PROCESS CHARACTERISTICS AND FUNCTIONALITY OF SARDINE (*SARDINA PILCHARDUS*) MUSCLE PROTEINS EXTRACTED BY A pH-SHIFT METHOD

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Abstract
The pH-shift method is a recent process used to isolate proteins from animal muscle with good functional properties. It can be used to recover proteins from cheap, underutilized sources and/or process byproducts. In this work, sardine (Sardina pilchardus) muscle proteins were extracted using acidic or alkaline-aided solubilization followed by isoelectric precipitation and compared to the traditional surimi process. This work also reports the comparison between centrifugation and filtration, as means of recovering the precipitated proteins. The highest solubilization of sardine proteins was recorded at pH 2.5 (94%) and 12 (98%), while optimum pH for the isoelectric precipitation of the solubilized proteins in both cases was 6. Protein recovery yields were 600g kg^{-1} for both acidic and alkaline processes, which are considerably higher than that of the traditional surimi process (400g kg^{-1}). When centrifugation was replaced by filtration, protein yields were significantly higher (730g kg^{-1} for the acidic and 800g kg^{-1} for the alkaline process), while fat reduction was decreased by 10%compared to the use of centrifugation. The proteins recovered after alkaline solubilization process showed better gelling properties compared to the ones recovered after acidic solubilization. The results of this work clearly show that the process of alkaline solubilization and filtration can successfully be employed to recover proteins with good functional properties from sardine mince.

Keywords: sardine, acidic and alkaline protein solubilization, protein yield, filtration

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1. INTRODUCTION

The surimi industry has changed dramatically over the past decade with reduced landings of several white muscle species beginning in 2000. As a result surimi prices have continuously increased prompting some researchers to explore the utilization of new species for surimi production (Chen et al. 1996; Park et al. 2003; Ramadhan et al. 2011). Expanding the range of species to include red muscle fish, however, has presented some obstacles due to proteolytic enzymes and color pigments (Park and Lin 2005). The pH-shift method is a recent process in which proteins are isolated from animal muscle using acid or alkaline solubilization followed by isoelectric precipitation to improve gelation properties and yield (Hultin and Kelleher, 1999). This method has several advantages, such as higher yield, higher protein quality, and efficient removal of insoluble impurities (lipids, membranes, skin, and bones) (Park and Lin, 2005). Almost all myofibrillar proteins and large proportion of the sarcoplasmic proteins are recovered, when the pH is adjusted to the isoelectric point after homogenization and solubilization at extreme pHs. Choi and Park (2002) reported that acidic or alkaline solubilization processes with Pacific whiting yielded 20% more proteins than the conventional 3-washing process. Alkali treated protein isolates have also shown better gel quality than those prepared from acid solubilization or conventional surimi processing (Yongsawatdigul and Park 2004). In addition, the use of high speed centrifugation removes neutral and membrane lipids, which result in significantly reduced oxidation and fishy odor. Lipid oxidation can affect the appearance, flavor, texture, and nutritional value of surimi, which ultimately leads to quality deterioration. The pH-shift method can also reduce lipid-soluble toxins such as diarrheic shellfish poisoning toxins (DSP), in the final product (Vareltzis and Undeland 2008). The process by which fish protein

isolate (FPI) are produced is distinctively different from conventional surimi production. Due to the nature of the process, FPI undergoes some conformational changes (unfolding and aggregation) during pH treatments. Fish protein isolate is currently ready to be used commercially as a functional ingredient, but has to be utilized differently from conventional surimi.

The two centrifugation steps involved in alkaline and acid solubilization processes is a controversial issue (Nolsoe et al. 2011). Centrifugation helps in a better separation of undissolved material, as well as membrane lipids. However, studies have shown that when the first centrifugation is avoided, the protein yields of acid and alkaline processes are increased. In addition, centrifugation is a quite costly industrial operation and should be avoided whenever possible (Nolsoe and Undeland 2008).

Sardine (*Sardina pilchardus*) is one of the main species caught off in southern Mediterranean waters and it is frequently under-utilized due to its low price in the fish auction. The use of small under-utilized pelagic fish, such as sardines, mackerel and others is also limited due to the large quantity of lipids and myoglobin in the muscle tissue. Generally, high quality surimi with improved gel strength and color can be obtained when dark muscle is removed as much as possible. However, abundant dark muscle in red-fleshed fish, such as sardine, is difficult to remove with a meat separator (Ochiai et al. 2001). Thus, it is of interest to compare the alkali- and acid-aided processes with the surimi traditional process. The main objective of this work was to determine the optimum pH conditions in order to solubilize the sardine proteins and then to recover them by iso-electric focusing. Second objective was to investigate some typical functional properties of the recovered proteins. Finally, it is of importance to investigate the possibility of replacing centrifugation during normal acid and/or alkaline solubilization process with filtration.

2. MATERIALS AND METHODS

Materials

Sardines (*Sardina pilchardus*) were purchased from a local retailer at Moudania, Greece. Their average size was 12±1.6cm in length. The total time elapsing between catch and processing ranged from 1 to 3 days. Chemicals were purchased from Sigma-Aldrich S.A. Solvents were purchased from Labscan Ltd. (Dublin, Ireland). All chemicals and reagents were of ACS grade and all solvents were of HPLC grade.

Methods

Sample preparation. Freshly caught sardines (*Sardina pilchardus*) were purchased from local retailer and transported to the Food Science Laboratory at Aristotelian University of Thessaloniki in insulated boxes filled with trimmed ice. The transportation time did not exceed 1h. Upon arrival the fish were manually eviscerated and filleted, rinsed with cold tap water and ground (meat grinder model 812 with 2.3 mm grinding plates, Biro, Marblehead, OH, USA). Eviscerated sardines were cut into smaller pieces prior to grinding.

Optimum solubilization and precipitation pH values. Ground sardine meat was transferred to a beaker placed in a container filled with crushed ice and was homogenized in nine volumes of cold distilled de-ionized water for 40 sec at 14,000 rpm using an Ultra Turrax T25 Basic homogenizer (IKA, Taquara, RJ, Brazil). The sardine homogenate was then divided into eight portions which were adjusted to pH values between 2.0 and 3.5 and between 10.5 and 12.0. These pH-ranges were chosen based on previous studies of pH-shift processing of fish (Marmon and Undeland 2010; Vareltzis and Undeland 2008) and on pre-studies with sardines. The pH adjustments were made using 0.5-2N HCl or 0.5-2N NaOH and a pre-calibrated Hamilton double pore electrode (Bonaduz, Switzerland) coupled to a pH-meter (Inolab WTW). The pH-adjusted homogenates were centrifuged at 8,000xg for 20 min in a Sorvall RC50 Plus centrifuge (DuPont, Newton, CT) set at 4^{o}C. The supernatant was separated from the sediment

and the floating top-layer through three layers of commercial cloth. Samples from the initial homogenate and supernatant were taken for protein analyses and calculations of relative solubility (see below).

To establish optimum precipitation pH-values, sardine homogenates prepared as previously described were adjusted directly to the optimum solubilization pH (2.5 and 12). After centrifugation the supernatant fractions were then each readjusted to a range of pH-values between 4.5 and 6.5. This was followed by a centrifugation at 8,000xg for 20 min and collection of the supernatant. Samples from the pH-adjusted supernatants were taken before and after the second centrifugation step for protein analyses and calculations of protein recovery.

Recovery of muscle proteins from filleted sardines by acid or alkaline solubilization process and the conventional washing process. The processing flow chart for the recovery of muscle proteins from sardines and the subsequent development of sardine protein-based gels is shown in Fig. 1. During the entire process to recover sardine muscle proteins, the temperature was carefully controlled at 1–4 ◦C to reduce the activity of endogenous proteases. To additionally minimize proteolysis, the processing time did not exceed 60 min.

In the acid process version, protein solubilization was carried out at pH 2.5, while for the alkaline process version was pH 12. After adjusting to the optimum pH the solution was subjected to filtration or centrifugation at 8,000xg for 20 min. In both processes, after centrifugation there were three layers: top – sardine fat; middle – sardine muscle protein solution; bottom – insolubles (bones, skin, insoluble proteins, membrane lipids, etc.).

The pH of the sardine muscle protein solution collected from the first centrifugation was adjusted to 6.0±0.05 with 0.5N and 2N HCl or 5N and 2N NaOH to precipitate sardine proteins previously solubilized at basic and acidic pH respectively. The acid (0.5N and 2N HCl) was used for the muscle protein solutions that had been solubilized at basic pH, while the base (0.5N and 2N NaOH) was used for the muscle protein solutions that had been solubilized at acidic pH. Once the desired pH was obtained, the precipitation reaction was allowed to take place for 10 min. The solution with precipitated proteins was centrifuged as described previously or filtered. The centrifugation resulted in two layers: top – process water; bottom – precipitated sardine protein. The proximate analysis was performed on the precipitated protein (see below).

Filtration as a method to separate dissolved from undissolved material was carried out with a 200 x 50 mm stainless steel test sieve (Retsch, SkanLab ApS, Slangerup, Denmark) with a 1 mm mesh opening.

To prepare protein isolate by the conventional washing process (PIWP), fish mince was washed with cold distilled water (4^{o}C) using a washing media/mince ratio of 3:1 (v/w). The mixture was stirred gently for 10min and the washed minced was filtered through a fiberglass screen. Washing was performed three times, where the third washing media contained 50mM NaCl.

Moisture content determination. Moisture determination was done according to the official AOAC method 985.14 using a Nuve FN500 oven (Ankara, Turkey).

Total lipid determination. The Lee et al. (1996) method as modified by Undeland et al. (2002) was employed for the determination of total lipids in raw materials and isolates. The ratio between chloroform and methanol used was 2:1.

Protein quantification. The protein content of the different samples was measured according to the method of Lowry as modified by Markwell et al. (1978). To measure the absorbance at 660 nm, a Heλios α UVA 090228 UV-visible spectrophotometer was used (Thermon Electron S.A., USA). Bovine serum albumin was used to obtain a protein standard curve in the range of 0-100 μg protein/mL. In order to calculate protein *solubility* (%) in the solubilization and precipitation steps, the protein concentration in the supernatant fraction was divided by the protein concentration in the non-centrifuged sample.

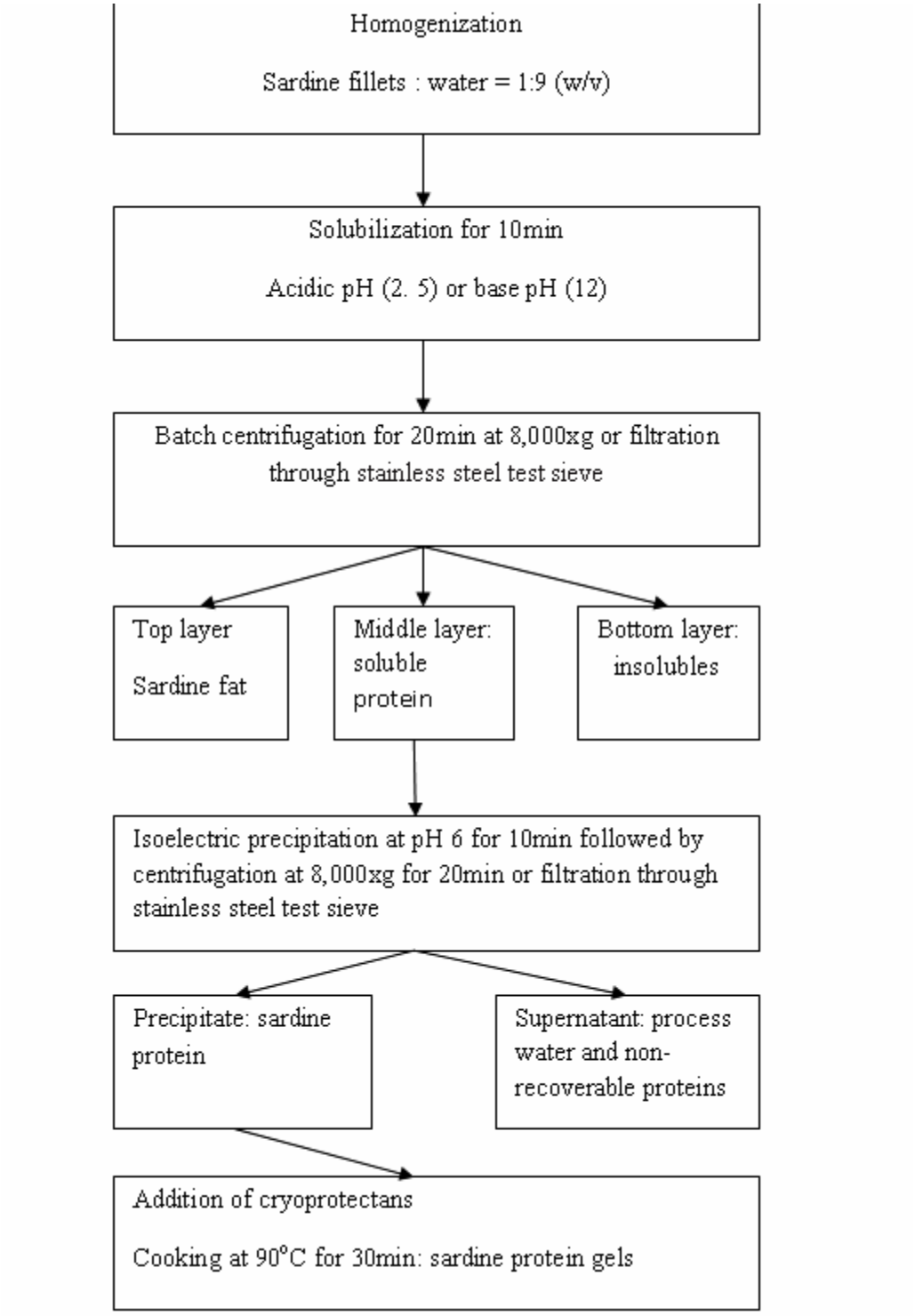

Fig 1 Flow chart for protein and lipid recovery from sardine fillets and subsequent development of sardine protein-based gels

Determination of protein recovery yield and fat reduction in recovered proteins. The protein recovery yield was calculated by determining the weight of the recovered sardine protein (dry basis) as g protein weight (dry basis) kg−1 protein in the raw material used.

The protein recovery yield was calculated by the following equation:

$$yield\ (g\ kg^{-1}) = \frac{wt\ recovered\ proteins}{wt\ protein\ in\ raw\ material}$$

The fat reduction was calculated by determining the weight of sardine fat (dry basis) removed from the recovered sardine proteins as g fat removed (dry basis) per kg fat in the raw material used. The fat reduction in the recovered sardine proteins was calculated by the following equation:

$$fat\ reduction(gkg^{-1}) = \frac{(fat\ in\ raw\ material) - (fat\ in\ recovered\ proteins)}{wt\ of\ fat\ in\ raw\ material}$$

Both fat and protein determination in recovered proteins were performed before the addition of the cryoprotectants for gel manufacturing.

Gel manufacturing. Gels were made from protein isolates as follows. Protein isolates were placed in a commercial cheese-cloth and water was manually squeezed out. The sample in the cheese-cloth was then placed in a refrigerator at 8°C without any applied pressure for 15 min to allow more water to be removed. Moisture content was then measured and if necessary adjusted with addition of pre-cooled distilled water to a final content of 83%. Sorbitol, sucrose, sodium tripolyphosphate and sodium bicarbonate at 5%, 4%, 0.3% and 0.4% (w/w) respectively were added to the samples which were then chopped at 8°C in a pre-cooled chopper to prevent the temperature of the protein isolates from exceeding 15°C throughout the mixing process. After the mixing, samples were placed in zipped-lock plastic bags and placed overnight in -20°C. Afterwards, the samples were partially thawed, and mixed with 2% NaCl in a pre-cooled mixer. The pH was adjusted to 7.1-7.3 if necessary with sodium bicarbonate and chopped in a pre-chilled chopper for about 5-6 minutes. The resulting paste was then stuffed in a plastic cylindrical bag and placed in 90°C water bath for 30 min. Finally, it was allowed to settle in an ice/water bath for 10min. Samples were kept in the refrigerator for 2 days before testing.

Color measurements. The color of the protein isolates was measured by determining the L*, a*, b* values with a colorimeter (Minolta Chroma Meter, CR-300, Minolta Corp., Ramsey, NJ) using the CIE Lab color scale.

Texture properties of sardine protein and surimi gels. At least 5 cylindrical gels (length 2.54 cm, diameter 1.9 cm) per treatment were used for TPA measurement. The gel samples were subjected to two cycle compression at 50% using the CT3 texture analyzer (Brookfield Engineering Laboratories, Inc, Middleboro, USA) with a 70 mm TPA compression plate attachment moving at a speed of 0.5 mm s^{-1}. From the resulting force–time curves, hardness, cohesiveness and elasticity were determined.

Foaming and emulsification properties. Foaming properties were evaluated as described by Liang and Kristinsson (2007). Relative overrun was calculated as V_0/V_1, where V_0 is the foam volume at 0min and V_1 the initial liquid volume. Liquid drainage was calculated as $1-\frac{(V_l - V_{l,30})}{(V_l - V_{l,0})}$ where $V_{1,30}$ is the liquid volume after 30 min and $V_{1,0}$ is the volume of liquid at 0 min. For foaming properties, protein isolate solutions were prepared at concentrations of 1% (w/v) by homogenizing the isolate in 100 mM NaCl solutions.

Emulsification activity index (EAI) was measured as described in Kristinsson and Hultin (2003).EAI was calculated as $\frac{2 \cdot 2.303 \cdot A}{l \cdot C \cdot \varphi} \cdot k$, where A is the absorbance, l is the path length of the cuvette (1cm), C is the protein concentration of an aqueous solution (5 mg/mL) and φ is the oil volume ratio (0.21= volume oil/total volume). Canola oil was used to form the emulsion and the units were m^2g^{-1}.

Statistical analysis. Data were analyzed by Excel (Microsoft Corporation, USA) and ANOVA using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Differences between treatment means at the 5% level were determined using the Duncan Multiple Range Test. Each process version was repeated at least three times (n=3) unless otherwise noted. The number of samples taken from each

process run for the different analyses was 2 or 3 (a=2-3). An average of the analytical readings-for these 2-3 samples was then used to calculate variations between different process runs.

3. RESULTS AND DISCUSSION

Sardine fillets proximate analysis. Sardine (*Sardina pilchardus*) is a nutritious source of proteins, poly-unsaturated fatty acids, vitamins A, D and B-12, as well as inorganic calcium, phosphorus and iron. Determination of protein, total lipids and ash content was performed on each new batch of fresh sardines (n=10). On a dry weight basis, sardines had 70.6±2.20% protein, 21.6±1.10% lipid and 5.7±0.10% ash content. Moisture content was 79.1±2.20%.

Optimum protein solubilization and precipitation pH values. The initial pH of the sardine-water homogenates was 6.32±0.1 (n=4). Following adjustments of the homogenates to pH-values in the ranges 2.0 to 3.5 (acid solubilization) and 11 to 12 (alkaline solubilization), the optimum pH for acid protein solubilization was found to be 2.5 (94% solubility) and for alkaline solubilization 12 (98% solubility) (Table 1).

TABLE 1 Solubility of sardine mince proteins at various pH (avg±SD, n=3)

pH	% solubility
Acidic solubilization	
2.0	94.1±0.50^{c}
2.5	94.4±1.30^{c}
3.0	91.1±1.31^{b,c}
3.5	85.5±1.40^{a}
Alkaline solubilization	
11.0	85.7±1.40^{a}
11.5	96.1±1.33^{b}
12.0	98.3±0.50^{b}

Numbers carrying different letters are significantly different ($p<0.05$

It was noticed that at pH around 12, the homogenate became very viscous and hard to handle at laboratory scale. Therefore pH values above 12 were not tested.

When searching for the optimal protein precipitation pH values following solubilization at pH 2.5 and 12, a range of pH values between 6.5 and 4.5 were tested. As shown in Fig. 2, the maximum recovery of solubilized proteins for both the acid and alkaline process was achieved at pH 6, where the recovery reached 90%. Batista et al. (2007) reported optimum precipitation pH for sardine myofibrillar proteins between 5 and 5.5.

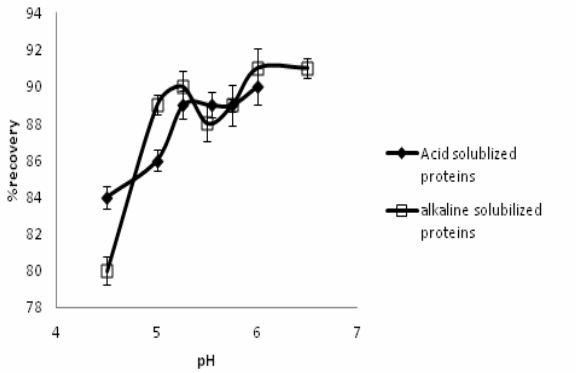

Fig 2 % recovery at different pH values of solubilized proteins at pH=2.5 (acid solubilized) or pH=12 (alkaline solubilized) (avg±SD, n=3)

Optimum precipitation pH values have been reported for other fish species in the range 5 to 6, such as cod (Stefansson and Hultin 1994), Pacific whiting (Choi and Park 2002), fish species from warm and temperate waters (Kristinsson and Demir 2003; Kristinsson and Ingadottir 2006; Kristinsson and Liang 2006) and rockfish (Yongsawatdigul and Park 2004). Undeland et al. (2002) working with herring light muscle checked different precipitation pH and chose pH 5.5 in the protein precipitation step because the optimal pH differed for the different characteristics during precipitation of acidified/alkalized herring proteins.

A small percentage of sardine proteins were not solubilized. This percentage probably consists of connective tissue proteins, as well as membrane lipo-proteins that precipitate out of the solution during centrifugation (Pacheco-Aguilar et al. 2001).

Composition of acid and alkaline produced protein isolates with or without centrifugation. Protein isolates from sardine fillets were produced by applying the acid and alkaline process using the optimum pH solubilization and precipitation values found previously. Since centrifugation and especially

at high speeds, like the ones that are commonly used in the pH-shift processes (>8,000xg), are avoided in the industry, it was necessary to study whether filtration could be employed instead. Protein, total lipids and moisture content of the protein isolates produced by different methods were determined and compared to the composition of protein paste produced by consecutive washes of sardine mince (surimi process) (Table 2).

TABLE 2 Protein, lipid and moisture content of surimi and protein isolates (PI) from acid and alkaline solubilization with centrifugation or filtration (avg±SD, n=4)

Sample	Protein (% dry wt)	Total lipids (% dry wt)	Moisture content (%)
AcPIC	71.0±3.51^{b}	1.7±0.30^{c}	90.9±0.31
ALPIC	68.2±4.60^{b}	2.6±0.42^{c}	90.6±0.32
AcPIF	63.9±4.21^{b}	4.8±0.10^{b}	89.5±0.34
ALPIF	62.1±3.20^{b}	4.2±0.10^{b}	90.1±0.28
PIWP	34.6±1.30^{a}	12.0±2.41^{a}	90.0±0.20

Numbers carrying different letters are significantly different ($p<0.05$)
AcPIC: acid produced protein isolate with centrifugation; ALPIC: alkaline produced protein isolate with centrifugation; AcPIF: acid produced protein isolate with filtration; ALPIF: alkaline produced protein isolate with filtration; PIWP: protein isolate produced by conventional washing process

Results indicate that the protein content in protein isolates from acid and alkaline solubilization processes with/without centrifugation was significantly higher than in PIWP. This is in agreement with other reports on acid and alkaline process performed on various species of fish (Batista et al. 2007; Cortes-Ruiz et al. 2001; Undeland et al. 2002). It is interesting to note that the use of centrifugation during acid or alkaline process resulted in protein isolates with only slightly, and not significantly, higher protein content compared to the treatments without centrifugation. In all samples lipid content was significantly lower compared to PIWP, indicating that the solubilization processes with/without centrifugation were effective in removing fat from sardine muscle. The use of centrifugation resulted in significantly lower lipid content in protein isolated compared to filtration.

Protein and fat recoveries from sardine mince during acid, alkaline and conventional surimi production processes. The protein recovery yield is a critical parameter to initiate determination of the economic feasibility of a new technology. As isoelectric solubilization/precipitation also allows recovery of fish oil, determination of fat recovery yield may aid in a more comprehensive understanding of the economic feasibility of this technology. Fish oil is often perceived by consumers as beneficial for human health, particularly the cardiovascular health associated with abundance of omega-3 fatty acids. At the same time, fish oil that is retained with the recovered protein may contribute to the development of rancidity. Therefore, determination of fat reduction in the recovered protein is also desirable. Protein recoveries by acid and alkaline solubilization method were both close to 600 g kg^{-1}, significantly higher than the conventional washing method (390 g kg^{-1}) (Fig. 3). This difference can be attributed mainly to the fact that by the pH-shift process recovers water soluble proteins by precipitating them, which are normally lost in the wash water of the conventional washing process (Batista et al. 2007). Cortes – Ruiz et al. (2001) reported 670 g kg^{-1} protein recovery when acid solubilization was applied to bristly sardines. In their review, Nolsøe and Undeland, reported on protein recoveries during acid ad alkaline processing of such materials to be on average 70% and 86%, respectively. However, when compared to other complex materials, then the yields especially for the alkaline procedure are comparable, and in certain cases better. Reported protein recovery for whole carp was 49 to 66% (Taskaya et al. 2009), 59 to 63% for Cape hake by-products (Ireneu et al. 2006), 57-59% from gutted herring (Marmon and Undeland, 2010), 45-50% from Antarctic krill (Chen et al. 2009) and 33% from shrimp processing waste (head and body shell) (Khumallambam et al. 2011). A slightly higher percentage of protein recovery was obtained by

the alkaline extraction (Fig. 3) however both the acidic and alkaline processes yields were considerably higher than the usually obtained in the traditional process. Park and Morrissey (2000) working with lean species also reported similar results. The yields reported by Undeland et al. (2002) on the protein recovery from herring were close to those obtained for sardine. However, in their work protein recoveries achieved in the acid process were slightly higher compared to the alkaline process. Kristinsson and Demir (2003) reported also similar findings for various species.

The use of filtration instead of centrifugation fro protein recovery significantly increased protein yield. For the acid solubilization process the yield from 606g kg^{-1} improved to 730g kg^{-1}, while for the alkaline solubilization process the yield improved from 621g kg^{-1} to 800g kg^{-1}. At the same time, there was a reduction in fat removal (Fig. 3).

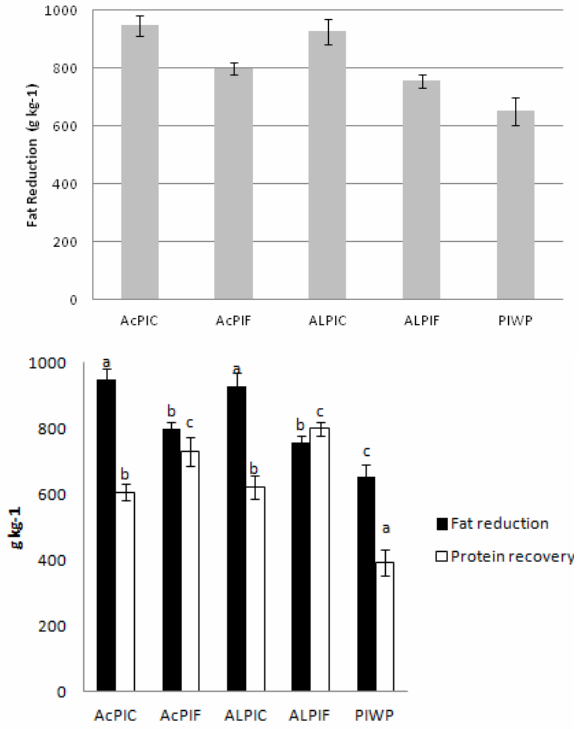

Fig 3 Recovery of proteins and reduction in fat in recovered proteins from sardines as influenced by different treatments (n =3)

AcPIC: acid produced protein isolate with centrifugation; AcPIF: acid produced protein isolate with filtration; ALPIC: alkaline produced protein isolate with centrifugation; ALPIF: alkaline produced protein isolate with filtration; PIWP: protein isolate produced by conventional washing process

Bars carrying different letters are significantly different ($p<0.05$)

Recently Nolsoe et al. (2011) showed that the replacement of centrifugation with filtration during acid and alkaline solubilization of pacific whiting substantially improved the protein yield by from about 38% to 62%, but also reduced the removal of lipid. They reported no significant effects on gel quality. Nolsøe et al. (2007), for the first time showed that filtration can be a promising alternative to centrifugation during the alkaline pH-shift processing of fresh cod, pollock and mackerel muscle. The increase in protein yield with sardine muscle tissue was up to 30%, and can be explained by the smaller fraction of the solubilized proteins being trapped in the filter cake, as compared to entrapment in the sediments derived from centrifugation. The present findings are in line with those previously reported by Nolsøe et al. (2007), although the average protein yields in that study generally were higher (90% with filtration and 71.7% with centrifugation). As expected, the lipid content of the protein isolates derived from filtration was higher than that found in protein isolates derived from centrifugation (Table 2). However, the literature indicates that, it is likely that the difference in lipid levels between isolates made with filtration vs. centrifugation is not likely to affect the development of rancidity. For example, Undeland et al. (2002) showed that painty odor and TBARS developed at equal rates in washed cod mince fortified with 3 µM Hb and 0, 7.5 or 15% non stabilized fish oil. That the lipid content of isolates made with filtration was higher than the lipid content of the raw material is consistent with the findings of Undeland et al. (2005) from a study in which they tested the acid process without any separation of insoluble matter at all. This is a consequence of a larger removal of proteins than lipids.

Results in Fig. 3 showed significant fat reduction. Both solubilization processes removed over 90% of initial lipids, while fat reduction by the conventional washing method was only 65%. Cortes-Ruiz et al. (2001) reported an 88% lipid reduction when acid solubilization was applied to fresh bristly

sardines. Batista et al. (2007) reported 65 and 51% fat reduction after alkaline and acid process on sardines respectively. These differences could be attributed to different methods used to calculate lipid content, as well as to seasonal variations in lipid and protein content within the species. Similar results, showing significant fat reduction after acid and especially after alkaline process have been reported for channel catfish, herring, Atlantic croaker, Antarctic krill, saithe (*Pollachius virens*) and blue mussels (Kristinsson et al. 2005; Undeland et al 2002; Perez-Mateos et al. 2004; Shaviklo et al. 2011; Vareltzis and Undeland 2008). Okada and Morrissey (2007) extracted oil from skin-on sardine fillets by adjusting the pH of the mince to the isoelectric point (pI) of sardine muscle followed by centrifugation at 10 000 × *g* for 20 min. Although there was no protein solubilization step, the pH adjustment to the pI increased oil recovery when compared with conventional heat-aided oil recovery. Liang and Hultin (2005) demonstrated that isoelectric solubilization/precipitation of cod muscle at basic and acidic pH allows efficient recovery of lipids, in particular when weak organic acids such as citric acid and its calcium salts are used.

Functionality of recovered proteins. On reviewing the literature, no information was available on functional properties of proteins isolated from sardine mince other than gel forming ability and gel color.

Emulsification activity index (EAI), which is a function of protein hydrophobicity (Xiong 1997), was higher in proteins from the conventional surimi process. There were no significant differences in liquid drainage, which is a destabilizing mechanism in protein foams. Finally, relative overrun, which is an index of protein foaming capacity, was higher in proteins produced by the conventional process (Table 3).

The color of protein isolates from the different processes is summarized in Table 4. L* value is significantly improved in isolated proteins compared to the sardine mince. This can be attributed to the removal of various constituents from raw material during acid and alkaline solubilization. Redness (a*) is similar in the mince and alkaline isolated meat, probably due to the fact that myoglobin is more stable at alkaline pH values.

TABLE 3 Functional properties of protein isolates (avg±SD, n=3)

Sample	EAI	Liquid drainage	Relative overrun
PI from acid produced proteins	0.53±0.009^{c}	0.20±0.09^{a}	0.063±0.001^{c}
PI from alkaline produced proteins	0.77±0.016^{b}	0.39±0.08^{a}	0.088±0.003^{b}
PIWP	0.86±0.027^{a}	0.30±0.02^{a}	0.192±0.012^{a}

EAI: Emulsification Activity Index; PI: protein isolate; PIWP: protein isolate from conventional washing process
Numbers carrying different letters are significantly different ($p<0.05$)

There was no significant difference in yellowness (b*) among the proteins from the raw material and the acid or alkaline solubilized proteins.

TABLE 4 Color determination of sardine mince and of protein isolates made with acid and alkaline processing (mean±SD, n=3)

Sample	a*-value	b*-value	L*-value
Sardine mince	6.6±0.6^{a}	16.9±1.7^{a}	51.4±0.3a
Acid process isolated meat	3.9±0.4^{b}	17.9±2.2^{a}	65.8±2.6^{b}
Alkaline process isolated meat	6.1±0.7^{b,a}	18.9±1.1^{a}	61.5±1.1^{b}

Numbers carrying different letters are significantly different ($p<0.05$)

It is necessary to stress out that gels were manufactured without any gel enhancers, like transglutaminase, bovine plasma protein etc. Taskaya et al. (2009) reports that carp-protein gels made without these additives had poor texture. Results from TPA are summarized in Table 5. In general, sardine proteins from basic solubilization resulted in better gel texture than their acidic counterparts.

TABLE 5 TPA of gels made by acid, alkaline and convetional washing process (avg±SD, n=5)

Sample	Hardness (g)	Cohesiveness	Deformation (mm)
Gel from acid produced proteins	186±13.5[b]	0.46±0.05[a]	5.4±0.10[b]
Gel from alkaline produced proteins	201±1.5[b]	0.37±0.02[a]	5.9±0.10[b]
Gel from PIWP	71±16.7[a]	0.34±0.03[a]	8.6±0.28[a]

Numbers carrying different letters are significantly different ($p<0.05$)

Chaijan et al. (2006) reported that the hardness and deformation of a gel from alkaline solubilized sardine proteins were 110g and 4,5mm respectively. These properties are considerably lower than the ones reported in the present study. The difference could be attributed to the optimized solubilization and precipitation pH values that were determined and used in our study, whereas Chaijan et al. (2006) used pH 10.8 for solubilization and 5,5 for precipitation of the proteins. The relatively poor gelation characteristics of the isolated proteins, and especially the acid aided ones, could be in part explained by the fact that some enzymes could be activated at the final isolate pH or additional proteolysis could also occur at the low solubilization pH in the case of the acid-aided process such as pointed out by Kristinsson and Liang (2006). In order to improve the gelling properties of recovered proteins, it would be advisable to control the exposure of proteins to the extraction medium, as concluded by Undeland et al. (2002). The utilization of microbial transglutaminase could also improve the gelling properties such as obtained by Pérez-Mateos et al. (2004).

Acid-aided isolates with poor gel-forming ability were also obtained from Pacific whiting (Choi and Park 2002), Spanish mackerel, catfish and mullet (Kristinsson and Demir 2003), rockfish (Yongsawatidgul and Park 2004) and Atlantic croaker (Pérez-Mateos et al. 2004). The acid-produced proteins from herring light muscle made gels with equal strain but lower stress values than those prepared from alkali-produced proteins (Undeland et al. 2002). However, good gels from acid aided protein isolates were obtained from cod and Atlantic mackerel (Hultin and Kelleher 1999), tilapia (Kristinsson and Ingadottir 2006) and Atlantic croaker (Kristinsson and Liang 2006). As pointed out by the latter authors, these contradictory results may indicate that the quality of gels from acid-aided proteins is highly species dependent.

Kristinsson et al. (2005) showed that basic pH solubilization induced less denaturation than acidic pH solubilization. Raghavan and Kristinsson (2007) measured the denaturation of isolated fish myosin as affected by acid solubilization and concluded that the viscoelasticity ($G\_$) increased with an increase in myosin denaturation and that the $G\_$ value and denaturation extent were highest at the lowest pH tested. Therefore the degree of denaturation during isoelectric solubilization/precipitation of fish proteins is likely only one of the factors that affect gel texture. Differences in gel forming ability between acid and alkaline produced gels might also result from the differences in protein integrity in formation of bonds during the thermal process and, in particular, the greater extent of disulfide bonds formed in the myofibrillar and sarcoplasmic proteins pools during alkaline process.

Abbreviations: TPA, texture profile analysis; PI, protein isolate; PIWP, protein isolate from conventional washing process; wt, weight; EAI, emulsification activity index

4. REFERENCES

[1] BATISTA I, PIRES C, NELHAS R (2007) Extraction of sardine proteins by acidic and alkaline solubilization. Food Sci Technol Int 13:189-194

[2] M, BENJAKUL S, VISESSANGUAN W, FAUSTMAN C (2006) Physichochemical properties, gel forming ability and myoglobin content of sardine (Sardinella gibbosa) and mackerel (Rastrelliger kanagurta) surimi produced by conventional method and alkaline solubilization process. Eur Food Res Technol 222: 58-63

[3] CHEN HH, LOU SN, CHEN TY, CHEN YW, LEE CH (1996) Gelation properties of horse mackerel (*Trachurus japaonicus*) surimi as affected by operation condition. Food Sci 23: 45-55

[4] CHEN Y, TOU JC, JACZYNSKI J (2009) Amino acid and mineral composition of protein and other components and their recovery yields from whole Antarctic krill (*Euphausia superba*) using isoelectric solubilization/precipitation. J Food Sci 74*:* H31-39
[5] CHOI YJ, PARK JW (2002) Acid-aided protein recovery from enzyme-rich Pacific whiting. J Food Sci 67: 2962-7
[6] CORTES-RUIS JA, PACHERO-AGUILAR R, GARCIA-SANCHEZ G, LUGO-SANCHES ME (2001) Functional characterization of a protein concentrate from bristly sardine made under acidic conditions. J Aq Food Prod Technol 10*:* 5-23
[7] HULTIN HO, KELLEHER SD (1999) Process for isolating a protein composition from a muscle source and protein composition. U.S.Patent 6,005,073
[8] IRENEU B, PIRES C, NELHAS R, GODINHO V (2006) Acid and alkaline aided protein recovery from Cape hake by-products. In: Luten, JB, Jacobsen C, Bekaert K, Saebo A, Oehlenschlanger J (eds) Seafood research from fish to dish, Wageningen Academic publishers, pp. 427-437
[9] KHUMALLAMBAM M, RAMA S, KARUPPANNAN RR, MANJABHAT SN (2011) Antioxidant activity of protein isolate obtained by the pH shift technique from hydrolyzed and unhydrolyzed shrimp processing discards. J Aq Food Prod Technol 20: 209-221
[10] KRISTINSSON H, HULTIN HO (2003) Effect of low and high pH treatment on the functional properties of cod muscle proteins. J Agric Food Chem 51*:* 5103-5110
[11] KRISTINSSON H, LIANG Y (2006). Effect of pH-shift processing and surimi processing on Atlantic croaker (*Micropogonias undulates*) muscle proteins. J Food Sci 70*:* C304–C302
[12] KRISTINSSON H, THEODORE AE, DEMIR N, INGADOTTIR B (2005) A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. J Food Sci 70*:* C298–C306
[13] KRISTINSSON H, DEMIR N (2003) Functional fish protein ingredients from fish species of warm and temperate waters: comparison of acid and alkali-aided processing vs. conventional surimi processing. In: Bechtel P Advances in Seafood Byproducts (ed) , Univ. Alaska Press, Fairbanks AK, pp. 277–295
[14] KRISTINSSON HG, INGADOTTIR B (2006) Recovery and properties of muscle proteins extracted from tilapia (*Oreochromis niloticus*) light muscle by pH shift processing. J Food Sci 70: E132–E141
[15] LEE C, TREVINO B, CHAIYAWAT M (1996) A simple and rapid solvent extraction method for determining total lipids in fish tissue. J AOAC Int 79: 487-492
[16] LIANG Y, HULTIN HO (2005) Separation of membranes from acid-solubilized fish muscle proteins with the aid of calcium ions and organic acids. J Agric Food Chem 53: 3008–3016
[17] LIANG Y, KRISTINSSON H (2007) Structural and foaming properties of egg albumen subjected to different pH-treatments in the presence of calcium ions J Food Res Int 40: 668-678
[18] MARKWELL M, HAAS S, BIEBER L, TOLBERT N (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87: 206-210
[19] MARMON S, UNDELAND I (2010) Protein Isolation from Gutted Herring (Clupea harengus) Using pH-Shift Processes. J Agric Food Chem 58: 10480-10486
[20] MONAHAN F, GERMAN J, KINSELLA JE (1995) Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. J Agric Food Chem 43: 46-52
[21] NOLSØE H, IMER S, HULTIN HO (2007) Study of phase separation by filtration instead of centrifugation affects protein yield and gel quality during an alkaline solubilization process- different surimi processing methods. Intern J Food Sci Technol 42: 139-47
[22] NOLSØE H, MARMON S, UNDELAND I (2011) Application of Filtration to Recover Solubilized Proteins During pH-Shift Processing of Blue Whiting (*Micromesistius poutassou*); Effects on Protein Yield and Qualities of Protein Isolates. The Open Food Sci J 5: 1-9
[23] NOLSØE H, UNDELAND I (2009) The acid and alkaline solubilization process for the isolation of muscle proteins state of the art. Food Bioprocess Technol 2: 1-27
[24] OCHIAI Y, OCHIAI L, HASHIMOTO K, WATABE S (2001) Quantitative estimation of dark muscle content in the mackerel meat paste and its products using antisera against myosin light chains. J Food Sci 66: 1301-1305
[25] OKADA T, MORRISSEY MT (2007) Recovery and characterization of sardine oil extracted by pH adjustment. J Agri Food Chem 55: 1808-1813
[26] PACHECO-AGUILAR R, RAMIREZ-SUAREZ JC, MAZORRA-MANZANO M (2001) Effect of alkaline and acidic wash treatments on functional properties and color of Monterey sardine (Sardinops sagax caerulea) minced flesh. J Aq Food Prod Technol 10: 85-99
[27] PARK J, JUNG C, KIM J, CHO D, CHO M, CHOI Y (2003) Surimi processing using acid and alkali solubilization of fish muscle protein. J Korean Soc Food Sci Nutr 32: 400-5
[28] PARK J, LIN T (2005) Surimi: manufacturing and evaluation. In: Park JW (ed) Surimi and surimi seafood, Taylor & Francis Boca Raton, FL, pp. 33-106

[29]PARK J, MORRISSEY MT (2000) Manufacturing of surimi from light muscle fish. In: Park JW (ed) Surimi and Surimi Seafood, Marcel Dekker Inc, New York, pp.23–58
[30]PÉREZ-MATEOS M, AMATO P, LANIER TC (2004) Gelling properties of Atlantic croaker surimi processed by acid or alkaline solubilization. J Food Sci 69*:* FCT328–FCT333
[31]RAGHAVAN S, KRISTINSSON H (2007) Conformational and rheological changes in catfish myosin as affected by different acids during acid induced unfolding and refolding. J Agric Food Chem 55: 4144–4153
[32]RAMADHAN K, HUDA N, AHMAD R (2011) Effect of number and washing solutions on functional properties of surimi-like material from duck meat**.** J Food Sci Technol. DOI: 10.1007/s13197-011-0510-1
[33]SHAVIKLO GR, THORKELSSON G, ARASON S, SVEINSDOTTIR K (2011) Characteristics of freeze-dried fish protein isolated from saithe (*Pollachius virens*). J Food Sci Technol. DOI: 10.1007/s13197-011-0285-4
[34]STEFANSSON G, HULTIN HO (1994) On the solubility of cod muscle proteins in water. J Agr Food Chem 42: 2656–2664
[35]TASKAYA L, YI-CHEN C, BEAMERC S, JACZYNSKI J (2009) Texture and colour properties of proteins recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*) using isoelectric solubilisation/precipitation. J Sci Food Agric 89: 349–358
[36]UNDELAND I, HALL G, WENDIN K, GANGBY I, RUTGERSSON A (2005) Preventing lipid oxidation during recovery of functional proteins from herring (*Clupea harengus*) fillets by an acid solubilization process. J Agric Food Chem 53: 5625-34
[37]UNDELAND I, KELLEHER S, HULTIN HO (2002) Recovery of functional proteins from herring (Clupea harengus) light muscle by an acid or alkaline solubilization process. J Agric Food Chem 50: 7371-7379
[38]VARELTZIS P, UNDELAND I (2008) Removal of lipids and the diarrhetic shellfish poisoning toxins (DSP) from blue mussels (*Mytilus edulis*) during acid and alkali solubilization. J Agric Food Chem 56: 3675–3681
[39]XIONG Y (1997) Protein denaturation and functionality losses. In: Erickson MNC , Hung YC (eds.) Quality in frozen food, Chapman and Hall, USA, pp.111-140
[40]YONGSAWATDIGUL J, PARK JW (2004) Effects of alkaline and acid solubilization on gelation characteristics of rockfish muscle proteins. J Food Sci 69*:* 499-505