 1.5	12.3 ± 2.3
total	150.6 ± 23.3	154.4 ± 8.8
monoenes		
C16:1	34.7 ± 6.8	31.8 ± 3.4
C18:1	68.3 ± 14.1	65.3 ± 3.6
C20:1	65.7 ± 8.2	61.7 ± 11.8
C22:1	39.9 ± 7.0	40.9 ± 1.3
total	208.7 ± 35.6	199.7 ± 20.1
polyunsaturates		
C18:2	14.5 ± 3.1	13.9 ± 1.3
C18:3	9.7 ± 2.8	8.9 ± 1.1
C18:4	17.8 ± 5.4	15.8 ± 1.7
C20:5	133.1 ± 12.7	125.8 ± 21.0
C22:6	86.8 ± 9.1	103.3 ± 1.3
total	262.0 ± 32.0	267.7 ± 26.4

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

to GSH-px, SOD, catalase (Mei et al., 1994), ascorbate, and ferroxidase (Hultin, 1994) are likely aqueous components contributing to this antioxidative effect. The lipid phase of the washing water did not give rise to a similar reduction in diene development.

The metal contents, namely Fe and Cu, decreased during washing by 43 and 38%, respectively (Table 3), whereas the corresponding figure for total aqueous pro-oxidative activity was 75% (Table 4). The properties of Fe as a strong initiator of lipid oxidation in biological tissues are well-known and include both low molecular weight (LMW) Fe (Tichivangana and Morrisey, 1984), and heme-bound Fe (Hultin, 1994). Strong pro-oxidative properties have also been observed for Cu (Tichivangana and Morrisey, 1984; Kadiiska et al., 1993), and to a great extent the mechanisms involved in Cu-catalyzed oxidation are believed to be the same as in Fe-catalyzed oxidation, for example, production of active oxygen species and a breakdown of preformed hydroperoxides. The lower oxidation states of Fe and Cu have often been recognized to be the most pro-oxidative forms (Tichivangana and Morrisey, 1984). Therefore, the washing-out of reducing compounds such as ascorbate and microsomal enzymes might also be of great relevance. Furthermore, washing-induced change in the types of complexing agents present may be a critical feature for the subsequent storage stability of the mince. Schaich (1992) has discussed a positive correlation between stronger complexation of a metal, that is, more ligands, and a lower catalytic activity. Table 4 illustrates that the total decrease in pro-oxidative activity during washing was not solely due to trace metal removal. From heating of the analyzed herring mince

buffer extracts, it could be seen that pro-oxidative enzymes were having a greater role than metal ions. This observation is further discussed under Precooking.

Table 3 also indicates that the total lipid content decreased by >70% due to washing. Even though positive correlations between the total lipid content and the degree of lipid oxidative changes have been pointed out (Yamaguchi et al., 1984), most studies indicate that the composition of the lipids is the most critical feature with regard to lipid oxidation. As shown in Table 3, the relative distribution of NL, PL, and FFA in the total lipids changed from 88, 10, and 2 to 78, 19, and 3%, respectively, after washing. According to Hultin (1988), the easier removal of NL, compared to PL, can be explained by the location of NL within globules, inside and between the muscle cells. PL, on the other hand, are converted into vesicles upon mechanical disruption and as such are retained in the muscle due to physical attraction to contractile elements. Previous studies on raw and cooked fish muscle (Halpin, 1984; Tichivangana and Morrisey, 1982) have shown a lower stability of PL as compared to NL, which concurs well with the present observations. One reason for this instability of PL is believed to be their high degree of unsaturation (Mai and Kinsella, 1979). This is reflected in Table 5, which shows that the proportion of C22:6 increased together with that of PL during washing. Another reason given for the instability of PL is their high polarity, which allows closer contact between lipids and aqueous pro-oxidants (Hultin, 1997). Apart from increasing the susceptibility of the lipid fraction to oxidation, it is

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believed that a high relative amount of PL also affects the type of oxidation products formed. As an example, the binding to free amino groups makes PL favor the formation of FP (Dillard and Tappel, 1973). This quality is reflected in Figure 3D, showing that the development of FP was more promoted by washing than the other lipid oxidation products. The higher level of FFA in fat from washed mince may be due to either increased lipolysis during the washing process itself (Ekstrand et al., 1993a) or vesicle formation, causing FFA retention, as discussed above. The ways in which this FFA increase influences lipid oxidation may be related either to the generally accepted view that FFAs are more susceptible to oxidation than esterified ones or to the capacity of FFAs to work as emulsifiers, thus increasing the contact surface between the aqueous and lipid phases. According to Shewfelt (1981), the impact of an increase in FFA on lipid oxidation depends on the origin of the FFAs; following lipolysis of triglycerides, a catalytic effect may be expected, whereas the effects may be the reverse after PL lipolysis.

In addition to the compositional changes discussed, it should be stressed that oxidation reactions taking place already during the washing procedure may be another explanation of the reduced storage stability of washed mince (Kelleher et al., 1992). Although no changes in the oxidation measures were seen, it cannot be excluded that formation of initiators and consumption of antioxidants may have facilitated the conditions for oxidation to occur.

Precooking. For all the measures of lipid oxidation, cooking time was the variable giving rise to the most strongly promoting main and interaction effects. The strong adverse influence of cooking time was in accordance with previous results reported in the case of lake herring (Wang et al., 1991), cod (Lovern, 1962), and ground beef (Mielche and Bertelsen, 1993). In relation to these studies, explanations such as greater nonenzymatic oxidation (Wang et al., 1991) have been offered. Against the promoting effects of cooking time was the retarding influence of cooking time × cooking temperature on FP (Figure 3D). This was probably caused by the proposed heat-induced polymerization of fluorescent compounds into yellow-brownish pigments (Pokorny et al., 1974). It is believed that this reaction is paramount after a certain time, when a sufficient level of FP is reached. In contrast to our results, retarding effects of cooking time were seen in two previous studies of sardine (Sen and Bhandary, 1978; Fujita et al., 1994). The explanations given were formation of antioxidants as well as destruction of "lipoxidase" and heme.

The main effect of cooking temperature was that it significantly promoted all responses, PV and A_{268} somewhat more than A_{234} and FP. Relationships between increased cooking temperatures and lower lipid stability have previously been seen in ground chicken (Smith et al., 1984), pork (Mei et al., 1994), and beef (Mielche and Bertelsen, 1993; Mei et al., 1994). These studies all pointed to a sharp increase in thiobarbituric acid reactive substances test (TBARS) when temperatures >60 °C were used during precooking. The sudden increase in TBARS correlated well with the inactivation of catalase and GSH-px, suggesting that these enzymes were strongly related to the development of oxidation (Mei et al., 1994). The drastic responses to cooking temperatures >60 °C may also be related to increased cell disintegration (Mielche and Bertelsen, 1993) and

to structural changes in hemoproteins. With regard to the latter, there have been previous reports of a sharp increase in Fe release from the hemoprotein porphyrin ring at 60 °C (Tichivangana and Morrisey, 1984) and, in addition, of increased heme group exposure (Cho et al., 1982), conversion of metmyoglobin into a porphyrin cationic radical (Harel and Kanner, 1985), and conversion of certain heme pigments into active pro-oxidative forms (Younathan and Watts, 1959).

Since enzyme inactivation and hemoprotein denaturation are the two dominating heat-induced changes affecting lipid oxidation (Mei et al., 1994; Slabyj and Hultin, 1982; Wang et al., 1991; Cho et al., 1989; Mielche and Bertelsen, 1993), we wanted to elucidate how these two reactions contributed to the present results. To do so, the in vitro study of buffer extracts from crude and washed mince (previously discussed under Washing) was extended to include pure hemoglobin and myoglobin solutions. Both extracts and hemoproteins were heated for 30 min at 55 °C and for 10 min at 100 °C prior to mixing with an LAE. Table 4 shows that ~60% of the pro-oxidative activity remained in crude mince after heating at 55 °C, whereas the extract from the washed mince was unaffected. The difference in sensitivity between the crude and washed mince extracts indicates a substantial removal of prooxidative enzymes, for example, lipoxygenases and microsomal enzymes, during washing. This removal, which is supported by previous studies of trout (Hsieh et al., 1988), sardines (Cho et al., 1989), and herring (Slabyj and Hultin, 1982), indicates that one of the benefits with precooking, that is, enzyme inactivation, is lost when this process is combined with washing. In accordance with this, Figures 4 and 5 show that the response minima obtained *without* washing (A panels) are lower than those obtained *with* washing (B panels) for A_{234} and FP. The crude mince extracts still had 60% of their pro-oxidative activity left at 100 °C, whereas the washed mince extracts retained only 48% activity after heating at this temperature. The latter observation may be due to a heat-induced formation of antioxidants or to the presence of enzymes more heat stable than those discussed above. With regard to the heat-induced response of hemoproteins, it can be seen in Table 4 that pure hemoglobin decreased its activity to 62% at 55 °C and to 36% at 100 °C, whereas pure myoglobin successively increased its activity during heating by 56% at 55 °C and by 78% at 100 °C. The observed behavior of hemoglobin may be related to decreases in heme exposure or quasi-lipoxygenase activity (Kühn et al., 1981), whereas that of myoglobin may be explained by Fe release (Rhee, 1988), conversion of metmyoglobin into a porphyrin cationic radical (Harel and Kanner, 1985), or increased heme group exposure (Cho et al., 1989). Most likely, the opposite ways in which hemoglobin and myoglobin responded to heat originate in structural differences between the two hemoproteins, for example, differences in size and heme group localization.

Finally, as was discussed in relation to washing, an acceleration of lipid oxidation reactions during the cooking process itself should not be excluded. This acceleration, originating in a general thermal increase in reaction rates (Mielche and Bertelsen, 1993), would then be more pronounced when high time−temperature settings are used and would thus, due, for example, to antioxidant consumption, impair the subsequent storage

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stability. The occurrence of such accelerations stresses an important difference between the present in situ and the in vitro experiments.

The present results point out that while a great benefit from precooking is inactivation of pro-oxidative enzymes, the inactivation of antioxidants, the activation of hemoproteins, and the general thermal acceleration of lipid oxidation negatively influence the lipid stability of herring mince subjected to cooking. It seems possible, though, to limit these side reactions by precooking at temperatures <60 °C. At this temperature, and especially when using low time settings, the precooked mince had a better oxidative stability than uncooked mince. Thus, under these mild conditions, it appears that a great part of the aqueous catalyzing enzymes in fish are inactivated without simultaneous denaturation of hemoproteins.

ABBREVIATIONS USED

a, number of analyses of each sample preparation; A_{234}, absorption at 234 nm; A_{268}, absorption at 268 nm; AAS, atomic absorption spectrophotometry; FFA, free fatty acids; FIA, flow injection analysis; FP, lipid soluble fluorescent lipid oxidation products; GSH-px, glutathione peroxidase; HDPE, high-density polyethylene; HPLC, high-performance liquid chromatography; IDF, International Dairy Federation; LAE, linoleic acid emulsion; LMW, low molecular weight; mequiv, milliequivalents; *n*, number of sample preparations; NL, neutral lipids; p.a., pro analysi; PL, phospholipids; PLS, partial least-squares regression analysis; PV, peroxide value; Q^2, fraction of the response variation that can be predicted by the PLS model; R^2, fraction of response variation explained by the PLS model; RSD%, relative standard deviation; SD, standard deviation; TBARS, thiobarbituric reactive substances test; te, precooking temperature; ti, precooking time; wa, washing; WW, washing water.

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