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Report

Report on physical, chemical and microbiological characteristic of rest raw materials (RRM) and wash water (WW)

[Sub Title]

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ABSTRACT
This report is a part of ReValue project and present the outcomes from activities within WP2 Valorization techniques. Deliverable D2.1. "Report on physical, chemical and microbiological characteristic of rest raw materials (RRM) and wash water (WW)" covers physical, chemical and microbiological characteristic of RRM)and WW used in the project for development and optimisation several technological solutions for better utilisation of valuable materials often considered as waste due to improper handling followed by lose of the quality. This deliverable indicates physical, chemical and microbiological characteristic of RRM) and WW collected and analysed in Norway, India and Spain. The work performed at CFTRI (India), AMITY (India), SINTEF/NTNU (Norway) and LEITAT (Spain). Performed tests and analyses indicated that different RRM obtained from surimi processing factories has a potential to be used as valuable ingredients in different formulations (both food and feed marked). Some variation in chemical composition as well as microbial quality gives indication that proper storage and handling methods and optimal processing technologies need to be applied in order to produce high quality final products. Summarised it can be stated that all analysed RRM obtained from surimi processing are rich in proteins (40-80 % of dry material) and are good source for production of protein rich products and ingredients.

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WP2 Valorization techniques

Deliverable D2.1. Report on physical, chemical and microbiological characteristic of rest raw materials (RRM) and wash water (WW)

This deliverable covers physical, chemical and microbiological characteristics of rest raw materials (RRM) and wash water (WW) used in the project for development and optimisation of several technological solutions for better utilisation of valuable materials often considered as waste due to improper handling followed by loss of the quality. The aim of this deliverable was to indicate physical, chemical and microbiological characteristics of RRM and WW collected and analysed in Norway, Spain and India. The work was performed at CFTRI (India), AMITY (India), SINTEF/NTNU (Norway) and LEITAT (Spain).

1 Raw material

The following RRM were analysed as shown in Table 1.

Table 1. *Overview over samples and characterisation performed with raw materials.*

Raw material	Collected (data)	Origin	Characterization			Notes
			Physical	Chemical	Microbiological	
Heads	23.01.2019	Kaiko surimi		+	+	HW
Skin and bone	23.01.2019	Kaiko surimi		+	+	SBW
Refined waste	23.01.2019	Kaiko surimi		+	+	RW
Pink perch whole fish	15.02.2019	Kaiko surimi		+	+	WF
Pink perch head & viscera	15.02.2019	Kaiko surimi		+	+	HV
Pink perch skin & bones	15.02.2019	Kaiko surimi		+	+	SB
Pink perch refiner waste	15.02.2019	Kaiko surimi		+	+	RW
Surimi	15.02.2019	Kaiko surimi		+	+	S
Wash water	15.02.2019	Kaiko surimi		+	+	WW
Pink perch: Head & Viscera	Produced: 30/9/19 Filleting: 3/10/19	Ulka sea food Pvt. Ltd	+	+		HV
Pink perch: Skin & bones	Produced: 30/9/19 Filleting: 3/10/19	Ulka sea food Pvt. Ltd	+	+		SB
Croaker: Head & Viscera	Produced: 30/9/19 Filleting: 3/10/19	Ulka sea food Pvt. Ltd	+	+		CHV
Croaker: Skin & bones	Produced: 30/9/19 Filleting: 3/10/19	Ulka sea food Pvt. Ltd	+	+		CSB
Reef cod: Skin & Bones	Produced: 19/9/19 Filleting: 19/9/19	Spanish market	+	+		SB
Croaker fish: Skin & bones	Produced: 19/9/19 Filleting: 20/9/19	Spanish market	+	+		SB
Croaker: Head & viscera	Produced: 19/9/19 Filleting: 20/9/19	Spanish market	+	+		HV

The RRM from production of surimi was received on January 23rd, 2019 from Kaiko surimi production plant which is based in Mumbai, India. The material was transported by plane from Mumbai to Bangalore, and then transported from Bangalore to Mysore by truck for approximately 4 hours. The material was received in frozen condition. When received at the lab at CFTRI, the material was put in a -20°C freezer. Three different fractions were received:

1) head waste (3 kg), which included head and viscera,
2) skin and bone (3 kg),
3) refined waste (2 kg), which consisted of connective tissue of the fish, small scales, small bones and some meat, illustrated in Figure 1.

Figure 1. *Thawed raw material received from Kaiko surimi production plant. Head waste, skin and bone waste and refined waste. 3 kg head waste, 3 kg skin and bone waste and 2 kg refined waste was received.*

Labelling of the different fractions are presented in Table 1. In this part of the work the definition of RRM was substituted by "waste" as this is the terminology applied in the corresponding factory. The raw material mainly consisted of RRM from the lean fish species commonly known as pink perch (*Nemipterus sp.).*

Another batch of RRM, WW and whole fish was collected from Refrigerated Distributors Pvt. Ltd (Kaiko surimi) in frozen form (15 February 2019). The samples were thawed at room temperature and homogenized in mixer grinder.

Whole pink perch and croaker from Ulka Seafood Pvt. Ltd (Produced on 30/9/19) were transported in frozen state from India to SINTEF Ocean (Norway) and stored in the freezer (-28˚C) until analysed. Then fish were thawed overnight in cold room (4ºC) and filleted on 3/10/19. All fractions were minced and frozen for further analysis and technological tests. Three different fractions were made by manual filleting in the lab (SINTEF Ocean): Figure 2 and Figure 3.
1) Head and viscera,
2) Skin and bone,
3) Fillet.

Figure 2. *Fractionation of pink perch.*

Figure 3. *Fractionation of croaker.*

Due to an easy execution of the task and to avoid customs problems for shipment of RRM from India to Spain, LEITAT decided to identify Species similar to the ones analysed in India from the Mediterranean sea (Table 2) and used as RRM for the firsts steps of the project. Once all the processes would be optimized, the processes will be validated with the Indian samples.

Table 2. *ReValue species list of fish used to produce surimi.*

Fish Type	Common Name	Scientific Name
Non-Histamine Forming	Pink perch	*Nemipterus sp.*
	Big eye	*Priacanthus harmur*
	Ribbon fish	*Lepuracanthus savala / Trichiurus lepturus*
	Lizard fish	*Saurida sp.*
	Croaker fish	*Johnius sp.*
	Reef cod	*Epinephelus sp.*
	Bronze croaker	*Otolithoides biaurituds*
Histamine Forming	Mamakari	*Sardinella brachysoma*

The species selected for analysis were reef cod (Epinephelus sp.) and croaker fish (Johnius sp.). The fish was bought from a Spanish market and transported to LEITAT laboratories by car. Samples were filleted and fractioned manually in the laboratory (Figure 4 and Figure 5) and then ground, dried, milled and stored at the freezer at -18ºC until they were analysed. The following fractions were obtained:

1) Head
2) Viscera
3) Bones
4) Skin

Figure 4. *Fractionation of reef cod. 1 Head, 2 Viscera, 3 Bones and 4 Skin.*

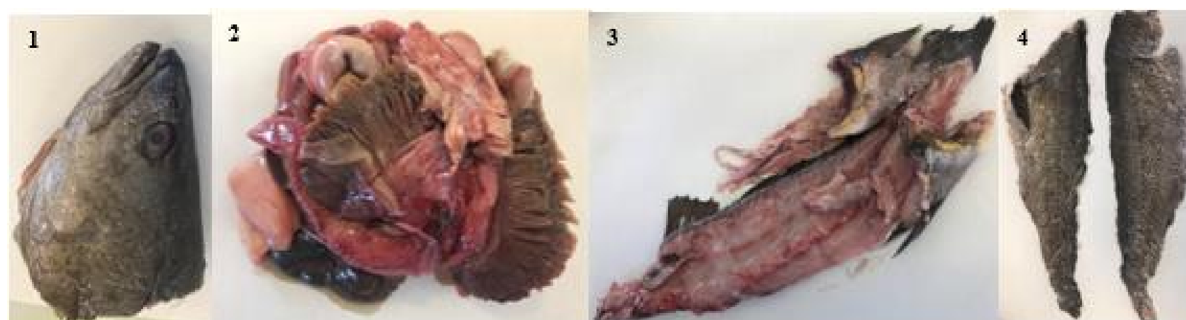

Figure 5. *Fractionation of croaker fish. 1 Head, 2 Viscera, 3 Bones and 4 Skin.*

1.1 Characterization of the raw material

1.1.1 Samples collected and analysed at CFTRI (India) and at SINTEF Ocean (Norway)

Raw material

Samples collected on 23 January 2019 and analyzed at CFTRI (India) and produced on 30 September 2019 and filleted on 3 October 2019 at SINTEF Ocean (Norway).

Preparation of samples

The moisture, fat, protein and ash content were determined for the different fractions of the raw material received from Kaiko surimi plant. The different fractions were thawed in water bath for

approximately 90 minutes and minced separately using a vertical cutter (robot coupe R 10 series 5) followed by a blender (Prestige PRO 250) in order to get the masses as homogenous as possible for further analysis.

Moisture

Moisture content was determined by the AOAC (1990) method. First, a glass petri dish was weighed, then 5-15 g sample from each fraction was placed on the petri dish and weighed before incubation at a temperature of 105°C for overnight. The weight of the petri dish with the dried sample was weighed the next day. The experiment was conducted in duplicates and the moisture content was calculated using following equation:

$$\%\,Moisture = \frac{total\ weight - final\ weight}{sample\ weight} * 100$$

The calculated moisture content was used to calculate dry matter of the sample.

Fat/Lipids

To determine the fat content, Soxhlet method following AOAC (1990) was used. First the empty round bottom flask was weighed, followed by weighing of the sample on a filter paper. The sample was wrapped in the filter paper and placed in the extraction tube. The tube was then filled with petroleum benzene with a boiling range of 60-80°C (Merck, India) until the solvent had circulated for one round in the Soxhlet extraction unit (Quest International). The extraction tube was then filled until the sample was covered with solvent and plugged loosely with cotton. The temperature was set at 60°C and the extraction was left for 6 hours. After 6 hours of the extraction, the round bottom flask with solvent was left for incubation at 105°C overnight. The round bottom flask was cooled before weighing, and the fat content was calculated using the following equation:

$$\%\,Fat = \frac{weight\ flask\ after\ drying - weight\ empty\ flask}{sample\ weight} * 100$$

The method by **Bligh and Dyer** (Bligh and Dyer, 1959) was used for the extraction of the lipids at SINTEF (Norway).

N-protein

For determining the total protein content, Dumas (1831) method was used that measures total nitrogen in the sample. Moisture free sample was crushed into a powder and 50 mg powder was weighed accurately and wrapped in aluminum cups. The rest of the analysis was carried out by a staff member at Central Instruments Facility & Services (CFS) at CFTRI. The detected nitrogen content was then multiplied with a nitrogen to protein conversion factor of 6.25.

Ash

Determination of ash was conducted following the AOAC method (1990). To determine the ash content in the raw material, the crucible was first weighed, followed by addition of 0.5-1 g sample and the crucible was placed in a desiccator. The samples were charred on a gas burner until only ash was left in the crucible. The samples were then placed in a muffle furnace at 550°C for 5 hours. The furnace was turned off and the samples were left in the furnace overnight to cool off. The analysis was performed in

duplicates, and the ash content was calculated using the following equation:

$$\% \; Ash = \frac{final \; weight \; crucible - empty \; weight \; crucible}{sample \; weight} * 100$$

Determination of amino acid composition

The amino acid profile of freeze-dried ground RRM was analyzed by a HPLC system (Agilent Infinity 1260, Agilent Technologies) coupled to an on-line post-column derivatization module (Pinnacle PCX, Pickering laboratories, Mountain View, CA, USA), using ninhydrin (Trione) as a derivatizing reagent and Na+-ion exchange column (4.6 x 110 mm, 5 µm). 18 standard amino acids, ammonia and taurine were quantified from standard curves measured with amino acid standards. Prior to the analysis, the samples were hydrolyzed in 6 M HCl containing 0.4% mercaptoethanol for 24 h at 110°C (HCl hydrolysis). Glutamine and asparagine were converted to glutamic and aspartic acid, respectively. Cysteine was quantified as cysteine (Cys-Cys). The samples were filtered via micro filter, the pH was adjusted to 2.2 and the samples were further diluted with a citrate buffer (pH 2.2) for the HPLC analysis. All buffers, reagents, amino acid standards and the column were obtained from Pickering laboratories (Mountain View, CA, USA). HCl and mercaptoethanol was obtained from Sigma-Aldrich. The analysis was conducted at SINTEF Ocean Trondheim.

Protein efficiency ratio (PER)

Quality of proteins in the samples was evaluated by Protein Efficiency Ratio (PER). PER of proteins in RRM was calculated by using following equations (Alsmeyer *et al.,* 1974; Lee *et al*., 1978):

PER 1: -0,684+0,456[Leu]-0,047[Pro]

PER2: -0,468+0,456 [Leu]-0,104 [Tyr]

PER3: -1,816+0,435[Met]+0,780[Leu]+0,211[His]-0,944[Tyr]

PER4: 0,08084[∑AA7]-0.1094, where ∑AA7=Thr+Val+Met+Ile+Leu+Phe+Lys

PER5: 0,06320[∑AA10]-0,1539, where ∑AA10=∑AA7+His+Arg+Tyr

Soy proteins and casein were used as reference samples for the PER determination. All measurements were performed in duplicates.

Proteolytic activity of head and viscera and skin & bones

Preparation of crude fish extracts: The sample (50 g) was weighed in centrifugation bottle (250ml). Sample was homogenized for 2 min by using distilled water in a ratio 1:2 (w/v). Sample was left in cold room for 10 min and centrifuged at 10400 x g for 20 min at 4ºC. After centrifugation supernatant was filtered through glass wool and pH was checked. Sample was stored in 4ºC for further analysis.

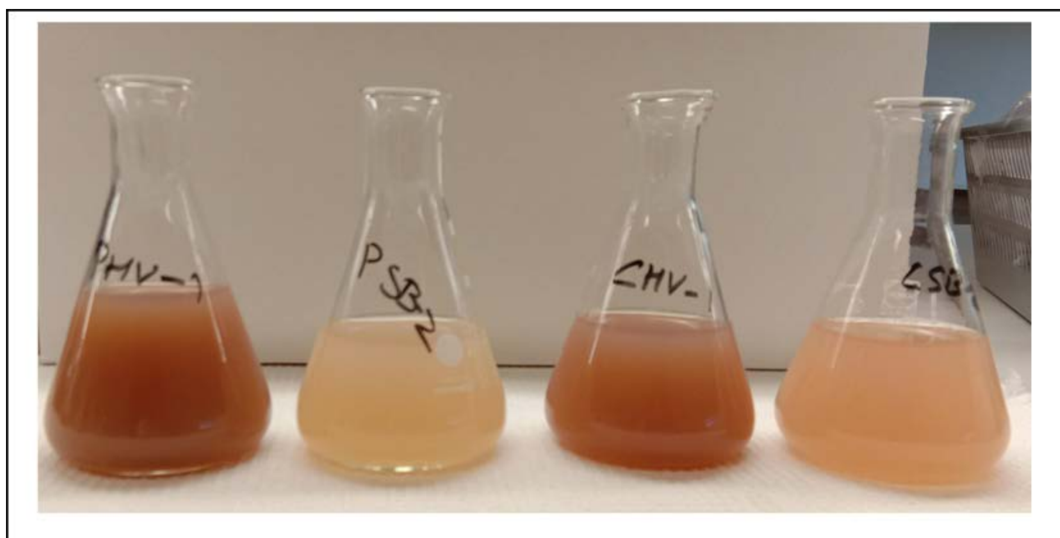

Figure 6. *Enzymatic extract of Pink perch and croaker.*

Proteolytic activity of Pink perch head and viscera extract (PHVE), Pink perch skin & bone extract (PSBE), Croaker head and viscera extract (CHVE), and Croaker skin & bone extract (CSBE). was determined by Lowery method (Barrett (1972) and Stoknes et al., (1993).

For 0- time: 1.2 ml buffer (0.1 M citric acid + 0.2 M Na_2HPO_4) and 0.4 ml substrate (1 % haemoglobin) were taken into test tube and mixed properly. 2.0 ml TCA (5%) was added in each tube**.** The tubes were placed on water bath at 50ºC for 2 min. then 0.4 ml fish extract was added to tube and incubated for 60 min at 50ºC in water bath. The solution was cooled down for 30 min at room temperature and filtered through 70mm filter paper. Solution was filtered twice. The protein content of the filtrate was determined by the Lowery method.

For fish extract: 1.2 ml buffer (0.1 M citric acid + 0.2 M Na_2HPO_4) and 0.4 ml substrate (1 % haemoglobin) were taken into test tube and mixed properly 2.0 ml. The tubes were preheated on water bath at 50ºC for 2 min. Then 0.4 ml fish extract was added to tube and incubated for 60 min at 50ºC in water bath. After incubation 2ml TCA (5%) was added to stop enzyme activity and mixed. The solution was cooled down for 30 min at room temperature and filtered through 70mm filter paper. Solution was filtered twice. The protein content of the filtrate was determined by the Lowery method.

1.1.2 Raw material collected from Kaiko surimi and analysed at AMITY University Uttar Pradesh (India)

The following work was executed:

- Samples (head and viscera; whole fish; skin and bones; refiner waste and wash water) were received from Refrigerated distributors Pvt. Ltd (Kaiko surimi) on 15 February 2019.
- The moisture content, fat content, protein content and ash content of above given RRM was determined.
- Microbiological characterization of RRM was performed for Aerobic plate count; *Salmonella* count; yeast and molds and Lactic acids bacteria (LAB)

Sample preparation

RRM, WW and whole fish was collected from Refrigerated Distributors Pvt. Ltd (Kaiko surimi) in frozen form. The samples were thawed at room temperature and homogenized in mixer grinder.

Proximate analysis of RRM, WW and whole fish

Moisture content

Moisture content was determined with slight modification of AOAC 2005**.** Sample was thawed in room temperature and uniformly homogenized in mixture grinder. 10g of sample was weighed in pre-weighed aluminum petri plate**.** Samples were kept in hot air oven at 105°C. Weight was measured at an interval of half an hour after 5 hours of drying till constant weight was obtained. Petri plates were kept in desiccator for 10 minutes for cooling prior to measurement of weight.

Calculation

$$\% \text{ Moisture content} = \frac{\text{Intial Weight} - \text{Final Weight}}{\text{Intial Weight}} \text{X } 100$$

Fat content

Fat Content was determined with slight modification of AOAC 2005**.** Thimbles were prepared using Whatman filter paper no 1and their weight was measured. 2gm moisture free samples were taken in thimbles and weight of thimble containing sample was taken. Thimbles were inserted in extractor. 200 ml of hexane was taken in receiving flask. It was ensured that water was running through condenser at all time. Apparatus was set up on heating mantel and a temperature of 70°C was maintained. Experiment was continued till clear hexane is collected in extractor. Thimbles were collected and kept in hot air oven at 40°C for drying. Hexane containing oil was collected and subjected to rotary evaporation to separate oil and hexane.

Calculation

$$\% \text{ Fat content} = \frac{\text{Weight of initial sample} - \text{weight of defatted sample}}{\text{weight of intial sample}} \text{X } 100$$

Protein content

Digestion

- The digestion unit was first switched on and temperature was set up to 350°C
- Fat free sample (2g liquid and 0.2 g of powder) was taken in digestion tube.
- 3g of pre-prepared catalyst (potassium sulphate: Copper sulphate in 5:1) was added in digestion tube followed by 10 ml of conc H_2SO_4.
- The sample containing digestion tube was loaded in digestion unit with manifold.
- Temperature of digestion unit was increased to 420°C.
- Digestion was carried for approximately 24 hours till fumes stopped coming
- Samples were kept in cooling rack for cooling.

Distillation and titration

- The solutions of 4% boric acids, 40% NaOH and 0.1 N HCl were prepared.
- Sample (dilute with 10 ml distilled water) was taken in distillation flask and 50 ml of 40% NaOH was added in it.
- 25 ml of boric acid was taken in 100 ml conical flask and 4 drops of indicator was added and place at the receiver end.
- Distillation was carried out for 3 hours. During the process liquid ammonia was collected in the boric acid and the color of boric acids changes due to indicator.

- After completion of the process conical flask was removed from the receiver end and then titrated with 0.1 N HCl.

Calculation:

$$\text{Nitrogen content (\%)} = \frac{14.01\ \text{X}\ 0.1\ \text{N X (TV}-\text{BV)X}\ 100}{\text{W X}\ 1000}$$

$$\text{Protein content (\%)} = \text{Nitrogen content (\%)}X\ 6.25$$

Where 14.01- Ammonia molecular weight, 0.1N- titration solution normality, TV- Titer value, BV-Blank value, W- Sample weigh.

Ash content

Ash content was determined with slight modification of AOAC 1990**.** Crucible was washed with nitric acid and dried in hot air oven at temperature 105°C. Sample was thawed at room temperature and homogenized in mixture grinder. 3 grams of sample was taken in dried crucible. Weight of both crucible and sample was noted. Sample was kept in muffle furnace till ashing was complete. Temperature for muffle furnace was adjusted for 550°C-600°C**.** Sample was cooled in desiccators and weight was recorded after sample reached room temperature.

Calculation

$$\text{Ash Content} = \frac{\text{Ash Weight (g)}}{\text{Initial weight of sample (g)}} * 100$$

Mineral composition analysis

Sample was coated on double sided carbon tape. Extra sample was dusted off to ensure fine layer of sample on carbon tape. Sample was then be coated with gold and palladium in a process called gold spluttering. This process was carried in vacuum for 4 minutes. It was then observed in scanning electron microscope. Working distance between the sample and lens was less than 10 mm. Elemental analysis was conducted on the cross section of the scale by EDX to verify the element content and distribution.

Microbiological analysis of RRM, WW and whole fish

The homogenized sample (1g) was serially diluted from 10^{-1} to 10^{-6} dilution. After that 100 µl of sample from dilutions was plated on different agar plates by spread plate techniques. Media used for Aerobic plate count, Yeast and Mold, Salmonella and Lactobacillus count were nutrient agar, CzapekDox agar, XLD agar and MRS agar respectively. Plates were incubated for 24 h at 37 ºC for Aerobic plate count, Salmonella and Lactobacillus and yeast and mold at 28° C for 3day. Total plate count was determined by below formula.

$$Total\ count\ (Cfu/ml) = \frac{No.of\ colonies\ X\ dilution\ factor}{Volume\ of\ sample\ (ml)}$$

1.1.3 Samples bought from Spanish market analysed at LEITAT (Spain)

Preparation of samples

The moisture, ash and protein content were determined for the different fractions of the RRM produced from the two fish species (croaker fish and reef cod) bought in a Spanish market, in LEITAT laboratory. After filleting, the four fractions (head, skin, spines, and viscera) were ground, dried at 50ºC using a heater PSELECTA® Digitronic and milled using a miller RETSCH ZM-200, which has sieves of 1.5 and 0.5 mm, to facilitate the correct sample analysis (Figure 7and Figure 8).

For analysis 50% reef cod was mixed with 50% croaker.

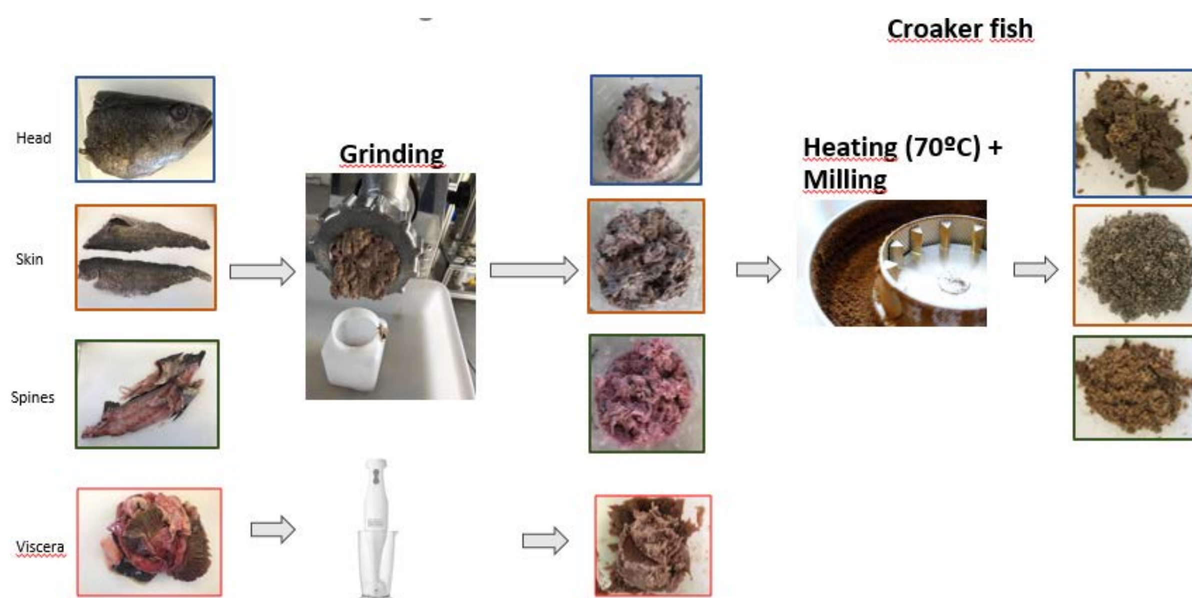

Figure 7. *Preparation of croaker fish samples.*

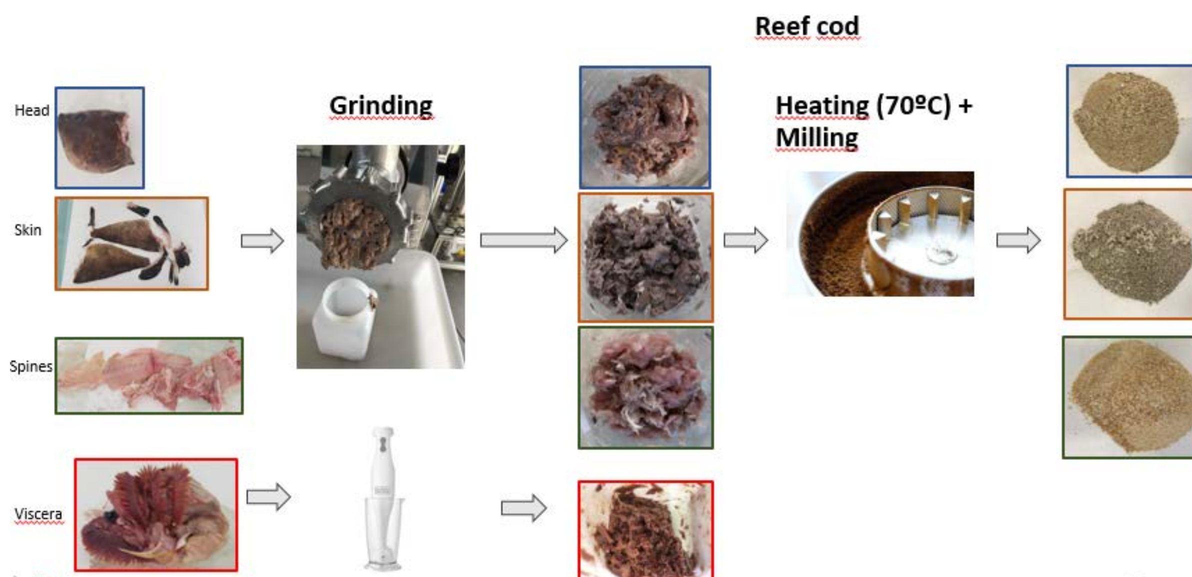

Figure 8. *Preparation of croaker fish samples.*

Moisture

Moisture content was determined by a variation of the Official Method of Analysis A.O.A.C. (15th edition 1990). First, an aluminum petri dish per sample was introduced in the heater PSELECTA® Digitronic at 105ºC for approximately 20 minutes to eliminate the moisture they have due to their storage at room temperature. Then all of them were placed into a desiccator for 10 minutes and weighed after they were cooled. After this, 2-7 g sample from each fraction was weighed and heated at 105ºC for 5 hours. The measurements were done in duplicates. Following this, aluminium petri dishes with samples were placed into the desiccator for 10 minutes for cooling them and weighed.

The moisture content was calculated using the following equation:

$$\% \, Moisture = \frac{Inicial\ sample\ weight - final\ sample\ weight}{sample\ weight} \, x\, 100$$

Protein content

Protein content was analyzed following the Kjeldahl method A.O.A.C. method (1990), which based on digesting the sample with concentrate sulfuric acid and alkalize with sodium hydroxide. Ammonia is released and driven by distillation and collected with boric acid. The following titration with hydrochloric acid allows us to know the percentage of nitrogen and, further, the initial protein content in the sample.
This process is divided in three different reactions:

1) Digestion
2) Neutralization and distillation
3) Titration

Calculation

$$Nitrogen\ (\%) = \frac{V\ HCl\ (ml) * [HCl] \left(\frac{mol}{L}\right) * Molecular\ weight\ N * HCl\ factor}{Sample\ weight(g) * 1000} \, x\, 100$$

$$Protein\ (\%) = \%\ Nitrogen\ x\ 6{,}25$$

For the calculation of nitrogen amount it was considered that molecular weight of Nitrogen is 14.01 g/mol, concentration used of HCl had been 1N and so the HCl factor is 1.8457.

Ash content

Ash content was determined following the A.O.A.C. method (1990). For doing this crucible (one per sample) were used. Crucible used were put in the heater (PSELECTA® Digitronic) at 105ºC to take out the moisture and then in the desiccator. Once cooled crucibles were weighed and, then, between 0.5g and 1.5g of sample were placed into the crucible. Crucibles used with samples were placed then to the muffle adjusted for 550ºC and were kept until ashing was complete.

Finally, crucibles with samples were cooled in desiccators and weight once samples were at room temperature. The ash content was calculated following this equation:

$$Ash\ Content = \frac{Final\ weight\ (g) - \ Crucible\ initial\ weight(g)}{Initial\ weight\ of\ sample\ (g)} \, x\, 100$$

2 Results

2.1 Characterization of the raw material: Samples analysed at CFTRI (India)

The composition of the different raw material received from Kaiko surimi production plant is presented in Table 3. Dry matter and ash content were determined in duplicates while fat and protein content only had one parallel.

Table 3. *Composition of the raw material received from Kaiko surimi production plant. Results are presented in wet weight. Dry matter and ash are presented as average n=2 ±SD, fat and protein as n=1. HW: head waste, SBW: skin and bone waste, RW: refined waste.*

	Dry matter (%)	Fat (%)	Protein (%)	Ash (%)
HW	21.4 ±0.1	1.4	12.5	7.0 ±0.4
SBW	17.3 ±0.9	0.8	9.5	7.3 ±0.7
RW	15.4 ±0.4	0.5	12.1	2.9 ±0.4

The samples had been frozen prior to the characterization of the raw material, and after thawing in water bath it was clear that it was quite a lot of water in the bags. The plastic bags had not been completely sealed at the surimi production plant, which was not discovered until after thawing of the samples. The fat content was found to be highest in heads fraction (1.4%) and lowest in refined waste (0.5%). Considering lean fish species, like pink perch stores fat in the liver, it makes sense that the head fraction, which includes viscera, had the highest fat content. A higher protein content was found in refined waste (12.1%) than the two other fractions (9.5 – 12.5%). These results are reasonable considering refined waste contained minor pieces of connective tissue and some meat which contains a lot of protein. Ash content was found to be higher in skin and bones (7.3 %) and head (7.1 %) fractions than refined waste (2.9 %) as these fractions had a high content of bones and bone like substances.

Amino acid composition: In order to determine the nutritional value of the dried raw material, the dried sediment from the hydrolysis and fish protein hydrolysate (FPH) from head waste, the total amino acid composition was investigated. Technological description of the hydrolysis together with yield and quality of hydrolysis products is not covered by this report. Amino acids composition of sediments and FPH is presented in order to illustrate distribution of amino acids after processing.

The samples were analyzed, and the results were calculated as the amount total amino acids (protein) in percentage of the samples. The total amino acid amount (protein) in dried sediments, FPH and head were obtained to be 46.7 %, 28.7 % and 71.5 %, respectively. The composition of amino acids was determined by evaluating each amino acid in the samples, as presented in Figure 9. Tryptophan is not included because it is not adapted to the method used. Most of the tryptophan will be damaged by processing and will not be detected by the HPLC system.

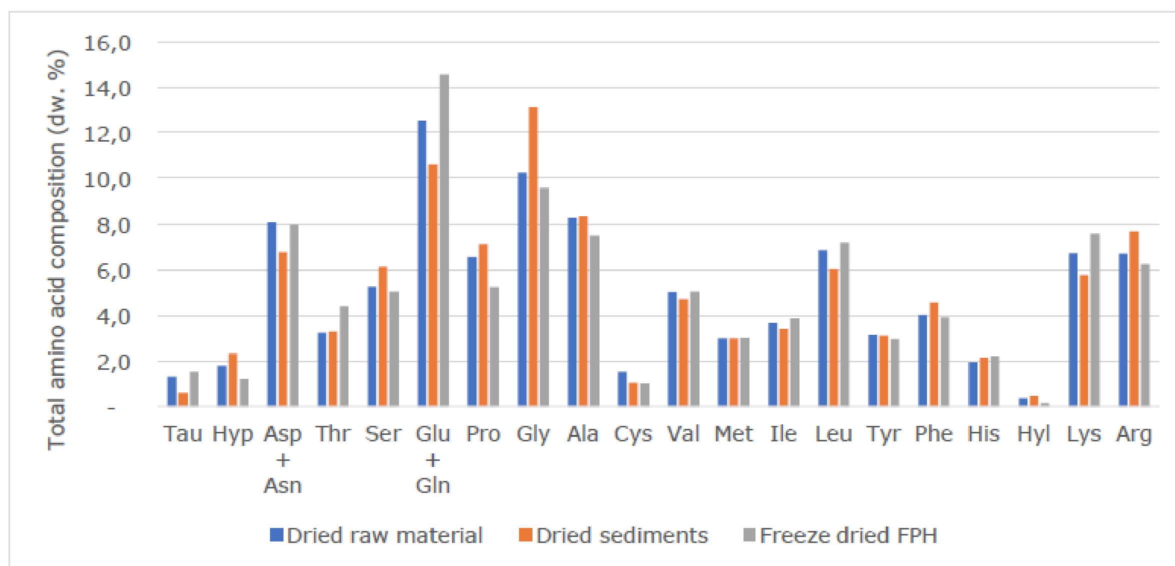

Figure 9. *Amino acid composition (dw %) in dried raw material (head waste), dried sediments and freeze dried FPH from the hydrolysis of head waste.*

The distribution of amino acids is similar for all the samples, with a high content of Glu+Gln, Gly, Asp+Asn, Ala, Leu, Lys and Arg. In a study by Liceaga- Gesualdo and Li-Chan (1999) the same amino acids were found to be dominating in FPH from herring. The amount of Glu+Gln ranged from 10.6-14.6 % with the highest amount found in FPH from heads fraction. The amount of Gly was also found to be high, with the highest content of 13.1 % in dried sediments.

The amount of (dw %) essential (His, Thr, Met, Val, Phe, Ile, Leu and Lys), hydrophobic (Ala, Tyr, Met, Val, Phe, Ile and Leu) and aromatic (Phe, Tyr and His) amino acids in the samples are presented in Table 4.

Table 4. *Essential, hydrophobic and aromatic amino acids in dried raw material, dried sediments and freeze dried FPH from head fraction. Values presented as percentage of all amino acids.*

Dw %	Dried raw material Head waste	Dried sediments	FPH (from head fraction)
Essential AA	34.4	32.8	37.1
Hydrophobic AA	33.9	33.1	33.4
Aromatic AA	9.1	9.8	9.1

Studies by Mendis et al. (2005) and Aluko (2015) have found that hydrophobic and aromatic amino acids can be linked to antioxidative activity of fish peptides. The samples were found to contain a high amount of hydrophobic amino acids (33.1-33.9 %) which can be linked to antioxidative properties of the samples. All the essential amino acids were present in the samples, except Tryptophan, whereas Lys and Leu had the highest content (5.8–7.6 % and 6.0–7.2 %). The content of essential amino acids is above the suggested content of 37 % essential amino acids of total amino acids in a product. This may indicate that the samples are of high nutritional value (Lee et al., 1978). However, hydrophobic amino acids are known to have a bitter taste which is not desirable in products for human consumption (Kirimura

et al., 1969) (Lalasidis and Sjoberg, 1978). Quality of proteins in different RRM and FPH from heads (PER, Table 5) varies from samples to samples, but quality of all evaluated proteins is in the line with the quality of soy and casein proteins.

Table 5. *Protein efficiency ratio (PER) of proteins in different RRM and FPH from heads. Soy and casein were used as reference proteins.*

	Dried raw material - heads	Dried sediments	FPH from heads	Soy	Casein
PER 1	2.13	1.72	2.34	2.84	2.99
PER 2	2.31	1.93	2.48	2.86	3.12
PER 3	2.28	1.68	2.77	1.82	1.96
PER 4	2.51	2.37	2.71	2.66	3.08

2.2 Characterization of raw materials from pink perch and croaker: samples collected in India and filleting/analysed at SINTEF Ocean (Norway)

The pink perch and croaker of weight 128±66g and 65±28g and length 17±4cm and 14±3cm respectively were used for RRM generation in laboratory. These measurements gave estimation that an average one unit of pink peach is approx. 126g and croaker is 84g in weight. Filleting of pink perch indicated that of 30% whole fish ends as skin & bones and 42% as head & viscera fractions. Even more RRM were obtained from croaker: skin & bone made up 20%, while head and viscera made up 59% of fish: Table 6.

Table 6. *Physical characterisation of RRM from pink perch and Croaker.*

	Pink perch		Croaker	
Parameters	**Weight, g**	**Length, cm**	**Weight, g**	**Length, cm**
	128±66	**17±4**	**65±28**	**14±3**
RRM after lab fractionation				
	Weight, g	**%**	**Weight, g**	**%**
Bones+skins	**649**	**30**	**331**	**20**
Heads+viscera	**896**	**42**	**995**	**59**
Fillet	**590**	**28**	**350**	**21**
RRM from 1 fish	**126**		**84**	

Chemical composition of RRM

Chemical composition of generated RRM as well as fillet of pink peach and croaker is presenter in Table 7. The *Pink Perch* head and viscera, skin and bones and fillet were having moisture content 78.2 %, 73.6 % and 81.9%; lipid content 2.2, 2.2 and 0.5 g lipid/100g raw material, protein content 17.4%, 23.5%, 17.2% and ash content 5.6 %, 7.8 % and 1.1 % respectively. Similarly, croaker head & viscera, skin & bone and Fillet were having moisture content 70.9%, 73.9% and 78.7%; lipid content 5.6, 4.2 and 1.6 g lipid/100g raw material; protein content 22.1%, 22.7% and 21.0%and ash content 8.5%, 5.5 % and 1.3% respectively.

Table 7. *Chemical composition of RRM from pink perch and Croaker. Values on wet weight basis presented as average value ± standard deviation.*

	Moisture content, %	Lipid content, g lipid/100g sample	Ash content, %	Protein content %
Pink perch head & viscera	**78.2±0.7**	**2.2±0.4**	**5.6±0.7**	**17.4±1.3**
Pink perch Skin & bones	**73.6±1.4**	**2.2±0.3**	**7.8±1.5**	**23.5±2.5**
Pink perch fillet	**81.9±0.3**	**0.5±0.2**	**1.1±0.0**	**17.2±0.0**
Croaker Head & viscera	**70.9±3.1**	**5.6±0.2**	**8.5±3.3**	**22.1±0.2**
Croaker skin & bones	**73.9±1.9**	**4.2±0.2**	**5.5±1.0**	**22.7±4.2**
Croaker fillet	**78.7±0.6**	**1.6±0.3**	**1.3±0.0**	**21.0±0.4**

Proteolytic activity

Proteolytic activity of fish head & viscera (HV) and skin & bones (SB) extracts were determined by using haemoglobin as substrate. Proteases from HV and SB break the haemoglobin into smaller peptides at optimum condition. Proteolytic activity was determined by measuring amount of soluble peptides before and after protease treatment (Yamashita and Konagaya, 1990). In this study pink peach samples show higher proteolytic activity than croaker RRM fractions evaluated after 60 min treatment at 50ºC (Figure 10). The proteolytic activity depends on reaction condition (temperature and pH) and reaction mixture (Stoknes and Rustad, 1995) and due to this optimal reaction conditions should be defined for each analysed fraction.

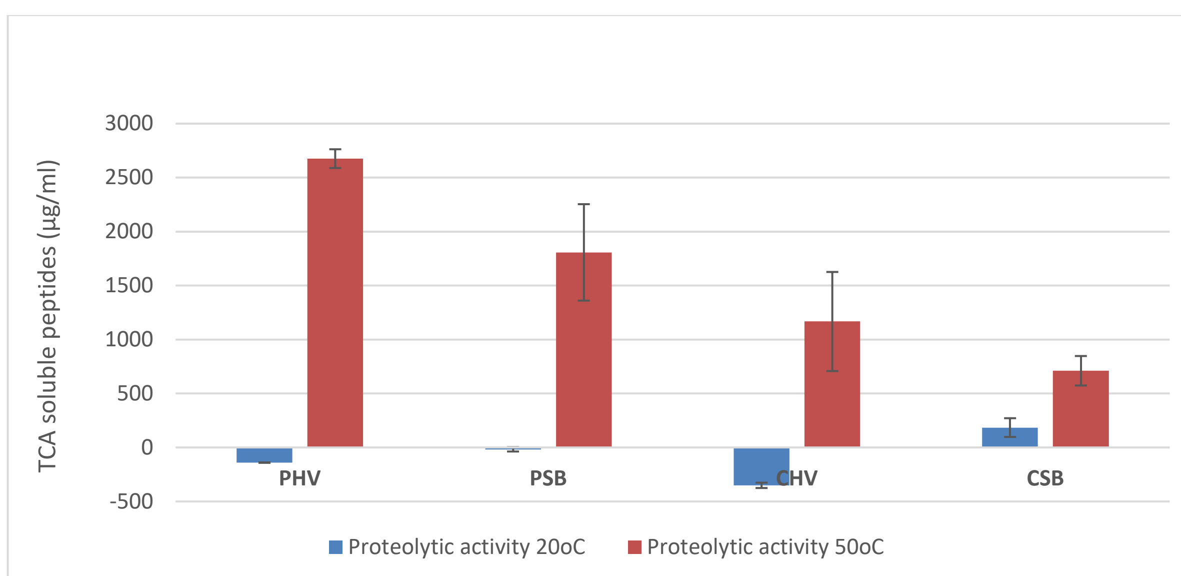

Figure 10. *Proteolytic activities of Pink perch (PHV and PSB) and Croaker (CHV and CSB) head & viscera (HV) and skin & bones (SV).*

Amino acids composition of pink perch

Amino acid analysis of pink perch head & viscera, skin & bones and fillet were performed by HPLC methods. The head and viscera, skin & bones and fillet fractions contain 17.5%, 18.3% and 36.6% of essential amino acids (such as threonine, valine, methionine, isoleucine, tryptophan, phenylalanine, histidine and lysine) and 27.3%, 31.1% and 41% of non-essential amino acids (such as taurine, methionine sulfoxide, hydroxyproline, arginine, hydroxylysine, cystine, alanine) as shown in Table 8 and Table 9.

Table 8. *Essential amino acid composition of Pink perch.*

	Amino acid (%) D/W		
Essential Amino acid	**Head & viscera**	**Skin & bones**	**Fillets**
Threonine	1.9±0.2	2.1±0.1	3.9±0.1
Valine	2.3±0.2	2.3±0.2	4.4±0.1
Methionine	1.3±0.1	1.4±0.1	2.7±0.1
Isoleucine	1.6±0.2	1.7±0.2	3.6±0.2
Leucine	3.1±0.3	3.2±0.3	6.7±0.3
Tyrosine	1.4±0.1	1.2±0.0	3.0±0.1
Phenylalanine	1.9±0.2	1.9±0.1	3.3±0.1
Histidine	0.7±0.1	0.8±0.0	1.2±0.0
Lysine	3.2±0.2	3.6±0.3	7.8±0.3
Tryptophan	0.0	0.0±0.2	0.0±0.0
Sum	17.5	18.3	36.6

Table 9. *Non-essential amino acids of pink perch.*

	Amino acid (%) D/W		
Non-Essential Amino acid	**Head & viscera**	**Skin & bones**	**Fillets**
Taurine	0.6±0.1	0.5±0.0	1.1±0.0
Methionine sulfoxide	0.0±0.0	0.0±0.0	0.0±0.0
Hydroxyproline	0.9±0.1	1.3±0.1	0.1±0.1
Aspartic acid + Asparagine	3.7±0.3	4.0±0.3	7.4±0.3
Serine	2.3±0.2	2.5±0.2	4.0±0.1
Glutamic acid + Glutamine	6.1±0.6	6.7±0.5	12.6±0.4
Proline	2.7±0.2	3.0±0.1	2.6±0.1
Glycine	4.7±0.3	6.0±0.3	3.8±0.2
Alanine	3.2±0.2	3.7±0.1	4.5±0.1
Cystine (Cys-Cys)	0.0±0.0	0.1±0.1	0.1±0.1
Hydroxylysine	0.0±0.0	0.0±0.0	0.0±0.0
Arginine	3.0±0.3	3.3±0.1	4.9±0.3
Sum	27.3	31.1	41.0

2.3 Characterisation of samples collected from Kaiko surimi and analysed at AMITY University Uttar Pradesh (India)

Proximate analysis of RRM, Whole Fish and Surimi

Proximate composition of RRM, whole fish, WW and Surimi obtained from Kaiko Surimi Industry. The sample was obtained in frozen form. Samples was thawed at room temperature. Moisture content, fat content, ash content and protein content were estimated to determined proximate composition of the samples. Microbiological analysis was done for Aerobic plate count, Yeast and Mold, *Salmonella* sp and *Lactobacillus* sp. The result of proximate analysis and microbiological analysis are shown in Table 10 and Table 12. The whole fish (Pink Perch), its head and viscera, skin and bones, refiner waste, and surimi were having 77%, 76.7%, 69.0%, 80% and 78.3% moisture content; 2.2%, 1.7%, 1%, 2% and 0.73% fat content; 8.7%, 6%, 10%, 9% and 7% protein contents respectively. Ash content of whole fish (Pink Perch), its head and viscera, skin and bones, refiner waste, and surimi were 4.6%, 4.3%, 10%, 3%, and 0.3% respectively. Wash water is water obtained after washing of fish mince. Moisture content, fat content, protein content and ash content of wash water is 98%, 0%, 1% and 0% respectively.

Table 10. *Proximate Analyses of RRM, Whole Fish and Surimi.*

Sample	Moisture, %	Protein, %	Fat, %	Ash, %
Whole Fish	77.0 ± 0.5	8.7 ± 3.0	2.2 ± 0.1	4.6 ± 0.1
Head and Viscera	76.7 ± 1.0	6.0 ± 0.9	1.7 ± 0.2	4.3 ± 0.4
Skin and Bones	68.6 ± 0.7	10.0 ± 4.7	1.0 ± 0.6	10.0 ± 0.2
Wash Water	98.3 ± 0.3	0.9 ± 0.1	0.3± 0.1	0.0
Refiner Waste	80.0 ± 1.0	9.0 ± 0.4	2.0± 0.1	3.0 ± 0.4
Surimi	78.3 ± 0.3	7.0 ± 0.5	0.7 ± 0.5	0.3 ± 0.5

Mineral content analysis, Electron Dispersive X-Ray (EDX) Analysis was conducted for whole fish, surimi, refiner waste and viscera and skin and bones which confirmed absence of any heavy metal. EDX also confirmed presence of calcium and phosphorus in maximum concentration in all the samples as shown in Table 11 and Figure 11.

Table 11. *Mineral elements present in RRM, Whole fish and Surimi.*

Elements	Weight (%)				
	Whole fish	Head & viscera	Skin & bones	Refiner waste	Surimi
Carbon	3.5±4.9	5.2±0.2	6.2±0.6	3.6±5.1	3.0±4.3
Oxygen	44.4±1.8	43.1±0.2	41.2±3.6	46.1±2.7	46.3±0.1
Sodium	2.6±0.9	1.7±0.9	1.2±0.1	1.5±0.4	17.2±4.1
Magnesium	1.6±0.1	1.0±0.1	1.0±0.1	1.5±0.3	2.1±0.9
Aluminum	0.0	0.2±0.2	0.0	0.0	0.0
Silicon	0.0	0.3±0.4	0.0	0.0	0.0
Phosphorus	18.7±1.9	17.9±0.6	17.8±0.8	18.5±1.1	17.3±2.3
Chlorine	0.0	1.2±0.5	0.2±0.2	0.4±0.1	0.0
Potassium	0.5±0.1	0.6±0.4	1.0±0.1	0.3±0.1	3.1±1.6
Calcium	28.2±4.6	28.4±1.6	31.6±5.4	28.2±0.5	10.5±5.5
Tungsten	0.6±0.8	0.6±0.8	0.0	0.0	0.6±0.8

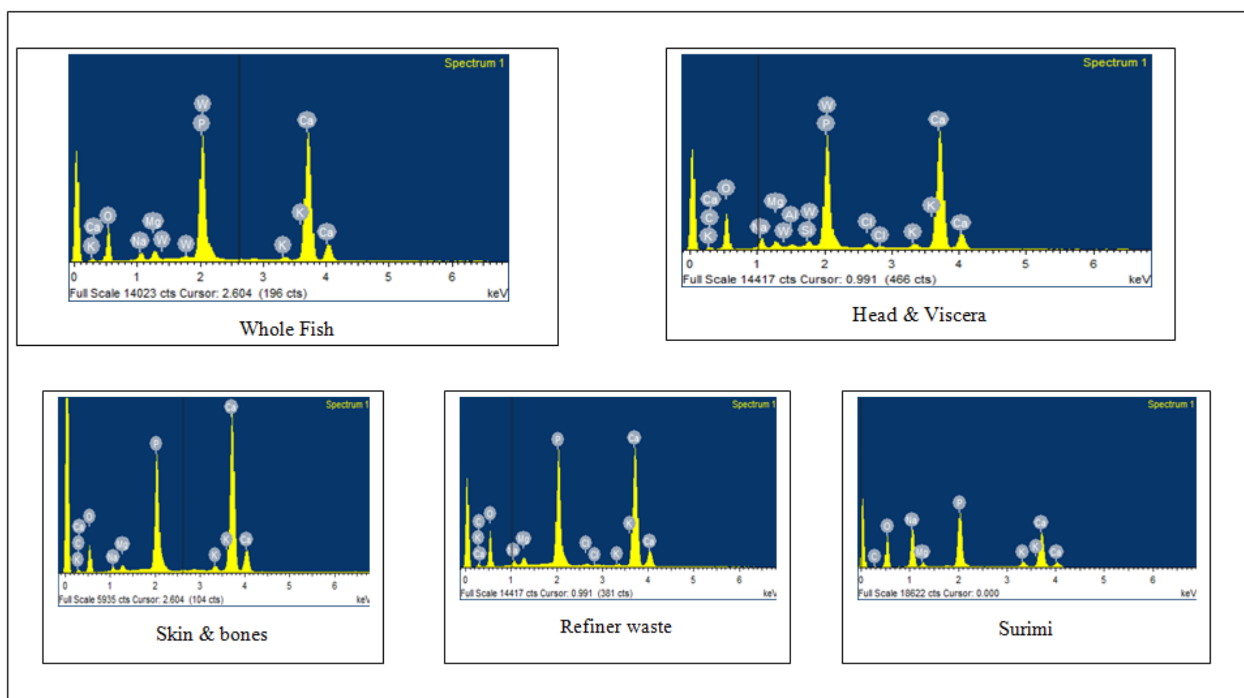

Figure 11. *Electron Dispersive X-Ray of RRM, Whole fish and Surimi.*

Table 12. *Microbiological Analysis of RRM, Whole fish and Surimi.*

Sample	Aerobic plate count (CFU/g)	Yeast and mold count (CFU/g)	*Salmonella* count (CFU/g)	*E. coli* (CFU/g)	*Lactobacillus* (CFU/g)
Whole fish	14×10^6	106×10^4	0.0	0.0	500
Head & Viscera	15.5×10^6	118×10^4	0.0	0.0	300
Skin and bones	14×10^6	3.1×10^6	0.0	0.0	700
Wash Water	5×10^5	3.9×10^6	0.0	0.0	150
Refiner Waste	5×10^6	0	0.0	0.0	0.0
Surimi	0.0	0	0.0	0.0	8600

Microbiological analysis of RRM, WW, whole fish and Surimi was conducted to analyse suitability of use RRM for potential use as food and feed (Table 12). Samples were analysed for Aerobic plate count, presence of *E. coli*, *Salmonella*, Yeast and mold, and *Lactobacillus* bacteria. No pathogenic bacteria were found in any RRM, whole fish and surimi, wash water. Few Lactobacillus sp were found in samples in which Surimi (8600 CFU/g) contain highest amount of Lactobacillus followed by skin & bones (700 CFU/g), whole fish (500 CFU/g), head & viscera (300 CFU/g) and wash water (150 CFU/g). whereas no Lactobacillus sp. was detected in refiner wastes. There is no regulation available for microbiological load of fish related raw material used in food industry. According to FSSAI permitted aerobic plate count in fish mince/surimi and analogues are 1 X 10^6 but yeast and mold should be absent. Yeast and mold count for dried fishery product can be 500 CFU/g.

2.4 Characterization of raw materials from reef cod and croaker fish: Samples collected analysed by LEITAT (Spain)

Entire reef cod and croaker fish weight (in fresh) 1355g and 2280g respectively. Manual filleting of the fishes provided information of fraction generated during manufacturing of surimi from reef cod bones and skin fraction made up 36% of the raw material by, while heads and viscera made up 35% of the whole fish In case of croaker fish, a bones and skin fraction made up 34% of the whole fish, while heads and viscera made up 28% of the whole fish, as is shown in Table 13. By analyzing this data, it could be assumed that approximately 72% from the entire reef cod is considered as RRM, moreover from croaker fish is a 63%.

Table 13. *Physical characterization of RRM from reef cod and croaker fish.*

	REEF COD		**CROAKER FISH**	
	Weight in fresh (g)		Weight in fresh (g)	
	1355		2280	
RRM obtained by laboratory fractionation				
	Weight in fresh (g)	**%**	Weight in fresh (g)	**%**
Bones + skin	476.4	**36**	753.3	**34**
Heads + viscera	467	**35**	620.5	**28**
TOTAL by-products/RRM (%)		**72**	TOTAL by-products/RRM (%)	**63**

Physico-chemical composition of lab-made RRM

The chemical composition of the RRM prepared in LEITAT laboratory is specified in Figure 12, Figure 13 and Figure 14. It is important to remember that for analysis50% fraction of RRM form reef cod and 50% fraction of RRM from croaker has been mixed.

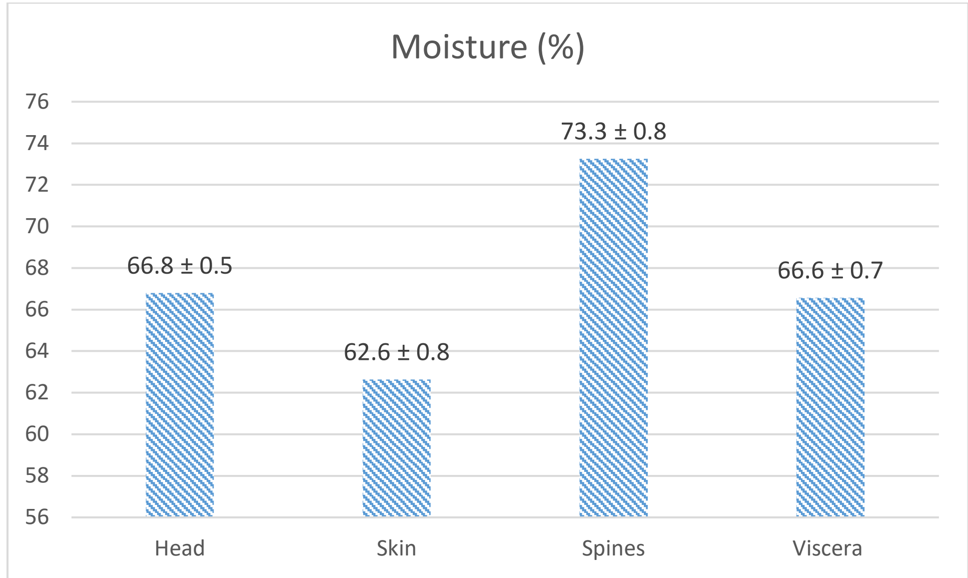

Figure 12. *Moisture content from RRM made from 50% reef cod and 50% croaker fish.*

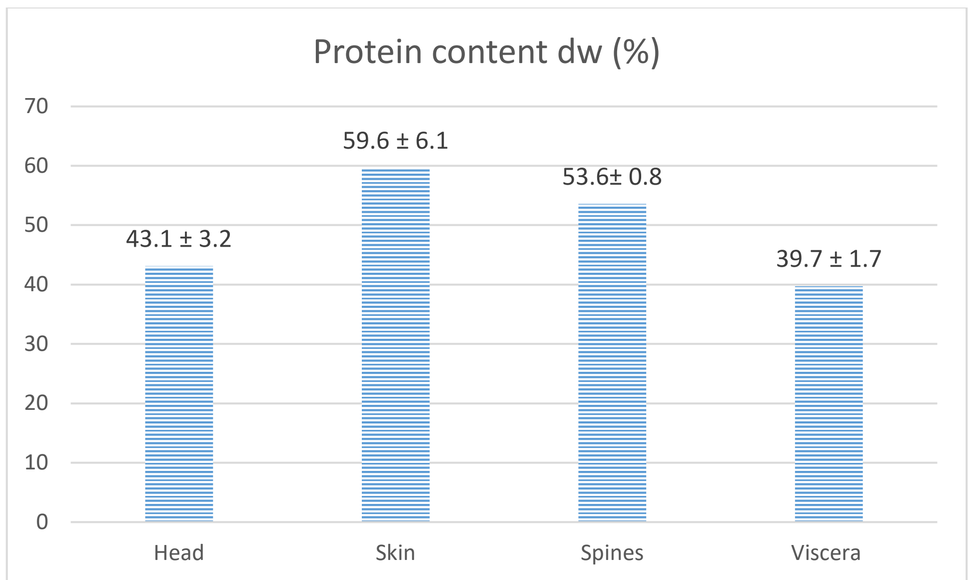

Figure 13. *Protein content from RRM made from dried 50% reef cod and 50% croaker fish.*

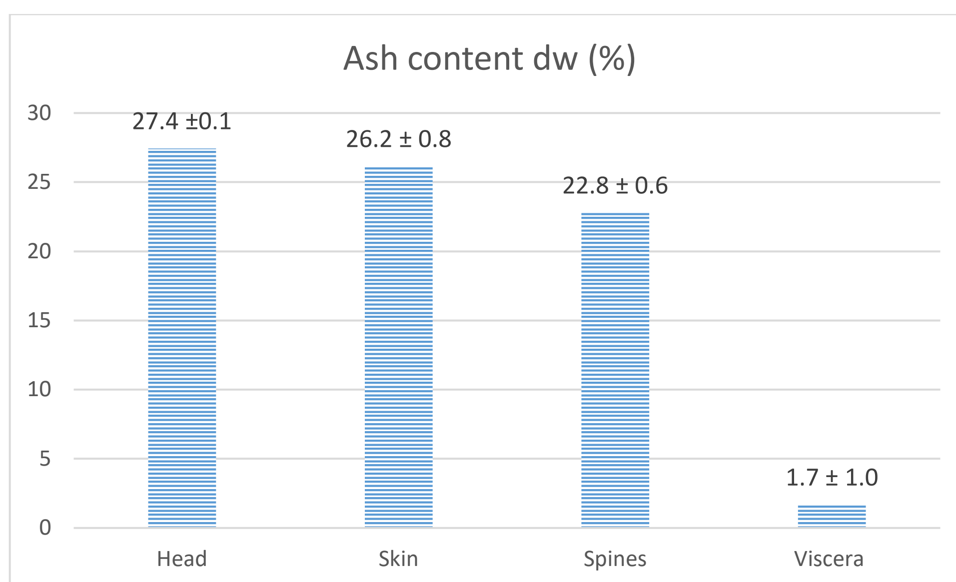

Figure 14. *Ash content from RRM made from dried 50% reef cod and 50% croaker fish.*

Physicochemical analysis shows a highest moisture content in the spines (73.3%). Protein content are similar in head and viscera (39-43%) and the highest protein content is observed in the skin (59.6%). In terms of ash content, the values were between 22-28% in heads, skins and spines, but viscera contained lowest amount of ash (1.7%).

3 Conclusions

Performed tests and analyses indicated that different RRM obtained from surimi processing factories has a potential to be used as valuable ingredients in different formulations (both food and feed marked). Some variation in chemical composition as well as microbial quality gives indication that proper storage and handling methods and optimal processing technologies needs to be applied in order to produce high quality final products. Conclusively, it can be stated that all analysed RRM and WW obtained from surimi processing are rich in proteins (40-80 % of dry material) and are potential source for production of protein rich products and ingredients.

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