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Preventing Lipid Oxidation during Recovery of Functional Proteins from Herring (*Clupea harengus*) Fillets by an Acid Solubilization Process

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It has previously been found that a process based on solubilization at pH 2.7 gives high yields of herring muscle proteins with good functionality. In this study, the development of lipid oxidation during acid processing of herring mince was studied. It was tested how modifications of the process conditions and/or additions of antioxidants could prevent lipid oxidation during the actual process and then during ice storage of the protein isolates. Processing parameters evaluated were prewash of the mince, exposure time to pH 2.7, inclusion or exclusion of a high-speed centrifugation, and addition of antioxidants. Antioxidants tested were erythorbate (0.2%, 9.3 mM), sodium tripolyphosphate (STPP; 0.2%, 5.4 mM), ethylenediaminetetraacetic acid (EDTA; 0.044%, 1.5 mM), and milk proteins (4%). The first three antioxidants were added in the prewash or during the homogenization step, whereas milk proteins were added to the final precipitate. At time 0, all isolates were analyzed for pH, moisture content, and thiobarbituric reactive substances (TBARS). Selected isolates were also analyzed for lipid and protein content. Stability during ice storage was followed in terms of odor, TBARS, and color (*a**/*b** values). Extensive lipid oxidation took place using the "control" process without high-speed centrifugation. This was not significantly ($p \leq 0.05$) affected by a prewash or varied exposure time to pH 2.7. Including high-speed centrifugation (20 min, 10000*g*) significantly ($p \leq 0.05$) reduced TBARS values, total lipids, *a** values and *b** values. Erythorbate alone, or in combination with STPP/EDTA, significantly ($p \leq 0.05$) reduced lipid oxidation during processing if added in the prewash or homogenization step. During ice storage, better stability was gained when antioxidants were added in both of these steps and when EDTA was used instead of STPP.

KEYWORDS: Functional; proteins; isolation; herring; *Clupea harengus*; solubilization; antioxidants; acid; lipids; oxidation; EDTA; STPP; erythorbate; TBARS

INTRODUCTION

Approximately 90% of the estimated 2.5 million metric tons of herring that is captured is directed toward nonhuman consumption as fish-meal and fertilizers. Isolation of pelagic fish proteins for food production would be a more responsible way of using a nutritious and abundant raw material. However, the seasonality, small size, and unfavorable composition of herring have made it difficult to extract stable and functional proteins for food applications (*1*, *2*).

Recently, new procedures were developed to recover functional proteins from low-value muscle sources such as herring (*3*−*6*). The principle is that minced muscle is homogenized with water (1:9) after which the muscle proteins are solubilized at low pH (≤3) or high pH (≥10.5). Oil and insoluble materials such as skin, bones, and, under favorable circumstances, even cellular membranes can be separated from the solubilized proteins by centrifugation. The soluble proteins, forming a supernatant between the floating oil layer and the sediment, are precipitated at pH 5.5 and then recovered by a second centrifugation. Using light muscle from herring caught along the coast of New England (*7*), 74 and 68% of the total proteins were recovered with a laboratory-scale acid and alkaline process, respectively. The acid- and alkali-produced proteins had good gelling capacity and color. Seventy percent of the muscle lipids and 50% of the phospholipids were removed, giving isolates with 0.04 and 0.02 g of total lipid and phospholipids/g of protein, respectively. Others have obtained total lipid levels down to

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0.007 g/g of protein (*4*, *8*, *9*) when subjecting acidified or alkalized fish muscle to high-speed centrifugation at 10000−127000*g*.

Due to the strongly pro-oxidative hemoglobin (Hb) present in fish (*10*, *11*), lipid oxidation can develop in fish muscle even having very low lipid contents (down to 0.1% wet weight) (*12*, *13*), particularly if the Hb is deoxygenated and/or oxidized (*14*). Deoxygenation and autoxidation are both accelerated by low pH, which is also true for a series of other changes that make Hb more pro-oxidative: subunit dissociation, unfolding (including heme-crevice opening), and/or heme group/Fe liberation (*15*). Thus, unless there is a 100% removal of lipids, the acid process can be critical from a lipid oxidation perspective. This was recently confirmed when using some tropical species of fish (catfish, tilapia, mullet, and Spanish mackerel) (*16*).

Among ways that could theoretically be used to overcome oxidation problems caused by Hb, one is to reduce the level of hemoproteins in the mince, for example, via the introduction of a prewash. Other ways are to reduce the exposure time to low pH and to add antioxidants. On the basis of previous findings (*17*, *18*), antioxidants should be added *early* in the process and should inhibit the pro-oxidative properties of both low molecular weight (LMW) Fe^{2+} and heme-bound Fe^{3+}. The latter can, for example, be an efficient lipid hydroperoxide decomposer (*19*). Good results have also been obtained with compounds that can scavenge radicals and/or volatile oxidation products (*20*). It is also of high interest to evaluate to what extent the high-speed centrifugation of acidified muscle affects the oxidative stability of the protein isolate, particularly from an economical aspect.

The aim of this work was to evaluate how the oxidative status (thiobarbituric reactive substances, TBARS) of herring protein precipitates made in a laboratory-scale process was affected by (i) prewashing herring mince, (ii) varying the exposure time to low pH, (iii) including or excluding a high-speed centrifugation step to lower lipids, and (iv) adding antioxidants [erythorbate, sodium tripolyphosphate (STPP), ethylenediaminetetraacetic acid (EDTA), and milk proteins] at various stages of the acid process (prewash, homogenization, or final precipitate). Using selected isolates, information on protein yields and lipid removal was also to be gathered. During ice storage of selected isolates, changes in odor, TBARS, and color were also followed.

MATERIALS AND METHODS

Herring Raw Material. Herring (*Clupea harengus*) was caught by local fishermen in Kattegat in November 2002. The fish were landed and filleted into "butterfly fillets" in Ellös, Sweden, and transported on ice to our laboratory (2 h). The estimated post-mortem age at the time of receiving the fish was ∼36 h: 12 h as whole fish and 24 h as filleted fish. The herring fillets were manually skinned and ground using a kitchen grinder (grind size of 4.5 mm), Ultra Power, model KSM90, KitchenAid, St. Joseph, MI). The mince was then divided into 120 g portions, which were "flattened out" to 10−15 mm thickness in zip-lock plastic bags and frozen at −80 °C. The average weight and length of the fillets were 17.2 ± 2 g and 12.3 ± 1.05 cm (mean ± STD, $n = 9$), respectively. The mince had a pH of 6.6 and a moisture content of 72%.

Chemicals. Erythorbate (isoascorbic acid), 98% purity, was from Acros Organics (Fair Lawn, NJ). STPP, practical grade (90−95% purity), was from Sigma-Aldrich (St. Louis, MO). EDTA, >99% purity, was from Fluka (Neu-Ulm, Switzerland). Milk protein concentrate was from NZMP Germany (Alapro 4560, Rellingen, Germany).

Acid Isolation of Proteins from Herring. The acid protein isolation process used is schematically outlined in **Figure 1**. Frozen herring mince (120 g portions) was thawed for 30 min under cold running water before it was subjected to isolation of proteins using the acid process. In the process here regarded as "control", that is, the process having the simplest conditions (see sample 1 in **Table 1**), the thawed herring mince was homogenized for 1 min with 9 volumes of ice-cold distilled water (DW) using an Ultra Turrax homogenizer (Ultra Turrax, IKA Werks, Intermed Labassco, speed 4.5). Proteins in the homogenate were solubilized by dropwise addition of 2 M HCl until a pH of 2.7 was reached. The protein suspension was then held at pH 2.7 for 75 min (the time required to complete the high-speed centrifugation process below), after which the solubilized proteins were precipitated by adjusting the pH to 5.5 using 1 or 2 M NaOH. The precipitated proteins were collected via centrifugation at 6000*g* (20 min, 4 °C). **Table 1** shows how the abovementioned process conditions were varied in order to evaluate how this would affect the level of lipid oxidation products (TBARS) in the final isolates. When isolate 2 was made, the acidified homogenate was centrifuged at 10000*g* (20 min, 4 °C) in a Sorval Superspeed centrifuge (model RC-2B, Instrument AB Lambda, Stockholm, Sweden) using an SLA-1500 rotor (sample 2). The supernatant of the centrifuged homogenate was separated from the floating oil layer by filtering these two phases through double cheesecloth. The floating layer and bottom sediment were weighed and then discarded. The supernatant was then taken for precipitation at pH 5.5 as described above. For isolates 3 and 4, the holding time at pH 2.7 was reduced from 75 min to 4 and 30 min. For isolates 5 and 6, the herring mince was first prewashed with 3 volumes of ice-cold DW (1 min of stirring, 15 min of leaching, followed by dewatering in a sieve). For isolates 7 and 8, the prewashing of mince was made in DW with 0.24% (6.5 mM) STPP and 0.24% (11.2 mM) erythorbate added (w/w). For isolates 7, 9, and 10, 0.2% (9.3 mM) erythorbate and 0.2% (5.4 mM) STPP were added into the 1080 mL of homogenization water. For

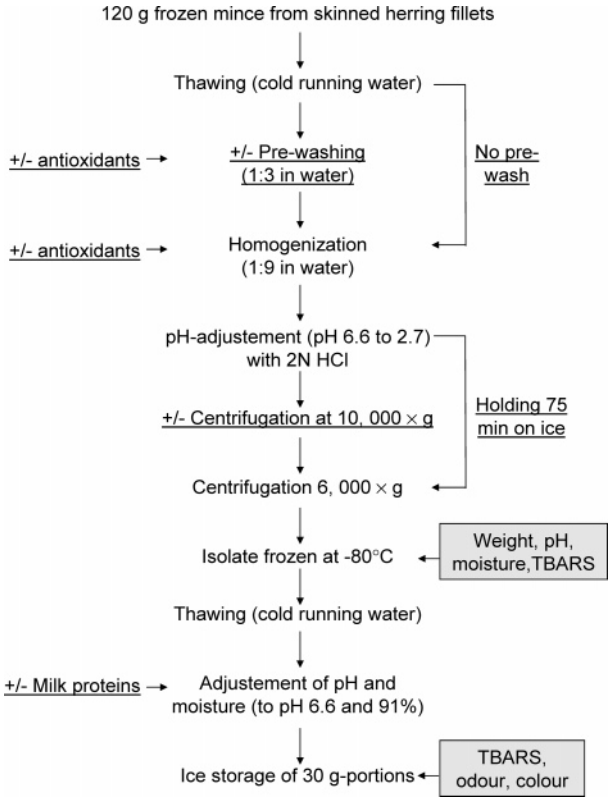

Figure 1. Schematic outline of the acid protein isolation process. Underlined steps were included or excluded according to **Table 1**. When used, antioxidants were added directly into the prewash water or homogenization water and consisted of erythorbate (0.2%, 9.3 mM) ± sodium tripolyphosphate (STPP; 0.2%, 5.4 mM) or ethylenediaminetetraacetic acid (EDTA; 0.044%, 1.5 mM). Milk proteins [4% (w/w)] were mixed into the protein isolate prior to ice storage. Gray boxes show at what stages samples were taken for analyses of weight, pH, moisture content, TBARS, odor, and color.

Table 1. Details about the Conditions Used To Produce Protein Isolates 1–16, Which Were Then Evaluated for Basic Composition and TBARS[a]

isolate	prewash	Ao in homogenization	HS-centr	time (min) at pH 2.7	milk protein	pH	Mc (%)	TBARS (μmol of MDA equiv/kg)	total yield (%, dw basis)
1	no	no	no	75		4.6	86	49.5 ± 0.1	78
2	no	no	yes	75		4.7	87	25.7 ± 1.6	58
3	yes	no	no	75		4.8	88	41.8 ± 1.2	62
4	yes	no	yes	75		4.7	91	24 ± 0.65	34
5	no	no	no	4		4.5	84	55.8 ± 13	76
6	no	no	no	30		4.7	85	62.2 ± 11	80
7	yes (+ Ao)	Ery + STPP[b]	no	75		4.9	76.5	4.9 ± 0.56	55
8	tes (+ Ao)	no	no	75		4.2	87	3.2 ± 1.3	61
9	no	Ery + STPP	no	75		4.8	80	5.4 ± 0.69	72
10	no	Ery + STPP	yes	75		4.6	90	5.8 ± 1.5	9
11	no	Ery	no	75		4.3	87	9.5 ± 0.7	80
12	no	Ery + EDTA	yes	75		4.7	90	4.6 ± 0.9	41
13	no	Ery + EDTA	no	75		4.4	86	6.8 ± 0.4	83
14	no	Ery + STPP[b]	yes	75		5	87	8.7 ± 0.9	49
15	no	no	no	75	yes	4.6	83	49.5 ± 0.1	78
16	no	Ery + EDTA	no	75	yes	4.4	83	6.8 ± 0.4	83

[a] An outline of the process is shown in **Figure 1**. Most of these isolates were also used in the subsequent ice storage trial. The prewash was done with 3 volumes of water (± antioxidants, Ao). When Ao [0.2% erythorbate (Ery) ± 0.2% STPP or 0.044% EDTA] were added in the homogenization step, they were predissolved in the 9 volumes of cold water. The high-speed centrifugation (HS-centr) was done for 20 min at 10000*g*, 4 °C. The holding time at pH 2.7 was varied from 4 to 75 min by keeping the homogenate on ice. Four percent Alapro milk proteins were added to two of the isolates (1 and 13) *after* processing, thus, right before starting the ice storage. The pH values, moisture contents (Mc), TBARS values, and isolate yields shown are those obtained right after processing. The minced herring used in the process had pH 6.6, 72% moisture, and 8 μmol of MDA/kg. [b] STPP was added after the high-speed centrifugation.

isolate 11, 0.2% erythorbate was added alone into the 1080 mL of homogenization water. For isolates 12 and 13, 0.044% (1.5 mM) EDTA and 0.2% erythorbate were added into the 1080 mL of homogenization water. For isolate 14, 0.2% erythorbate was added during the homogenization step, whereas 0.2% STPP was added into the supernatant from high-speed centrifugation. In all cases, the final protein isolate was weighed and tested for pH, moisture, and TBARS before freezing it in 30 g portions at −80 °C.

Total Lipid Analyses. Total lipids were analyzed in the mince, in the prewashed mince, and in isolates 1−4. The method used was from Lee et al. (*21*) with chloroform and methanol (1:1) as the extraction solvent. Results are expressed as percent on a wet weight and dry weight basis.

Quantification of Total Proteins. When samples 1 and 2 were produced, total proteins were measured on the acidified homogenate, the first supernatant, and the second supernatant. It was also done on the prewash water obtained in process 3−4. Measurements were done according to the method of Lowry et al. (*22*) as modified by Markwell et al. (*23*). Protein solubility (percent) at pH 2.7 and 5.5 was expressed according to the following formula: (protein concentration in supernatant after centrifugation/protein concentration in homogenate before centrifugation) × 100.

Quantification of Hemoglobin (Hb). Hemoglobin (Hb) was extracted from unwashed and prewashed herring mince. Four grams of mince was homogenized for 30 s in 50 mL of cold buffer (10 mM Tris, 1 mM EDTA, 80 mM KCl, pH 8) (*24*) using an Ultra Turrax homogenizer (Ultra Turrax, IKA Werks, Intermed Labassco, speed 4.5). The homogenate was centrifuged at 37000*g* for 40 min at 4 °C after which the supernatant was filtered through a Whatman no. 1 filter. Quantification of Hb levels was done according to the method of Brown (*25*) as modified by Richards and Hultin (*26*). Standard curve was made with bovine Hb.

Analysis of Moisture Content and pH. The moisture content of mince and isolates was measured using an HA300 moisture balance (Precisa balance 310M). The pH was recorded with an Orion PerpHect Sure-Flow electrode (Orion Research Inc., Beverly, MA) in conjunction with a pH-meter (model MA235, Mettler Toledo, New York). To analyze the pH in solid samples, they were stirred manually with 9 parts of Milli-Q water.

Ice Storage of Protein Isolates. To study the effect of ice storage on lipid oxidation, the frozen protein precipitates were thawed under cold running water for 5 min and adjusted from their original moisture (see **Figure 1** and **Table 1**) to 91% moisture with ice-cold DW. Also, the pH was adjusted from the original pH of the isolates (pH 4.2−5) (see **Table 1**) to 6.55 by dropwise addition of 1 and 0.1 N NaOH. To prevent bacterial growth, streptomycin (200 ppm on a moisture basis) was added to all isolates under manual stirring with a stainless steel spatula. Two samples, one made without antioxidant (sample 1) and one with antioxidants (sample 13) (**Table 1**), were at this stage fortified with 4% (w/w) milk proteins (samples 15 and 16). Twenty-five grams of each of the 16 protein samples was then flattened out in the bottom of screw-capped 250 mL glass Erlenmeyer flasks and stored on ice for up to 17 days. The sample thickness was ∼6 mm. At regular intervals during the storage, the samples were analyzed for changes in odor, color, and TBARS (see below). To limit the number of samples for sensory analyses, three isolates were excluded from the ice storage trial. These were the samples for which exposure time to pH 2.7 had been shortened without effect (samples 5 and 6) and the one where STPP had been added during the homogenization (sample 10), which largely reduced recovery.

Sensory Analysis. During the first sensory session, seven trained assessors from an analytical sensory panel were trained how to evaluate odor attributes of the herring protein isolates. The attributes were four odors described as “painty”, “ocean”, “fishy”, and “mineral”. The assessors were also trained in how to utilize a 100 mm long continuous scale. The scale was anchored with low and high intensity at 10 and 90 mm, respectively, from the left end. During the storage trial, the assessors sniffed the headspace above the 17 samples by uncapping the 225 mL screw-capped Erlenmeyer flasks. The sample set was split into three sets of five or six sample bottles each, and the panelists were rested for 45 min between the evaluation of each set. The panelists shared the same sample sets for sensory evaluation. The sample flasks were therefore held on ice for 1 h between each panelist so that the volatiles could reach a new equilibrium between the herring proteins and the headspace above. The evaluations took place once a day during days 1−6 and then on days 7, 9, 11, 14, and 17.

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

TBARS Analyses. TBARS were analyzed in the herring mince, prewashed mince, and all isolates shown in **Table 1**. During the ice storage trial, 1-g sample "plugs" were taken from the precipitates for TBARS analyses using a hollow cylinder. In this way, the sample plugs had a constant surface-to-volume ratio. The plugs were frozen in aluminum foil at −80 °C until the entire ice-storage trial was completed. TBARS were analyzed according to the method of Lemon et al. (*27*), after the extraction of the 1 g sample with 6 mL of trichloroacetic acid solution (7.5% TCA, 0.1% propyl gallate, and 0.1% EDTA in Milli-Q water). The standard curve was made fom malondialdehyde (MDA), and results are expressed as micromoles of MDA equivalents per kilogram of wet isolate.

Replication and Statistics. The process used to produce samples 1 and 2 (**Table 1**) was run five times in total. Two of the five times, samples were taken for calculation of protein recovery and lipid removal in the different steps. The other 12 "versions" of the acid process (3−14, **Table 1**) were run twice, with the exception of processes 5 and 6, which were run only once. Duplicate samples ($n = 2$) from all isolates were analyzed for moisture, pH, proteins, and TBARS. Selected samples were also analyzed for total lipids. All analyses were made in duplicate ($a = 2$). An average of the two analyses was used for calculations of sample-to-sample variations or shown as is.

During the ice storage trial, the use of sensory analysis limited the possible number of samples to 18 (3 sets with 6 samples each). To still get an estimate of the sample-to-sample variation, we chose to make triplicate sample bottles ($n = 3$) of sample 1, whereas the others were stored as single samples ($n = 1$). Standard deviations (SD) from the triplicated sample were calculated using (Excel 2000, Microsoft Corp., Seattle, WA), and are expressed as coefficient of variance (percent). To find whether the analyzed characteristics of the samples differed significantly before and during the storage, ANOVAs were applied on the resulting data. Two-way ANOVAs with samples and assessors as fixed effects were used on the sensory data. One-way ANOVAs were applied on color data (*a* and *b* values) and on TBARS data with samples or groups of samples as fixed factors. Tukey's multiple-comparison test was performed on attributes for which effect-significant differences were found in ANOVA (SYSTAT 10.0, SPSS Inc.). Differences between samples, or groups of samples, are regarded as significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

To date, several studies have been published on the use of acid or alkaline solubilization techniques to extract fish muscle proteins. The species tested include herring (*7*), Pacific whiting (*28*), jack mackerel, white croaker (*29*, *30*), catfish, tilapia, mullet, and Spanish mackerel (*16*). To date, the primary focuses have been on yield and functionality of the resulting proteins. When using dark muscle fish species such as mackerel or herring, an important aspect to consider is, however, the development of lipid oxidation during acid and alkaline processing. The pH cycle through which the proteins are taken is critical, for example, for the activation of muscle-derived pro-oxidants, especially the heme-proteins. We have here evaluated the development of lipid oxidation during acid extraction of proteins from frozen herring mince. Strategies for reducing lipid oxidation have also been tested. Some of these strategies have been based only on changes in the process conditions (**Figure 1**; samples 1−6, **Table 1**), others on the addition of antioxidants at different stages (**Figure 1**; samples 7−16, **Table 1**).

Initially, some basic data on solubility, protein recovery, lipid removal, moisture content, and pH changes from the processes used are given and discussed. Thereafter, development of lipid oxidation during the actual processing and during subsequent frozen storage are highlighted.

Solubility and Recovery of Proteins during Acid Processing of Herring. Solubility and recovery of proteins were calculated when using processes 1 and 2 (**Table 1**), that is, no prewash or antioxidant additions, but with and without the high-speed centrifugation (**Figure 1**). For the other processes, only the final weight of the isolate was recorded. It is expressed on a dry weight (dw) basis as weight percent of the starting material (see **Table 1**).

When the herring mince homogenate was brought from its original pH 6.6 to pH 2.7, 84.3% of the proteins were solubilized. When the pH was raised to 5.5, without applying a high-speed centrifugation at 10000*g* (sample 1), the protein solubility was reduced to 3.1%. Ninety-seven percent of the proteins were then recovered in the centrifugation at 6000*g*. When the acidified homogenate (pH 2.7) was subjected to a high-speed centrifugation at 10000*g* (sample 2), 4.7 and 9% (w/w) of the first supernatant was trapped into the sediment and floating layer, respectively, whereas only 72.5% of the initial proteins could be recovered. When the pH of the collected supernatant was raised from 2.7 to 5.5, the protein solubility was reduced from 84.3 to 4.5%. The recovery of proteins in the second centrifugation at 6000*g* was 96.1%. Also in this step, some supernatant was trapped in the sediment. The total recovery of sample 2 hereby became 69.7% (0.725×0.961).

The recovery of total isolate (i.e., *total* weight, not only proteins) was 78 and 58% when the high-speed centrifugation was excluded and included, respectively (samples 1 and 2). For the other processes (3−14, **Table 1**), the recovery of total isolate varied from 9 to 58% and from 55 to 83% in the presence and absence of high-speed centrifugation, respectively. Thus, lower recoveries when using high-speed centrifugation have to be weighed against the higher purity obtained (see below). The lowest isolate recovery of all, 9%, was obtained when 0.2% erythorbate and STPP was added in the homogenization step after which the homogenate was subject to high-speed centrifugation. The solubility of proteins at pH 2.7 was severely reduced in the presence of 0.2% STPP, so that very little proteins could be recovered in the first supernatant. Similarly, it was found previously that 10 mM pyrophosphate reduced the solubility of herring light muscle proteins from 76 to 20% at pH 2.7 (unpublished data). STPP easily undergoes hydrolysis to, for example, pyro- and monophosphate (*31*). Pyrophosphate can, just like ATP, dissociate actomyosin (*32*), which could lower the solubility of the actin. In this study, the problem of reduced solubility was circumstanced by first adding 0.2% erythorbate alone in the homogenization and then 0.2% STPP *after* the high-speed centrifugation (sample 14). The recovery of isolate was then 49%. However, some protection against oxidation during acidification/centrifugation was then lost as erythorbate was not as efficient alone as when combined with a chelator (see below and **Figure 2B**). It should be noted here that the different processes (1−14) were run primarily in a qualitative manner; thus, the weight data should be used only for rough comparisons of yields among the different process versions.

Lipid Removal, Moisture Content, and pH Changes during Prewashing and Acid Processing. The original lipid content of the mince was 22.5% on a dw basis [6.1% on a wet weight (ww) basis], which was reduced by 39% in the prewash with water. When unwashed and prewashed minces were processed *with* high-speed centrifugation (samples 2 and 4, **Table 1**), 51 and 56%, respectively, of the lipids in the mince were removed, and the isolates obtained contained 11 and 10%, respectively, on a dw basis (1.3 and 0.9%, respectively, on a ww basis). In cases when unwashed and prewashed minces were processed without the high-speed centrifugation (samples 1 and 3), the lipid content of isolates became 33.2 and 32.2%, respectively, on a dw basis (4.3 and 3.9%, respectively, on a

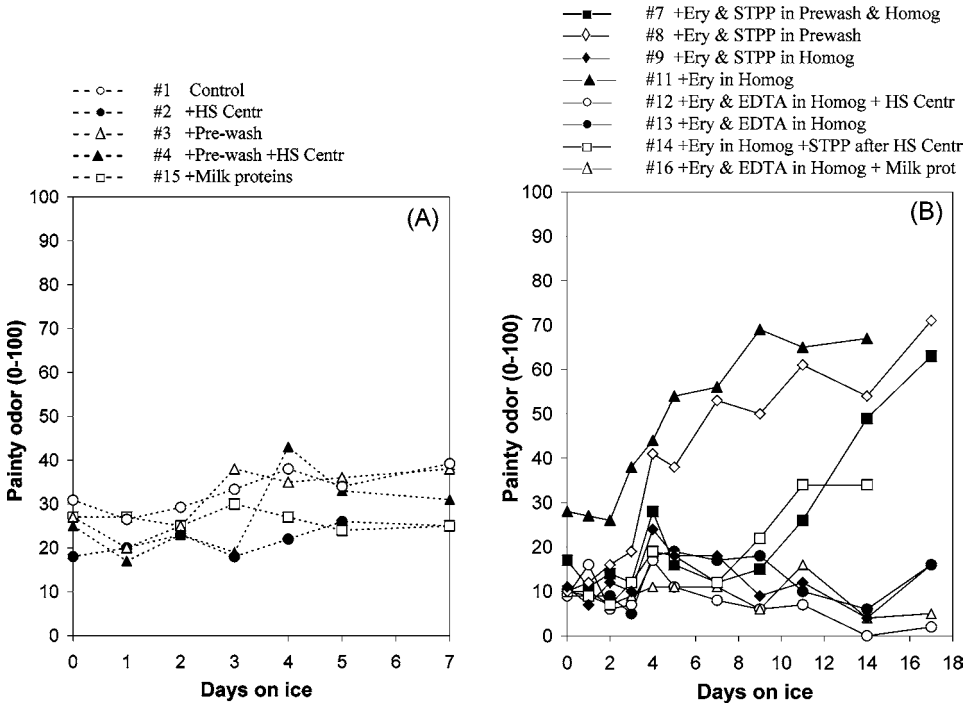

Figure 2. Development of "painty" odor during ice storage of the protein isolates. Panel **A** shows isolates with the process conditions varied, but without antioxidants added *during* processing (samples 1–4 and 15, **Table 1**). No significant differences ($p \leq 0.05$) were present between samples or between storage points for the same sample. Panel **B** shows isolates produced in the presence of antioxidants (samples 7–9 and 11–14, **Table 1**). Samples 9, 12, 13, and 16 developed significantly less ($p \leq 0.05$) painty odor than the other samples. All isolates were adjusted to 91% moisture and pH 6.55. Streptomycin (200 ppm) was also added. Two samples (15 and 16, **Table 1**) were fortified with 4% (w/w) milk proteins. Details about the storage conditions and sensory evaluation are given under Materials and Methods. HS Centr, high-speed centrifugation (10000*g*, 20 min at 4 °C); prewash, prewashing of the herring mince (1:3) in cold distilled water with/without added antioxidants; Homog, homogenization of the mince (1:9) with cold distilled water with/without added antioxidants; Ery, erythorbate (0.2%, 9.3 mM); STPP, sodium tripolyphosphate (0.2%, 5.4 mM); EDTA, ethylenediaminetetraacetic acid (0.044%, 1.5 mM).

ww basis). Thus, on the dw basis, the lipid content of isolates made without high-speed centrifugation *increased* by 47 and 44% compared to the whole mince, which was because more proteins than lipids were removed. It was previously shown (*7*) that a high-speed centrifugation step (18000*g*, 20 min) reduced the total lipid and phospholipid contents in acidified herring light muscle by 70 and 45%, respectively (expressed on protein basis).

The moisture content increased slightly during prewashing, from 73 to 80 and 76% with only water and with an antioxidant mix (erythorbate + STPP), respectively. Erythorbate slightly reduced the pH from 6.6 to 5.9, which may have been the reason for lower water holding of the proteins. In the final isolates, the moisture ranged from 77 to 91% (**Table 1**). Usually the isolates subjected to high-speed centrifugation had moisture contents a few percentage points higher than the noncentrifuged ones.

Although the precipitation was made at pH 5.5, the final pH of the isolates recovered after centrifugation at 6000*g* was usually lower than this, pH 4.3−5. There was a tendency that slightly higher pH values were obtained in isolates containing STPP (**Table 1**). Generally, the reduction in pH may be due to poor buffering capacity of the isolates around pH 5.5 after extensive dilution of naturally buffering compounds of the herring muscle.

Development of TBARS during Processing. The initial level of TBARS in the herring mince was 9.3 ± 1.9 μmol of MDA equiv/kg (mean ± SD, $n = 3$). After prewashing of the mince with distilled water or antioxidants, the TBARS were 6.3 and 8.2 μmol of MDA equiv/kg of sample, respectively.

Table 1 shows TBARS of acid-produced proteins from unwashed and prewashed minces without additions of antioxidants (samples 1−6) and with antioxidants added (samples 7−16). Isolates made without antioxidants had increased significantly ($p \leq 0.05$) in TBARS during the processing; they contained ~49 and 25 μmol of MDA equiv/kg when the high-speed centrifugation at 10000*g* was excluded and included, respectively. That high-speed centrifugation lowered TBARS in the isolates by 38% could be because the TBA-reacting substances themselves and/or compounds contributing to their formation were removed into the floating oil layer and membrane-containing sediment. Shortening of the exposure at pH 2.7 to 4 or 30 min (samples 5 and 6, **Table 1**) still gave high TBARS, 55 and 62 μmol of MDA equiv/kg of sample, respectively.

It is believed that the extensive and fast lipid oxidation that took place during the acid process in the absence of antioxidants most likely was due to the pro-oxidative action of acid-activated hemoproteins (*11*, *13*, *26*) (**Table 1**). When the pH was lowered from 6.6 to 2.5, it was shown that fish hemoglobins are converted to the more catalytically active deoxy and met forms (*33*, *34*). It has been suggested (*35*) that deoxy-Hb acts as a stronger oxidation catalyst than oxy-Hb because it has a heme crevice that is more accessible (*36*). The iron atom inside the crevice is also kicked out of the plane of the porphyrin group when deoxygenation occurs (*37*). This allows the iron to more easily interact with lipid hydroperoxides and, for example, hydrogen peroxide, creating more free radicals to facilitate both lipid oxidation and Hb autoxidation. Heme autoxidation can influence lipid oxidation by giving rise to oxygen radicals ($O_2^{\bullet -}$, $^{\bullet}OH$) (*12*), protein radicals [e.g., hypervalent ferryl-

hemoglobin (Fe^{4+}=O)] (*38*), and lipid radicals (LOO• and LO•) (*39*). Acid can also catalyze hemoglobin dissociation (*40*), which further accelerates autoxidation (*41*).

The unfolding and pro-oxidative activities of trout (*42*) and flounder (*34*) oxy-Hb were evaluated in great detail between pH 7.5 and 1.5. The major unfolding took place between pH 3.5 and 4.5, and the protein was maximally unfolded at pH 2.5. Concurrently with the unfolding, the pro-oxidative activity increased when tested in a linoleic acid emulsion (*34*) and in a washed cod mince model (*43*). In the cod mince model, trout Hb (5.8 μM) that had been exposed to pH 2.5, 3, 3.5, and 7 gave rise to TBARS development after 0, 0, 5, and 25 h, respectively, at 5 °C. It was further found that Hb unfolding caused increased surface hydrophobicity, which could facilitate interaction with lipids. There was a loss of contact between the highly hydrophobic heme group and the proximal histidine upon reduced pH, which could make the heme more detachable and partition into the nonpolar phase of membranes (*43*).

Kristinsson and Hultin (*42*) found that when neutralizing trout Hb that had been acidified to pH 1.5, recovery of native structure was proportional to the extent of unfolding occurring during the acidification. The less refolded the Hb was, the more hydrophobic it was. In the present study, there was no difference between 4, 30, or 75 min of exposure of the herring homogenate to pH 2.7 on the degree of lipid oxidation (**Table 1**). In agreement with this, there was no difference in the pro-oxidative activity between trout Hb that had been exposed to pH 2.5 for either 9 s or 20 min before adjustment to pH 7 (*42*).

Extreme acidification can also affect the susceptibility of the muscle itself to oxidation (*43*). Washed cod mince subjected to pH 2.5 for 20 min oxidized more quickly than native cod in the presence of Hb. When preacidified Hb was incubated with native and with preacidified washed cod, more and faster oxidation developed with the native washed cod. Thus, it was concluded that acid-induced damage to the Hb was more critical for lipid oxidation than acid-induced changes of the mince (*43*).

Washing of fish mince, such as in conventional surimi-making, has been a common strategy to remove water-soluble pro-oxidants (*17*, *44*). Here, the use of a 1:3 prewash in distilled water did not reduce oxidation during the process (**Table 1**), although it removed 50% of the total heme. Obviously, the 10 μM heme that remained in the washed mince was still enough to start lipid oxidation. This amount of trout Hb quickly caused oxidation in a washed cod mince model during ice storage at pH 6.5 (*45*). It was also seen previously that herring mince that had been prewashed (1:3) in 50 mM NaCl oxidized more quickly than unwashed herring mince during frozen storage, although 40% of the total iron was removed (*46*). When the wash water residual was tested in a linoleic acid emulsion (pH 6.8, 20 °C), a net antioxidative effect was found (*46*). It has also been found that an aqueous fraction of herring light muscle (press-juice, PJ) inhibited LMW-Fe-mediated oxidation of isolated herring microsomes (*47*). Thus, a likely reason for our results is that water-soluble antioxidants are removed together with pro-oxidants during a prewash, with the net effect from the former being dominating.

When 0.2% erythorbate and 0.2% STPP were added into the prewashing step (sample 8), TBARS of the final protein precipitate was significantly reduced ($p \leq 0.05$) to 3.2 μmol of MDA equiv/kg. This was despite the fact that less Hb was removed with the more acidic antioxidant solution (pH 5.9) compared with water alone (pH 6.6), 26 versus 50%. Increased adhesion of Hb to myofibrillar proteins/membranes after acid-induced denaturation may lower the efficiency of Hb removal. Adding the same antioxidant mix also into the homogenization step (sample 7) did not further improve the stability (TBARS was 5 μmol of MDA equiv/kg). Equally low TBARS (5.4−5.8 μmol of MDA equiv/kg) were obtained when the prewash step was excluded but 0.2% erythorbate and 0.2% STPP were added in the homogenization (samples 9 and 10).

When 0.2% erythorbate was added alone in the homogenization (sample 11), precipitates contained 9.5 μmol of MDA equiv/kg. This was significantly less ($p \leq 0.05$) than the corresponding isolate made without erythorbate (sample 1). There was no significant difference ($p \leq 0.05$) when 0.2% erythorbate was added alone in the homogenization, and 0.2% STPP was added *after* the high-speed centrifugation, that is, directly into the collected supernatant. When STPP was replaced by 1.5 mM EDTA in the homogenization step (samples 12 and 13), TBARS of the precipitate also became significantly lower ($p \leq 0.05$) than in the corresponding isolates made without antioxidants (1 and 2), 4.6 and 6.8 μmol of MDA equiv/kg, with and without the high-speed centrifugation, respectively.

These results show that the combination of a LMW-Fe^{2+} chelating agent and a heme-Fe^{3+} reducing agent efficiently prevented oxidation from taking place during acid protein isolation. The findings were in agreement with those of Kelleher et al. (*17*). If added during each of four washes, 0.2% sodium ascorbate and 1.5 mM EDTA efficiently prevented oxidation during conventional surimi-making from mackerel mince. Ascorbic acid at the level of 0.2% was ascribed dual effects as an antioxidant because it can both reduce heme-Fe^{3+} and scavenge free radicals in the aqueous phase (*17*). It is believed that the iso-form of ascorbate (erythorbate) possessed similar effects in our process and that this is the reason for its good stabilization.

Changes in Odor, TBARS, and Color during Ice Storage of Protein Isolates. It was evaluated how process modifications and addition of different antioxidants at various stages of the process affected the conditions for lipid oxidation to take place during subsequent ice storage. We particularly wished to elucidate the role of process-induced alterations in the activity and the level of pro-oxidants (e.g., Hb) and antioxidants. Therefore, it was decided to normalize other factors known to influence lipid oxidation, moisture (*48*) and pH (*33*, *34*). All isolates were adjusted to 91% moisture (the highest value obtained in the processes) and pH 6.55 ± 0.05, the value commonly found in postmortem herring tissue.

Changes in Odor. "Ocean", "mineral" "fishy", and "painty" odors were followed. There were no significant initial differences ($p \leq 0.05$) between the samples regarding ocean odor. However, in all samples, the intensities of this descriptor slowly fell over time. For half of the samples (1, 3, 4, 6, 9−11, and 14) this decrease was significant ($p \leq 0.05$). The intensity of the mineral odor did not change significantly ($p \leq 0.05$) in any sample over time, but samples with EDTA (12 and 13) had significantly higher ($p \leq 0.05$) mineral intensities than the others at several occasions. During the first 8 days, there were no differences between the samples regarding fishy odor. From day 9 to day 11, samples with antioxidants only in the prewash (8) or with erythorbate alone in homogenization (11) were significantly higher ($p \leq 0.05$) in fishy odor intensity than the others. In general, there were large variations in fishy odor, and the data were difficult to interpret. The descriptor discriminating the samples the most, in terms of both their processing history and storage time, was painty odor. This descriptor also co-varied the strongest with the TBARS values and was therefore chosen as the primary sensory indicator of oxidative changes during

ice storage. **Figure 2A** shows the painty odor intensities in samples produced without antioxidants, but where other parts of the processing had been varied. Sample 1 was also stored with 4% milk proteins added (15). In this period, there were no significant differences ($p \leq 0.05$) between the samples and also not over time. Between days 7 and 9 the painty odor increased significantly ($p \leq 0.05$) in sample 2 (data not shown). Between days 8 and 14, there was also a 2-fold increase in painty odor in sample 5. From the one sample being triplicated during storage (1), it was found that the coefficient of variation regarding assessment of painty odor in three individual samples of the same kind was between 6 and 18% during days 0−7.

Figure 2B shows all samples produced in the presence of antioxidants, one (sample 13) of which was stored also with 4% milk proteins (sample 16). All samples, except that with only erythorbate, had initial painty odor intensities of <20. Four of the samples stayed on intensities of <20 throughout the entire 17 days on ice. At the end of the storage, these four stable samples had intensities significantly lower ($p \leq 0.05$) than the samples made without antioxidants. Three of the stable samples were treated with erythorbate and EDTA in the homogenization step, with and without the inclusion of a high-speed centrifugation or milk protein addition (samples 12, 13, and 16). One sample had STPP instead of EDTA (sample 9). The sample with erythorbate and STPP added *both* in the prewash and in homogenization steps (sample 7) stayed on intensities of <20 for 9 days, but then increased to 62, which significantly differentiated ($p \leq 0.05$) this sample from the most stable samples. The sample to which STPP was added *after* the high-speed centrifugation (sample 14) behaved in a similar manner, but the increase after day 9 did not distinguish it significantly ($p \leq 0.05$) from the four stable samples. Samples to which erythorbate alone had been added in the homogenization step (sample 11) and for which erythorbate plus STPP had been added in the prewash but not in the homogenization (sample 8) reached intensities that were significantly higher ($p \leq 0.05$) than the other samples already after 5 days. At the end of the storage, their intensities were between 60 and 70. Thus, although antioxidant addition in the prewash alone was enough to obtain good stabilization during the process, it did not yield an isolate with good storage stability. Possibly the antioxidant levels became too low in the subsequent dilution during homogenization, or the net effect from losing natural antioxidants into the prewash water dominated.

Lee and Lian (*20*) reported that milk proteins can act both as radical scavengers and as sinks for volatile oxidation products. In this study, no effect could be seen from adding 4% milk proteins into one nonstabilized (sample 15) and one antioxidant-treated isolate (16) prior to storage. Corresponding samples without milk proteins (1 and 13) were either already too oxidized to change further during the ice storage or did not change at all during the storage (**Figure 2**). The antioxidative role of milk proteins will be evaluated further in samples that initially are low in oxidation products but that tend to oxidize during the storage.

Changes in TBARS. Selected samples were taken for TBARS analysis to relate the sensory changes to a chemical measure of lipid oxidation. As shown in **Figure 3**, all samples without antioxidants started off at relatively high TBARS values (25−60 μM MDA equiv/kg), sample 1 being significantly higher ($p \leq 0.05$) than the antioxidant-treated samples. All samples then declined somewhat during the storage. This decline indicated that lipid oxidation probably had "peaked" in the herring mince already during the acid processing. The sample

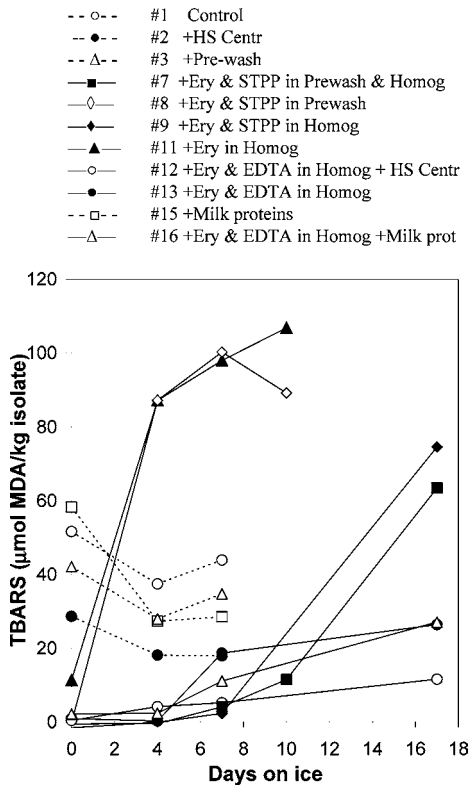

Figure 3. Development of TBARS during ice storage of protein isolates made with the process conditions varied and with/without antioxidants added. The development rate for TBARS grouped the samples in four significantly different groups ($p \leq 0.05$). The rate declined in the following order: samples 8 and 11 > samples 7 and 9 > samples 12, 13, and 16. Samples 1–3 and 15 instead decreased in TBARS over time. All isolates were adjusted to 91% moisture and pH 6.55. Streptomycin (200 ppm) was also added. Two samples (15 and 16, **Table 1**) were fortified with 4% (w/w) milk proteins. TBARS is expressed as micromoles of MDA equivalents per kilogram of isolate. Other details about the storage conditions and TBARS analyses are given under Materials and Methods. Abbreviations are explained in the caption of **Figure 2**.

that was only prewashed with antioxidants (8) and the sample to which erythorbate alone had been added in the homogenization (11) started off at low values, but during the first 4 days increased to values that were significantly higher ($p \leq 0.05$) than for the other antioxidant-treated samples (80 μmol MDA equiv/kg). After 7 days, samples with erythorbate and STPP in both prewash and homogenization (7) or in homogenization alone (9) obtained significantly higher values ($p \leq 0.05$) than the three most stable samples, those with erythorbate and EDTA (samples 12, 13, and 16). The latter did not change significantly ($p \leq 0.05$) during the 17 days on ice.

EDTA thus seemed to create a less pro-oxidative complex with Fe^{2+} than did STPP. Previous studies have given very conflicting data as to whether EDTA prevents or encourages lipid oxidation (*49*, *50*). Engelmann et al. (*51*) suggested that such confusion on EDTA comes from the fairly narrow "window" of ratios between LMW-Fe and EDTA where the EDTA-chelate is an efficient Fenton reagent and thus promotes oxidation. When ratios from 1:0 to 1:500 were tested at pH 7.4, Fenton activity was found for ratios between 1:0.25 and 1:2.5 (*49*). Herring mince contains 180 μmol of total Fe/kg in the aqueous phase (*46*); thus, the ratio of total Fe to EDTA was at least 1:8.3 in this study. However, as the mince contained 27 μmol of Hb/kg in the aqueous fraction, each Hb molecule having four iron atoms, a rough estimation of the

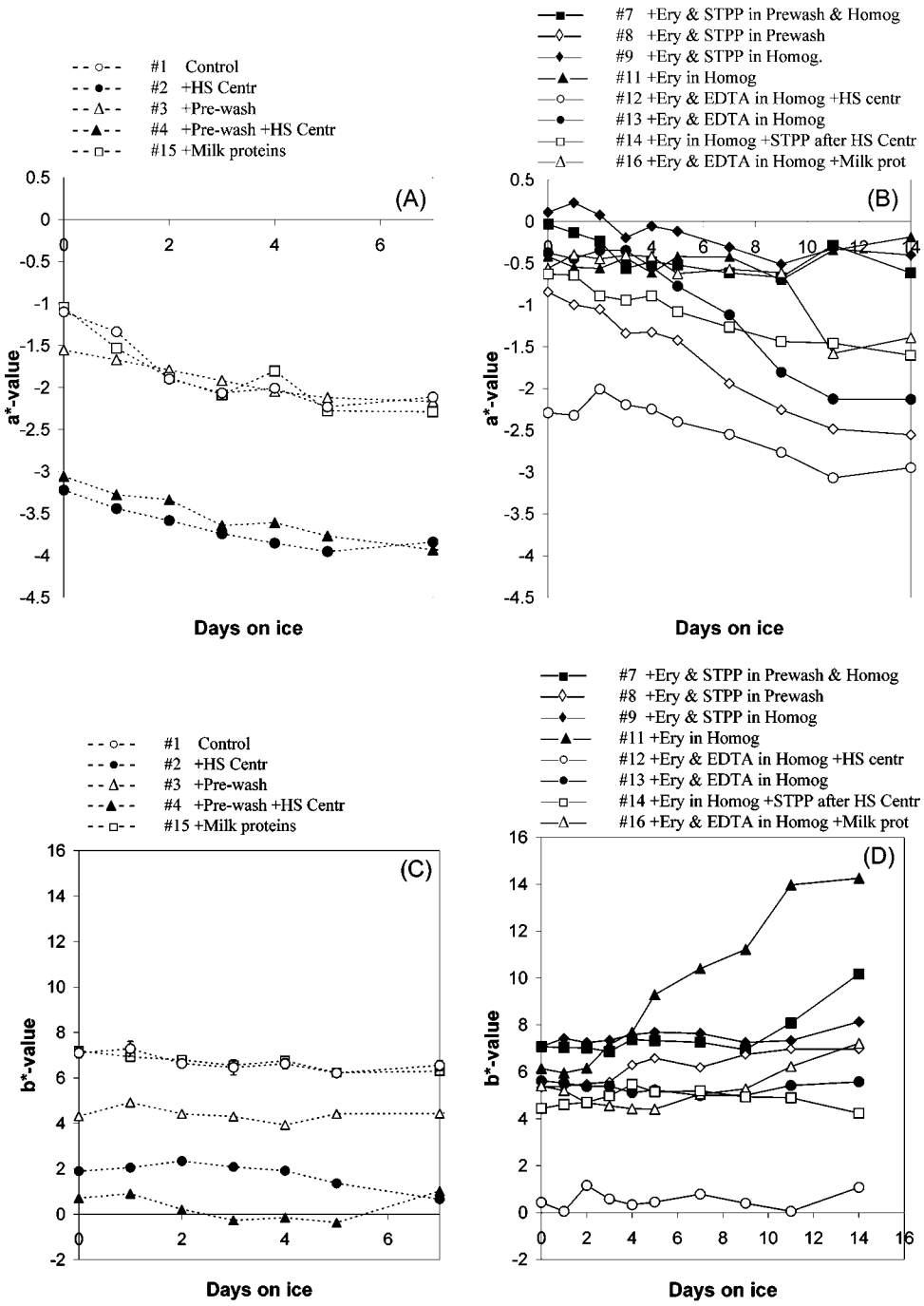

Figure 4. Changes in redness (*a** values) (panel **A**, **B**) and in yellowness (*b**) (panel **C**, **D**) during ice storage of the protein isolates. Panels **A** and **C** show isolates with the process conditions varied, but without antioxidants added *during* processing (samples 1–4 and 15, **Table 1**). Panels **B** and **D** show isolates produced in the presence of antioxidants (samples 7–9 and 11–14, **Table 1**). In panel **A**, significant differences ($p \leq 0.05$) were only found regarding absolute *a** values. Samples 1, 3, and 15 were higher than samples 2 and 4. In panel **B**, three sample groups differed significantly ($p \leq 0.05$) in their absolute *a** values and in their rate of *a** value loss: samples 7, 9, 11, 14, and 16 versus samples 8 and 13 versus sample 12. In panel **C** significant differences ($p \leq 0.05$) in absolute *b** values were found between samples 1, 3, and 15 versus samples 2 and 4. In panel **D**, sample 12 had significantly lower ($p \leq 0.05$) absolute *b** values than the others, and sample 11 increased significantly ($p \leq 0.05$) more quickly than the others over time. All isolates were adjusted to 91% moisture and pH 6.55. Streptomycin (200 ppm) was also added. Two samples (15 and 16, **Table 1**) were fortified with 4% (w/w) milk proteins. *a** and *b** values were followed with a colorimeter. Other details about the storage conditions and colorimetric evaluation are given under Materials and Methods. Abbreviations are explained in the caption of **Figure 2**.

actual ratio LMW-Fe/EDTA is 1:21. This is outside the "critical pro-oxidative window" and could explain the stability gained.

Color (a and b* Values).* **Figure 4** shows *a** values (redness) and *b** values (yellowness) in isolates made in the absence (**Figure 4A**,**C**) and presence (**Figure 4B**,**D**) of antioxidants, respectively. It was unexpected that initial *a** values (redness) of the isolates had not been lowered in isolates made with a prewashing step included. A likely reason could be that the heme molecule was so oxidized after acid processing that its quantity had no effect on the redness. All precipitates, except some with added antioxidants, were grayish at the end of the acid process. However, both initial *a** values and *b** values were significantly reduced by the high-speed centrifugation. Among the pigments that could have partitioned into the sediment and floating layer

are heme and melanin. Reduced b^* values were likely due to reduction in phospholipids, which contain free amino groups. Interactions between amino groups and, for example, aldehydes, can yield Shiff bases that further may polymerize into yellow pigments (*52*).

There was a clear loss of redness (a^*) in the herring mince during acid processing, regardless of the actual conditions used (from $a^* = 2.4$ to $a^* = 0.2--3.2$). The a^* values grouped the protein isolates into two significantly different groups ($p \leq 0.05$), one containing high-speed centrifuged isolates and one the noncentrifuged ones (**Figure 4A**,**B)**. The former had a^* values of -1 to -1.5, and the latter, values of -2 to -3. Both groups decreased further during subsequent storage. After 8 days, the isolate made using prewashing with erythorbate and STPP (8) and the isolate made with erythorbate and EDTA in homogenization (13) reached values significantly lower ($p \leq 0.05$) than the other antioxidant-treated samples (**Figure 4B**). The relative sample-to-sample variation (expressed as coefficient of variance) in a^* values between the triplicates of sample 1 varied from 1 to 13% during the 7 days on ice.

Yellowness (b^*) went from 7.1 in the original mince to 0.2−7 after processing. The b^* values were lower during the whole storage in samples subjected to high-speed centrifugation ($b^* < 2$) than in noncentrifuged samples ($b^* = 4-7$) (**Figure 4C**,**D**). No significant changes ($p \leq 0.05$) took place in b^* values during the storage, except in the sample made with only erythorbate (11); this increased significantly ($p \leq 0.05$) between days 4 and 13. A likely reason was polymerization of Schiff bases as described above (*52*). Nonchelated reduced LMW metals may efficiently catalyze hydroperoxide breakdown into aldehydes that can be substrates for Schiff bases.

On the basis of the triplicated sample (1), the coefficient of variance regarding b^* values was 1.6−6% during days 0−7.

In conclusion, this study shows that TBARS developed during the isolation of proteins from herring mince with an acid solubilization process. Reducing the exposure time at pH 2.7 to 4 min, or including a 1:3 prewash with water, did not minimize oxidation. Including high-speed centrifugation (10000*g*) reduced initial TBARS levels in the precipitates by 50%. Process-induced oxidation was efficiently prevented by early addition of a metal-reducing agent (erythorbate) and a metal chelator (STPP or EDTA). STPP was suitable only in the absence of the high-speed centrifugation. To gain good ice storage stability, antioxidant had to be added in the homogenization step; EDTA here seemed to be more efficient than STPP. No effect was detected from adding milk proteins (4%) to the precipitate, but this will be further evaluated.

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