CHAPTER – 4

METHODOLOGY
A. Processing of the fresh water fingerlings major carp *Labeo rohita* for study.

1) Live and healthy fresh water fingerlings major carp *Labeo rohita* of both sexes were collected from the local fish farms.

2) The fingerlings were transported in polythene bag containing O$_2$ saturated water and brought to the aquarium of the size of $4^{'} \times 2^{'} \times 2^{'}$ nearly 125 to 160 fingerlings of *Labeo rohita* and were kept as stock.

3) Aquarium containing normal fresh water, commercial fish food was given twice a day.

4) Dissolved Oxygen level and pH of water was maintained in the laboratory. Experiments were commenced after acclimatizing the fishes to the lab condition for a period ranging from 15 to 20 days.

5) Different concentration of Zinc, Cadmium, Copper, Nickel and Cobalt were made by dissolving appropriate amount of analytical grade Zinc chloride, Cadmium chloride, Copper chloride, Nickel sulphate and Cobalt sulphate in the fresh water.

6) Amino acids were separated from the fish tissue with the help of centrifuge.

7) For the qualitative and quantitative analysis of amino acids two dimensional ascending paper chromatographic techniques was used.

8) Live and healthy fresh water fingerlings exposed to different concentration of Zinc chloride, Cadmium chloride, Copper chloride, Nickel sulphate and Cobalt sulphate.

9) After exposing to different concentration for different duration 2–3 fingerlings were remove and sacrifice for blood smear study.

10) The thin blood smears were prepared for observing the cellular alterations in the blood cells. The smears were stained with Leishmann’s stain and the observations were noted in a tabular form.
B. Preparation of heavy metal samples and processing of fingerlings.

1) COPPER CHLORIDE:
   a. Different concentration of Copper was made by dissolving appropriate amount of analytical grade Copper chloride in the fresh water. These sets of different concentration of Copper chloride (1, 2, 3 ppm) were prepared for treatment.
   b. The fish were divided into different group of 3 individuals each in separate polythene boxes. Commercially available fish food was provided twice a day and water medium with Copper chloride was changed every alternate day.

2) ZINC CHLORIDE:
   a. Different concentration of Zinc was made by dissolving appropriate amount of analytical grade Zinc chloride in the fresh water. These sets of different concentration of Zinc chloride (40, 50, 60 ppm) were prepared for treatment.
   b. The fish were divided into different group of 3 individuals each in separate polythene boxes. Commercially available fish food was provided twice a day and water medium with Zinc chloride was changed every alternate day.

3) CADMIUM CHLORIDE:
   a. Different concentration of Cadmium was made by dissolving appropriate amount of analytical grade Cadmium chloride in the fresh water. These sets of different concentration of Cadmium chloride (5, 10, 15 ppm) were prepared for treatment.
   b. The fish were divided into different group of 3 individuals each in separate polythene boxes. Commercially available fish food was provided twice a day and water medium with Cadmium chloride was changed every alternate day.
4) **NICKEL SULPHATE:**  
   a. Different concentration of Nickel was made by dissolving appropriate amount of analytical grade Nickel sulphate in the fresh water. These sets of different concentration of Nickel sulphate (40, 55, 70 ppm) were prepared for treatment.  
   b. The fish were divided into different group of 3 individuals each in separate polythene boxes. Commercially available fish food was provided twice a day and water medium with Nickel sulphate was changed every alternate day.

5) **COBALT SULPHATE:**  
   a. Different concentration of Cobalt was made by dissolving appropriate amount of analytical grade Cobalt sulphate in the fresh water. These sets of different concentration of Cobalt sulphate (10, 20, 30 ppm) were prepared for treatment.  
   b. The fish were divided into different group of 3 individuals each in separate polythene boxes. Commercially available fish food was provided twice a day and water medium with Cobalt sulphate was changed every alternate day.

C. **PREPARATION OF BLOOD SMEAR:**

Live and healthy fresh water fish subjected to different concentration of Copper chloride (1, 2, 3 ppm), Zinc chloride (40, 50, 60 ppm), Cadmium chloride (5, 10, 15 ppm), Nickel sulphate (40, 55, 70 ppm) and Cobalt sulphate (10, 20, 30 ppm). The blood smear was prepared for observation.

After an interval of 15 days fish were removed from each of container and the blood was drawn from the dorsal aorta or from pressing of gills. A drop of blood was taken on a glass slide and a thin smear was made. It was allowed to dry at room temperature. The slide was then stained with Leishmann’s stain for 10 minutes. Then it was washed with
distilled water and allowed to dry at room temperature and studied under light microscope.

After an interval of 15 days. 3 fish were taken from each of the container and made inactive by general hypothermia. The muscle tissues were used for the estimation amino acids using paper chromatography.

D. CHROMATOGRAPHIC TECHNIQUE:

**Principle:**
The technique is based on the fact that the paper used is made up of cellulose; it is moderately polar and has a strong affinity for water present in the solvent phase. As the solvent flows through a section of paper containing the solute. A partition of this occurs between the mobile organic phase and the stationary water phase. Some of the solutes leaves the paper and enter the organic phase. When the mobile phase reaches a section of paper containing no solute partition occurs. This time solute is transferred from the organic phase into the paper phase. This results in the transfer of a solute from the point of application to the paper to a point of some distance along the paper in the direction of flow.

**Choice of filter paper:**
Whatman filter paper No. 1.

The chemical composition of Whatman filter paper No. 1 consist of $\alpha$ – cellulose $\rightarrow$ 98 – 99 %; $\beta$ – cellulose $\rightarrow$ 0.3 – 1 %; Pentosans $\rightarrow$ 0.4 – 0.8 %; Ash $\rightarrow$ 0.07 – 0.1%; Ether soluble matter $\rightarrow$ 0.015 – 0.1 %.

**Preparation of sample:**
Extraction of free amino acids from the fish tissues:
Weighed tissues (300 mg), were homogenized in glass homogenizer in 80 % alcohol and was left for 24 hours at 0 – 4 °C. The homogenous mixture was centrifuged after 24 hours and alcoholic supernatant was filtered and evaporated over water bath in porcelain crucible at 80 °C. The dried residue was extracted with 1 ml of 10 % aqueous ethanol. These samples were used for chromatography for the determination of free amino acids.

**Choice of solvent system:**

Selection of the solvent for the chromatography is most important for separation of the compounds. The solvent consisted of n – Butanol: Glacial Acetic Acid: Distilled water in the proportion of 4:1:5. The mixture was thoroughly shaken in a separating funnel and after the formation of two layers, lower layer was discarded and upper layer was used for chromatography.

**Location reagent:**

0.2 % ninhydrin in acetone was used as location reagent.

**Procedure:**

Note: Chromatographic paper was handled with care after washing hands with soap and water because direct contact may introduce some organic compounds which may interfere with chromatography.

1. Whatman No. 1 chromatograph sheet was used and the origin was marked from it with a pencil, 3cm away from one end.
2. Four equal distance spot were marked with a pencil as control, 15, 30 and 45 days respectively.
3. Using micropipette 10µL of the sample was loaded on the respective spot at the origin.
4. The paper was allowed to dry and reloading was done when necessary.
5. The developing solvent was poured into the chromatography chamber and covered with airtight lid and was allowed to stand for 30mins. To saturate the internal environment.
6. The lid of the chamber was opened and loaded chromatogram was hanged vertically with its lower end dipping into the developing solvent. The lid was tightly sealed with grease to make it airtight.

7. The solvent front was allowed to ascend upwards along the paper by capillary action till it reaches 2/3 of the length of the paper. Time of development was about two to three hours depending upon the component being investigated.

8. After the development was over the lid was opened to remove the chromatogram and the solvent front was marked with a pencil. The chromatogram was allowed to air dry at room temperature.

9. The paper was then ready for locating the position of separated components.

10. The locating reagent (Ninhydrin) was sprayed on the chromatogram uniformly and the paper was allowed to air dry.

11. The chromatogram was heated in hot air oven at 105ºC for four minutes.

12. The amino acid location appeared as pinkish violet spots. The spots were circled with a pencil. The individual spots were cut out from the chromatograms and kept in numbered test tubes separately. Coloured spots from paper pieces in test tubes were extracted with 0.42 % Sodium bicarbonate in 48 % ethanol and kept undisturbed overnight. In the morning the test tubes were shaken and after the filter paper pieces settled down the coloured solutions were decanted in colour Imation tubes. The colour intensity of known and unknown spots of chromatograms was determined with the help of photo colorimeter using green filter. Then optical densities of amino acids were measured with the help of photo colorimeter.

**Preparation of stain:**

(1). **Ninhydrin solution**: Ninhydrin is prepared by dissolving 2 g of ninhydrin in 25ml of water. To this solution, 25 ml of 0.2 M acetate buffer (pH=5.5) is added. The mixture is protected from light.
(2). Leishmann’s stain:

Solution 1.
Methylene blue ----- 1 gm.
Sodium carbonate (0.5 % aqueous solution) -----100 ml.
Eosin (0.1 % aqueous solution) -----100 ml.
Methyl alcohol ----- 100 ml.

Dissolve Methylene blue in the sodium carbonate solution. Heat at 65°C for 12 hours, cool and allow the mixture to stand for 10 days. Add equal volume (100 ml) of eosin solution, mix well and allow standing for 6 to 12 hours. Filter and collect the precipitate. Wash the precipitate with several changes of distilled water until no more colours are extracted. Dry the precipitate at 37°C. Grind to powder in a mortar.

Weigh 0.15 gm of the powder and grind it in a mortar with methyl alcohol. Pour off the supernatant into a volumetric flask and add more methanol until all the powder is dissolved. Make up the volume up to 100 ml with methanol. Store in a tightly stopper bottle and allow to stand for 24 hours before use.

Solution 2.
Buffer solution (pH – 6.8).

a) Dissolve 9.47 gm of Disodium hydrogen phosphate (anhydrous) powder per liter of distilled water. Make it to M/15 solution.

b) Dissolve 9.08 gm of Potassium di-hydrogen phosphate (anhydrous) powder per liter of distilled water. Make it to M/15 solution. To prepare the buffer, mix 49.6 ml of solution (a) and 50.4 ml of solution (b). Check the pH.

Staining technique:
1) Prepare a thin layer and dry in air.
2) Cover the smear completely with Leishmann’s stain.
3) Stain for 1 to 2 minutes.
4) Dilute the stain with twice its volume of the buffer solution. The slide will almost be completely flooded.

5) Stain for 10 minutes.

6) Wash the slide with buffer solution. Use tap water if not too acidic or alkaline.

7) Drain and dry in air by keeping it in a slanting position.