Leaching and heating process as alternative to produce fish protein powder from Kilka (*Clupeonella cultiventris caspia*)

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Abstract. *Rahmanifarah K, Shabanpour B, Shaviklo AR, Aalami M. 2014. Leaching and heating process as alternative to produce fish protein powder from Kilka (*Clupeonella cultiventris caspia*). Nusantara Bioscience 6: 1-6.* The effect of protein extraction procedures (leached mince and heated suspension) on selected properties of fish protein powder (proximate composition, pH, color, density, viscosity, fat adsorption, emulsifying capacity, emulsifying stability, foaming capacity, foaming stability, WBC, protein solubility in water, hygroscopicity, Trichloroacetic acid (TCA)-soluble peptides and free sulfhydryl groups) was investigated. Results showed that Fish protein powder (FPP) produced by leaching mince (LM) have higher protein, moisture, ash, pH, L*, viscosity, emulsion capacity, emulsion stability, foam capacity, foam stability, water binding capacity (WBC), protein solubility, hygroscopicity, TCA soluble peptides and free sulfhydryl group content than heated suspension (HS) (P<0.05). However, HS had higher fat and density in comparison with LM (P<0.05). No significant differences between a*, b* and fat adsorption were observed (P>0.05). Overall, it was observed that high temperature during heating of suspension in HS method makes possible protein denaturation and aggregation. Consequently, based on functional, chemical and physical properties, extraction of fish protein by leaching process was found to be suitable for the production of fish protein powder.

Keywords: Fish protein powder, functional properties, kilka, leaching, suspension

INTRODUCTION

Common kilka (*Clupeonella cultiventris caspia*) is one of the most important economic fishes in the Caspian Sea (Figure 1). It belongs to the Clupeidae family (Nelson 1998). The processing of small pelagic fish is associated with difficulties. Kilka has a dark and sensitive muscle, small size and high value of fat that cause difficulty in processing. Therefore, because of these unsuitable properties, more than 95% of the Kilka resource in Iran has no direct human applications. Methods for processing small pelagic fish such as Kilka that counteract these difficulties and facilitate utilization of their valuable proteins have been missing. Typically, most kilka is dried at high temperature and powdered for livestock and poultry feed.

The functional properties of proteins are a major interest as they affect the usability of the proteins in different food applications. Sathivel et al. (2004) reported that protein powders from herring and arrow tooth were good sources of high-quality fish protein with many desirable functional properties. Fish

is regarded as an excellent source of high-quality protein, particularly the essential amino acids lysine and methionine (Sathivel and Bechtel 2006). Many protein-rich seafood byproducts have a range of dynamic properties (Phillips et al. 1994) and can potentially be used in foods as binders, emulsifiers, and gelling agents (Sathivel et al. 2004). The FPP, kept above 0ºC has many advantages in food trade such as ease of handling, low distribution costs, convenient storage and ease in mixing with other ingredients (Shaviklo

Figure 1. Common kilka (*Clupeonella cultiventris caspia*) (photo: Yuriy Kvach)

et al. 2010).

The minced fish can be dried via different drying procedures. Cordova-Murueta et al. (2007), Huda et al. (2001) and Shaviklo et al. (2010) applied hot air drying, vacuum drying and spray drying, respectively to dry the minced fish. However, these methods have some advantage of fish drying, but freeze drying is the best method for drying fish mince (Cordova-Murueta et al. 2007; Huda 2001).

Extraction of fish proteins to produce FPP can be turned into a solvent extraction method (Liston and Pigott 1971), pH shifting method (Hultin and Kelleher 1999), Enzyme/acid hydrolysis (Hoyle and Merritt 1994) and Mince leaching (Shimizu 1965) (surimi) by adding four times the weight of water and has excellent functional properties such as the ability to form kamaboko gels (Huda et al. 2001; Niki et al. 1983; Shaviklo 2013). Moreover, Sathivel et al. (2003) introduce the new method to extract fish protein that heated the fish suspension and dried after separation. In this study, we compare the leaching process with heated suspension to investigate some functional and chemical properties of dried leached mince and heated suspension.

MATERIALS AND METHODS

Materials
Kilka fish was caught from Caspian Sea and transported to Amir Abad beach in the Caspian Sea Water (CSW) system. Afterward, the fish was packed in polystyrene boxes with ice and brought to the laboratory.

FPP production
Samples of Kilka were washed, gutted, headed and deboned with bone separator (Bone Separator Farayazan Andishan, Iran). After mincing, fish mince was split into two parts. One part was used to prepare leached mince. The ratio of mince to water was 1: 5 and the temperature was kept under 10 $^{\circ}$C during processing. The mixture was stirred well with a stainless steel spatula for 15 min. The slurry was passed through cheesecloth (Shimizu 1965). At this stage leached mince was prepared.

Another protein extraction method was done according to the method of Sathivel et al. (2003). A 500 g portion of each groundfish part was mixed with an equal volume of distilled water and homogenized in an Ultraturrax homogenizer (IKA, T25, digital Germany) for 2 min. The mixture was continuously stirred for 60 min at 85 $^{\circ}$C. The heated suspension was centrifuged at 2560 g for 15 min, resulting in three separate phases: the semi-solid phase at the bottom containing insoluble protein, bone, and skin; the heavy liquid phase in the middle containing soluble proteins, and the light liquid phase at the top, containing crude lipids. The heavy liquid middle layer was separated and collected.

Leached mince (LM) and heated suspension (HS) were mixed with 2% (w/w) sucrose and 0.2 % (w/w) sodium tripolyphosphate as lyoprotectants using a silent cutter (Saya, Pars Khazar, Iran) for 5 min. After mixing, protein

extracts were freeze-dried for 72 h. The resulting FPP samples Milled and placed in zip lock plastic and stored at -80 °C until analyzed.

Proximate composition
Crude protein, ash, moisture and lipid content of samples were analyzed by the method of AOAC (1990). The 5 g of samples were dried in an oven at 105°C until constant weights were achieved and moisture content was calculated. Samples were then extracted using a Soxhlet extraction (416 SE, Gerhardt, Germany) with petroleum ether to determine oil content. Protein content was determined using the Kjeldahl method (Gerhardt, Vap 40, Germany). Ash content was determined by holding samples overnight at 550°C.

pH
pH of FPP was determined by blending 5 g of samples with 20 mL of distilled water for 30 seconds using an Ultra Turrax tissue homogenizer (T25 IKA-Ultra-Turrax, Germany). The pH of suspension was recorded by using a combined glass electrode with a digital pH meter (728 pH Lat Stirrer, Metrohm).

Color
FPP sample color was evaluated using the colorimeter (Lovibond CAM-system, England 500). CIE (Commission Internationale de l'Eclairage) L* (lightness), a* (red to green), and b* (yellow to blue) were measured. All samples were kept at room temperature in a plastic bag for more than 2 h to eliminate the effects of various temperatures at measurement.

Density
Density was determined in triplicate for each sample by placing the sample in a pre-weighed 10 mL graduated cylinder up to the 10 mL mark with gentle tapping. The graduated cylinder weighed again and the density was calculated as g powder per mL volume (Venugopal et al. 1996).

Viscosity
The viscosity of FPP was measured using a Brookfield synchro-lectric viscometer model LVT (Brookfield Ltd, Cooksville, ON, Canada). A sample solution containing 10 % protein was prepared from each FPP and homogenized with Ultra Turrax homogenizer (T25 IKA-Ultra-Turrax, Germany). The solution was kept refrigerated overnight. It was homogenized the day after, before running viscosity measurements. The viscosity was measured routinely at 60 rpm using spindle No. 3. The values were recorded after 30 s of rotation of the spindle in the dispersion.

Fat adsorption
The fat adsorption capacities were determined by the methods of Shahidi et al. (1995). This test was performed in triplicate and fat adsorption was expressed as the volume (mL) of fat adsorbed by 1 g of protein.

Emulsification properties

Emulsifying capacity was measured using the procedure described by Yatsumatsu et al. (1972). The powder (1 g) was added to 25 mL of distilled water and 25 mL of sunflower oil. The mixture was then mixed with Ultra Turrax homogenizer (T25 IKA-Ultra-Turrax, Germany) for 1 min and transferred to the 50-mL calibrated centrifuge tube. The tube containing the sample was then centrifuged at 7500 g for 5 min. The emulsifying capacity was calculated by dividing the emulsion volume after centrifugation by the original emulsion volume and then multiplied by 100. Emulsifying stability was determined by the same procedure, except that, before centrifugation, the emulsion was heated at 90 °C for 30 min followed by cooling in tap water for 10 min (Yatsumatsu 1972).

Foaming properties

Determination of foaming capacity was done following the method of Miller and Groninger (1976) with slight modification. Forty milliliters of 1 % protein aqueous dispersion was mixed thoroughly using an Ultra Turrax (T25 Ika-Ultra-Turrax, Germany) at 10,000 rpm for 2 min. The total volume of the protein dispersion was measured immediately after 30 sec. The difference in volume was expressed as the volume of the foam. Foam stability was determined by measuring the fall in volume of the foam after 1 h.

Water binding capacity

WBC was measured using the method described by the American Association of Cereal Chemists (AACC 1981) with slight modifications. 1 g FPP was weighed into centrifuge tubes. Subsequently, 40 mL deionized water was added and mixture was left to stand for 30 min at room temperature and then the sample was centrifuged at 5000 g for 15 min. The weight of supernatant after centrifuge was recorded. WBC was expressed as the ratio of weight gained per unit weight of fish protein powder (AACC 1981).

Protein solubility in water

To determine protein solubility, 1 g FPP was dispersed in 20 mL of deionized water. The mixture was stirred at room temperature for 30 min with intermittent stirring and then the sample was centrifuged at 5000 g for 15 min. Protein content in the supernatant and in the sample was determined. Protein solubility was calculated as follows:

$$Solubility = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content\ in\ sample} \times 100$$

Hygroscopicity

For the hygroscopicity, about 1 g of FPP was placed in a desiccator containing a saturated solution of Na_2SO_4 to establish a relative humidity of 81 % (Jaya and Das 2004). After keeping the sample at 25°C for a week, the hygroscopic moisture (%) was calculated using the equation:

$$Hygroscopicity = \frac{\frac{b}{a}}{1}$$

Where a (g) was the amount of the sample, W_i was the moisture content in the powder before the measurement and b (g) was the powder weight increase. All the measurements were made in three replicates.

TCA-soluble peptides

TCA-soluble peptides were determined according to the method of Visessanguan et al. (2004). FPP samples (1 g) were homogenized with 29 mL of cold 5% (w/v) TCA with an Ultra Turrax (T25 Ika-Ultra-Turrax, Germany) and kept at 4°C for 1 h, followed by centrifugation at 12,000 g for 15 min at 4°C. TCA-soluble peptides in the supernatant were measured by the method of Biuret using bovine serum albumin (BSA) as a standard. The results are the average of three determinations and expressed as micromole tyrosine/ gram sample.

Free sulfhydryl groups

The concentration of free sulfhydryl groups (SH) of the FPP samples was determined using Ellman's reagent (50, 5-dithiobis (2-nitrobenzoic acid), DTNB) (Sigma-Aldrich. Milan, Italy). Changes in free sulfhydryl groups were measured in triplicate as reported by Beveridge et al. (1974). Briefly, FPP 1.5 g was diluted to 10 mL with 1% (p/v) NaCl in Tris-glycine buffer (10.4 g tris, 6.9 g glycine, 1.2 g EDTA per liter, pH 8.0). A volume of 2.9 mL of 0.5% SDS in Tris-glycine buffer was added to 0.1 mL of diluted egg white and 0.02 mL of Ellman's reagent (4 mg/mL DTNB in Tris-glycine buffer) to develop a color. After 15 min, absorbance was measured at 412 nm using a UV-VIS spectrophotometer. The concentration of free sulfhydryl groups (1 mg^{-1}) was calculated from the following equation:

$$\mu M\ SH/g = \frac{73.53 A_{412} \cdot D}{C}$$

Where A_{412} is the absorbance at 412 nm; C is FPP concentration (mg/mL); D is the dilution factor; and 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 is the molar absorptive and 10^6 is the conversion factor from molar basis to $\mu M/mL$ and mg solid to g solid respectively (Ellman 1959).

Data analysis

All data presented are means ± standard deviations. Assays were conducted in triplicate and the statistical significance of differences between means ($P<0.05$) was determined by Student's t-tests. The analysis was carried out using SPSS 16.0 for Windows software package.

RESULTS AND DISCUSSION

Table 1 depicts proximate composition and pH value of trial group. Results show that LM has higher protein content than HS ($P<0.05$) but the fat content in the LM was

lower than HS (P<0.05). Better protein extraction in LM than HS could be due to higher fat depletion during leaching process. Hence fat reduction leads to an overall increase in protein content in LM. Fat content of HS in this work is similar to the fat content of insoluble fraction in the study of Sathivel et al. (2008). Sathivel et al. (2006) observed protein extraction with solubilizing leads to increase lipid scattering in suspension and therefore fat reduction in extracted protein. Kahn et al. (1974) reported a number of variables influencing solubility and protein extraction efficiency from fish tissues, including concentration and particle size of suspended tissues, extraction time, temperature, pH, and the type and concentration of salts used for extraction. Moisture and ash content in the LM was higher than HS (P<0.05). Higher moisture might be partly due to higher cohesion of leached mince during freeze-drying in comparison with heated suspension. Heating process in HS extracting method leads to protein denaturation and aggregation. Lower ash value in HS may be as results of better separation process during protein extraction. However, in LM some bone section probably causes an increase in ash content. Additionally, pH in the LM was higher than HS (P<0.05).

As can be seen in Table 2, the lightness of LM was higher than HS (P<0.05). Millard reaction during heating and lower moisture content throughout drying could be the reason for lower lightness of HS group. Redness and yellowness in the HS group were higher than in the LM group, but these differences were not significant (P>0.05). Density of HS was higher than LM (P<0.05) because of lower pores. The higher the processing temperature, the higher the shrinkage of the material leading to lower levels of pores (Rahman et al. 2002). Density of freeze-dried Kilka protein powder in this study was higher than the freeze-dried lizard fish, threadfin bream and purple spotted big-eye prepared by Huda et al. (2001). In the current study LM had a higher viscosity than HS (P<0.05). Higher viscosity in LM attributes better protein quality in comparison with HS. The viscosity of all FPP samples was lower than that of Saithe reported by Shaviklo et al. (2010). This may be as results of lower quality in functional properties of Kilka protein than Saithe. Fat adsorption is an important functional characteristic of ingredients used in the meat and confectionery industries. Results show no significant differences among different extraction method (P>0.05) (Table 2). Fat adsorption capacity values have been reported that ranged from 3.9 to 11.5 mL of oil/g

protein for herring protein powders (Sathivel et al. 2004), 3.7 to 7.3 mL of oil/g protein for hydrolyzed herring by-product proteins (Sathivel et al. 2003) and 2.86 to 7.07 mL of oil/g protein for Atlantic salmon protein hydrolysate (Kristinsson and Rasco 2000). The mechanism of fat binding capacity is thought to be mainly because of the physical entrapment of the oil (Sathivel and Bechtel 2008).

The ability of proteins to form stable emulsions is important for interaction between proteins and lipids in many food systems. It has been reported that proteins with both hydrophilic and hydrophobic residues act as emulsifiers and when the protein has a balance between these residues the emulsion capability is optimal (Damodaran 2008). Emulsion capacity and emulsion stability are shown in Figure 2. LM had higher emulsion capacity than HS (P<0.05). It could be explained by more protein denaturation during protein extraction in HS procedure while protein exposed to 85˚C for 60 min. Emulsion capacity and stability of Kilka protein powder in this work are lower than Arrow tooth Flounder and Herring reported by Sathivel et al. (2004). Gauthier et al. (1993) stated that factors such as protein solubility and hydrophobicity also play major roles in emulsifying properties.

Both FPP had lower foam capacity in comparison with other reports (Shaviklo et al. 2010). The molecular

Table 1. Proximate composition and pH of leached mince (LM) and heated suspension (HS)

Sample	Protein %	Fat %	Moisture %	Ash %	pH
LM	74.85±1.6[a]	8.17±1.7[b]	3.53±0.3[a]	7.08±0.3[a]	7.93±0.1[a]
HS	68.35±0.1[b]	24.66±0.6[a]	2.31±0.1[b]	3.61±0.3[b]	7.51±0.1[b]

Different letters (a-b) represent significant differences between protein extraction method (P<0.05, n = 3). Values are means±SD.

Table 2. Color, density, viscosity and fat adsorption of leached mince (LM) and heated suspension (HS)

Sample	L*	a*	b*	Density g/ml	Viscosity Pa	Fat adsorption oil/g
LM	68.20±0.1[a]	4.30±0.0[a]	2.46±0.4[a]	0.39±0.1[b]	1.93±0.1[a]	3.67±0.3[a]
HS	62.20±0.9[b]	4.83±0.5[a]	2.70±0.1[a]	0.43±0.1[a]	1.20±0.1[b]	3.2±0.4[a]

Different letters (a-b) represent significant differences between protein extraction method (P<0.05, n = 3). Values are means±SD.

Table 3. WBC, protein solubility, hygroscopicity, TCA soluble peptides and free sulfhydryl group content of leached mince (LM) and heated suspension (HS)

Sample	WBC (%)	Protein solubility (%)	Hygro-scopicity (%)	TCA-soluble peptides (micromole tyrosine/g)	Free sulfhydryl group (micromole/g)
LM	637.1±38.3[a]	30.21±6.0[a]	3.05±0.1[a]	5.49±0.3[a]	0.86±0.3[a]
HS	226.3±13.7[b]	25.46±2.7[b]	2.22±0.1[b]	4.73±0.2[b]	0.29±0.1[b]

Different letters (a-b) represent significant differences between protein extraction method (P<0.05, n = 3). Values are means±SD. WBC: water binding capacity

properties relevant to foaming are similar to those required for emulsification (Panyam and Kilara 1996). Foamability is an important functional property of proteins by which proteins form a flexible cohesive film to entrap air bubbles. In this study, LM shows higher foam capacity than HS (P<0.05) (Figure 2). Proteins that rapidly unfold and adsorb at the freshly formed air/liquid interface during bubbling exhibit improved foamability (Damodaran 1997). Foam expansion is mainly related to the solubility of proteins (Kinsella 1979). The more the proteins are soluble, the more the protein available to form the flexible cohesive film to entrap the air and hence the higher foam expansion. In this study extraction method in the HS group had lower protein solubility; therefore higher foam capacity of LM could be explained. Foam stability of FPP reported by Shaviklo et al. (2010) was higher than observed in this work (Figure 3). Formed foam was dropped after about few min and slight foam was absorbed in 1 h.
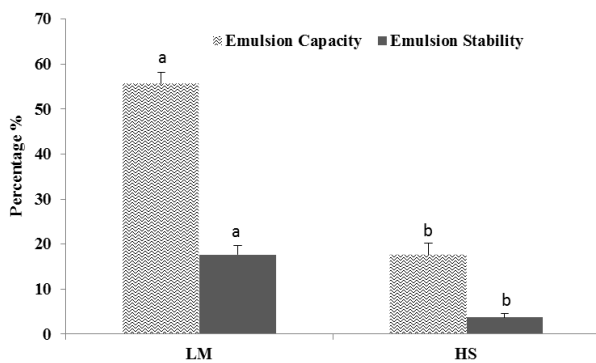

Figure 2. Emulsion capacity and emulsion stability of leached mince (LM) and heated suspension (HS). Different letters (a-b) represent significant differences between same factor (P<0.05, n = 3). Values are means±SD.
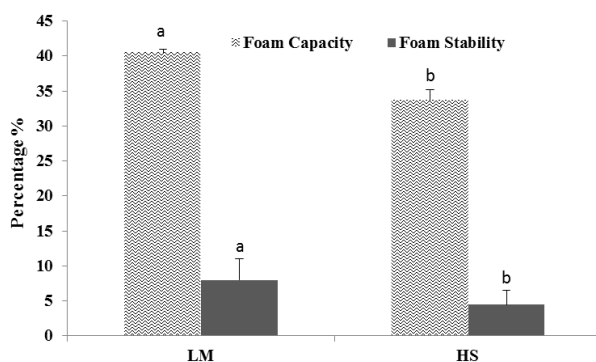

Figure 3. Foam capacity and foam stability of leached mince (LM) and heated suspension (HS). Different letters (a-b) represent significant differences between same factor (P<0.05, n = 3). Values are means±SD.

Results of current study depicted higher amount of WBC and protein solubility in the LM group than HS (P<0.05) (Table 3). At higher drying temperatures, WBC and protein solubility could be decreased as a result of protein denaturation (Huda et al. 2000). High temperature

during heating of suspension in HS method makes possible protein denaturation and aggregation. From other studies, it is clear that WBC is closely related to fish species (Huda et al. 2001), amount of Lyoprotectants (Matsuda 1971), different techniques and processes used for drying, and the interaction between these factors (Roos 2002). Mean WBC of LM in this study was 637 % while in other study reported by Shaviklo et al. (2010) on freeze dried and spray dried fish protein powder on Saithe ranged between 300-350 % (Shaviklo et al. 2010). Hygroscopicity of fish protein powder in LM group was higher than HS group (P<0.05). The hygroscopicity can be defined as the ability of a food to absorb the moisture from a high relative humidity environment and has been related either to the porosity of the powder (Nadeau et al. 1995) or the amorphous glassy state of the sugars present in the food (Roos 2002). With regard to the density of FPP, it obviously appeared that LM has more porous than HS, therefore, porosity could be the reason for higher hygroscopicity of LM in this work.

LM had higher TCA-soluble peptides than HS (P<0.05). TCA-soluble peptides for LM and HS were 5.49 and 4.73 respectively. High TCA-soluble peptide content indicated greater hydrolysis and degradation of muscle proteins. Leached mince extraction might produce small peptides, resulting in an increase in TCA-soluble peptide content. Myofibrillar protein degradation, especially myosin, resulted in reduction in molecular weight and the loss of structural domains, which are essential for molecular interaction and binding (Visessanguan and An 2002). However, lower TCA-soluble peptide content in HS than LM, could be explained by higher aggregation of HS protein affected by heating denaturation.

Sulfhydryl groups are considered to be the most reactive functional group in proteins. Free sulfhydryl groups of trial treatments (Micromole/g) are depicted in Table 3. The free sulfhydryl group method has been widely used in order to evaluate protein oxidation and more precisely cysteine oxidation. Cysteine oxidation can induce protein cross-links by the formation of intermolecular disulfide bridges; thus, the higher the free sulfhydryl groups the lower cysteine oxidation (Lara et al. 2011). Free sulfhydryl groups in current work show significant differences between trial groups (P<0.05). The free sulfhydryl groups content of the LM and HS were 0.86 and 0.26 respectively. Protein oxidation is also associated with a decrease in sulphydryl groups, which are converted into disulfides (Batifoulier et al. 2002). Cysteine residues in proteins occur as the free sulphydryl form or oxidized system. Oxidation-induced by a massive free radical production had the edge on the reduction process leading to the decrease of the free sulfhydryls (Soyer et al. 2010). Heat treatment could induce the SH/S-S exchange reaction and lead to an increase in S-S group level during suspension heating. Visschers and De Jongh (2005) had reported that cysteine residues and disulfide bonds had important contributions to the aggregation of proteins. Increase in the S-S group level during processing could induce changes in myofibrillar proteins structure and lead to protein aggregation.

CONCLUSION

The overall results of this study have shown that protein extraction without heating process gives better quality in fish protein powder. Extraction of protein with the leaching process shows better fat suspension and therefore higher protein extraction. Functional and physicochemical properties of FPP extracted with leached process were higher than for the heated suspension procedure. Consequently, based on functional, chemical and physical experiments, extraction of fish protein by leaching process was found to be suitable for the production of fish protein powder.

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