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ARTICLE

Assessment of Washing with Antioxidant on the Oxidative Stability of Fatty Fish Mince during Processing and Storage

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Fatty fish have been recognized as potential raw material for the production of surimi; however, they can easily oxidize. The ability of antioxidants added in the washing water to reduce oxidation during the washing and subsequent storage needs to be evaluated. Horse mackerel (*Trachurus trachurus*) mince was washed three times with 3 volumes of cold water (W) or the antioxidant solutions caffeic acid (CA) or propyl gallate (PG), at concentrations of 100 mg/kg, or spermine (SP), at a concentration of 400 mg/kg. Accumulation of antioxidant in the mince at each washing step was evaluated. The obtained washed minces were characterized and stored for 5 days at 5 °C. Lipid oxidation was followed by measuring primary and secondary lipid oxidation products (peroxides and volatiles, respectively). Characterizations of the physicochemical properties of protein and protein oxidation were also performed. Results indicated that the antioxidants were accumulated differently, but all antioxidants tested were able to prevent lipid oxidation in fatty fish mince during washing and subsequent storage. The ranking in terms of oxidative stability of the washed minces was CA = PG > SP > W. The antioxidants tested also showed some protection of the protein during processing and storage,; however, the results were more difficult to explain and indicated complex interactions between protein and antioxidant. The chemical structures of the antioxidant and its functional groups, its properties, and its interaction with the protein matrix are important parameters that need to be carefully evaluated to reveal to what extent antioxidants are able to protect protein from oxidative damage.

KEYWORDS: Washed fish mince; horse mackerel; lipid oxidation; protein oxidation; spermine; propyl gallate; caffeic acid; fatty fish

INTRODUCTION

Washing fish mince to remove the water-soluble protein and to concentrate the myofibrils is a common process used for the production of fish protein gels such as surimi and surimi-like products. Surimi products are popular in Asia and in southern Europe and are usually prepared with lean fish species such as Alaska pollock or hake, which after successive washings are processed into gels (*1*). Due to the decline in marine resources and the general concern about overfishing and sustainability, other abundant fish species have been tentatively used for the production of washed mince, for example, small fatty pelagic fish such as mackerel and sardine (*1*−*3*). However, their utilization for the production of surimi can be challenged due to their high content of sarcoplasmic protein, the low susceptibility of their protein to gelation, and their high proportion of dark muscle. Nevertheless, small fatty fish species have the advantage of being rich in polyunsaturated omega-3 fatty acids eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3), which have been shown to have several beneficial health effects (*4*,*5*). However, due to their high content of polyunsaturated fatty acids, which are very susceptible to oxidative damage, washing fatty fish can induce lipid and protein oxidation, resulting in quality loss (*6*, *7*).

Horse mackerel (*Trachurus trachurus*) has been shown to be a good alternative to lean fish species for the preparation of surimi, provided that the polyunsaturated long-chain omega-3 fatty acids are protected from oxidation during processing (*3*, *6*). Indeed, processing and storage of fatty fish have been shown to result in severe oxidative damage of both the lipid and protein fractions due to several factors such as changes in the prooxidant and antioxidant balance (*3*,*6*,*7*). However, washed minces with good quality can be prepared if processing of fatty fish can take place under lipid-stabilizing conditions by using either low oxygen availability, low temperature, or antioxidants during the mincing and washing steps (*8*, *9*).

Introduction of antioxidants either in the washing step or in the grinding step has been a successful strategy to prevent oxidation in fatty fish such as mackerel fillets and mince (*9*, *10*). Several antioxidants, both natural and synthetic, have been tested in fish products and in general have been shown to provide good antioxidative protection (*11*−*13*). The antioxidants evaluated included, among others, tocopherols, ascorbate, polyphosphates, synthetic phenolics such as butylated hydroxytoluene or

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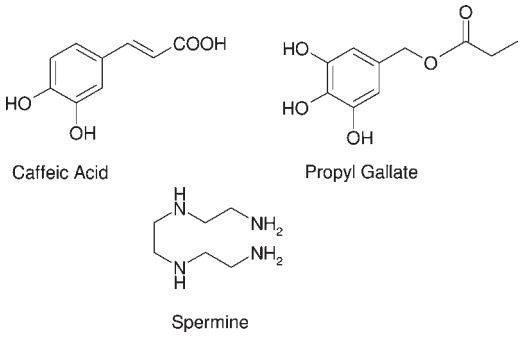

Figure 1. Molecular structures of tested antioxidants.

butylated hydroxyanisole (BHT and BHA, respectively), and propyl gallate as well as the naturally occurring phenolics such as caffeic acid, chlorogenic acid, and ferulic acid (*9*−*13*). Natural compounds such as plant phenolics have received a lot of attention because they are secondary metabolites of fruits and vegetables, are natural alternatives to synthetic antioxidants, and can be easily obtained from fruits or plants as natural extracts. The polar phenolic compound caffeic acid has been shown to be a very promising candidate for delaying significantly lipid oxidation in fish muscle as it has been shown to be efficient even at very low concentrations (*13*).

Another class of natural polar compounds that has also received increasing attention is the polyamines, such as spermine and spermidine (*14*). They are ubiquitously present in almost all cells and have been reported to be able to scavenge free radicals and chelate metals ions, thereby protecting DNA and lipids from oxidative damage (*14*, *15*). The antioxidative activity of spermine has not been investigated thoroughly in food systems. So far, only a few reports have investigated the effect of adding any of these antioxidants during the washing step in surimi production.

The aim of this study was to investigate the effects of washing with antioxidant on the overall quality of horse mackerel during both processing and storage and to compare the antioxidative activity of natural compounds with different functional groups and different properties, spermine and caffeic acid, with the synthetic phenolic compound propyl gallate (**Figure 1**). Their antioxidant activities were evaluated and compared during washing of horse mackerel mince by adding the different antioxidants directly to the washing water. In addition, the oxidative stability of the obtained washed horse mackerel minces was also followed. The performance of the different antioxidants added in the washing water was evaluated and compared in relationship to their antioxidant properties as reported in the literature.

MATERIALS AND METHODS

Materials. Chemicals and standards were obtained from Sigma-Aldrich (St. Louis, MO). Solvents were of analytical grade and from Lab-Scan (Slangerup, Denmark). Deionized water (Millipore A/S, Copenhagen, Denmark) was used throughout. Gel and standards for SDS-PAGE were from Invitrogen (Carlsbad, CA).

Sample Processing and Storage. Horse mackerel (*T. trachurus*) used was caught at the end of July 2006 during a scientific cruise of DANA, the Danish scientific vessel of DTU AQUA. Fish were immediately filleted, skinned, vacuum packed in portions of approximately 2 kg, and stored at −80 °C. Upon arrival at the laboratory, the frozen fillets were stored at −40 °C until processing. A second experiment was performed using commercial freshly caught horse mackerel purchased in Denmark. The fish arrived on ice and were immediately filleted, skinned, vacuum packed, and stored at −40 °C until processing. The experiment was performed twice using two different batches of fish, and the obtained results showed the same trend; therefore, results are given for a typical experiment. However, samples for analysis of accumulation of the antioxidant in the mince were obtained from only a single experiment. Prior to each experiment horse mackerel samples were transferred to −20 °C the day before the experiment, and the next day they were thawed for approximately 1.5 h in cold water. All processing steps were performed in a 10 °C room. Fillets were minced using a grinder plate with 3 mm holes (ATOM TL22, ceg, Cardano al Campo, Italy). The obtained mince was washed three times with 3 volumes of cold (2 °C) distilled water (W) or with either propyl gallate (PG) or caffeic acid (CA) solutions at a concentration of 100 mg/L or with spermine (SP) solution at a concentration of 400 mg/L. The antioxidants were added to the washing water as ethanolic stock solutions, with ethanol representing 1% (v/v) of the total volume. For the water wash (W) the same amount of ethanol (1%, v/v) was added to the washing water. In all washing steps, the fish to washing water ratio was 1:3 (w/v). Mince and washing solutions were slowly stirred for 30 s every 2 min for 4 min and then allowed to settle for 6 min. Total time spent for each washing step was approximately 10 min. Following each washing, the minces were dewatered for approximately 5 min through a polyamide net (Sefar Nitex 03-100/44, Sefar AG, Heiden, Switzerland). A total of three washing steps were performed, and after the last washing/dewatering step, the products were centrifuged for 20 min at 5 °C and 24500*g* (Sorvall RC 5B *Plus*, SLA 1500, DuPont, Norwalk, CT). For the storage experiment the obtained washed mince were divided into five portions of approximately 260 g and placed in aluminum boxes (18.5 × 13.5 cm) closed with plastic lids. Immediately after processing, t0 samples for the four washed minces (W, PG, CA, and SP) were vacuum packed in plastic bags and stored at −80 °C. The remaining samples from W, PG, S, and CA were stored at 5 °C in a temperature -controlled room in the dark for 12, 24, 60, and 96 h. At each time point the content of the aluminum boxes was mixed with a fork for homogeneity, vacuum packed in plastic bags, and stored at −80 °C until further analysis. Samples of washed minced and water were collected after each washing step, vacuum packed, and stored at −80 °C until further analysis. Unless otherwise stated, all analyses were performed at least in duplicate for each sample and at each time point.

Lipid Content. The lipids were extracted in triplicate from 10 g of fish or washed mince sample with methanol/chloroform (1:2, v/v) according to the Bligh and Dyer (*16*) method. The lipid content in the Bligh and Dyer lipid extract was determined gravimetrically, and the results are expressed as percent of wet weight.

Antioxidant Measurements. *Tocopherols*. Tocopherol content was determined on the Bligh and Dyer lipid extracts by HPLC using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA), equipped with a fluorescence detector. A fraction of the lipid extract was evaporated under nitrogen and redissolved in 2 mL of *n*-heptane, and an aliquot (40 μL) was injected on a Spherisorb s5w column (250 mm × 4.6 mm) (Phase Separation Ltd., Deeside, U.K.). Elution was performed with an isocratic mixture of *n*-heptane/2-propanol (100:0.4; v/v) at a flow of 1 mL/min. Detection was done using fluorescence with excitation at 290 nm and emission at 330 nm and according to the AOCS (*17*). Results are expressed in milligrams of tocopherol per kilogram of lipid.

Propyl Gallate and Caffeic Acid. Washed mince samples were chopped in a hacker (Knifetec 1095 Sample Mill, Foss Tecator, Foss Hillerød Denmark). Subsequently, samples of 5 g were extracted with 15 mL of 96% ethanol using an Ultra-Turax (Janke & Kungle, IKA-Werk, Staufeb, Germany) for 1 min at 17500 rpm. The samples were centrifuged for 10 min at 1620*g*, filtered on cotton wool, and subsequently filtered on Ministart SRP 15 filter (Sartorius AG, Gottingen, Germany). For propyl gallate extraction, the sample was extracted three times and twice for caffeic acid extraction. A 10 μL aliquot of the ethanolic fraction was injected in duplicate onto an HPLC (Agilent 1100 series Agilent Technology, Palo Alto, CA) equipped with a UV diode array detector. The aliquots were applied to a C18 (Thermo Hypersyl, ODS) column (250 mm × 4.6 mm, 5 μm). For CA elution was performed using a mixture of solvent water/acetic acid/acetonitrile and detection was performed at 324 nm. For PG elution was performed with an isocratic mixture of water/acetonitrile/acetic acid and detection was performed at 275 nm. Quantification was performed using standard curves prepared with authentic standards, and the results are expressed in milligrams per kilogram of wet weight.

Spermine. Samples were frozen in liquid nitrogen and reduced to a fine powder using a blender (Waring, Torrington, CT). The resulting powder (7.0 g) was homogenized in 15 mL of trichloroacetic acid (TCA; 7.5% w/v)

with an Ultra-Turax (Janke & Kungle, IKA-Werk) for 1 min at 17500 rpm. The samples were centrifuged for 10 min at 1620*g* and filtered on paper, and the precipitate was then washed twice with 15 mL of TCA. The TCA fractions were injected onto a HPLC (Agilent 1100 series, Agilent Technology) equipped with a Hypersil ODS column (200 mm × 3 mm, 5 μM) (Chrompack, Middelburg, The Netherlands), and samples were eluted with ammonium acetate/acetronitrile. Postcolumn derivatization with orthophthalic aldehyde (OPA) was performed according to the method of Seiler and Knodgen (*18*) and modified after Veciana-Nogués (*19*). Detection was performed using fluorescence with excitation at 354 nm and emission at 430 nm, and quantification was done using standard curves prepared with authentic standards; the results are expressed in milligrams per kilogram of wet weight.

Iron Content. The total iron content of fish was determined using an atom absorption spectrophotometer 3300 (Perkin-Elmer, Waltham, MA). The practical conditions used under the preparation of the mince and the washing steps are expected to result in unavoidable contamination. However, for analysis purposes all glassware used was treated with hydrochloric acid prior analysis to avoid contamination during sample preparation. Horse mackerel or washed mince sample (2.0 g) was mixed with 5.0 mL of concentrated HNO_3 and 2.0 mL of H_2O_2, and the samples were digested using a microwave oven (CEM, MOS 81D, Matthews, NC). After acid digestion, the samples were analyzed according to the protocol from the Nordic Committee on Food Analysis (*20*).

Lipid Oxidation. *Peroxide Values*. Lipid hydroperoxide (PV) content was determined in duplicate on the Bligh and Dyer lipid extracts according to the method of Shantha and Decker (*21*). Results were expressed in milligrams of peroxide equivalents per kilogram of lipids.

Volatile Compounds. The volatile compounds were sampled by dynamic headspace according to the method of Refsgaard et al. (*22*). The mince samples were cut into pieces, frozen in liquid nitrogen, and reduced to a fine powder using a blender (Waring). Deionized water (10 mL) was added to 5 g of powder, and the aqueous suspension of mince powder was placed at 37 °C for 30 min and was purged with a nitrogen flow of 340 mL min^{-1}. The volatiles were collected on Tenax-GR traps (Chromapack, Bergen op Zoom, The Netherlands). All collections were made in triplicate. A Perkin-Elmer (Norwalk, CT) ATD-400 automatic thermal desorber system was used, and helium was used as a carrier gas. The gas flow from the trap to the transfer line to the capillary column in the gas chromatograph (GC) was split in the ratio of 5.0 mL min^{-1}/1.3 mL min^{-1}. The transfer line of the ATD was connected to a Hewlett-Packard 5890 IIA gas chromatograph (Palo Alto, CA) equipped with a HP 5972A mass-selective detector. A DB 1701 column (30×0.25, 1.0 μm; J&W Scientific, Folsom, CA) with a flow of 1.3 mL of helium/min was used. The mass selective detector used ionization at 70 eV in EI mode and 50 μA emission. Scans were performed in the mass range of 30−350 atomic mass units with a repetition rate of 2.2 scans s^{-1}.

Compounds were identified by MS library searches and by comparing retention time and spectra with MS runs of external standards. A calibration curve was made for each standard using the HP Chemstation software.

Chelation of Fe^{2+}. The ability of the different antioxidants to chelate Fe^{2+} ions was evaluated according to the method described by Dinis et al. (*23*). The chelating capacity was calculated as follows: Fe^{2+} chelating activity (%) = ((blank − sample)×100%)/blank.

Color Measurement: *a, *b**, *L** Values.** Changes in *a** value (redness), *b** value (yellowness), and *L** value (lightness) were followed during storage of the washed minces. Measurements were performed at each time point at the surface of each sample before mixing, and a minimum of six measurements per mince was performed using a Minolta Chromameter (Cr-200, Minolta, Osaka, Japan). The results are given as the mean of at least six measurements per sample ± standard deviation.

Protein Solubility. Samples (0.5 g) were homogenized in 10 mL of 0.6 M KCl in 50 mM (pH 7.4) Tris-HCl buffer with a Polytron PT1200CL (Kinematica AG, Lucerne, Switzerland) for 1 min. The homogenate was centrifuged at 14000*g* for 15 min at 5 °C (Sorvall RC 5B *Plus*, SS24 rotor, DuPont). The supernatant was diluted 10-fold with 0.6 M KCl, and protein determination was performed using the BCA kit (Pierce, Rockford, IL). The change in solubility during storage was expressed as percentage of the initial protein solubility at time zero.

Protein Oxidation. *Determination of Sulfhydryl Groups*. Total free thiol group content was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to Ellman's method (*24*) with some modifications. A 0.5 g sample was homogenized in 10 mL of 0.05 M phosphate buffer (pH 7.2) with a Polytron PT1200CL (Kinematica AG) for 30 s. Subsequently, 1 mL of the homogenate was mixed with 9 mL of 0.05 M phosphate buffer (pH 7.2) containing 0.6 M NaCl, 6 mM EDTA, and 8 M urea. The mixture was centrifuged for 15 min at 14000*g* at 5 °C (Sorvall RC 5B *Plus*, SM34 rotor, DuPont). To 3 mL of the supernatant was added 0.04 mL of 0.01 M DTNB solution in 0.05 M sodium acetate, and the mixture was incubated at 40 °C for 15 min (Shimadzu UV 160A, Kyoto, Japan). The absorbance was measured at 412 nm, and the SH content was calculated using a molar extinction coefficient of 13600 M cm^{-1} (*24*). Results were expressed in micromoles of SH per gram of mince.

Spectrophotometric Determination of Protein Carbonyls. Protein carbonyls were measured as described by Levine et al. (*25*). A sample of mince (0.5 g) was homogenized in 10 mL of Tris buffer (pH 7.4, 50 mM, 1 mM EDTA) containing 0.01% BHT, and 100 μL of the homogenate was precipitated with TCA %. After centrifugation (12000*g*, 3 min), the pellet was incubated with dinitrophenylhydrazine (DNPH) in 2 M HCl in the dark for 30 min. The samples were precipitated with TCA, and the pellets were washed three times with 1 mL of ethanol/ethyl acetate 1:1 (v/v). The pellet was redissolved in 6 M guanidine chloride in 20 mM KH_2PO_4, and after centrifugation (3 min at 12000*g*), the carbonyl content was determined in the supernatant by measuring the absorbance at 370 and 280 nm. Results are expressed in nanomoles of carbonyl per milligram of soluble protein.

Gel Electrophoresis and Immunoblot. Samples of washed mince (0.5 g) were homogenized in 4 mL of Tris buffer (pH 7.4, 50 mM, 1 mM EDTA). The protein concentration was determined using the BCA kit (Pierce), and the mince homogenates were adjusted to 10 mg/mL. Subsequently, the samples were derivatized as previously described (*26*), and 5 μL was loaded onto the gel (10% NuPage Bis-tris, Invitrogen. After the run, one gel was stained with Coommasie Brilliant Blue G-250, and one gel was used for immunoblotting. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane 0.2 μM (Millipore) using a Mini Cell SureLock, equipped with an XCell II blot module (Invitrogen A/S) for 60 min. After transfer, the membrane was incubated with a 1:15000 dilution of rabbit anti-DNP (DAKO Denmark AS, Glostrup, Denmark) and with a 1:7500 dilution of the secondary antibody, peroxidase-conjugated swine anti-rabbit (DAKO). The blot was developed using the ECL+ kit (Invitrogen). Chemiluminescence was detected on hyper-film ECL (Amersham Health A/S, Princeton, NJ).

Statistical Analysis. One-way ANOVA followed by Bonferroni multiple-comparison test was performed using GraphPad Prism (GraphPad Software, Inc.) to evaluate differences between sample treatments at specific time points. The applied level of significance was $p < 0.05$, and significant differences are reported using letters in the tables.

RESULTS AND DISCUSSION

Characterization of Washed Horse Mackerel Minces. After each washing step, the different antioxidants were retained in the mince differently, as illustrated in **Figure 2**. PG and SP accumulated in the mince and reached 585 and 4010 mg/kg, respectively, after the third wash, revealing their affinity for muscle protein. In a glassing experiment, phenolics extract from grape has been shown to be quickly transferred from the aqueous phase into the fish muscle and to have high affinity for the fish muscle membrane (*27*). Of the three antioxidants tested, PG was the most hydrophobic and might have accumulated in hydrophobic cavities of fish proteins or associated with hydrophobic amino acids. Similarly, binding of hydrophobic flavonoids to BSA has been shown to occur within the hydrophobic pockets of BSA (*28*). Caffeic acid content did not increase to the same level as PG and SP in the mince, reached a maximum after the second wash (141 mg/kg), and remained constant thereafter. Binding of the antioxidants to fish protein matrices might also be due to the presence of reactive functional groups, carbonyl and primary

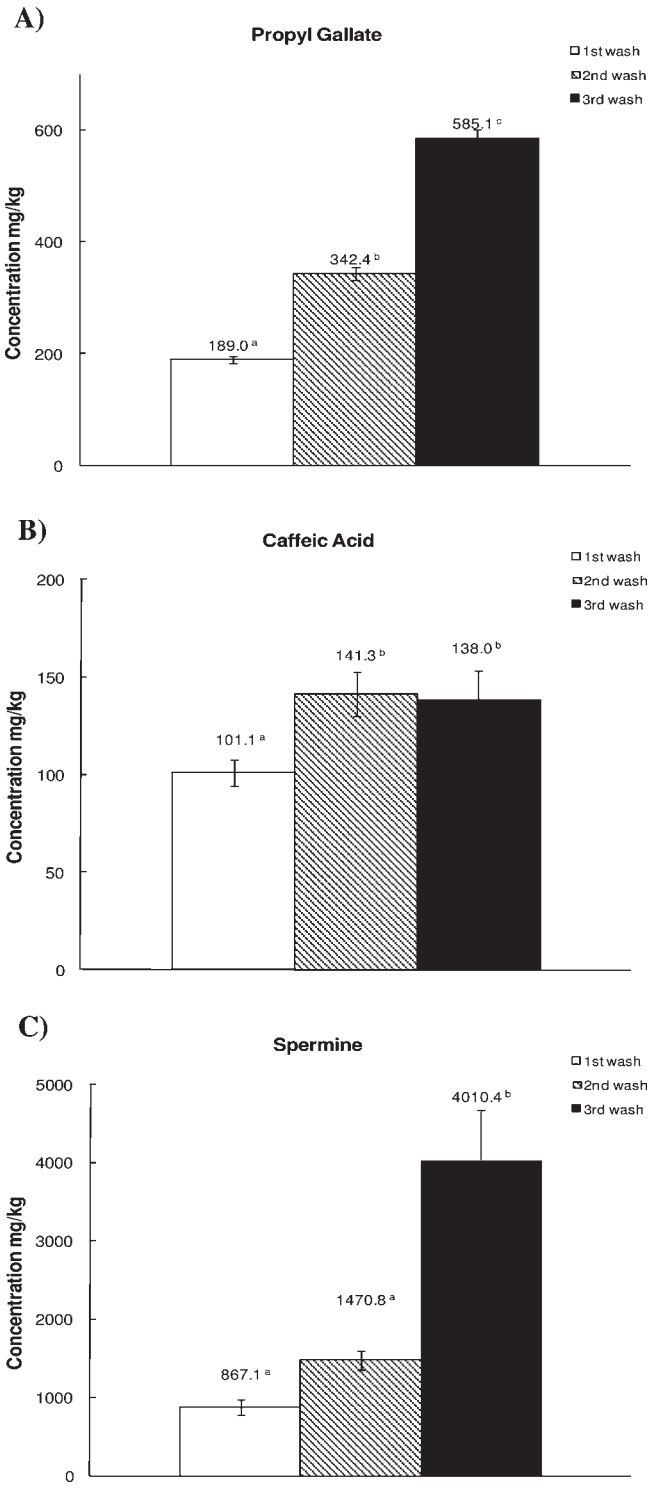

Figure 2. Concentration of antioxidant in the mince after the first, second, and third washes with (**A**) propyl gallate (PG), (**B**) caffeic acid (CA), or (**C**) spermine (SP) (averaged values of triplicate measurements; error bars show standard deviation). Different letters indicate significant differences between washes.

amine groups, in PG and SP, respectively. Interaction between carbonyl group on the protein side chain and the SP primary amine group might have resulted in the reversible formation of Shiff bases and accumulation of the SP in the washed mince. In addition, interaction between phenols and protein has been reported to be related not only to their polarity (*29*) but also to the amino acid composition and the structure of the protein

Table 1. Characterization of the Horse Mackerel Mince Used and of the Washed Minces Obtained after Washing with Water (W), Caffeic Acid (CA), Propyl Gallate (PG), and Spermine (SP)[a]

	mince	W	CA	PG	SP
pH	6.0 a (±0.1)	6.0 a (±0.1)	6.0 a (±0.1)	6.1 a (±0.1)	8.2 b (±0.1)
lipid (% total wt)	3.81 b (±0.5)	1.94 a (±0.01)	2.83 ab (±0.08)	2.03 a (±0)	2.56 a (±0.03)
PV (mequiv/kg of lipid)	1.4 a (±0.3)	6.8 b (±0.1)	1.0 a (±0.1)	0.8 a (±0.1)	1.7 a (±0)
α-tocopherol (mg/kg)	6.44 a (±0.03)	0.30 a (±0.01)	5.92 bc (±0.19)	5.29 b (±0.36)	nd
iron (g/kg)	10.2 a (±1.1)	13.6 a (±2.3)	29.2 bc (±3.3)	31.8 c (±1.0)	22.8 b (±1.5)

[a] pH, lipid, peroxide value (PV), α-tocopehrol, and iron content presented as mean ± standard deviation. nd, not detected. Different letters for an attribute in the same row indicate significant differences between samples (with $p < 0.05$).

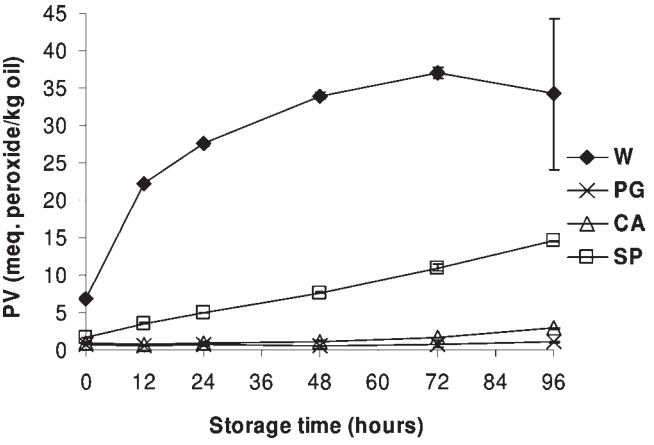

Figure 3. Development of peroxide (PV) during storage at 5 °C of horse mackerel mince washed with water (W), propyl gallate (PG), caffeic acid (CA), or spermine (SP). Averaged values of triplicate measurements; the error bars show the standard deviation.

involved (*30*). However, other parameters such as solubility, diffusion, and affinity to protein or lipid of antioxidants might also need to be considered when one is working with complex food matrices.

During washing, the lipids present in the mince were removed as also reported by others (*6*, *7*), and the lipid contents obtained in the main experiment after the third wash ranged from 2.5 to 1.9%, with the lowest lipid content in the water (W) and PG minces (**Table 1**).

The initial peroxide value in the mince was 1.4 mequiv/kg, and washing with W resulted in a significant development of lipid oxidation, which was characterized by a significant increase in peroxide value (PV) to 6.8 mequiv/kg. This is in accordance with previous investigations, which also showed that washing fatty fish mince with water resulted in a significant increase in oxidation (*3*, *6*, *8*). In the presence of antioxidant in the washing water, the development of oxidation during processing was minimized and resulted in washed minces with low levels of peroxides (**Figure 3**; **Table 1**). This was further confirmed by the measurement of the initial level of volatile oxidation products: 1-penten-3-ol, 2-penten-1-ol, hexanol, heptanal, hexanal, octanal, 2-heptenal, 4-heptenal, 2-hexenal, and 2,4-heptadienal. All volatiles followed

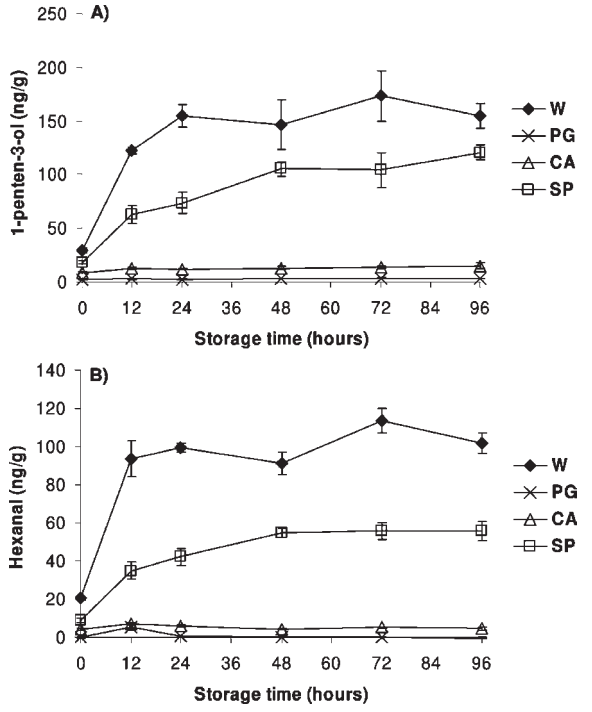

Figure 4. Formation of (**A**) 1-penten-3-ol and (**B**) hexanal during storage at 5 °C of the horse mackerel mince washed with water (W), propyl gallate (PG), caffeic acid (CA), or spermine (SP) (averaged values of triplicate measurements; error bars show standard deviation).

the same tendency, and results are presented for only 1-penten-3-ol and hexanal, which represent oxidation products of omega-3 and omega-6 fatty acids, respectively (**Figure 4**). Similarly, Kelleher et al. (*9*) prepared surimi from mackerel using antioxidants such as ascorbate, tripolyphosphate, or propyl gallate either in the grinding step or in the washing water and showed that it was possible to significantly reduce oxidation during processing.

The initial mince had an α-tocopherol content of 6.4 mg/kg (**Table 1**). Washing with W and SP resulted in a significant loss or degradation of α-tocopherol, whereas washing with either CA or PG spared the α-tocopherol. However, the higher α-tocopherol content after washing with CA and PG could be due to a potential antioxidant activity of CA and PG during processing preserving the α-tocopherol. Interestingly, even though SP prevented oxidation during processing, no α-tocopherol was detected in the mince after washing with SP. This finding may indicate a different mechanism for the antioxidative activity of SP compared to CA and PG. The antioxidative mechanisms of CA and PG have previously been shown to result in regeneration of α-tocopherol (*31*, *32*). In contrast, the antioxidant mechanism of SP is still unclear but has been reported to be related to its free radical scavenging activity and its iron chelating activity (*14*, *33*). Interaction between SP and tocopherols is not very well understood, but an early investigation showed that spermine antioxidant activity depends on the presence of tocopherols (*34*). The tocopherolyl radical has been shown to be regenerated by the amine group of spermine. An intervention study also showed correlation between spermine and other nutrients such as carotenoid and tocopherol concentration in blood plasma (*35*). In that respect, the interaction between SP and other cellular antioxidants should be further investigated.

Surprisingly, washing with antioxidants also resulted in a higher amount of iron in the mince. The iron level in the mince was initially 10.2 mg/kg, but washing with antioxidant resulted in a significant increase in iron content in all washed minces

Table 2. Color Values for the Different Minces after Washing with Water (W), Caffeic Acid (CA), Propyl Gallate (PG), or Spermine (SP), Immediately after Washing (t0) and after 96 h of Storage at 5 °C (t96)[a]

	W	CA	PG	SP
t0				
*a**	0.75 a (±0.14)	1.31 bc (±0.08)	2.38 d (±0.04)	1.49 c (±0.18)
*b**	7.74 c (±0.18)	6.11 b (±0.37)	5.40 a (±0.09)	8.64 d (±0.27)
*L**	62.89 b (±1.26)	62.87 b (±1.19)	60.26 a (±0.38)	61.54 ab (±1.13)
t96				
*a**	−0.7 a (±0.10)	0.5 b (±0.13)	2.23 c (±0.10)	0.54 b (±0.18)
*b**	8.48 b (±0.52)	5.48 a (±0.18)	4.95 a (±0.14)	9.34 c (±0.41)
*L**	62.96 b (±1.08)	60.51 ab (±2.18)	59.07 a (±0.97)	62.37 b (±0.81)

[a] Different letters for an attribute in the same row indicate significant differences between samples (with $p < 0.05$).

(**Table 1**). This might be due to impurity and contamination during washing and is more likely related to the ability of the different antioxidants tested to chelate metal ions, resulting in accumulation of iron in the matrices. Medina et al. reported that CA and PG were good metal chelators (*12*). The chelating properties of the different antioxidants were tested in an in vitro assay, and the data showed that at the concentration used in the washing water, SP had a high Fe^{2+} chelating activity (90%) compared to CA and PG (approximately 0−5%) (data not shown). Polyamines have been shown previously to be strong chelators of metals (*14*, *33*). However, our results also indicated that oxidation is not necessarily related to the level of iron present in the matrix, but rather to its availability and its oxidation state as reported by others (*36*). The results also point out that interactions between the different components in the food matrix are important in relation to the oxidative stability of foods (*37*).

The washed minces had different colors as reported in **Table 2** by *a**, *b**, and *L** values. The *a** value or redness was low for the mince washed with water, but significantly higher for minces washed with antioxidants, indicating a redder mince. Even if most of the hemoglobin is washed out, due to the presence of a large portion of dark muscle in horse mackerel mince, myoglobin is expected to be retained in the muscle, giving the mince a slightly reddish or brownish appearance. The mince washed with PG had a high *a** value, indicating a redder color, and also had lower *b** and *L** values compared with the other washed minces. This could indicate that PG prevented the oxidation of the heme proteins, hemoglobin and myoglobin, which are red in their reduced from and brown in their oxidized ferric form. However, Pazos et al. reported in a model system that PG did not effectively protect heme protein from oxidation and thereby did not prevent discoloration (*27*). However, phenolic extracts from capers have been found to effectively prevent activation of myoglobin to its hypervalent state ferryl myoglobin (*38*), indicating a potential interaction between some phenolic compounds and heme protein redox reactions. Therefore, the interaction between heme proteins, in their different oxidative states, and phenolic compounds deserves further attention.

SDS-PAGE presented in **Figure 5A** did not reveal any major difference in the protein pattern for the washed mince at t0

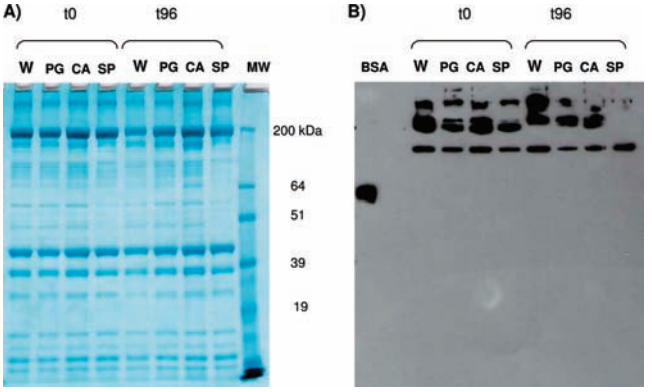

Figure 5. (**A**) SDS-PAGE and (**B**) Western blot against protein carbonyl groups of washed horse mackerel minces at t0 and after 96 h of storage at 5 °C. W, washed with water; CA, washed with caffeic acid; PG, washed with propyl gallate; SP, washed with spermine; MW, molecular weight marker; BSA, carbonylated BSA standard.

Table 3. Protein Carbonyl Group and Thiol Group Contents for the Different Minces after Washing with Water (W), Caffeic Acid (CA), Propyl Gallate (PG), or Spermine (SP) Immediately after Washing (t0) and after 96 h of Storage at 5 °C (t96)[a]

	W	CA	PG	SP
carbonyl groups (nmol/mg of protein)				
t0	5.0 a (±0.9)	3.5 a (±1.1)	8.9 a (±4.4)	10.9 a (±2.4)
t96	6.8 a (±2.0)	9.8 ab (±0.4)	6.7 a (±0.7)	13.6 b (±0.8)
thiol groups (nmol/mg of protein)				
t0	78.5 a (±3.0)	95.2 b (±6.1)	104.3 b (±1.6)	74.8 a (±6.7)
t96	86.5 a (±6.2)	104.1 b (±1.3)	108.6 b (±2.5)	82.6 a (±8.8)

[a] Different letters for an attribute in the same row indicate significant differences between samples (with $p < 0.05$).

irrespective of the washing solution. Initial protein oxidation in the washed mince was measured as protein carbonyls and protein thiol groups (**Table 3**). Free thiol group content reached its highest levels for PG and CA, indicating a protective effect of the phenols on the thiol groups. Interaction between caffeic acid oxidation and thiol groups has been previously reported, but the mechanisms remain unclear (*39*). However, the antioxidant efficiency on protein thiol groups was ranked in the following order PG = CA > W = SP. These results did not correlate well with the results obtained for protein carbonyl groups, which showed highest protein carbonyl groups for PG and SP, even if the results are not significantly different. Indeed, according to the carbonyls results SP and PG seemed to promote protein oxidation, indicating no protective effect on the protein. In beef patties, plant phenolics from rosemary extract were not able to prevent protein oxidation, whereas in liver pate the same plant phenolics were shown to be good antioxidants (*40*, *41*). The antioxidant activity of phenolic compounds on protein has been attributed to their ability to prevent lipid oxidation by chelating metals and also to their ability to bind to proteins. Phenolic compounds bind to protein either through non-covalent or covalent interactions. Rohn et al. (*42*) reported that covalent binding between protein and quercetin resulted in less antioxidant activity of the phenolics on the protein. However, others have reported that complexation of phenolics to protein could enhance their antioxidative activity toward proteins (*43*). Hence, reports on interactions between phenolic compounds and proteins show that the consequences of the interaction for the antioxidative properties of the phenolics on both lipid and protein need further investigation. The partly contradicting findings for thiol groups and carbonyls could be due the fact that these two methods are measuring different reactions, which could be affected differently by interactions between proteins and antioxidants.

Immunoblot against protein carbonyl groups was also performed (**Figure 5B**) and revealed for all samples that proteins with molecular mass above 120 kDa were oxidized. Oxidized proteins were detected on the blot at approximately 120, 200, 220, and 350 kDa, but were not further characterized. However, it can be speculated that one of the bands at 200−220 kDa represents myosin. W and CA presented heavier bands at 120 and 200−220 kDa compared to SP and PG, indicating that the former were more oxidized than the latter and contradicting the spectrophotometric data showing that CA and W washed minces were less oxidized (**Table 3**). The results might be partly explained by non-covalent binding of antioxidants to the protein, leading to an overestimation of the protein carbonyls. For example, this can result in the introduction of carbonyl groups via binding of the antioxidant molecule containing carbonyl groups such as PG to the protein. Such binding might not be stable under electrophoresis conditions. In contrast, interactions of the amine group of SP might have indirectly hindered the interaction between carbonyl groups and DNPH by indirectly blocking the carbonyl groups. Indeed, the formation of Schiff base between the carbonyl group on the protein and the primary amine group of SP may have resulted in fewer carbonyl groups on the blot. Others have reported formation of Schiff bases between protein carbonyl groups and the amine group of peptides blocking protein carbonyls on the protein, which would result in lower levels of protein carbonyls measured by the DNPH assay (*44*). In fact, the DNPH assay has recently been shown (*45*) to be rather unspecific. Our results are difficult to explain, and more studies are needed to clarify the relationship between protein carbonyl, thiol group content, and protein oxidation. The matrix/protein composition and properties as well as the phenolic structure and properties are likely to affect the abilities of phenolic compounds to protect both the lipids and the proteins in muscle foods. In addition, it is important to point out that the reactive group of the tested antioxidants might have interfered with the selected assays for assessing protein oxidation, thereby affecting the results and making them more difficult to interpret.

Stability of Washed Minces during Storage. The four washed products obtained presented very different antioxidative statuses at the beginning of the storage period, and oxidation developed differently in the different washed minces. During storage, oxidation was highest in W as observed in **Figure 3**, presenting a PV that reached approximately 35 mequiv/kg after 96 h. This confirmed previous results indicating severe oxidation with washing (*6*). SP was to some extent able to prevent lipid oxidation; however, its PV increased steadily and reached 10 mequiv/kg after 96 h. In contrast, CA and PG prevented the development of PV during storage and seemed to completely delay lipid oxidation. Others have also reported very good antioxidant capacity for both CA and PG in different food systems including fish muscle (*13*, *32*, *40*). Volatiles indicated that SP could only slightly prevent oxidative damage during storage and confirmed that CA and PG were very effective antioxidants (**Figure 4**). Others have also reported that phenolic compounds were effective at preventing lipid oxidation in muscle food during storage (*32*, *39*), but the precise mechanism needs to be investigated further, especially with respect to interaction with other constituents of

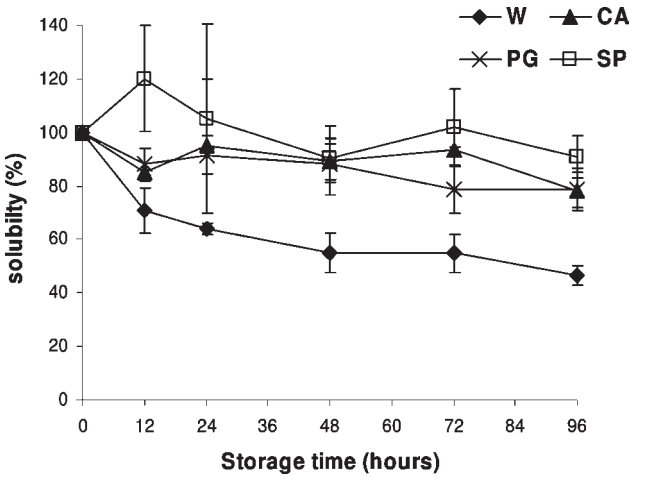

Figure 6. Protein solubility during storage at 5 °C of the horse mackerel mince washed with water (W), propyl gallate (PG), caffeic acid (CA), and spermine (SP) and expressed as percentage of solubility at t0 (averaged values of triplicate measurements; error bars show standard deviation).

the matrix. As for SP, some studies have indicated that SP is able to delay oxidation in model systems (*14*, *15*), but it had never been tested in muscle system. Our results show that SP is a poor antioxidant in such systems. The poor effect of SP may be attributed to the washing steps, which might have resulted in the loss of SP's antioxidative capacity. Testing of SP by adding it directly to the muscle might have resulted in different conclusions, and more systematic investigations are needed to clarify this issue.

Changes in color during storage are presented in **Table 2**. After 96 h, redness, *a**, decreased in all minces except for PG, implicating a possible effect in PG on autoxidation of heme proteins as previously mentioned. To date, no precise and detailed investigation about the effect of phenolic compounds on the autoxidation of heme proteins and the reduction of the hypervalent state exists, and this might deserve further attention. Yellowness, *b**, increased in W and SP, perhaps indicating rancidity and the formation of secondary oxidation products. However, a decrease in *b** was observed with time for CA and PG, whereas for all samples lightness, *L**, was unchanged.

For the protein part, SDS-PAGE (**Figure 5A**) did not reveal major differences between samples in their protein composition during storage. However, the intensity of the myosin band at 200 kDa for W seemed to be significantly weaker compared to its intensity at t0 and compared to the other samples at t96. This might reflect the decrease in solubility of myosin over time as a result of protein cross-linking as also previously suggested (*6*, *46*). Protein solubility over time is presented in **Figure 6** and confirmed the decrease in solubility over time with a significant 50% reduction after 96 h for the W samples as previously reported. However, all of the tested antioxidants seemed to prevent the drastic decrease in protein solubility occurring after 96 h of storage that was observed for W, but no difference between the antioxidants was observed. The results from the protein carbonyl and thiol groups are presented in **Table 3**, and no significant changes were observed during storage for any of the samples except for CA, which had a higher level of protein carbonyl groups after 96 h of storage compared to that at t0. This was not reflected in the carbonyl protein blots (**Figure 5B**). Similarly, our previous investigation showed little change in the protein carbonyl group measured spectrophotometrically during storage for washed mince compared to unwashed mince (*6*). However, from the blots it is interesting to note that the W sample presented a very heavily oxidized band at approximately 350 kDa. This finding supported the formation of high molecular weight oxidized protein, probably as the result of protein cross-linking as already reported previously (*6*, *46*). PG and CA samples appeared to have fainter oxidized protein bands compared to t0, but for SP the high molecular weight band completely disappeared. The disappearance of the high molecular weight band in SP samples may be attributed to a slow reaction between spermine and myosin, resulting in reversible Schiff base formation, which may have impaired carbonyl group detection. However, from the blot after 96 h, it seemed clear that all antioxidants tested reduced protein oxidation when compared to W. It is also worth noting that after 96 h, the band present at 200 kDa in all samples was converted to a slightly higher molecular mass of approximately 220 kDa. This might be an indication of myosin modification and cross-linking as a consequence of oxidation or interaction with the antioxidants. However, more detailed investigations are needed to reveal how important protein oxidation is in muscle food and to what extent it can affect quality and how antioxidants can prevent these changes. Indeed, very few papers have examined oxidation of both protein and lipid in food matrices.

In conclusion, washing fatty fish mince with antioxidants is a good alternative to prevent oxidative deterioration during processing and subsequent chilled storage. The antioxidants tested accumulated differently in the washed products, but effectively prevented lipid oxidation with a ranking of CA = PG > SP. In contrast, the results obtained for protein oxidation were more difficult to explain. It is suggested that the chemical structure of the antioxidant, its functional groups, and its properties as well as its interaction with the protein matrix might be important parameters that need to be further investigated in relation to protection of the protein from oxidation. In addition, to what extent protein oxidation affects fish quality during processing and storage needs to be clarified.

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