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Recovery and Characterization of Sardine Oil Extracted by pH Adjustment

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A new oil extraction method involving pH adjustment was developed and compared with the traditional heat extraction method. Sardine oil was obtained by adjusting the pH to the isoelectric point (p*I*) of sardine muscle (pH 5.5) using HCl or food-grade organic acids [with/without calcium (Ca)] followed by centrifugation. Oil extracted with citric acid plus Ca showed the best quality, along with good recovery, the lowest haze value, and the highest n-3 polyunsaturated fatty acid content. All oils extracted by pH adjustment exhibited high stability against oxidation supported by low conjugated dienes and thiobarbituric acid reactive substance level with fewer impurities compared to the heat process. There was a significant beneficial effect of Ca addition prior to pH adjustment in terms of lipid oxidation stability and color of the final products. The pH adjustment method is a novel process for oil extraction and a promising method that can be utilized for high-oil pelagic species such as Pacific sardines.

KEYWORDS: Pacific sardines; oil extraction; isoelectric point; n-3 PUFA; oxidation

INTRODUCTION

The need for high-quality fish oils is increasing due to growing nutraceutical markets as well as increased demand in the aquaculture industry. A number of methods, such as physical fractionation, low-temperature solvent fractionation, and supercritical fluid extraction, have been used to produce fish oils. However, wet reduction, which includes cooking, pressing, and/or centrifugation, is the traditional method used to produce fish oil (*1*). The processing of pelagic fish is normally carried out by cooking the fish in large continuous cookers to temperatures of 95−100 °C for 15−30 min (*2*).

The chemical and physical properties of edible oils depend primarily on the composition of original materials and processing temperatures. The crude oil obtained by traditional heat processing tends to be dark in color and to contain a large amount of impurities. Therefore, refining steps including degumming, neutralization, bleaching, and deodorization are necessary. However, the high temperatures and chemical treatments utilized during the refining steps may cause isomerization of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), producing trans isomers and resulting in decreased nutritional value (*3*). It is generally recognized that high temperatures influence fish oil deterioration and can accelerate lipid oxidation. When fish oils oxidize, they produce unstable intermediary compounds, such as free radicals and hydroperoxides, which are susceptible to further decomposition into products such as aldehydes and ketones. These decomposition products adversely affect flavor, taste, nutritional value, and the overall quality of fish oils. Volatile compounds such as pentanal can also develop, resulting in strong fishy odors in the final product (*4*). Typically, these impurities and decomposed products can be partially removed in the traditional oil refining process, although high temperature tends to be required for the process.

The aims of oil processing are to maximize yield while maintaining high quality and to produce highly stable oils by eliminating undesirable compounds. The high levels of PUFAs in sardine oil are subject to rapid and extensive oxidation by exposure to air, light, or heat during processing. Oil extraction by non-heat processes would be expected to result in higher quality compared to heat-based extraction by minimizing the decomposition of PUFAs and the formation of various oxidation products. In addition, separated protein and phospholipids could be recovered with further purification steps and utilized. The objective of this study was to develop an oil extraction method using pH adjustment to the isoelectric point (p*I*) and to compare this method with the heat-processing method in terms of oil recovery and quality. The use of acids in pH adjustment, as well as the addition of calcium, was also investigated.

MATERIALS AND METHODS

Fish and Sample Preparation. Sardines (*Sardinops sagax*) were harvested off the northwestern Oregon coast by commercial purse seiners. The sardines were pumped from the seine nets directly into refrigerated seawater holds aboard the vessels and rapidly cooled to <4°C. The sardines were off-loaded within 8 h of capture at a local processing plant in Astoria, OR. Sardines were randomly chosen, packed in ice, and delivered to the Oregon State University Seafood Laboratory on the day of catch. The head, viscera, and backbone were removed from each fish, and skin-on fillets were obtained. The fillets were mixed together and minced with a vertical-cutter/mixer (Stephan Food Processing Technology, Hamburg, Germany) for 2 min and kept in

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vacuum-sealed bags at −30 °C for further analysis. The lipid content of sardine mince sample was determined according to Association of Official Analytical Chemists (AOAC) method 934.01 (*5*).

Determination of p*I* of Sardine Muscle. Citrate−phosphate buffers of various pH values (5.0−5.9) were prepared using 0.1 M citric acid ($C_6H_8O_7 \cdot H_2O$, J. T. Baker Chemical Co., Phillipsburg, NJ) and 0.2 M dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$, Matheson Coleman & Bell, East Rutherford, NJ). Sardine mince (5 g) was mixed with citrate−phosphate buffers (15 mL) at various pH values, and the final volume of homogenates was brought to 25 mL. The mixture was centrifuged at 20000*g* at 4 °C for 30 min, and three layers were obtained: the supernatant lipid fraction in the upper layer, an aqueous fraction in the middle, and a residue fraction in the bottom layer. Soluble protein concentration in the aqueous layer was determined according to the Bradford dye binding method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The absorbance was measured at 595 nm (UV−vis spectrophotometer; UV 2401 PC, Shimadzu Corp., Kyoto, Japan) using bovine serum albumin as the standard.

Oil Extraction. Sardine mince was mixed with deionized water (1:3, v/v), and the pH was adjusted to the p*I* of sardine muscle protein with either 2.0 M hydrochloric acid (HCl), 2.0 M tartaric acid (TA), or 2.0 M citric acid (CA). It has been reported that Ca^{2+} ions facilitate the separation of the oil and water phases in fish mince (*16*). To test this, a separate set of mince samples was treated with either calcium tartarate (1.5% of volume) followed by TA (Ca-TA) or calcium citrate (1.5% of volume) followed by CA (Ca-CA) for pH adjustment. Oils were separated by centrifugation at 10000*g* for 20 min. After centrifugation, the upper and middle layers containing oil and aqueous fractions, respectively, were transferred to a separatory funnel, and the oil was removed and weighed. In the control group, sardine mince was heated with deionized water (1:3, v/v) at 95 °C for 30 min and then centrifuged at 10000*g* for 20 min. The resulting supernatant was transferred to a separatory funnel, and the oil was removed and weighed. All extracted oils were filtered under vacuum with hardened ashless filter paper (type 542, Whatman Inc., Brentford, U.K.) to remove contaminants and particles from the final products. Oil recovery (percent) was determined by dividing the removed oil in the different phases by the total lipid content in sardine muscle. Six replicates were run, and the extracted oils were stored under nitrogen at −80 °C until further analysis. Oil content in the precipitated residue after oil extraction was determined by AOAC method 934.01 (*5*) to measure the oil retained in the centrifuged residues. The oil content (percent) in the emulsion layer was calculated by subtracting the sum of the recovered oil and precipitated residue oil from 100%.

Color Analysis. Color analysis of extracted oils was carried out using a spectrophotometer (Hunter lab Color Quest, Hunter Associates Laboratory, Inc., Reston, VA). The color of oils was determined based on 10° standard observation and D 65 illuminants. Values were expressed as *L**, *a**, *b**, and haze. The *L** value indicates lightness (100, white; 0, black), the *a** value indicates either redness (+) or greenness (−), and the *b** value indicates either blueness (+) or yellowness (-). Haze represents cloudiness of the products possibly as a result of particles such as pigments or contaminants. Hue angle, which represents spectral distribution (actual color), and chroma, which evaluates the intensity of the color, were also calculated.

Thin-Layer Chromatography (TLC) Separation. Extracted sardine oils were subjected to TLC separation for lipid composition determination. Lipids were separated into triacylglycerol (TG), diacylglycerol (DG), monoacylglycerol (MG), free fatty acids (FFA), and phospholipids (PL). Samples were dissolved in hexane (10% solution, v/v), and an aliquot of 5 μL of sample mixture was loaded onto the silica plate (K6 silica gel 60, 200 × 200 × 0.25 mm, Whatman Inc.). The developing solvent was hexane/diethyl ether/acetic acid (60:40:2, volume basis). Spots were visualized by spraying 5% sulfuric acid solution followed by drying at 100 °C on a hot plate for 30 min. Quantification was carried out with densitometry ChemDoc with Quantity One software (Bio-Rad, Hercules, CA) using a commercially available TLC standard (Nu Check Prep, Inc., Elysian, MN), and each fraction level was expressed as weight/weight percent.

Determination of Fatty Acid Composition by Gas Chromatography (GC). Fatty acids in sardine oils were converted into fatty acid methyl esters (FAME) according to AOAC method 991.39 (*5*), and their composition was determined by GC. A Shimadzu GC-2010 (Shimadzu Corp.) equipped with a flame ionization detector and capillary column (Omegawax 250 capillary column, 30 m × 0.25 μm × 0.25 μm film thickness; Supelco, Bellefonte, PA) was used for analyzing FAME. Injector and detector temperatures for the GC system were set at 250 and 270 °C, respectively. The column temperature was set at 170 °C with 8 min of hold time and gradually heated to 245 °C at a rate of 1 °C/min with 2 min of hold time. Helium was used as a carrier gas. The fatty acid concentrations were calculated by comparison of their retention times and area of each peak with those of the reference standards (Supelco).

Protein Content Determination in Extracted Oils. Extraction of protein from oils was carried out according to a method of Zitouni et al. (*6*) with a slight modification. A volume of 2 mL of 50 mM phosphate-buffered saline solution was added to 5 g of extracted sardine oils, and the mixture was maintained at 60 °C for 24 h. After centrifugation at 1500*g* for 15 min, 2 mL aqueous layers were collected and dialyzed (dialysis membrane with 12000−14000 Da MW; 45 mm flat; 20 μm wall thickness; Fisher Scientific, Pittsburgh, PA) against deionized water for 24 h. The protein concentration was determined with the Lowry method (*7*) using bovine serum albumin as the standard.

Conjugated Diene Determination. Conjugated dienes were measured as indicators of free radical production. The progress of the peroxidation reaction was estimated by measuring absorbance (Abs) at 234 nm for conjugated dienes using a spectrophotometer (UV 2401PC, Shimadzu Corp.). Abs was measured using 10 mm path length quartz UV cuvettes with reference readings made on spectroscopic grade *n*-hexane with a 1% oil dilution.

Thiobarbituric Acid (TBA) Measurement. Degree of oxidation based on secondary oxidation products was determined by using the TBA method (*8*). Extracted oils (approximately 0.05 g) in antioxidant solution were mixed with 3 mL of TBA reagent, and the mixture was heated in boiling water for 30 min. After cooling, 5 mL of chloroform was added, and the mixture was shaken vigorously. The mixture was centrifuged at 3500 rpm for 10 min, and the absorbance of the upper layer was measured at 535 nm by spectrophotometer (UV 2401PC, Shimadzu Corp.). TBA values were determined at day 0 and then every 2 weeks over an 8 week time period. The samples were stored at 0 °C in a glass tube container covered with Parafilm. The results were expressed as milligrams of malonaldehyde per kilogram of sardine oil.

Statistical Analysis. All analyses were run in triplicate, and the results were presented as averages and standard deviations. Statistical comparisons were made between treatments by analysis of variance (ANOVA) and Tukey's test using SPSS software. The results were presented in terms of p values ($p < 0.05$).

RESULTS AND DISCUSSION

p*I* of Sardine. Solubility of sardine protein at pH 5.0−5.9 is shown in **Figure 1**. Solubility ranged from a high of 5.22 mg/g of sardine tissue (pH 5.0) to a low of 3.77 mg/g of sardine tissue (pH 5.5), indicating a p*I* of 5.5 for sardine muscle protein. In general, the p*I* of fish muscle ranges between pH 4.5 and 5.5 depending on species. Several other studies have shown similar p*I* values for fish muscle protein. Pacific whiting and rockfish muscle protein had a p*I* of 5.0 (*9*, *10*), and mackerel and cod muscle protein had a p*I* of 5.5 (*11*, *12*).

The pH-shift method has been shown to be a feasible process for recovering protein from various fish species (*13*). By this method, protein is first solubilized at extreme pH and then precipitated at the p*I* and removed by centrifugation. It was expected that this method might also be practical for oil recovery from sardines. However, preliminary experiments showed that pH shifting to either extreme acid or alkaline endpoints (pH 2−3 or 10−11, respectively) resulted in lower oil recovery due to the presence of white membrane-like emulsions formed at the oil−water interface. This was perhaps due to high emulsifier

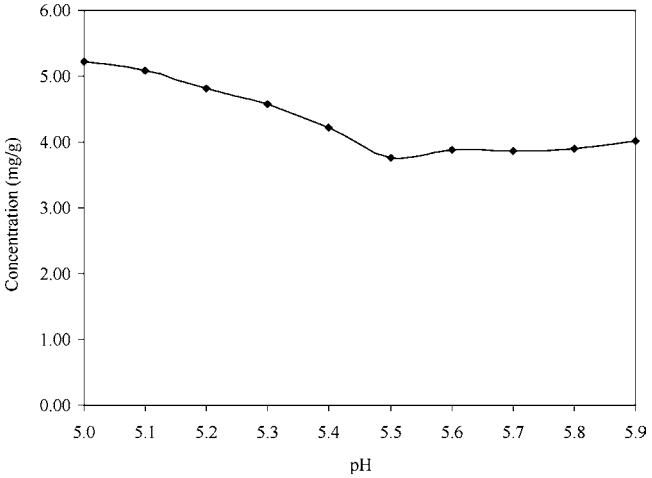

Figure 1. Changes in solubility of sardine muscle protein at different pH values.

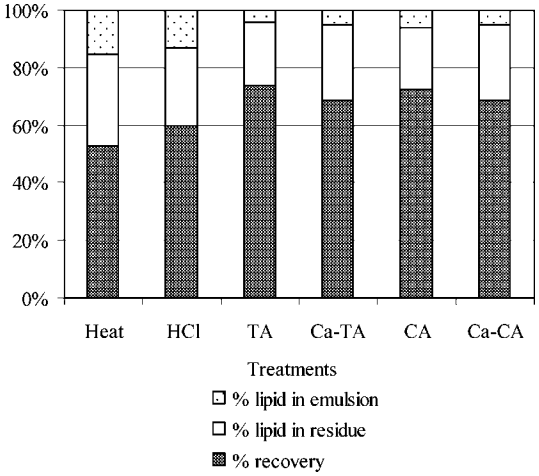

Figure 2. Percent oil recovery from sardine mince and lipid distribution in centrifuge residues and emulsions.

effects of partially denatured proteins exposing both hydrophilic and hydrophobic amino acid sequences. By going directly to the p*I*, electrostatic charges can be minimized, and the proteins quickly aggregate and begin to precipitate out of solution, thereby limiting emulsion-type interactions (*14*). Because the emulsion capacity of sardine protein is minimal at the p*I*, separation of the oil and water phases was greatly facilitated in the present study by adjusting a sardine mince/water slurry to pH 5.5 without first solubilizing the proteins.

Oil Recovery and Lipid Distribution. The oil recoveries (percent) of different extraction methods are shown in **Figure 2**. The oil extracted by adjusting the pH to the p*I* of sardine muscle protein with TA and CA showed the highest recoveries (73.73 and 72.24%, respectively) followed by Ca-TA and Ca-CA. Furthermore, oil separation after adjustment of the pH to the p*I* with organic acid, with or without calcium, was notably easier than in the heating process due to fewer particles and contaminants at the oil−water interface. This may be due to effective removal of emulsifiers including PL and protein residues in the pH adjustment method, demonstrating advantageous effects on emulsion breaking and clear separation of the oil and water phases.

Emulsified oil can be localized, with particle-like aggregations of compatibly charged molecules existing in an incompatible aqueous phase. Substances in the slurry such as amphiphilic proteins and phospholipids can act as aids in forming emulsions. Coagulants including metal salts have been used for removal of emulsified oil from large amounts of oily wastewater generated by various industries (*15*). Likewise, it was reported that the addition of calcium ions in the presence of organic acid greatly facilitated removal of PL from cod muscle homogenates (*16*). To achieve high oil recovery, emulsifiers including PL should be removed. If emulsions are already formed, addition of cations, including organic acid and calcium, as emulsion-breaking agents can disrupt the charge attraction of the hydrophobic molecules and coagulate oil droplets via charge neutralization, resulting in fewer emulsions at the oil−water interface (*17*). This was most likely the reason that pH adjustment methods demonstrated effective removal of emulsifiers including PL and protein residues, facilitating separation of the oil and water phases and allowing for higher oil recovery compared to heat extraction. In contrast, oil extraction by heat resulted in more emulsion layers at the oil−water interface, and the lowest oil recovery (52.95%) was observed. The results of lipid analysis of centrifuged residues also suggest increased efficiency of oil removal with pH adjustment methods. Centrifuged residues from heat extraction contained the highest amount of lipid (31.83%) followed by residues from HCl treatment (27.36%), whereas pH adjustment methods with organic acids resulted in lower lipid in the residues, ranging from 21.87 to 25.90%. Lipid content in the emulsion layer also showed significantly higher levels in heat-processed oil (15.22%) as well as in oil extracted with HCl (12.99%) compared to oils extracted by pH adjustment with organic acids (range = 4.04−5.89%).

Color Analysis. The color of oil is a factor in determining quality because dark-colored oils require high-cost processing to achieve an acceptable light-colored product. Color analysis (**Table 1**) indicated that all sardine oils had high *L** values, suggesting light color characteristics of the products. All oils had a negative *a** value, indicating a slight greenish color, and a positive *b** color, indicating yellowish colors. Chroma represents the intensity of the color and also showed similar values among treatments. Hue angle values were in the range of 98.33−99.02°, which implies greenish yellow color of the products.

There were significant differences found in haze values among treatments.

Haze represents cloudiness of the oils, perhaps as a result of particles such as pigments. The lowest haze value (4.58) was

Table 1. Color Analysis of Sardine Oils Extracted by Heat and pH Adjustment Methods[a]

treatment	*L**	*a**	*b**	chroma	hue	haze
heat	95.89 ± 0.01 b	−1.91 ± 0.01 a	12.01 ± 0.01 a	12.16 ± 0.01 a	99.02 ± 0.04 d	7.56 ± 0.10 b
HCl	96.14 ± 0.01 d	−1.80 ± 0.01 d	12.13 ± 0.01 b	12.26 ± 0.01 b	98.43 ± 0.03 ab	16.76 ± 0.57 e
TA	96.08 ± 0.01 c	−1.77 ± 0.01 e	12.12 ± 0.01 b	12.25 ± 0.01 b	98.33 ± 0.06 a	15.16 ± 0.89 d
Ca-TA	95.93 ± 0.01 b	−1.84 ± 0.01 c	12.30 ± 0.01 c	12.44 ± 0.01 c	98.52 ± 0.04 b	10.30 ± 0.25 c
CA	95.85 ± 0.04 a	−1.87 ± 0.01 b	12.59 ± 0.01 e	12.73 ± 0.01 e	98.47 ± 0.07 b	10.62 ± 0.03 c
Ca-CA	95.85 ± 0.02 a	−1.91 ± 0.02 a	12.48 ± 0.03 d	12.62 ± 0.02 d	98.69 ± 0.0 8c	4.58 ± 0.03 a

[a] Values are mean ± SD of triplicates. Values in the same row labeled with different letters are significantly different ($p < 0.05$).

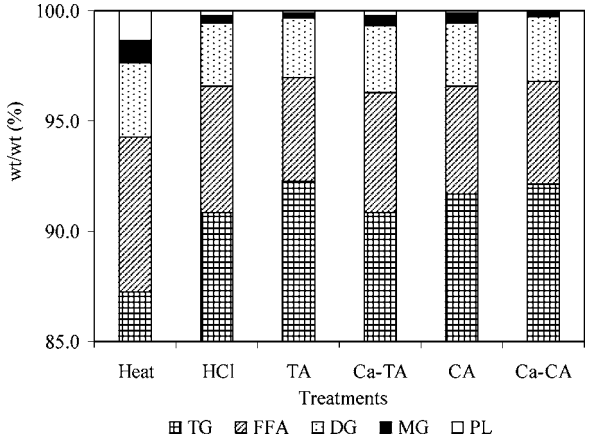

Figure 3. Lipid compositions of sardine oils extracted by heat and pH adjustment methods.

recorded for the oil treated with Ca-CA, suggesting that this treatment results in a relatively clear oil final product.

Lipid Composition. Lipid compositions (TG, DG, MG, FFA, and PL) of extracted oils are shown in **Figure 3**. Oil extracted by heat showed significantly higher levels of FFA, DG, MG, and PL (7.29, 3.41, 0.99, and 1.33%, respectively) with a lower level of TG (86.98%) compared to oils extracted by pH adjustment. This is in agreement with a study on oil separation from precooked and raw skipjack tuna heads (*1*), which showed that oils prepared at high temperatures had high resulting FFA levels. The high levels of FFA as well as DG and MG in oil extracted by heat suggest that hydrolysis of the ester bonds of TGs might have occurred during the heat extraction process, as oil can undergo hydrolysis in the presence of moisture and heat. There were no significant differences found in TG levels among the pH adjustment methods, and the levels were significantly higher than those in heat extraction, ranging from 90.92 to 92.24%. PL was lowered in all non-heat treatments and nondetectable in oil extracted with Ca-CA, showing efficient removal of PL from the final products.

Cationic salts, such as calcium chloride, aluminum sulfate, and ferric chloride, are used extensively by wastewater treatment plants for the removal of phosphorus (*18*). Calcium ions have also been used for whey protein purification by promoting the aggregation and precipitation of phospholipoproteins that originate from fat globule membranes in cheese whey (*16*). Cations are known to form precipitates with phosphorus, creating an insoluble material (*19*). A study on membrane sedimentation from acid-solubilized cod protein also showed that significant amounts of membrane PL were removed from muscle homogenates by adding Ca^{2+} in the presence of CA prior to solubilization at pH 3 (*16*). This observation was similar to results of the present study, and a possible mechanism suggested was that the polycarboxylic groups of CA induce membrane removal, whereas Ca^{2+} ions allow for close contact between the vesicles through the formation of anhydrous calcium−phospholipid complexes.

Fatty Acid Profiles. GC analysis showed that oil extracted from sardines harvested off the Oregon−Washington coast had high levels of n-3 PUFAs. PUFAs represented the highest oil fraction, ranging from 219.19 to 264.40 mg/g of oil, followed by the saturated fatty acid (SFA) fraction, which ranged from 185.70 to 219.95 mg/g of oil (**Table 2**). The major fatty acids in sardine oils were 16:0, 16:1n-7, 20:5n-3, and 22:6n-3, with 20:5n-3 being the highest, in agreement with other studies (*20*, *21*). These results show relatively similar amounts of fatty acids in all extracted oils in terms of weight/weight percent; however, there were differences found in terms of fatty acid milligrams per gram of oil. Oil extracted by heat and HCl showed the lowest levels of total fatty acids (513.49 and 515.02 mg/g of oil, respectively) and n-3 PUFAs (207.40 and 209.54 mg/g of oil, respectively). The pH adjustment procedure with CA, TA, Ca-CA, and Ca-TA showed higher amounts of total fatty acids, ranging from 530.36 to 597.54 mg/g of oil, with n-3 PUFAs ranging from 221.18 to 361.62 mg/g of oil. Oil extracted with Ca-CA contained the highest levels of 20:5n-3 and 22:6n-3 (146.75 and 63.12 mg/g of oil, respectively), whereas oil

Table 2. Fatty Acid Profiles of Sardine Oils Extracted by Heat and pI Adjustment Methods[a]

FFA	heat	HCl	TA	Ca-TA	CA	Ca-CA
C14:0	43.48 ± 2.27 ab	40.66 ± 1.30 a	46.23 ± 0.56 b	42.46 ± 0.26 a	42.43 ± 0.90 a	43.93 ± 0.93 ab
C15:0	1.08 ± 0.01 a	1.35 ± 0.35 a	1.65 ± 0.60 a	1.12 ± 0.06 a	1.70 ± 0.37 a	1.63 ± 0.50 a
C16:0	109.12 ± 4.36 a	113.82 ± 7.68 a	132.86 ± 2.88 c	118.30 ± 0.56 ab	120.94 ± 1.92 abc	128.34 ± 2.74 bc
C17:0	9.33 ± 0.82 a	8.44 ± 1.94 a	9.58 ± 0.24 a	9.46 ± 0.90 a	9.87 ± 0.62 a	10.56 ± 0.69 a
C18:0	22.69 ± 0.62 a	24.22 ± 4.29 a	29.43 ± 0.68 a	23.53 ± 0.38 a	24.24 ± 0.64 a	28.93 ± 1.08 a
SFA	185.70 ± 6.44 a	188.49 ± 15.59 a	219.75 ± 4.80 c	194.86 ± 2.16 ab	199.19 ± 3.40 abc	213.38 ± 4.98 bc
C14:1n-5	0.20 ± 0.02 a	0.19 ± 0.01 a	0.23 ± 0.00a	0.19 ± 0.01 a	0.19 ± 0.00 a	0.20 ± 0.02 a
C16:1n-7	59.35 ± 1.22 ab	57.06 ± 2.73 a	63.80 ± 1.41 b	59.17 ± 0.38 ab	60.39 ± 0.97 ab	63.68 ± 1.34 b
C17:1n-9	7.62 ± 0.20 ab	7.45 ± 0.47 a	8.25 ± 0.16 b	7.64 ± 0.05 ab	7.64 ± 0.13 ab	8.22 ± 0.19 ab
C18:1n-9	27.30 ± 0.56 ab	26.39 ± 2.09 a	29.46 ± 0.68 ab	27.29 ± 0.19 ab	28.26 ± 0.39 ab	30.45 ± 0.57 b
C20:1n-9	9.83 ± 0.07 a	9.82 ± 1.19 a	11.41 ± 0.39 ab	10.01 ± 0.13 a	10.56 ± 0.16 ab	11.77 ± 0.21 b
C22:1n-9	4.20 ± 0.02 a	4.32 ± 0.66 a	4.97 ± 0.16 ab	4.34 ± 0.11 ab	4.66 ± 0.11 ab	5.29 ± 0.10 b
C24:1n-9	0.09 ± 0.02 a	0.11 ± 0.00 ab	0.12 ± 0.01 bc	0.11 ± 0.00 b	0.12 ± 0.00 bc	0.14 ± 0.01 c
MUFA	108.59 ± 2.07 ab	105.34 ± 7.10 a	118.24 ± 2.72 b	108.75 ± 0.86 ab	111.83 ± 1.67ab	119.75 ± 2.39 b
C18:2n-6	8.12 ± 0.21 a	8.02 ± 0.74 a	9.29 ± 0.32 b	8.18 ± 0.10 ab	8.49 ± 0.10 ab	9.32 ± 0.20 b
C18:3n-6	1.93 ± 0.06 ab	1.90 ± 0.20 a	2.23 ± 0.06 b	1.91 ± 0.01 a	1.98 ± 0.04 ab	2.17 ± 0.05 ab
C18:3n-3	2.69 ± 0.07 ab	2.60 ± 0.27 a	3.02 ± 0.09 b	2.71 ± 0.01 ab	2.81 ± 0.04 ab	3.09 ± 0.06 b
C20:2n-6	0.49 ± 0.01 a	0.49 ± 0.06 a	0.57 ± 0.01 ab	0.50 ± 0.01 a	0.54 ± 0.01 ab	0.60 ± 0.01 b
C20:3n-6	1.26 ± 0.06 a	1.24 ± 0.18 a	1.01 ± 0.39 a	1.15 ± 0.03 a	1.14 ± 0.01 a	1.27 ± 0.04 a
C20:3n-3	3.34 ± 0.03 a	3.26 ± 0.28 a	3.66 ± 0.13 ab	3.39 ± 0.03 a	3.56 ± 0.05 ab	3.85 ± 0.05 b
C20:5n-3	123.09 ± 0.91 a	124.08 ± 14.0 a	141.69 ± 4.66 ab	127.09 ± 1.47 ab	132.46 ± 2.00 ab	146.75 ± 2.78 b
C22:5n-3	27.70 ± 0.17 a	28.11 ± 3.56 a	32.35 ± 1.13 ab	28.84 ± 0.50 a	30.57 ± 0.40 ab	34.23 ± 0.61 b
C22:6n-3	50.58 ± 0.37 a	51.48 ± 6.64 a	59.60 ± 2.07 ab	52.98 ± 0.91 a	55.89 ± 0.72 ab	63.12 ± 1.10 b
PUFA	219.19 ± 0.80 a	221.18 ± 25.92 a	253.43 ± 8.23 ab	226.76 ± 3.09 ab	237.44 ± 3.34 ab	264.40 ± 4.77 b
n-3 PUFA	207.40 ± 0.46 a	209.54 ± 24.74 a	240.32 ± 8.06 ab	215.01 ± 2.93 ab	225.29 ± 3.19 ab	251.04 ± 4.50 b
total FA	513.49 ± 9.31 a	515.02 ± 48.42 a	591.43 ± 14.66 b	530.36 ± 6.11 ab	548.46 ± 7.41 ab	597.54 ± 11.99 b

[a] Values (w/w %) are mean ± SD of triplicates. Values in the same row labeled with a different letter are significantly different ($p < 0.05$).

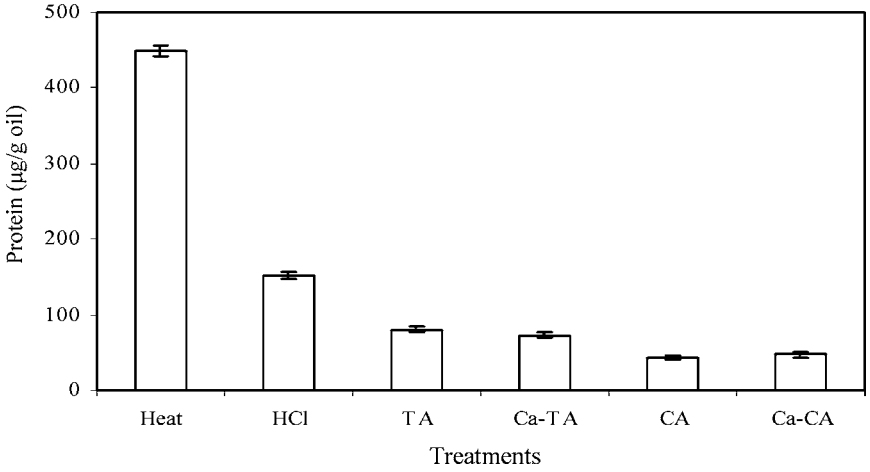

Figure 4. Protein content in sardine oils extracted by heat and pH adjustment methods.

Table 3. Conjugated Dienoic Acid Content (Percent in Oil) Extracted by Heat or pH Adjustment Methods[a]

treatment	conjugated dienes (%)
heat	12.75 ± 0.00 e
HCl	13.32 ± 0.10 f
TA	8.92 ± 0.01 a
Ca-TA	12.12 ± 0.01 d
CA	10.43 ± 0.00 b
Ca-CA	11.86 ± 0.01 c

[a] Values are mean ± SD of triplicates. Values in the same column labeled with a different letter are significantly different ($p < 0.05$).

extracted by heat showed significantly lower levels of 20:5n-3 and 22:6n-3 (123.09 and 50.58 mg/g of oil, respectively), demonstrating reduced purity of heat-extracted oil.

Edible oils are complexes of glycerides and other minor components, including hydrocarbons, waxes, sterols, alcohols, vitamins (tocopherols), pigments, terpenic acids, and other contaminants such as proteins and phenols, and each of these components can alter the texture and/or flavor of the oil (*22*). The higher total fatty acids content, and therefore lower contaminant levels, found in oil extracted by pH adjustment with organic acids, especially with Ca-CA, suggests higher oil quality.

Protein Content in Extracted Oils. Protein contents of sardine oil extracted by both heat and pH adjustment methods are shown in **Figure 4**. Oil extracted by heat showed the highest protein content (448 μg/g of oil) followed by oil extracted with HCl (151 μg/g of oil). The lowest protein content was found in oil extracted with CA (43 μg/g of oil) and Ca-CA (47 μg/g of oil) followed by oil extracted with TA and Ca-TA (80 and 73 μg/g of oil, respectively). Traditionally, minor oil components, such as proteins, have been considered to be impurities (*23*). Other nitrogen-containing intermediates including trimethylamine oxide, a hydrolysis product of phospholipids, have been identified in the oils. These intermediates react with hydroperoxides, giving formaldehyde and dimethylamine, which are responsible for fishy odors (*24*). To achieve high-quality oil recovery from sardines, protein contaminants should be removed during the extraction process; however, during conventional oil extraction processes, partial proteins derived from original material (sardine mince) can be solubilized and remain in the final product. Protein content in refined/crude oil ranges from 0.1 to 148 μg/g of oil depending on the nature of the source and the processing method (*23*). It was demonstrated that extraction with CA and Ca-CA could result in higher quality oils among all treatments with fewer impurities, as supported by low levels of protein contamination in the final products.

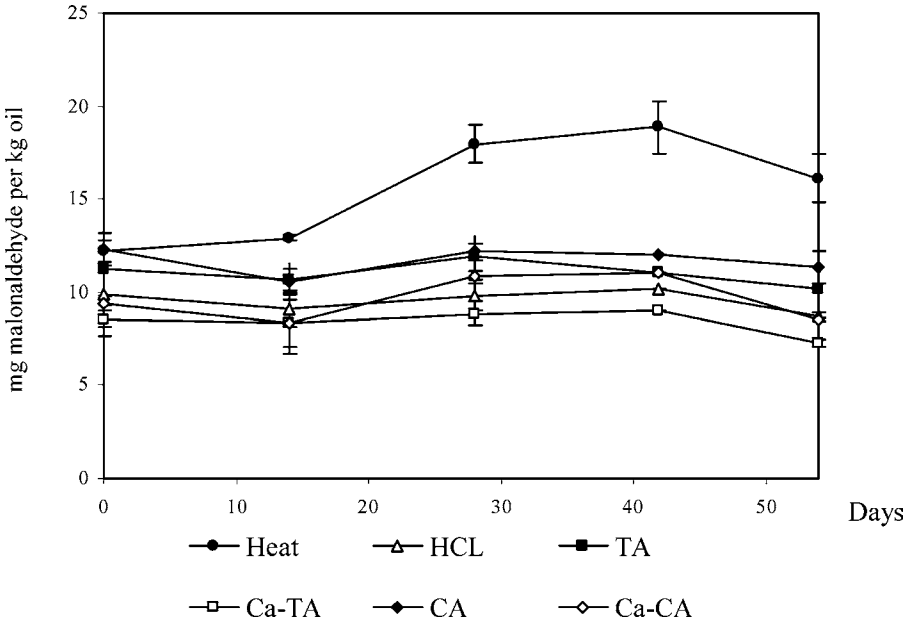

Figure 5. Changes in TBA values of sardine oil extracted by heat and pH adjustment methods over the storage period.

Conjugated Dienes. Results of conjugated diene measurements are shown in **Table 3**. Conjugated dienes are typically generated during initial oxidation processes, when *cis*-methyl interrupted double bonds are converted into conjugated double bonds (*25*). These conjugated double bonds have strong UV absorption at 234 nm, and therefore relative levels of conjugated dienes can be determined by measuring the absorbance (Abs) of the sample at this wavelength.

Conjugate dienes (percent) were found to be the highest in oil extracted by HCl (13.32%) followed by heat (12.75%). Among the oils extracted by pH adjustment, the lowest values were found in oil extracted with TA (8.92%) followed by oil extracted with CA (10.43%).

TBA Values. At day 0, the oil extracted by heat showed the highest TBA value (12.24 mg of malonaldehyde/kg of oil), whereas the lowest TBA value was found in the oil extracted by Ca-TA (8.50 mg of malonaldehyde/kg of oil) followed by Ca-CA [(9.40 mg of malonaldehyde/kg of oil) (**Figure 5**)]. TBA values of oil extracted by heat steadily increased with storage time and reached the highest level at day 42 (18.87 mg of malonaldehyde/kg of oil). On the other hand, TBA values of oil extracted by pH adjustment remained low throughout the storage period. TBA values were significantly reduced with the addition of calcium in both the CA and TA treatments.

Organic acids including CA and TA interact with metals, such as copper, iron, magnesium, and calcium, to form a wide range of metallic salts that exhibit antioxidant properties in oils by reducing metal-catalyzed oxidation through chelation of trace metals (*24*). Lipid oxidation in fish is often associated with PL, which are usually in cellular membranes and can be in direct contact with aqueous pro-oxidants. Researchers (*16*) have shown that calcium ions and organic acids help to promote the separation of membranes from acid-solubilized fish muscle, thus lowering the oxidation potential. The low TBA values in **Figure 5** correspond to the low PL levels shown in **Figure 3** showing that removal of PL from the oil improves its stability. Approaches to inhibit oxidation of fish oil also include maintaining minimal temperatures during processing, which lessen the oxidation reaction and help to retain natural antioxidants by avoiding conditions that destroy them (*26*).

LITERATURE CITED

(1) Chantachum, S.; Benjakul, S.; Sriwirat, N. Separation and quality of fish oil from precooked and non-precooked tuna heads. *Food Chem.* **2000**, *69*, 289−294.

(2) Sobstad, G. E. Marine oil separation, purificatin technology. *INFORM* **1992**, *3*, 827−830.

(3) Toyoshima, K.; Noguchi, R.; Hosokawa, M.; Miyashita, K.; Fukunaga, K.; Nishiyama, T.; Takahashi, R. Separation of sardine oil without heating from surimi waste and its effect on lipid metabolism in rats. *J. Agric. Food Chem.* **2004**, *52*, 2372−2375.

(4) Eymard, S.; Carcouët, E.; Dumay, J.; Chopin, C.; Rochet, M. J.; Genot, C. Development of lipid oxidation during manufacturing of horse mackerel surimi. *J. Sci. Food Agric.* **2005**, *85*, 1750−1756.

(5) AOAC. *Official Methods of Analysis of AOAC International*; Arlington, VA, 1998.

(6) Zitouni, N.; Errahali, Y.; Metche, M.; Kanny, G.; Moneret-Vautrin, D. A.; Nicolas, J. P.; Fremont, S. Influence of refining steps on trace allergenic protein content in sunflower oil. *J. Allergy Clin. Immunol.* **2000**, *106*, 962−967.

(7) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(8) Sinnhuber, R. O.; Yu, T. C. The 2-thiobarbituric acid reaction, an objective measure of the oxidative deterioration occuring in fats and oils. *J. Jpn. Oil Chem. Soc.* **1977**, *26*, 259−267.

(9) Choi, Y. J.; Park, J. W. Acid-aided protein recovery from enzyme-rich Pacific whiting. *J. Food Sci.* **2002**, *67*, 2962−2967.

(10) Yongsawatdigul, J.; Park, J. W. Effects of alkali and acid solubilization on gelation characteristics of Rockfish muscle proteins. *Food Chem. Toxicol.* **2004**, *69*, C499−539.

(11) Park, J. D.; Jung, C. H.; Kim, J. S.; Cho, D. M.; Cho, M. S.; Choi, Y. J. Surimi processing using acid and alkali solubilization of fish muscle protein. *J. Korean Soc. Food Sci. Nutr.* **2003**, *32*, 400−405.

(12) Stefansson, G.; Hultin, H. O. On the solubility of cod muscle proteins in water. *J. Agric. Food Chem.* **1994**, *42*, 2656−2664.

(13) Hultin, H. O.; Kelleher, S. D. Advanced Protein Technology, assignee. Process for isolating a protein composition from a muscle source and protein composition. U.S. Patent 6,005,073, Dec 21, 1999.

(14) Pace, C. N.; Treviño, S.; Prabhakaran, E.; Scholtz, J. M.; Franks, F.; Wilson, K.; Daniel, R. M.; Halling, P. J.; Clark, D. S.; Purkiss, A. Protein structure, stability and solubility in water and other solvents. *Philos. Trans. R. Soc. London, Ser. B, Biol. Sci.* **2004**, *359*, 1225−1235.

(15) Suzuki, Y.; Maruyama, T. Removal of emulsified oil from water by coagulation and foam separation. *Sep. Sci. Technol.* **2005**, *40*, 3407−3418.

(16) Liang, Y.; Hultin, H. O. Separation of membranes from acid-solubilized fish muscle proteins with the aid of calcium ions and organic acids. *J. Agric. Food Chem.* **2005**, *53*, 3008−3016.

(17) EPA. Wastewater technology fact sheet chemical precipitation. EPA 832-F-00-018, 2000; http://www.epa.gov/owm/mtb/uv.pdf.

(18) Georgantas, D. A.; Grigoropoulou, H. P. Phosphorus removal from synthetic and municipal wastewater using spent alum sludge. *Water Sci. Technol.* **2005**, *52*, 525−532.

(19) Zvomuya, F.; Rosen, C. J.; Gupta, S. C. Phosphorus sequestration by chemical amendments to reduce leaching from wastewater applications. *J. Environ. Qual.* **2006**, *35*, 207−215.

(20) Shirai, N.; Terayama, M.; Takeda, H. Effect of season on the fatty acid composition and free amino acid content of the sardine *Sardinops melanostictus*. *Comp. Biochem. Physiol.* **2002**, *131*, 387−393.

(21) Leonardis, A. D.; Macciola, V. A study on the lipid fraction of Adriatic sardine fillets (*Sardina pilchardus*). *Nahrung/Food* **2004**, *48*, 209−212.

(22) Ruiz-Gutierrez, V.; Perez-Camino, M. C. Update on solid-phase extraction for the analysis of lipid classes and related comounds. *J. Chromatogr. A* **2000**, *885*, 321−341.

(23) Hidalgo, F. J.; Zamora, R. Peptides and proteins in edible oils: stability, allergenicity, and new processing trends. *Trends Food Sci. Technol.* **2006**, *17*, 56−63.

(24) Debruyne, I. Soybean oil processing: quality criteria and flavor reversion. *Oil Mill Gazet.* **2004**, *110*, 10−11.

(25) Vaya, J.; Aviram, M. Nutritional antioxidants: mechanisms of action, analyses of activities and medical application. *Curr. Med. Chem.: Immunol. Endocr. Metab. Agents* **2001**, *1*, 99−117.

(26) Hultin, H. O. Oxidation of lipids in seafoods In *Oxidation of Lipids in Seafoods*; Hultin, H. O., Ed.; Blackie Academic and Professional: New York, 1994; pp 49−74.

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