Chapter 3

MATERIALS AND METHODS
MATERIALS AND METHODS

The present study was conducted in Environmental Fish Biology and Nutrient Profiling Lab of Directorate of Coldwater Fisheries Research (ICAR), Bhimtal for a period of 2009-2011. The experimental fishes *Tor putitora, Neolissocheilus hexagonolepis, Oncorhynchus mykiss* and *Schizothorax richardsonii* were chosen for the present study.

3.1 EXPERIMENTAL FISH

3.1.1 Golden mahseer (*Tor putitora*)

Taxonomically it belongs to-

- Kingdom: Animalia (Linnaeus, 1758)
- Phylum: Chordata (Bateson, 1885)
- Subphylum: Vertebrata
- Superclass: Gnathostomata
- Class: Osteichthyes
- Subclass: Actinopterygii (Cope, 1887)
- Division: Teleostei
- Order: Cypriniformes
- Family: Cyprinidae
- Genus: Tor
- Species: putitora (Hamilton, 1822)

3.1.1.1 Species character

Body color usually greenish above, light pink at sides, with a silvery white abdomen; a broad light grayish blue or purplish lateral band present; a pair of rostral and a pair of maxillary barbells almost equal to the diameter of eye present; length of head considerably greater than depth of body; lips thick with a continuous groove in the lower jaw; dorsal fin originating midway
between tip of mouth and of the tail base; paired fins grayish green, other fins shot with pink (Talwar and Jhingran, 1991).

3.1.2 Chocolate mahseer (*Neolissocheilus hexagonolepis*)

Kingdom: Animalia (Linnaeus, 1758)
Phylum: Chordata (Bateson, 1885)
Subphylum: Vertebrata
Superclass: Gnathostomata
Class: Osteichthyes
Subclass: Actinopterygii (Cope, 1887)
Division: Teleostei
Order: Cypriniformes
Family: Cyprinidae
Genus: *Neolissocheilus* (Rainboth, 1985)
Species: *hexagonolepis* (Mc Clelland, 1839)

3.1.2.1 Species character

In general, body deep anteriorly, head broad, Eyes moderate, lips thick, but not hypertrophied; barbells are of two pairs and are longer than orbit, both the pairs longer than the diameter of eye; Dorsal fin inserted nearer to the tip of the snout than to the base of caudal fin in younger stages, but in adults it is equidistant or often nearer to the base of caudal fin; scales large; Sides of snout and cheeks studded with large tubercles. color is olive-green on back, silvery white below with a golden yellow lateral band in the middle; each scale above the lateral line coppery-colored at the end, deepening to bronze-green at the base; scales pale slate colored fading to pure white on belly; iris bright coppery red. Dorsal fin spine strong and smooth, its length equal to the distance from tip of the mouth to the front margin of the eye, fins mainly slaty gray paling towards their margins (Talwar and Jhingran, 1991).
3.1.3 Rainbow trout (*Oncorhynchus mykiss*)

Kingdom: Animalia (Linnaeus, 1758)
Phylum: Chordata (Bateson, 1885)
Subphylum: Vertebrata
Superclass: Gnathostomata
Class: Osteichthyes
Subclass: Actinopterygii (Cope, 1887)
Division: Teleostei
Order: Salmoniformes
Family: Salmonidae
Genus: *Oncorhynchus*
Species: *mykiss* (Walbaum, 1792)

3.1.3.1 Species character

The color of the rounded body is variable, but the upper part is predominantly olive green, with many rounded dark spots on the body, head and fins; the spots on the tail fin. There is an iridescent band of color, mainly pink, along the lateral line, and the lower part of the body is silver. The mouth is large and set obliquely, with well developed teeth in the jaws, on the palate and in the throat. The rear of the upper jaw is in line with the rear edge of the eye. The skin, covered with very small scales and slime, feels smooth. Body comparatively short and deep, more elongated in males than females; color variable depending on sex, age and character of water; back bluish above, silvery on sides with irregularly located dark spots and sides showing a red band and blotches; belly merely plain. The skin of rainbow trout from back to mid-lateral region is covered with numerous small black star-shaped spots. The adult has an iridescent reflecting rose-colored band on the lateral side which is more prominent in the males during the breeding season (Talwar and Jhingran, 1991).
3.1.4 Snow trout (*Schizothorax richardsonii*)

Kingdom: Animalia (Linnaeus, 1758)  
Phylum: Chordata (Bateson, 1885)  
Subphylum: Vertebrata  
Superclass: Gnathostomata  
Class: Osteichthyes  
Subclass: Actinopterygii (Cope, 1887)  
Division: Teleostei  
Order: Cypriniformes  
Family: Cyprinidae  
Subfamily: Schizothoracinae  
Genus: *Schizothorax*  
Species: *richardsonii* (Gray, 1832)

3.1.4.1 Species character

Snow trout has a conical head with slender, elongated and strong body to resist the strong water current of the hill streams and rivers. The body is covered with minute silvery scales and the abdomen with lighter brown scales. Snout bears nuptial tubercles and the size and number are well developed in males. They are greyish black on the dorsal side and silvery on belly and sides. A distinct suctorial disc in addition to 4 barbells is present on the chin for attachment to stones (Talwar and Jhingran, 1991).

3.2 Sampling Stations and collection

Ten to fifteen samples of each species (*Tor putitora, Neolissocheilus hexagonolepis, Oncorhynchus mykiss, Schizothorax richardsonii*) were collected from Kosi river, Uttarakhand (latitude 29°25´ to 29°39´ N, longitude 78°44´ to 79° 07´ E and 1960 mts. asl); Mahseer hatchery complex, Bhimtal (latitude 29°20´40” N, longitude 79°36´16” E and 1371 mts. asl), Uttarakhand; Kameng river (latitude 27°48´36”, longitude 92°26´38” and 2443 mts. asl) in West
Kameng district, Arunachal Pradesh; Champawat experimental fish farm of DCFR, Champawat (Latitude 29°20’N, Longitude 80°06’E), Uttarakhand; Shergaon trout farm, West Kameng District (latitude 27° to 27° 15’ N and longitude 92° to 92°30’E), Arunachal Pradesh; Alaknanda river, Nandprayag (Latitude 30.33°N, Longitude 79.33°E), Uttarakhand and Tenga river, West Kameng District, (Latitude 27.444N and longitude 92.480E, 6500 feet above sea level), Arunachal Pradesh, India.

Table 3.1: Name of Sampling Stations

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of Fish Species</th>
<th>Sampling Stations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tor putitora</td>
<td>Kameng river, West Kameng district, Arunachal Pradesh; Kosi river, Uttarakhand</td>
</tr>
<tr>
<td>2</td>
<td>Neolissocheilus hexagonolepis</td>
<td>West Kameng district, Arunachal Pradesh; Mahseer hatchery complex, Bhimtal, Uttarakhand</td>
</tr>
<tr>
<td>3</td>
<td>Oncorhynchus mykiss</td>
<td>Shergaon trout farm, West Kameng District, Arunachal Pradesh; Champawat experimental fish farm of DCFR, Champawat, Uttarakhand</td>
</tr>
<tr>
<td>4</td>
<td>Schizothorax richardsonii</td>
<td>Tenga river, West Kameng District, Arunachal Pradesh; Alaknanda river, Nandprayag, Uttarakhand</td>
</tr>
</tbody>
</table>

The samples of golden mahseer (Tor putitora), chocolate mahseer (Neolissocheilus hexagonolepis), rainbow trout (Oncorhynchus mykiss) and snow trout (Schizothorax richardsonii) used in this study were collected based on three different seasons (season 1= February-May, season 2= June-September and season 3= October-January) in a year (Piggot and Tucker, 1990).
3.3 Fish measurements

Immediately after catching, the fishes were stored in an insulated ice box and transported to the Directorate of Coldwater Fisheries Research (DCFR, ICAR) laboratory at Bhimtal, Uttarakhand, India for analyses. After that length and weight measurement were recorded. Golden mahseer were about 14-52 cm in length and 110-700 gm in weight while chocolate mahseer were about the 20-40 cm in length and 150-500 gm in weight. Rainbow trout samples were about 10.5-54 cm in length and 100-1200 gm in weight while snow trout were about 9.5-35 cm in length and 80-300 gm in weight.

3.4 Sample Preparation and Preservation

After morphometric measurements, the fresh fish were washed immediately and the bone and flesh were separated from the fish. The pieces such as head, skin, fins, scales and viscera, which are not edible were removed. Fish flesh was then washed until it was free from blood. Boneless muscles from all the representative individual fish were pooled together and then mashed. Mashed muscle sample was mixed thoroughly in grinding mixer (Bajaj, G x7) and are taken for analysis. For analysis, a minimum of 150gm composite meat should be available. The very small fishes were homogenized as such after cleaning and stored in a plastic bag and then kept in a refrigerator at -20°C (Celfrost). The dried samples were ground with mortar and pestle into fine powder and stored in labeled polythene bags until required for analysis. All analyses were performed in triplicates.

3.5 Fish composition

3.5.1 Proximate composition analysis

Proximate composition of fish is generally the percentage composition of the four basic constituent viz. water, protein, fat, and ash. The processor, the
nutritionist, the cook and the consumer all have a direct interest in the composition of fish. The processor needs to know the nature of the raw material before he can apply correctly the techniques of chilling, freezing, smoking or canning. The nutritionist wants to know what contribution fish can make to the diet and to health and the cook must know for example whether a fish is normally lean or fatty in order to prepare it for the table. The consumer is interested not only in whether a particular fish tastes good, which is a matter of opinion, but also in whether it is nutritious. While the consumer is interested mainly in the edible part of the fish, that is the flesh or muscle, the fishmeal manufacturer is concerned with the composition of the whole fish, and the processor of fish oils wants to know what is in the liver. Measurement of constituents of fish products is sometimes necessary to meet specifications or to comply with regulations. For example, the fish content of fish cakes or the oil content of fishmeal may need to be known in order to meet certain commercial or legal requirements. Fish is one of the most valuable sources of high grade protein available to man in this hungry world, and knowledge of its composition is essential if the fullest use is to be made of it.

3.5.1.1 Moisture

Fish contains more than 65% moisture. It is very important to estimate the quantity of water in fish since it is directly related to spoilage and quality of fish may affect the fish product during processing. The moisture and low volatile materials are removed by heating at 95-100°C under partial vacuum. It is applicable to fish and fish products, including frozen scallops, block frozen, and fish by-products. The water in fresh fish muscle is tightly bound to the proteins in the structure in such a way that it cannot readily be expelled even under high pressure. After prolonged chilled or frozen storage, however, the proteins are less able to retain all the water, and some of it, containing dissolved substances, is lost as drip. The moisture content was determined
according to the method of Association of Official Analytical Chemistry (AOAC, 1995).

3.5.1.1.1 Apparatus:

1. Hot air oven (U-TECH)
2. Weighing Balance (0.00000 gm, DENVER)

3.5.1.1.2 Procedure:

Step 1: Petridish was cleaned and kept in hot air oven for drying.
Step 2: Weight of Petridish (W1) was taken and noted.
Step 3: Weight of sample in pre weighed Petridish and note down the weight (W2).
Step 4: Transfer the Petridish in a hot air oven at 100 °C temperature until the samples are free from moisture.
Step 5: Cooled down the hot petridish at room temperature using a desiccator.
Step 6: Weight the Petridish and record the reading (W3).

Calculation:

\[
\text{Moisture content (gm/100gm)} = \frac{W2 - W3}{W2 - W1} \times 100
\]

Where,

- W1 - weight of petridish
- W2 - weight of Petri dish with fish meat
- W3 - weight of petridish with fish meat after drying

3.5.1.2 Crude Protein

Nitrogen is a necessary component of all living things. Amino acids are the building blocks of proteins. When Amino acid was discovered it was found that each amino acid contained Nitrogen. Nitrogen determination has a long
history in the area of analytical Chemistry. Johan Kjeldahl first introduced Kjeldahl Nitrogen method in 1883 at meeting of the Danish Chemical Society. As a Chairman of Chemistry department of the Carlsberg laboratorium near Copenhagen, Kjeldahl was assigned, to scientifically observe the processes involved in beer making. While studying proteins during production he developed a method of determining Nitrogen content that was faster and more accurate than any method available at that time. An Italian Scientist by name Dumas developed a method in 1830, which dealt with the Pyrolytic distraction of sample with the Nitrogen being reduced to Nitrogen gas. From the volume of Nitrogen gas produced the Nitrogen content of the sample is calculated. But Dumas method was too slow and gave incomplete results too. There are number of secondary methods for determination of protein or nitrogen. But all have to be calibrated against a standard method and with no other option the standard method is Kjeldahl. The Kjeldahl method is a means of determining the Nitrogen content of organic and inorganic substances. Although the technique and apparatus have been altered considerably over the past 125 years, the basic principles introduced by John Kjeldahl endure even today. From 1889 onwards several Chemists have made improvements in the method. These include the use of metallic Catalyst which produced a faster and greater recovery of Nitrogen, addition of potassium Sulphate which raised the digestion temperature and shortened the digestion time, etc. Later another Scientist – a Scandinavian agricultural Chemist by name Rozen Mosseberg’s invented the block digester for more economical, accurate, safe, efficient and speedy way of Analysis, based on Kjeldahl Chemistry. The AOAC (1995) method was used.

**Three main steps of Kjeldahl method**

**Digestion:** The decomposition of Nitrogen in organic samples utilizing a concentrated Acid Solution. This is accomplished by boiling a homogenous
sample in concentrated sulphuric acid and digestion catalyst. The end result is an ammonium Sulphate solution.

**Distillation**: Distillation involves adding base to the acid digestion mixture to convert NH4+ to NH3. This is followed by boiling. Finally NH3 gas is condensed and trapped in a receiving solution (H3BO3)

**Titration**: Quantifying the amount of Ammonia ions in the receiving solution the percentage of Nitrogen can be calculated.

The protein content in the food is calculated by estimating the nitrogen content and multiplying with a factor (6.25) calculated based on the nitrogen content of the food. Nitrogen content of most fish meat protein is 16%. Hence 1 g nitrogen equivalent of protein is 100/16 or 6.25.

**3.5.1.2.1 Apparatus:**

1. KEL PLUS automatic micro six sample digestion system (model kes 06, regular version). The Hazardous acid fumes liberated during digestion are efficiently dissolved in water through giant aspiration filter pump and sent to the drain without polluting the atmosphere.
2. Kjeldahl digestion flask
3. Kjeldahl distillation apparatus
4. Burette for titration
5. Hot air oven (U-TECH)
6. Weighing Balance (0.00000 gm, DENVER)
7. Deionised distilled water (Millipore)
3.5.1.2.2 Reagents:

1. Con. Sulfuric acid
2. Digestion mixture: Combination of copper sulphate (CuSO₄·5H₂O) and potassium sulphate (K₂SO₄) in the ratio 1:8 in finely powdered form
3. 40% Sodium hydroxide: 40 g sodium hydroxide crystals dissolved in 100 ml distilled water
4. Boric acid 2% solution: 20 g boric acid dissolved in 500 ml hot distilled water, cooled and made up to 1 liter
5. N/I00 Sulfuric acid: Standardized
6. Tashiro's indicator:
   - Stock solution: (a) 0.2% alcoholic methyl red; (b) 0.1% alcoholic methylene blue mixed in 2:1 ratio

3.5.1.2.3 Procedure:

Procedure for Digestion:

Step 1: Preheat the digestion system to 300 °C
Step 2: Wet sample (0.2 g approx) was weighed and putted in the in Kjeldahl digestion flask
Step 3: One pinch of digestion mixture was added into it
Step 4: 10 ml concentrated sulfuric acid was added
Step 5: Placed the tubes in the digestion chamber along with manifolds.
Step 6: Manifolds are fitted properly and switched on the scrubber system immediately
Step 7: Increased the temperature at 420 °C, Sample color was dark black
Step 8: After some time color was turned to bluish green or white
Step 9: Removed the digestion tubes and placed them in the cooling stand for cool down
Step 10: Added distilled water and made up total volume 100 ml, ready for distillation.

Procedure for Distillation:

Step 1: 10 ml of boric acid was taken into a conical flask and added few drops of Tashiro's indicator, turned in whitish to pink color.

Step 2: It was kept in such a way that the tip of the condenser was slightly immersed in boric acid.

Step 3: Approx 5 ml of the sample was pipette out in to the distillation flask.

Step 4: 10 ml of 40% NaOH was added into distillation flask.

Step 5: The distillation unit was made airtight. The content was steam distilled till the boric acid solution got doubled in the flask or for 5 minutes. The color of the solution turned pink to green.

Step 6: Finally it was ready for titration.

Procedure for Titration:

Step 1: The content was titrated against N/100 Sulfuric acid until the original pink color was restored.

Step 2: The volume of acid used for titration was noted.

Step 3: Distillation and titration was repeated to get concordant value.

Calculation:

1000 ml 1 N H₂SO₄ N/100 Sulfuric acid = 14 g N₂

1 ml 1 N H₂SO₄ = 0.014 N₂

1 ml 0.01 N (N/100) H₂SO₄ = 0.00014 g N₂

If the titer value of the sample after subtracting blank is ‘X’, then,

Protein content (gm/100gm) = \( \frac{X \times 0.14 \times V \times 6.25 \times 100}{1000 \times V1 \times W} \)
Where,

\[ V \] - Total volume of digest
\[ V_I \] - Volume of the digest for distillation
\[ W \] - Weight of sample for digestion

### 3.5.1.3 Crude fat

Crude fat has been analyzed by Soxhlet apparatus. A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapor cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is
removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble and is usually discarded.

Fat soluble in organic solvents can be extracted from moisture free samples by using solvents like petroleum ether, ethyl ether etc. The solvent is evaporated and fat is estimated gravimetrically. The fat soluble in the hot petroleum ether, except phospholipids, is extracted from the sample and quantified. Oils and Fats are the triglycerides of fatty acids in which one molecule of glycerol has combined with three molecules of long chain fatty acids with the elimination of water. In addition to triglycerides, the oils and fats may contain a total of 2% free fatty acids, and sterols, phospholipids coloring material and hydrocarbons. Oil is a liquid at the ordinary temperature whereas a fat is usually a solid. Extraction is a physical separation method used when the substance to be extracted is soluble in a solvent. The condensed solvent is continually contacting the sample, the solutes affinity for the solvent will continue until the extraction is complete. The solvent flask contains solvent as soluble. In gravimetric analysis the solvent is driven off. The solute must be removed and weighed for analytical measurement. The AOAC (1995) method was used.

3.5.1.3.1 Apparatus:
Soxhlet apparatus, Vacum rotary evaporator (U-TECH), Cellulose thimbles (19mm X 90mm, Qualigens), Hot air oven (U-TECH), Weighing Balance (0.00000 gm, DENVER)

3.5.1.3.2 Reagent: Petroleum Ether (60-80°C) is required to extract the fat content from fish muscles.

3.5.1.3.3 Procedure:
Step 1: 3-5 g (W1) of moisture free dried sample was weighed and kept in a thimble.
Step 2: Top of the thimble was covered with cotton plug.

Step 3: The thimble was placed in a thimble holder.

Step 4: Now weighed the empty flat round bottom flask and let the weight be W2. This is initial flask weight.

Step 5: 250 ml of petroleum ether was added in to the pre weighed flat round bottom flask.

Step 6: Open the tap water to the condenser

Step 7: The complete unit was made airtight and it was distilled for 16 hours at 60-80°C.

Step 8: Cooled the apparatus

Step 9: After distillation, apparatus was rinsed with small quantities of ether and added in the flask.

Step 10: All the flasks were taken out from the system and removed all the thimbles from thimble holder.

Step 11: Ether was removed through vacuum rotary evaporator by evaporation.

Step 12: Putted the flask with fat was dried at 80 – 100°C in a hot air oven.

Step 13: After 20-30 minutes, taken out all the flasks and placed them in a desiccator about 10-15 minutes for cooling up to the room temperature.

Step 14: After cooling weighted all the flasks, this the final weight of the flask (W3).

Calculation:

Crude Fat content on Dry wet basis (gm/100gm) = \frac{W3 - W2}{W1} \times 100

Where,

- W1 - weight of dry sample taken for extraction
- W2 - weight of empty round bottom flask
- W3 - weight of round bottom flask with fat

Crude Fat content on Wet basis (gm/100gm) = \frac{Value \times Dry \ matter}{100}
3.5.1.4 Ash

In analytical chemistry, ashing is the process of mineralization for pre-concentration of trace substances prior to chemical analysis. Solid residue of combustion is termed as ash. When organic burns, it becomes carbon and when the carbon burnt it becomes ash. The white or grayish powder remaining after something has been thoroughly burned. The chemical composition of an ash depends on that of the substance burned. Ash is the name given to all non-aqueous residue that remains after a sample is burned, which consists mostly of metal oxides. Ash content may be listed in nutrition labels, such as for pet food.

Ash is one of the components in the proximate analysis of biological materials, consisting mainly of salty, inorganic constituents. It includes metal salts which are important for processes requiring ions such as Na\(^+\) (Sodium), K\(^+\) (Potassium), Ca\(^{2+}\) (Calcium). The ash fraction contains all the mineral elements jumbled together. It would be more useful to know the amounts of different individual elements. This method consists of oxidizing all organic matter in a weighed sample of the material by incineration and determining the weight of the ash remaining. Note that the high temperature may cause the volatilization of certain elements (particularly K, Na, Cl, and P) and may also cause the mineral matter to melt and fuse. Ash content of fish was determined according to method described by AOAC (1995).

3.5.1.4.1 Apparatus:

- Heater
- Muffle furnace (MAC)
- Hot Air Oven (U-TECH)
- Weighing Balance (0.00000 gm, DENVER)

3.5.1.4.2 Procedure:

Step 1: Moisture free crucible was weighed (W1)
**Step 2:** Approx 1g of moisture free sample was weighed in a crucible (W2)

**Step 3:** The crucible with sample was kept in a heater and it was heated till the fumes stop.

**Step 4:** The charred material was kept inside the muffle furnace at 600°C temperature for 6 hours till a white or grayish white ash was obtained.

**Step 5:** Cooled the instrument. Now take out the crucible from instrument.

**Step 6:** It was placed in desiccator for 30-45 min and taken final weighed (W3)

**Calculation:**

\[
\text{Ash content on DM basis (gm/100gm) = } \frac{W3 - W1}{W2} \times 100
\]

Where,
- \(W_1\) - weight of crucible
- \(W_2\) - weight of sample
- \(W_3\) - weight of sample with crucible after ashing

\[
\text{Ash content on Wet basis (gm/100gm) = } \frac{\text{Value} \times \text{Dry matter}}{100}
\]

### 3.5.1.5 Crude fiber

The residue left behind after treating the sample with acid and alkali digestion is termed as crude fibre. Crude fibre is a measure of the cellulose and lignin content mainly. This method was originally proposed at the Weende experiment station. Hence this method on determination of crude fibre is known as Weende’s method. When the residue is ignited the organic matter gets oxidized leaving the inorganic residue or ash. Thus the difference in weight of the residue before and after ashing gives the weight of crude fibre. Crude fibre was analyzed following the procedure of AOAC (1995).
3.5.1.5.1 Apparatus:
1. FIBRA PLUS- Two Places Automatic Fiber Estimation System (MODEL FES 2)
2. Fibrastat
3. Hot Air Oven (U-TECH)
4. Weighing Balance (0.00000 gm, DENVER)
5. Muffle furnace (MAC India)

3.5.1.5.2 Reagents:
1. Sulfuric acid 1.25% - To prepare 1.25% (W/V) H$_2$SO$_4$ solution, 12.5 g of H$_2$SO$_4$ (100%) is to be added to distilled water to make the volume 1000 ml.

   Volume of H$_2$SO$_4$ to be taken = \( \frac{12.5 \times 100}{178.5} = 7 \text{ml} \)

   7 ml concentrated H$_2$SO$_4$ (specific gravity 1.84 and 97% concentration) in a 1000 ml volumetric flask half-filled with distilled water. Shake well and add distilled water to make volume upto the mark.

2. Sodium hydroxide 1.25 % - 13.16 g of NaOH (95% NaOH) in one liter distilled water and shake well.
3. Distilled Water

3.5.1.5.3 Procedure:

Step 1: Prepared dilute acid and alkali for the extraction.
Step 2: Boiled the acid and alkali.
Step 3: Weigh the samples accurately and noted down the weights (W)
Step 4: Transfer the weighed samples into oven dried crucibles
Step 5: Place the crucibles into the metal adapters of Fibra Plus hot extraction unit by lowering the handle.

Step 6: Ensured proper sealing of crucibles with the adapter rubber.

Step 7: Opened the tap water to the condenser.

Step 8: kept the individual valves in close position.

Step 9: Kept the vaccum/pressure valve in vaccum position.

Acid wash:

Step 1: Poured 150ml of hot 1.25 % H2SO4 into the extractors from the top for each sample.

Step 2: Checked any vacant place without crucible.

Step 3: Switched on the instrument and set the initial temperature to 500°C

Step 4: After boiling starts, reduced the temperature to 400°C

Step 5: Allowed the samples to boil for 45 minutes in acid.

Step 6: After 45 minutes boiling, drain the acid and wash the samples twice or thrice with distilled water.

Step 7: During draining, ensured that the knob is in vaccum mode.

Step 8: If the draining is not effective due to clogging of sample in the crucible, then, kept the knob in pressure mode, pressed the pressure button twice or thrice and immediately turned the knob to vaccum mode.

Alkali Wash:

Step 1: Poured 150ml of hot 1.25% NaOH into the extractors from the top for each sample.

Step 2: Checked any vacant place without crucible.

Step 3: Switched on the instrument and set the initial temperature to 500°C

Step 4: After boiling starts, reduced the temperature to 400°C

Step 5: Allowed the samples to boil for 45 minutes in alkali.

Step 6: After 45 minutes boiling, drained the alkali wash the samples twice or thrice with distilled water.
Step 7: During draining, the knob was in vacuum mode.

Step 8: If the draining is not effective due to clogging of sample in the crucible, then, kept the knob in pressure mode, pressed the pressure button twice or thrice and immediately turned the knob to vacuum mode.

Step 9: After alkali wash removed crucibles and dried them in hot air oven @ 100°C until the crucibles are free from moisture.

Step 10: Cooled down the hot crucibles to room temperature using a desiccator.

Step 11: Weighed the crucibles and recorded the readings (CWBA=W1).

Step 12: Placed all the crucibles in the muffle furnace at 400°C for ashing.

Step 13: Cooled down the hot crucibles after ashing to room temperature using a desiccator.

Step 14: Now weighed the crucibles and recorded the readings (CWAA=W2)

Calculation:

Crude fiber (gm/100gm) = \(\frac{(W_1 - W_2)}{W} \times 100\)

Where,

\(W_1\) = weight of crucible with sample before ash
\(W_2\) = weight of crucible with sample after ash
\(W\) = Sample weight

3.5.1.6 Carbohydrate

The carbohydrate content in the case of fleshly samples is determined by Merril and Watt method (1973). The total of the components is subtracted from 100 to get the carbohydrate content by using the following equation:

Calculation:

Carbohydrate (gm/100gm): \((100 - (\text{Protein} + \text{crude fat} + \text{Ash} + \text{Moisture}))\)
3.5.2 Fat extraction

The wet sample is homogenized with 2:1 mixture of chloroform and methanol. The chloroform-methanol mixtures extract the total lipid from the tissue into a single phase of solvent. Disturbing the equilibrium between chloroform and methanol separates the chloroform soluble fat. The process of total fat estimation was carried out based on the methodology of Folch method (1957).

3.5.2.1 Apparatus:

1. Homogenous blender (Bajaj)
2. Buchner funnel 250ml (Borosil)
3. Separating funnel 500 ml (Borosil)
4. Vaccum rotary evaporator (U Tech)

3.5.2.2 Reagents:

Following reagents are used for the analysis of fat extraction:
- Chloroform : Methanol Mixture = 2:1
- Sodium Sulphate Anhydrous

3.5.2.3 Procedure:

Step 1: Weighed 30 gm of minced fish meat and kept in a 250 ml conical flask.
Step 2: Added 150 ml of pre cooled chloroform and methanol mixture in 2:1 ratio.
Step 3: Covered the mouth with aluminium foil and kept in refrigerator for overnight.
Step 4: Removed the chloroform and methanol mixture in a volumetric flask.
Step 5: Macerated the sample in homogenous blender for 5 minutes.
Step 6: Filtered the extract through filter paper (Whatman No. 1) using a Buckner Funnel with suction.
Step 7: Collected the meat and again added 300 ml of pre cooled chloroform and methanol mixture.

Step 8: Repeated the process upto filtration.

Step 9: Measured the volume of extract.

Step 10: Added 20% of distilled water of total volume of extract.

Step 11: Mixed both the liquid properly and the filtrate was transferred to a separating funnel. It was kept for overnight.

Step 12: The lower clear phase was drained into a 250 ml round-bottom flask and passed through sodium sulphate anhydrous.

Step 13: Concentrated with a rotary evaporator at 40°C.

Step 14: Collected the sample in a test tube and added chloroform and methanol mixture and make the volume 10 ml.

Step 15: Put 1 ml of aliquot in a pre weighed glass vial.

Step 16: Dried in hot air oven at 750°C.

Step 17: Cooled the vial in a desiccator and weighed.

Step 18: Recorded the weight of dried lipid.

Calculation:

Fat content (gm/100gm meat) = \( \frac{W2 \times V1 \times 100}{V2 \times W1} \)

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

3.5.3 Minerals Analysis

Minerals like sodium, potassium and calcium are estimated using Flame photometer after dissolving the ash in dilute hydrochloric acid (Shearer, 1984). Spectrophotometry is a common method used in analytical chemistry to
measure the concentration of an ion in a solution. It is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength (http://en.wikipedia.org/wiki/spectrophotometry).

There are mainly three methods to calculate the concentrations of an ion in the solutions:

1. Atomic Emission Spectrophotometry (Flame Photometer)
2. Atomic Absorption Spectrophotometry
3. Inductively Coupled Plasma Atomic Emission Spectrophotometry

Flame photometer is the origin of analytical chemistry in determining the ion concentration of aqueous solutions. It is a branch of atomic spectroscopy in which the species examined in the spectrometer are in the form of atoms. First known use of flame photometer was in the year 1945. Flame photometer is a device used in inorganic chemical analysis to determine the concentration of certain metal ions, among them sodium, potassium, calcium and lithium, an instrument that uses flame emission spectrophotometry to measure the intensity and other properties of light. In principle, it is a controlled flame test with the intensity of the flame color quantified by photoelectric circuitry. The sample is introduced to the flame at a constant rate. Filters select which colors the photometer detects and exclude the influence of other ions. Before use, the device requires calibration with a series of standard solutions of the ion to be tested. The liquid sample is introduced, at a constant rate, to the flame. Absorption techniques measure the absorbance of light due to the electrons going to a higher energy level. Emission techniques measure the intensity of light that is emitted as electrons return to the lower energy levels. Flame photometry is suitable for qualitative and quantitative determination of several cations, especially for metals that are easily excited to higher energy levels at a relatively low flame temperature. This technique uses a flame that evaporates the solvent and also sublimates and atomizes the metal and then excites a valence electron to an upper energy state. Light is emitted at
characteristic wavelengths for each metal as the electron returns to the ground state that makes qualitative determination possible. Flame photometers use optical filters to monitor for the selected emission wavelength produced by the analyte species.

It consists of 4 fundamental parts. They are

- The Atomizer
- The Burner
- The Optical System
- Photosensitive Detectors

**The Atomizer**

Atomizer is the part where the ions of atoms meet with the flame. Firstly, in the flame, the solvent of the solution is evaporated. The metal ions get into a gaseous phase. As the atoms gain energy because of the heat and the reducing fuel, the atoms become reduced as well. “These molecules are then dissociated by the heat of the flame into free atoms; some of these atoms recombine reversibly with other components of the flame, such as OH, and still others lose an electron and become ionized.” The electron on the outer shell moves to a higher energy shell. Still, there is too much energy the produced energy from the fuel will be taken up by the atoms. As modern theory of atom implies, when atoms get energy, they release it in the form of photon. An example chemical reaction for atomization and reduction is given below:

\[
K^+(aq) \rightarrow K^+(g) \\
K^+(g) + e^- \rightarrow K(g)
\]

To summarise, the atomizer is the part in which the atoms meet with the flame and start to emit light.

**The Burner**

The flame that atomizer needs, is obtained by the burner. Gases like butane, propane reacted with air are the most suitable gases that is needed to
heat the atomizer. They give an approximate heat of 2000K. The burner has an air entrance, which enables the reaction of fuel with air or oxygen. Specific gases give good flame and heat whereas some gases give lesser heat. If a gas that gives cooler flame is used then the atoms couldn’t get excited. So, ion concentration of aqueous solutions cannot be determined effectively. A gas that gives a hotter flame can also be a problem as the energy can break some parts of the photometer and it will be much harder to cool it down. Another problem might be that atoms can get excited too much that they form 2+ ions instead of 1+ ions, releasing more radiation. A good flame giving gas must be used in a flame photometer to determine the ion concentration of aqueous solution.

Table 3.2: A list for the specific gases and temperature in flame photometer

<table>
<thead>
<tr>
<th>Fuel-oxidant mixture</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coal gas-air</td>
<td>1840</td>
</tr>
<tr>
<td>Propane-air</td>
<td>1925</td>
</tr>
<tr>
<td>Butane-air</td>
<td>1930</td>
</tr>
<tr>
<td>Acetylene-air</td>
<td>2050</td>
</tr>
<tr>
<td>Hydrogen-air</td>
<td>2115</td>
</tr>
<tr>
<td>Hydrogen-oxygen</td>
<td>2690</td>
</tr>
<tr>
<td>Acetylene-oxygen</td>
<td>3110</td>
</tr>
<tr>
<td>Hydrogen-perchloryl fluoride</td>
<td>3300</td>
</tr>
<tr>
<td>Hydrogen-fluorine</td>
<td>4000</td>
</tr>
<tr>
<td>Cyanogen-oxygen</td>
<td>4850</td>
</tr>
</tbody>
</table>
The Optical System

An optical system is required to focus the light emitted from the atoms to the photo detector of the photometer. The optical system consists of convex mirror, lens and filter. The convex mirror is in the part where the atomizer is. It surrounds the atomizer, so that the light emitted from the atoms are focused to the lens. If there is not a mirror then concentration cannot be determined because most of the light would be dispersed in the system and a little amount of light would reach the photo detectors, but that amount would be too small to measure it. Lens is the second part of this system. The convex lens’ job is to focus the light on a point which is called slit. It collects the light emitted directly from the atoms and the reflections from the mirror. After the light passes from the slit, it goes to the filter. Filter as the name implies, filters the light at a specific wavelength that is required. It doesn’t let the wavelengths different from the filter’s wavelength pass to the photo detectors. For accurate results, there should be a perfect optical system.

![Distribution of light rays through the optical system in flame photometer](image)

**Figure 3.1**: Distribution of light rays through the optical system in flame photometer

Photodetector

Photodetector is the final destination of the emitted light. These photodetectors have photomultipliers. When the light comes, they produce an electrical signal. The photomultipliers produce electrical signals directly
proportional to the intensity of light. From this light intensity, the ion concentration of aqueous solutions can be determined.

The intensity of the light emitted could be described by the Scheibe-Lomakin equation:

$$I = k \times c^n$$

Where:
- $c$ = the concentration of the element
- $k$ = constant of proportionality
- $n \approx 1$ (at the linear part of the calibration curve), therefore the intensity of emitted light is directly proportional to the concentration of the sample.

Because of the very narrow and characteristic emission lines from the gas-phase atoms in the flame plasma, the method is relatively free of interferences from other elements.

A gas that gives a flame that is sufficient enough to excite atoms should be chosen. Gases like propane and butane reacting with air gives a temperature of 2000K in the flame. This is the optimum level for a metal like sodium as they can ionize quickly and easily. Using an empty sample, called blank solution, which contains deionized water, will do the standardization of the flame photometer. As the solutions are put into the flame photometer, solutions will be taken up by the aspirator and then sprayed on the flame. A fuel is used to give a flame. That fuel will be propane reacted with air. When the atoms are excited, they will emit light. From the intensity of light emitted, ion concentration in the solutions will be determined.

**Principles of operation**

Flame photometry relies upon the fact that the compounds of the alkali and alkaline earth metals can be thermally dissociated in a flame and that some of the atoms produced will be further excited to a higher energy level. When
these atoms return to the ground state they emit radiation, which lies mainly in the visible region of the spectrum. Each element will emit radiation at a wavelength specific for that element.

Table 3.3: Measurable atomic flame emissions of the alkali and alkaline earth metals in terms of the emission wave length and colour in flame photometer

<table>
<thead>
<tr>
<th>Element</th>
<th>Emission of Wavelength (nm)</th>
<th>Flame Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (Na)</td>
<td>589</td>
<td>Yellow</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>766</td>
<td>Violet</td>
</tr>
<tr>
<td>Barium (Ba)</td>
<td>554</td>
<td>Lime Green</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>622</td>
<td>Orange</td>
</tr>
<tr>
<td>Lithium (Li)</td>
<td>670</td>
<td>Red</td>
</tr>
</tbody>
</table>

Figure 3.2: A schematic figure of a flame photometer
It can be seen that if the light emitted by the element at the characteristic wavelength is isolated by an optical filter and the intensity of that light measured by a photo-detector, then an electrical signal can be obtained proportional to sample concentration. Such an electrical signal can be processed and the readout obtained in an analogue or digital form.

Flame photometry has many advantages. It is a simple, relatively inexpensive, high sample throughput method used for clinical, biological, and environmental analysis. On the other hand, the low temperature makes this method susceptible to, particularly, interference and the stability (or lack thereof) of the flame and aspiration conditions. Many different experimental variables affect the intensity of light emitted from the flame. Fuel and oxidant flow rates and purity, aspiration rates, solution viscosity, concomitants in the samples, etc. affect these.

3.5.3.1 Apparatus:
- Flame photometer 128, the main unit (Systronics)
- FPM Compressor 126 (Systronics)
- Muffle furnace (MAC India)
- Weighing Balance (0.00000 gm, DENVER)

3.5.3.2 Requirements:
- Filters (Regular): Na (589 nm), K (786 nm), 10 nm bandwidth (typical).
- Filters (optional): Ca (622 nm), 10 nm bandwidth (typical).
- Minimum sample: Approx. 3 ml per element.
- Operating air pressure: 0.45 Kg/cm² (typical), regulated
- Fuel Gas: LPG (Liquid petroleum gas), regulated.
- Power supply: 230V ±10%, 50Hz
- Ashless filter paper (Whatman 41, 125 mm Ø)
- Tripled distilled water
3.5.3.3 Preparation of standard solutions:

- Sodium 1000 ppm – Dissolve 2.5416 g NaCl in one liter of glass-distilled water.
- Potassium 1000 ppm – Dissolve 1.9070 g KCl in one liter of glass distilled water.
- Calcium 1000 ppm – Dissolve 2.497 g CaCO$_3$ in 300 ml glass distilled water and add 10 ml conc. Hydrochloric acid. Dilute to 1 liter.
- 60 ppm – 6 ml stock solution (1000 ppm) and 94 ml distilled water (100ml)
- 40 ppm – 4 ml stock solution (1000 ppm) and 96 ml distilled water (100ml)
- 20 ppm – 2 ml stock solution (1000 ppm) and 98 ml distilled water (100ml)
- 10 ppm – 1 ml stock solution (1000 ppm) and 99 ml distilled water (100ml)

3.5.3.4 Procedure:

Step 1: Weighed 1 g of moisture free sample in a crucible

Step 2: The crucible with sample was kept on heater and it was heated till the fumes stop

Step 3: The charred material was kept inside the muffle furnace at 600°C temperature for 6 hours till a white or grayish white ash was obtained

Step 4: The crucible was cooled in Desiccators

Step 5: A few drops of concentrate HCl was added into sample for dissolving

Step 6: Added distilled water and the total volume was made 100 ml

Step 7: Filtered through ashless filter paper (Whatman 41, 125 mm Ø)

Step 8: 2 ml of filtered sample was taken and 18 ml distilled water was added in to it (total volume 20ml.)

Step 9: The sample was analysed using Flame photometer (Systronics 128)
Step 10: The reading was noted

Step 11: Result was calculated using formula

Calculation:

\[
\text{Mineral content (gm %)} = \frac{\text{Reading} \times 100 \times 10 \times 100}{1000 \times 1000 \times \text{Weight of sample}}
\]

Mineral content = gm% X 1000
Mineral content = mg/100 gm

Where,
100 = Made up sample
10 = Dilution factor

3.5.4 Trace elements analysis

Trace elements like Iron (Fe), Zinc (Zn), Manganese (Mn) and Selenium (Se) are estimated by Atomic Absorption Spectrophotometer (AAS) (AOAC, 1975). Atomic absorption spectrometer is a spectro analytical procedure for the quantitative determination of chemical elements employing the absorption of optical radiation (light) by free atoms in the gaseous state. In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples employed in pharmacology, biophysics and toxicology research. It is mostly used to measure the concentrations of metals like Al, Se, Fe, Mn, Pb, Cu and Zn. Atomic absorption spectrometer was first used as an analytical technique, and the principles were established in the second half of the 19th century by Robert Wilhelm Bunsen and Gustav Robert Kirchhoff, both professors at the University of Heidelberg, Germany. The modern form of AAS was largely developed during the 1950s by a team of Australian chemists. Atomic
absorption spectrophotometer is a way that works inversely as flame photometry. In this spectrophotometry, the solutions are atomized in a flame. Second part of this, comes from the fact that metal atoms absorb UV lights. So they were given UV radiation from a bulb. The metals absorb these light waves. The remaining light will give an absorption by which the concentration can be determined. The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law. In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law. In their elemental form, metals will absorb ultraviolet light when they are excited by heat. Each metal has a characteristic wavelength that will be absorbed. The AAS instrument looks for a particular metal by focusing a beam of UV light at a specific wavelength through a flame and into a detector. The sample of interest is aspirated into the flame. If that metal is present in the sample, it will absorb some of the light, thus reducing its intensity. The instrument measures the change in intensity. A computer data system converts the change in intensity into an absorbance. As concentration goes up, absorbance goes up. The researcher can construct a calibration curve by running standards of various concentrations on the AAS and observing the absorbances. The computer data system will draw the curve. Then samples can be tested and measured against this curve. Since samples are usually liquids or
solids, the analyte atoms or ions must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption. Applying the Beer-Lambert law directly in AA spectroscopy is difficult due to variations in the atomization efficiency from the sample matrix, and non-uniformity of concentration and path length of analyte atoms (in graphite furnace AA). Concentration measurements are usually determined from a working curve after calibrating the instrument with standards of known concentration. (http://www.files.chem.vt.edu/chem-ed/spec/atomic/aa.html).

**Instrumentation**

**Light source**

The light source is usually a hollow-cathode lamp of the element that is being measured. Lasers are also used in research instruments. Since lasers are intense enough to excite atoms to higher energy levels, they allow AA and atomic fluorescence measurements in a single instrument.

**Atomizer**

AA spectroscopy requires that the analyte atoms be in the gas phase. Ions or atoms in a sample must undergo vaporization in a high-temperature source such as a flame or graphite furnace. Flame AA can only analyze solutions, while graphite furnace AA can accept solutions, slurries, or solid samples. Flame AA uses a slot type burner to increase the path length, and therefore to increase the total absorbance. Sample solutions are usually aspirated with the gas flow into a nebulizing/mixing chamber to form small droplets before entering the flame. The graphite furnace has several advantages over a flame. It is a much more efficient atomizer than a flame and it can directly accept very small absolute quantities of sample. It also provides a
reducing environment for easily oxidized elements. Samples are placed directly in the graphite furnace and the furnace is electrically heated in several steps to dry the sample, ash organic matter and vaporize the analyte atoms.

**Light separation and detection**

AA spectrometers use monochromators and detectors for UV and visible light. The main purpose of the monochromator is to isolate the absorption line from background light due to interferences. Simple dedicated AA instruments often replace the monochromator with a band pass interference filter. Photomultiplier tubes are the most common detectors for AA spectroscopy (http://www.files.chem.vt.edu/chem-ed/spec/atomic/aa.html).

The sample is atomized at a very high temperature (2500-3000 °C) and the free atoms have line spectrum. It means that they can only absorb the energy of light at discrete energy levels according to the excitations of electrons. Excitation energies in this case are determined by the difference between the energy level of the ground state and one of the excitation states of their electrons. Only a light with a concrete wavelength belongs to each of these excitation energies and when this light is absorbed it is missing from the continuous spectra of the electromagnetic radiation: a black line appears in the absorption spectrum of the atom. There are no vibration or rotation energy levels that would widen the lines to bands in the spectrum (like it happens in the case of UV-Vis spectrophotometry, when molecules and ions are measured. Using AAS free atoms are “lit” by monochromatic light (called “resonance radiation” that has got a special wavelength) that belongs to one line of their spectrum and therefore it has the suitable excitation energy mentioned above. Only the examined atoms can absorb it. As a result of absorption, the intensity of light decreases, this is proportional to the number of the examined atoms being present. That makes very sensitive quantitative measurements possible. To produce the proper monochromatic light necessary for the AAS, so called “hollow cathode lamps” are used. The cathode of this sort of lamp is made of
the metal under investigation (or its alloy). It means that different lamps are used for the determination of each element. It is named after the cylindrical shape of the cathode that gives direction to emerging beam, and helps re-deposit sputtered atoms back on cathode. The anode is made of tungsten and the electrodes are surrounded by noble gases. At high voltage the cathode produces electrons that speeding up in the electric filed cause the ionization of noble gas atoms. These high-speed noble gas ions bombard the cathode and therefore sputtering occurs, dislodging atoms from the surface of cathode.

Solid samples should be accurately weighed and then dissolved, often using strong acids. The solvent of the solution is evaporated and all materials present in the sample are vaporized and dissociated to atoms at the very high temperature. (The process in the reality is a bit more complicated, since ions and oxides are also produced, decomposition and association reactions take place too.) The source of atoms is usually flame (“flame atomization”). The sensitivity could be increased when the light travels for longer in the flame. Therefore most of the burners are about 5-10 cm long. The accuracy is very good, about 1-2%. The sample solution is sprayed (“nebulized”) continuously into the flame (similarly to the flame photometer).

**Three steps are involved in turning a liquid sample into an atomic gas:**

1. Desolvation – the liquid solvent is evaporated, and the dry sample remains

2. Vaporizations – the solid sample vaporizes to a gas

3. Volatilization – the compounds making up the sample are broken into free atoms.
Figure 3.3: A schematic diagram of Atomic absorption spectrophotometer

The flame is arranged such that it is laterally long (usually 10cm) and not deep. A beam of light is focused through this flame at its longest axis (the lateral axis) onto a detector past the flame. The light is focused into the flame is produced by a hollow cathode lamp. Inside the lamp is a cylindrical metal cathode containing the metal for excitation, and an anode. When a high voltage is applied across the anode and cathode, the metal atoms in the cathode are excited into producing light with a certain emission spectra. The type of hollow cathode tube depends on the metal being analyzed.

3.5.4.1 Apparatus:

- KEL PLUS automatic micro six sample digestion system (model kes 06, regular version). The Hazardous acid fumes liberated during digestion are efficiently dissolved in water through giant aspiration filter pump and sent to the drain without polluting the atmosphere.
- Kjeldahl digestion flask
- Weighing Balance (0.00000 gm, DENVER)
- Deionised distilled water (Millipore)

3.5.4.2 Standard solutions

Fe
- Standard 1-1 ppm
- Standard 2-5 ppm
- Standard 3-10 ppm

Se
- Standard 1-0.5 ppm
- Standard 2-1 ppm
- Standard 3-5 ppm

Mn
- Standard 1-1 ppm
- Standard 2-1.25 ppm
- Standard 3-2.50 ppm
- Standard 4-5.00 ppm
- Standard 5-10 ppm

Zn
- Standard 1-1 ppm
- Standard 2-5.00 ppm
- Standard 3-10 ppm
3.5.4.3 Flame parameters

**Fe**
- Flame type: Air-C2H2
- Burner height: 7.0mm
- Fuel flow: 0.9 L/min
- Wavelength: 248.3nm

**Se**
- Flame type: N2O-C2H2
- Burner height: 11.0mm
- Fuel flow: 4.0 L/min
- Wavelength: 196.0nm

**Mn**
- Flame type: Air-C2H2
- Burner height: 7.5mm
- Fuel flow: 1.0 L/min
- Wavelength: 279.5nm

**Zn**
- Flame type: Air-C2H2
- Burner height: 7.0mm
- Fuel flow: 1.2 L/min
- Wavelength: 213.9 nm

3.5.4.4 Supplying of Gas:
1. Acetylene for Mn, Zn, Fe
2. Acetylene and Nitrous Oxide for Se
3.5.4.5 Procedure:

**Step 1:** weighed 1g of moisture free sample in digestion tube

**Step 2:** 15 ml concentrate HNO₃ and 5 ml H₂SO₄ was added for dissolving

**Step 3:** Digest the sample at 150°C for 1 hour

**Step 4:** At 200°C for 1 hour

**Step 5:** At 250°C for 1/2 hour

**Step 6:** At 300°C for 1/2 hour

**Step 7:** At 350°C for 1/2 hour

**Step 8:** Digest the sample at 400°C for 1/2 hour till white or light yellow color developed

**Step 9:** After digestion add distilled water into the sample and the total volume was made 100 ml

**Step 10:** Filtered through ashless filter paper

**Step 11:** Run the sample through Atomic Absorption Spectroscopy

**Step 12:** The reading was noted

**Step 13:** Result was calculated using formula

**Calculation:**

Trace element content (mg/100 gm) = \( \frac{A \times B}{C} \)

Where,
- A - Reading
- B - Made up Volume
- C - Weight of sample

3.5.5 Statistical analysis

The results are presented as Mean ± standard deviation in tables and figures of determinations for triplicate samples. The results obtained were analyzed statistically by performing ANOVA and Tukey test where there were significant differences (Zar, 1998). Statistical significance is indicated with
appropriate letters on the data tables. Significance level was set to an alpha level of 0.01. The level of significance between the treatments using the statistical package- SAS, version 9.2 for Windows (SAS, 1989). Statistical Analysis System (SAS) is an integrated system of software products provided by SAS Institute Inc. SAS Institute is one of the largest privately held corporations in North Carolina and in the software business.
Experimental fish species

Plate 3.1: *Tor putitora* (Hamilton, 1822)

Plate 3.2: *Neolissocheilus hexagonolepis* (Mc Clelland, 1839)

Plate 3.3: *Oncorhynchus mykiss* (Walbaum, 1792)

Plate 3.4: *Schizothorax richardsonii* (Gray, 1832)
Figure 3.4: Location Map of Sampling Stations in Arunachal Pradesh

Figure 3.5: Location Map of Sampling Stations in Uttarakhand
Plate 3.5: Collection site at Kameng river, Arunachal Pradesh

Plate 3.6: Collection site at Tenga river, Arunachal Pradesh

Plate 3.7: Collection site at Shergaon trout farm, Arunachal Pradesh
Plate 3.8: Collection site at Bhimtal hatchery, Uttarakhand

Plate 3.9: Collection site at Kosi river, Uttarakhand

Plate 3.10: Collection site at Champawat experimental fish farm, Uttarakhand
Plate 3.11: Length weight measurement of fish sample

Plate 3.12: Removal of head, scale and alimentary canal

Plate 3.13: Digestion of sample for crude protein analysis
Plate 3.14: Analysis of crude protein through Kjeldahl apparatus

Plate 3.15: Analysis of crude fat through Soxhlet apparatus

Plate 3.16: Analysis of ash through Muffle furnace
Plate 3.17: Analysis of crude fiber through Fibra plus

Plate 3.18: Analysis of total fat through separating funnel

Plate 3.19: Drying the sample through Vacuum rotary evaporator
Plate 3.20: Dissolving ash sample for mineral analysis

Plate 3.21: Analysis of mineral through flame photometer

Plate 3.22: Analysis of trace elements through AA