23. Profiling of macro and micronutrients in seafood

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Seafood is one of the highly traded food which provide essential food and hold a major share in the economy of many countries. It has been regarded as an excellent source of various nutritional compounds like proteins, healthy fats and rich source for a great number of nutritive and important components. The high amount of long-chain polyunsaturated fatty acids of the n-3 series such as Eicosapentanoic (EPA 20:5n-3) and Docosahexanoic acid (DHA 22:6n-3); the well-balanced content of essential amino acids; the high amount of taurine; the presence of antioxidants such as tocopherols; the exceptional concentrations of essential elements such as selenium and iodine; and the good digestibility of fish protein due to low amounts of connective tissue are some examples of the many benefits seafood offers, when consumed. These compounds are having preventive effects over many heart diseases and autoimmune disorders.

Proximate composition of Sea foods

The proximate composition comprises the percentage of the four basic constituents' viz. water, protein, fat and ash. The chemical composition of fish varies widely between species and among the individual fishes within the same species depending on age, sex, environment and season**.** Protein and ash content do not register much variation whereas lipid content shows remarkable variation and displays an inverse relationship with water content.

1. Moisture

It is estimated that over 35% of our total water intake comes from the moisture in the foods we consume. The difference in weight after heating the finely ground fish at a particular temperature for a defined duration gives the water content present in the sample. It is represented as g per 100g meat. Place a clean dry petri dish, kept in an oven at 105° C for 2 hours, cooled in a desiccator and weighed. About 10-20g portion of meat was taken in the pre-weighed petri dish, kept in an oven maintained at 105° C overnight. The petri dish was cooled in a desiccator and weighed again. The petri dish was again kept in an oven for half an hour, cooled as above and weighed again to get reproducible weights

$$\text{Moisture (\%)} = \frac{\text{Weight of moisture in thesample}}{\text{Weight of the wet sample}} * 100$$

Crude Protein

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Fish provides about 14% of the world's need for animal proteins and 4%-5% of the total protein requirement. Both the amino acid composition and the digestibility of fish proteins are excellent. Fish is regarded as an excellent source of high-quality protein, particularly the essential amino acids lysine and methionine. Protein analysis is highly important for the food industry, including the fish industry. Both the content and the properties of the proteins are important for the value and the quality of the products. Protein deficiency leads to various clinical and sub-clinical syndromes, such as impaired health, lowered resistance to infection and susceptibility to diseases.

The total content of proteins is usually determined by the Kjeldahl method. It is also possible to determine the nitrogen content using elemental analysis. The method includes sample digestion, neutralization, distillation, and trapping of ammonia and titration steps. The nitrogenous compounds in the sample are converted in ammonium sulfate by boiling with concentrated sulfuric acid. Upon distillation with excess alkali, the ammonia is liberated which is estimated by titration with standardized sulfuric acid. The advantage of this method is that it gives accurate results for all types of samples. Crude protein content was determination involves the following steps.

1. *Digestion*: 0.1-0.2g of wet sample was weighed in to a Kjeldahl flask. A pinch of digestion mixture (copper sulphate and potassium sulphate were mixed in the ratio 1:8 and finely powdered) and 10 ml of concentrated sulfuric acid was added. It was then digested over a sand bath by heating slowly till the solution starts boiling and then vigorously until the solution becomes colorless. The sample was then cooled and made up to the desired volume (100ml) according to the protein content of the sample. A blank was kept with distilled water.
2. *Distillation*: A conical flask containing 10 ml of boric acid with few drops of Tashiro's indicator (pink in color) was placed at the receiving end of the distillation apparatus in such a way that the tip of the condenser is slightly immersed in boric acid.5ml or any convenient volume of the made up sample was pipette out in to the distillation apparatus.10ml or known volume of 40% NaOH as shown excess by phenolphthalein indicator was added in to the distillation unit followed by rinsing with little distilled water. The unit was made air tight. The content was steam distilled till the boric acid solution in the flask doubles or for 5minutes. The color of the solution turns green. The flask was lowered and the condenser tip was washed with little water.
3. *Titration:* The solution in the receiving flask is green at this stage. The content was titrated against N/100 sulfuric acid until the original pink colour is restored. The volume

of acid used for titration was noted. The distillation and titration process was repeated to get concordant value.

1ml 0.01N/100 Sulfuric acid = 0.14mg Nitrogen

$$\text{Protein Content} = \frac{X * 0.14 * V * 6.25 * 100}{V1 * W * 1000}$$

X=Titre value of the sample

V=Total volume of digest

V1=Volume of digest taken for distillation

W=Weight of sample taken

Crude Fat

Fat soluble in organic solvents can be extracted from moisture free samples. The solvents commonly used includes petroleum ether, ethyl ether etc., the solvent is evaporated and fat is estimated gravimetrically. 5-10g of dried sample was weighed accurately in to a thimble and cotton plugged. The thimble was then placed in a Soxhlet apparatus and 1and half volume of ether was added and distilled for 16 hrs.

Figure 1: Soxhlet Apparatus

The apparatus was cooled and the solvent was filtered in to a pre-weighed conical flask. The flask of the apparatus was rinsed with small quantities of ether and the washings were added to the above flask. The ether was removed by evaporation and the flask with fat was dried at 80-100 °C, cooled in a desiccator and weighed

$$\text{Fat content}\left(\frac{g}{100g}\right) = \frac{\text{Weight of fat}}{\text{Weight of sample}} * 100$$

Ash

Ash is the residue obtained after incineration of the dry material at high temperature and appears as grey-white coloured powder. Silica crucible was heated to 600 °C in a muffle furnace for one hour, cooled in a desiccator and weighed. 2g of dried sample was weighed accurately in to a crucible and heated at low flame by keeping on a clay triangle to char the organic matter. The charred material was then placed inside the previously set (600 °C) muffle furnace and heated for 6-8 hrs which gave a white or grayish white ash. The crucible was cooled in a desiccator and weighed. The crucible was heated again for further 30mins to confirm

completion of ashing, cooled and weighed again.

$$\text{Ash content}\left(\frac{\text{g}}{100\text{g}}\right) = \frac{\text{Weight of ash}}{\text{weight of sample}} * 100$$

Figure 2: Muffle Furnace

Lipids in Sea foods

Lipids are heterogeneous group of compounds and can be defined as the fraction of any biological material extractable by solvents of low polarity. Any material extracted with 'fat solvents' like ethyl alcohol, ether, chloroform, hexane, petroleum ether etc. is classified as a lipid. The important type of compounds included in this group are fatty acids, glycerides, phosphoglycerides, sphingolipids, aliphatic alcohols and waxes, steroids and combination of the above type of compounds with proteins, peptides carbohydrates etc. In the case of fish tissues, the major components of lipids are triacylglycerol and phosphoglycerides, both containing long chain fatty acids.

Phospholipids, another important constituent of lipids are essential components of cell membranes. It is the lipid-globular protein mosaic structure that determines important functions like permeability of cell membranes, transport of various substances into and outside the cell. Various types of phospholipids are essential for the proper functioning of the cell. Unlike in the case of depot fat, the proportions of phospholipids do not show wide variation. Normally it is in the range of 0.5 to 1% of tissue.

In fish muscle, lipids are the third major constituent in quantity. Fat varies between species and also within the species between different organs. Fish with fat content as low as 0.5% and as high as 18-20% are common and the major fish lipids are triacylglycerol and

phosphoglycerides containing long chain fatty acids. Squalene and wax esters are the other components seen in high concentration in certain fish meat.

Determination of total lipids are generally based on solvent extraction followed by gravimetric determination. The wet muscle is homogenized with 2:1 mixture of chloroform and methanol. The chloroform-methanol mixture extracts the total lipid from the tissue in to a single phase of solvent and disturbing the equilibrium between chloroform and methanol separates the chloroform soluble fat. Depending on the fat content, extract about 25-50 g meat with about 15 volumes of chloroform-methanol mixture. Filter the extract using a Buckner funnel with Whatman No.1 filter paper applying little vacuum and the extraction, filtration is carried out thrice. Take the combined extract in to a separating funnel. Add 20% of the volume water, mix well and allow to separate overnight. Concentrate lipid to a known volume, say 10 ml, by evaporating the solvent in a vacuum flash evaporator and keep under nitrogen. Take one ml of aliquot in a pre-weighed test tube and allow it to dry. Cool the test tube in a desiccator and weigh.

$$Fat\ content\ \left(\frac{g}{100g}\right) = \frac{W2 * V1 * 100}{V2 * W2}$$

Where V1 =Total volume of extract

V2=Volume of extract taken for drying

W2=Weight of dried lipid

W1=Weight of sample for fat extraction

Analysis of Fatty acid

The analysis of fatty acids in a fish tissue involves mainly three steps: lipid extraction, preparation of fatty acid derivatives, and gas chromatographic (GC) analysis. For decades, GC has been the most applied method for fatty acids analysis. The success of GC with flame ionization detector (FID) for the analysis of fatty acids is based on the ability of this technique to separate dozens of fatty acids depending on the type and the length of the column, and on the economical accessibility of the GC instrumentation that is actually present in most analytical laboratories.

Saponification of fats liberates fatty acids from triglycerides. The fatty acids are derivatised into their corresponding fatty acid methyl esters by reflexing with BF_3 methanol reagent and the fatty acid profile analysed using Gas Liquid Chromatography.

a. Extraction of fatty acids

Weigh 2g oil into a round bottom flask and add 10 ml alcoholic KOH. Reflux for 20 min and cool to room temperature. Extract non-saponifiable matter with 10 ml portions of hexane or petroleum spirit. Acidify the aqueous fraction and re-extract with petroleum either to separate fatty acids. Wash the fatty acid portion repeatedly with water. Pass the fatty acid portion through anhydrous Sodium sulphate and evaporate to dryness.

b. Preparation of methyl esters

Add 5 ml of BF_3- CH_3OH reagent to the extracted free fatty acids. Reflux for another 2 min. Add to the mixture sufficient saturated sodium chloride (10 ml) to separate the fatty acid methyl esters. Extract the contents of the flask into ether layer. Dry ethyl layer over anhydrous sodium sulphate and evaporate the petroleum ether fraction to 2 ml.

Inject into Gas Chromatogram for analysis and the operating conditions are set for separation of fatty acid methyl esters using Gas Chromatography -FID method. The Gas Chromatograph is set at required temperature with optimum flow of carrier gas. Programme of GC Injector 260°C; FID-275°C; Capillary column, PE Elite 225 (30 m, 0.25 mm 1.d, .25 um) Carrier gas- Nitrogen at 0.6m/min; Air 30ml/min and Hydrogen 30ml/min for FID Temperature programme-110°. After initial hold of 4 min temperature is programmed to raise at 2.7"C/min to 240°C and maintained at that temperature for 5 min; Split flow 12ml. Samples are identified by retention time by comparing with respective standards using software. Area of each component is obtained from the computer-generated data and concentration calculated using the software by external standard method.

Figure 3: Perkin Elmer Clarus 580- Gas chromatograph -FID

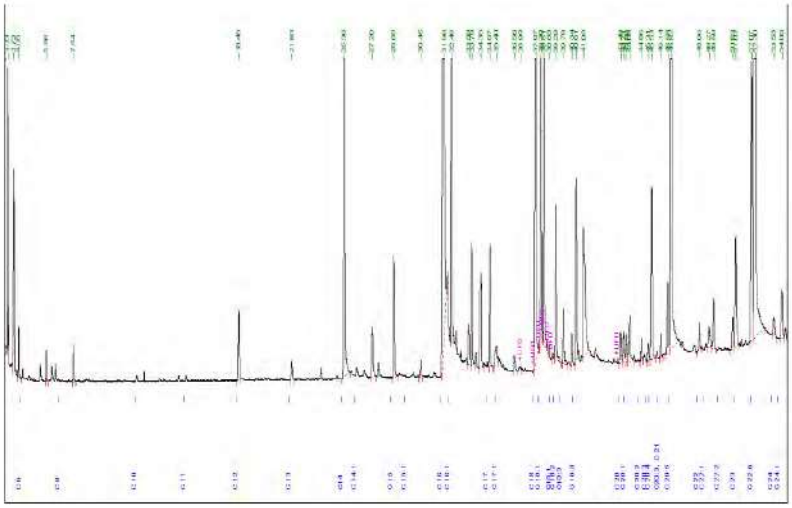

Figure 4: Chromatogram of Fish

The fatty acid composition of fish, shellfish, seafood products, and encapsulated fish oils products have been extensively studied. Clinical and epidemiological studies indicated that the consumption of fish and fish oils renewed interest in investigating the lipid content and the fatty acid composition of fish and seafood products. Marine-based fish and fish oil are the most popular and well-known sources of n-3 polyunsaturated fatty acids (PUFAs), namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These n-3 PUFAs are known to have variety of health benefits against cardiovascular diseases (CVDs) including well-established hypotriglyceridemic and anti-inflammatory effects as well as antihypertensive, anticancer, antioxidant, antidepression, antiaging, and antiarthritis effects.

Amino acid Profiling

Proteins and amino acids are important biomolecules which regulate key metabolic pathways and serve as precursors for synthesis of biologically important substances and amino acids are building blocks of proteins. Fish is an important dietary source of quality animal proteins and amino acids and play important role in human nutrition. Seafood contains all nine essential amino acids. It is an excellent choice for meeting our daily protein needs and the protein in seafood is highly digestible. This advantage makes seafood an excellent food choice for people of all ages.

Dietary protein must be balanced in composition with all essential amino acid in proper proportion. Hence analysis of dietary proteins for amino acids become essential. Most modern technique for amino acid analysis is High performance liquid chromatography (HPLC). Inadequate uptake of quality proteins and calories in diet leads to protein energy malnutrition (PEM) (or protein-calorie malnutrition, PCM) which is the most lethal form of malnutrition/hunger. Kwashiorkor and marasmus, the extreme conditions of PCM mostly

observed in children, are caused by chronic deficiency of protein and energy, respectively. PCM also occurs in adults who are under chronic nutritional deficiency.

Total Amino acid

Protein is hydrolysed to constituent amino acids by 6N Hydrochloric acid. The amino acids are separated in a HPLC equipped with an ion exchange column. Two buffers (pH 3.2 and pH 10) with a pH gradient is used to elute the amino acid from the column and the individual amino acids are estimated by their fluorescence intensity, imparted on to the individual amino acids by reaction with O-phthalaldehyde in the presence of hypochlorite solution, using a fluorescence detector.

Weigh about 100 mg of finely homogenized fish mince in to a test tube and add 10 ml of 6N HCI in to the test tube. Seal the tube after filling nitrogen and digest the contents of the tube by keeping at 120°C for 24 hours in an oven. Cool the test tube and filter the contents using Whatman No 1 filter paper. Rinse the tube with distilled water and filter. Evaporate filtrate in a vacuum flash evaporator. Add deionized water in to the tube and continue evaporation until the contents are acid free. Dissolve the free amino acids in buffer A and inject in to HPLC. The amino acids are separated in a HPLC equipped with an ion exchange column. Two buffers (pH 3.2 and pH 10) with a pH gradient is used to elute the amino acid from the column. Individual amino acids are estimated by their fluorescence intensity, imparted on to the individual amino acids by reaction with O-phthalaldehyde in the presence of hypochlorite solution, using a fluorescence detector. The separation and quantification of amino acids are carried out with HPLC with an ion exchange column. Filter the samples using 0.45µm syringe filter and inject appropriate quantities in to the HPLC system as per the specifications of the injector.

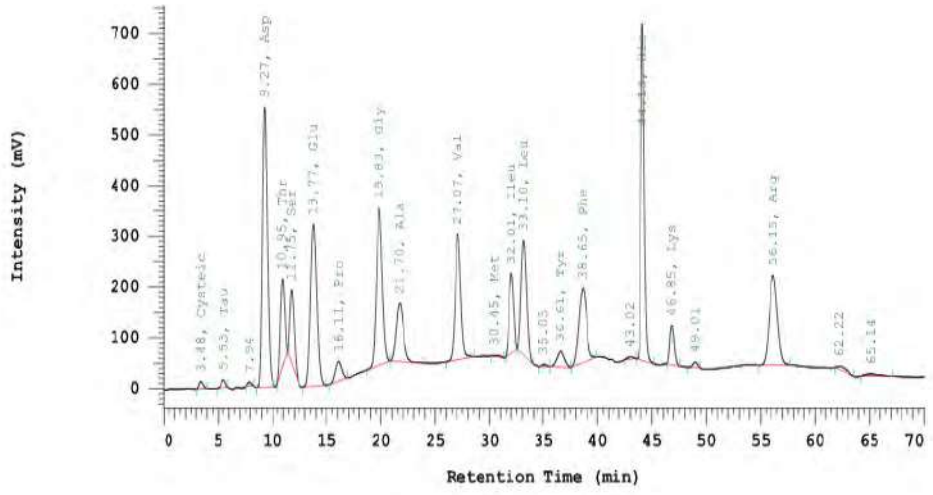

Figure 5: Chromatogram of amino acids

Figure 6: Hitachi Amino acid Analyser

Estimation of Tryptophan

Tryptophan being labile to the conditions of hydrolysis is estimated spectrophotometrically after alkali hydrolysis of the protein. Under acidic conditions of reaction, the 5-hydroxy furfural resulting from sucrose forms pale green colored condensation product with thioglycolic acid, which reacts with tryptophan in the hydrolyzed protein giving a pink coloured complex which is measured is measured at 500 nm.

Weigh about 200 mg of finely homogenized fish mince in to a test tube. Add 10 ml of 5% NaOH in to the test tube. Seal the tubes after filling nitrogen and digest the contents of the tube by keeping at 120°C for 24 hours in an oven. Neutralize the contents after hydrolysis to pH 7.0 using 6N HCI. Total volume is made to 100 ml and filter through Whatman No.1 filter paper. Add 0.1 ml 2.5 % sucrose and 0.1 ml 0.6 % thioglycolic acid successively in to a test tube containing 4 ml of 50% H_2SO_4. Keep the tubes in a water-bath at 45-50°C and cool. Add the aliquots (0.1-0.8ml) sample to the test tube and mix. Make the volume of the test tube to 5 ml with 0.1N HCI and leave aside for five min. Measure the colour intensity at 500 nm. For standards, add tryptophan standard solution in to a series of test tubes instead of sample and perform experiment as above, Calculate the concentration using a standard graph or by regression.

Vitamins

Fish is a rich source of vitamins, particularly vitamins A, D and E from fatty species, as well as thiamine, riboflavin and niacin (Vitamins B1, B2 and B3). Vitamin A from fish is more readily available to the body than from plant foods. Among all the fish species, fatty fish contains more vitamin A than lean species. Vitamin A is also required for normal vision and for bone growth. As sun drying destroys most of the available vitamin A, better processing methods are required to preserve this vitamin. Vitamin D present in fish liver and oils is crucial

for bone growth since it is essential for the absorption and metabolism of calcium. It also plays a role in immune function and may offer protection against cancer. Oily fish is the best food source of unfortified vitamin D. Vitamin D is not found in many foods and tends to be a vitamin that many vulnerable groups go short of, such as teenage girls and the elderly people. Fish is also a good source of the B vitamins and can provide a useful contribution to the diet. The B group of vitamins is responsible for converting food to energy in the cells of the body and they help with the function of nerve tissue.

High Performance Liquid Chromatography (HPLC) is now used regularly for the analysis of fat soluble Vitamins in a wide range of foods. It offers many advantages over traditional methods of analysis in particular with regard to speed, sensitivity and selectivity. An extraction step prior to chromatographic determination is required for clean-up and concentration of vitamins.

Sample Preparation

Grind fish tissue (20g) with anhydrous sodium sulphate and extract oil using 2:1 chloroform: methanol after adding BHA or BHT as antioxidants (Folch's method). To about 2g oil in a RB flask, add 25 ml alcohol, and 1.5 ml of 150% KOH. Reflux in a water bath for 30 min. Transfer the contents in to a 250 ml separating funnel after cooling; wash the flask with 50 ml petroleum ether and add to the separating funnel; shake the content of the separating funnel thoroughly and allow to separate. Extract the aqueous layer twice more and the pool solvent layer. Wash the solvent layer with two 20 ml portions of water to make it alkali free. Concentrate non-saponifiable matter in the ether fraction using a flash evaporator at 30-40°C to a definite volume. NSM is filtered through 0.45µ syringe filter and stored under refrigeration.

Chromatographic analysis

The HPLC consisting of a quaternary gradient pump, programmable variable wave length UV detector is used for the analysis. The column used is C18 RP 5µ 250 x 4 mm Atlantis (Waters Corporation) or related. The mobile phase - water with 1% TFA (A) and acetonitrile with 1% TFA (B) at 1 ml per min. The fat soluble Vitamins elute from the column in the order Vitamin A, Vitamin D, Vitamin E and Vitamin K. The wavelength used for eluting different Vitamins is as follows. 265nm for vitamin D_1, 325nm for Vitamin A, 291nm for Vitamin E and 250nm for Vitamin K. The Vitamin content in the unknown sample is determined from the linear graph drawn for the standard.

Figure 7: Shimadzu HPLC PDA

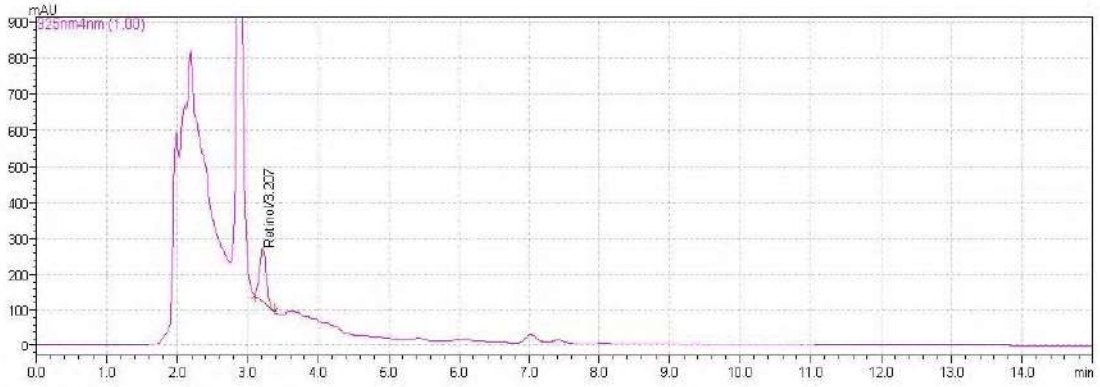

Figure 8: Chromatogram Vitamin A

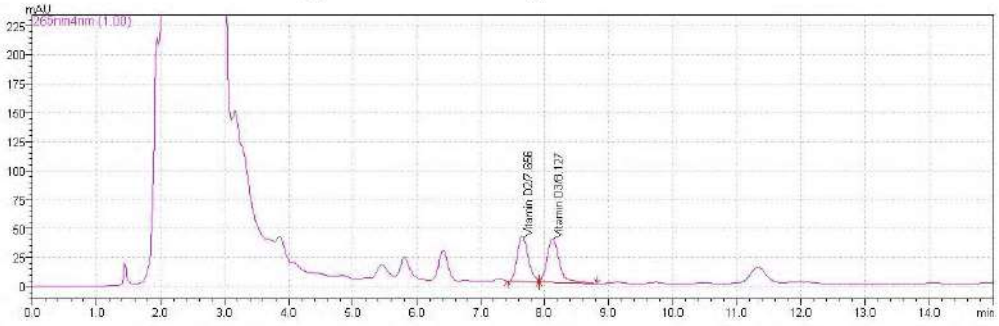

Figure 9: Chromatogram Vitamin D

Minerals

Minerals are inorganic elements necessary in the diet for normal body functions. They can be divided into two groups: macro-minerals and micro-minerals based on the quantity required in the diet and the amount present in fish. The common macro-minerals are calcium, phosphorus, magnesium, sodium, potassium, chloride, and sulfur. These minerals regulate osmotic balance and aid in bone formation and integrity Micro minerals or trace minerals are required in small amounts as components in enzyme and hormone systems. Common trace minerals are copper, chromium, iodine, zinc and selenium.

Fish is a good source of almost all the minerals present in seawater. Calcium and phosphorus account for more than 75% of the minerals in the skeleton. Besides forming a part of skeleton, phosphorous has many metabolic and physiological roles in fish. Elements of special nutritional significance such as iodine and fluorides are also present in fish. Sulphur is present in the form of amino acids as fish is a good source of sulphur containing amino acids, cysteine and methionine. Copper and iron are associated with muscle tissues. Cobalt is present in the form of Cyanocobalamin (Vitamin B_{12}). It should be noted that the sodium content of fish meat is relatively low which makes it suitable for low-sodium diets. Fish can absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet. Fish contains most of the 90 naturally occurring elements. The average ash content in the edible part of the fish may range from 0.5-1.8% and it is an indication of total minerals.

Under mineral profiling the determination of alkali metals, viz; Na, K, and Ca are normally determined by flame photometer method. The metals in any other biological samples can be analysed by atomic absorption spectrophotometry. The technique makes use of absorption of light by the particles or atom to assess the concentration of an analyte in a sample and depends on the Beer-Lambert law. The electrons of the atoms in the atomizer are promoted to higher orbitals for a short period of time by absorbing a set quantity of energy from a light of a given wavelength depending on the metal used which gives the technique its elemental selectivity. The signal generated in the flame is proportional to the concentration of the element being measured.The ash estimated after proximate analysis is dissolved in 100 ml 6N HCl quantitatively. The solution is appropriately diluted and aspirated in to the Photometer for quantification.

Figure 10: Atomic absorption spectrophotometry

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