Chapter-VII

RESIDUE ANALYSIS
RESIDUE CONFIRMATION IN TISSUES OF THE FISH LABEO
ROHITA EXPOSED TO SUBLETHAL CONCENTRATIONS OF
CYHALOTHRIN

Aquatic organisms are affected by the pesticides leaching into the
waters through agricultural run off, although often the aquatic environment is
not the primary site of application of pesticides. Two factors ultimately
contribute to the concentration of the pesticides in the aquatic eco-system:
persistence of pesticides in the soils and long-range transport of the pesticides
in the atmosphere. As soils receive the major part of the globally used
pesticides, and residues are transported eventually to the water bodies.
Persistence in the soil ultimately constitutes a threat to the aquatic
environment.

Persistent pesticides like hydrocarbon compounds have a tendency to
concentrate in living organisms. The residues of such widely used
hydrocarbon insecticides have been reviewed by Murty (1986). Among these
are specimens even from remote areas, where pesticides have not been known
to be used. The residue levels in the animals vary from a fraction or part per
million to as high as several thousand parts per million. It was also reported
that there is a general correlation between the pesticide residue level and
position of animal in the food chain (Walker, 1970).

Stringent regulations have been introduced in many countries for the
purpose of minimizing the hazards from the use of pesticides, which may
leave behind residues or degradation products or metabolites after the desired
purpose has been accomplished or when they reach some part of the
environment other than intended target. Efforts are also made for development
of
a) Chemical pesticides which do not have toxic residue problems
b) Pesticides of less persistent which are safe to be introduced
c) Measures for promoting rapid degradation of pesticides including
   metabolites are being stepped up.
As an outcome of these studies, selection and measurement of these pesticides is made as an indispensable ingredient of any comprehensive programme taken up to assess or abate their deleterious effects on the biota of various ecosystems.

According to Kenaga (1974), pesticides in animals may be due to contact, with the treated environment, or through food. Regardless of the sources, pesticides enter the blood stream of the animals and if stable, they are concentrated in specific tissues and excreted depending on the nature of the pesticides reflecting in terms of its chemical and physical properties. It was reported that new generation pesticides are not expected to be biomagnified through food chain due to their high adsorption and immobility in soil (Casida, 1980; Rawn, 1981; American Cyanamid Company, 1981; Veeraiah 2002, Veeraiah and Durga Prasad, 1998) and they are rapidly eliminated from tissue after termination of exposure. However, their persistence in aquatic systems may be sufficient to cause environmental impact from direct field applications because of their extremely high toxicity and fast acting nature (Spehar et al., 1982).

New generation pesticides such as synthetic pyrethroids and nicotinoids are relatively non-persistent and do not accumulate in the environment. However, some are bioaccumulated by various organisms during exposure to sub-lethal concentrations but levels rapidly return to normal after exposure ceases. Generally, the use of new generation insecticides at recommended rates of application have no deleterious long-term effects on microorganisms or microbial processes. Similarly, higher vertebrates are also relatively insensitive to these pesticides. Generally crustaceans, mollusks and fish are most sensitive to cyhalothrin followed by amphibians, reptiles, birds and mammals (Larson et al., 1985; Hollingsworth 2001; Smith 2001; Ware 1982, Elbert et al., 2000; Yaragi et al., 2000).

Fish are often considered to be much better and sensitive indicators of pesticide residues in the aquatic environment, in which they are present in very small quantities to be analyzed chemically than analysis of water
samples, because residues in the fish tissue are in higher orders of magnitude and are much easier to be analyzed (Edwards, 1973). Hence, an attempt has been made to know the presence of cyhalothrin residues in different tissues viz., brain, liver muscle, gill and kidney of *Labo rohita* exposed to sublethal concentrations 0.0056 mg/L (1/10 of static 48 h LC$_{50}$) for 24, 48, 96 hours and 8 days.
MATERIALS AND METHODS

In the present investigation three kinds of chromatographic methods were employed. They are column chromatography for clean up, thin layer chromatography (TLC) for the confirmation of pesticide residues and Gas liquid chromatography for quantitative analysis, whereby the residue is made free from co-extractives or impurities by differential adsorption running through a column containing different lengths of adsorbents.

Extraction:

After exposure to sublethal concentration, the fish were sacrificed, tissue were extracted and analyzed for the identification and quantification of residues. The control fish were treated with pure acetone.

The residues from the fish tissue of brain, liver, muscle, gill and kidney were extracted by the modified method of Mills and Olney (1977). 10 gram of muscle and gill tissue was blended with 40 gram of anhydrous sodium sulphate (Na₂SO₄) (pre-extracted in a soxhlet column with analytical grade hexane); to get a free flowing powder. This powder was extracted with 5 ml of hexane and acetone in ratio of 2:1 for each gram of powder. Soft tissues like liver, brain, kidney were homogenized in a tissue homogenizer with minimal quantity of all glass triple distilled water. The homogenized tissue was extracted with 2:1 hexane: acetone at a ratio of 5 ml for each gram of tissue. When very small quantity of tissue was available, a minimum of 6 ml of extracting solvent was used. Extraction was carried out for one hour in horizontal shaker bath, at gentle speed to avoid spillage.

Clean-up and removal of the co-extractives:

After extraction, acetone was washed out and the hexane extract was dried out over Na₂SO₄ (AR grade). The extract was stored in stoppered glass vials and kept in the refrigerator for further processing. The hexane extract was concentrated to about 1 ml (when the volume of extract was 4 ml or more) and transferred directly on to a florisil column prepared according to the PR grade
and was heated overnight at 130°C and after cooling, it was deactivated with grades of increasing polarity using hexane and acetone.

**Thin layer chromatographic analysis:**

The method of Moats (1966) was followed and is as follows:

The silver nitrate impregnated silica gel plates were prepared by adding silver nitrate (0.2%) to the silica gel. The slurry was prepared and spread on the plates to give 250 μ thick TLC plates. The plates were dried for one hour and activated at 80°C in hot air oven for 15 minutes, taken out of the oven, cooled and stored.

The concentrated extract and technical pesticide standard were spotted with a sample spotter and micropipettes (5-20 μl) on thin layer chromatographic plates (5 x 20 cm size). The spotted plates were developed in a TLC chamber until the solvent front reached 10 cm mark from the original point. The plates were removed from the chamber, air dried and exposed to short-wave-length UV light, with intermittent exposure to steam to locate the spots. The exposure to steam improved the resolution of the spots. The appearance of brown spot on the TLC plates was taken as confirmation of the presence of residue in the particular tissue.

**Gas liquid chromatographic analysis**

Gas chromatography was carried out on GC-MS 6890N, Agilent Technologies coupled with 5975 / L Mass solutions detector with standard operating procedure. The column temperature was 280°C, injector and detector temperatures were 300°C and 300°C respectively. Nitrogen was used as the carrier gas at 50 ml/min. An online integrator provided the retention time and area of each peak. 5 μl of each sample was analyzed thrice with and without adding known amounts (100 mg/g of sample) of cyhalothrin as an internal standard. The analytical conditions were attenuation 8, slope
sensitivity 0.5 area rejection in lakhs, carrier gas Nitrogen at 2 kilo Pascal’s per cm². Peak retention times and areas were calculated using a microprocessor connected to the GLC instrument.

**Estimation**

5 µl of standard cyhalothrin solution was injected into the instrument and also the samples under test. Then using the graph measured the areas of cyhalothrin on IS (Internal Standard) peaks in each case was observed and compared to the cyhalothrin content with standard.

**Calculation**

\[
\text{Cyhalothrin content per cent by mass} = \frac{m_1 \times A_1 \times A_3 \times P}{m_2 \times A_2 \times A_4}
\]

Where,

- \( m_1 \) = mass in grams of the standard cyhalothrin
- \( m_2 \) = mass in grams of the sample taken for test
- \( A_1 \) = Area of internal standard peak in standard solution
- \( A_2 \) = Area of internal standard peak in sample solution
- \( A_3 \) = Area of cyhalothrin peak in sample solution
- \( A_4 \) = Area of internal standard peak in standard solution
- \( P \) = Percentage purity of standard cyhalothrin

The estimated cyhalothrin was expressed as µg/g wet weight of the tissue tested. High recovery values were obtained on extraction of the tissue with hexane and column chromatography.
RESULTS AND DISCUSSION

Results of the appearance of brown spot on the TLC plates in different tissue at different time period of exposure was presented in table VI.1. The results of the 100 x Rf values of cyhalothrin technical grade in four different solvent systems were given in Table VII.1. The results of the gas liquid chromatographic analysis in the tissues of brain and liver of the fish, *Labeo rohita* were given in Table VII.2 and Fig. 2-9.

After 24 h exposure period the residue spots appeared prominently in brain and gill. In other tissues the residue spots were traced very little, whereas, in kidney no residue was identified. In the other tissues, i.e., liver, muscle and kidney little amount of residues were traced. At 96 hrs exposure, in almost all the tissues, the residues appeared prominently except in kidney where in the residue spot is not very prominent. In 8 days exposure also, same trend was noticed.

In the present thin layer chromatographic study, it was observed that the prominence in residue spots increased steadily throughout the investigation period. This may be due continuous bathing of the fish in the toxicant medium. Though the excretion and metabolism processes are continuous, the uptake will lead to the maintenance of residue load. Hence, the residues were found to appear throughout the study period whereas in terrestrial organisms, the dose will be given intraperitonially and the residue levels decrease, with the increase in the duration of exposure.

In the gas chromatographic analysis, residues were detected in less quantities due to the fixation of detector sensitivity at mg/kg and the test fish were exposed at ng/gram. The protocols fixed by the analyzing agency by following the pre-fixed standard protocols made the analysis, and hence clear quantities at ppb levels were not quantified.(vide chromatograms provided by the agency which analyzed the samples). In all the test organs of the test fish *Labeo rohita* the residues reported were less than mg/Kg.
This may depend on the metabolism of the animal or degradation of the pesticide. Bradbury et al., (1986) reported high levels of fenvalerate residues in bile, followed by gill and kidney tissues after a 48 hours exposure in rainbow trout. Moizuddin and Menzer (1986) reported that residues of $^{14}$C labeled fenvalerate are highest in fat, followed by skin, liver, heart, kidney, lung and brain in Japanese quail. Koteswara Rao (2003) reported chlorpyrifos residues in the brain, liver, kidney, muscle and gill of the Indian major carp Catla catla, Labeo rohita and Cirrhinus mrigala analyzed by GLC.

Accumulation studies, conducted under laboratory conditions with constant concentrations, showed that rapid uptake takes place in fish (accumulation factor approximately 1000-2000). However, in the presence of soil and suspended sediment, the bioaccumulation factors were greatly reduced - to 19 in the case of fish and 194 in the case of Daphnia. When exposed fish and Daphnia were placed in clean water, the residues declined rapidly, with half-lives of 7 days and 1 day, respectively. The concentrations of cyhalothrin and lambda-cyhalothrin that are likely to arise in water from normal agricultural application will be low. Because the compound is rapidly adsorbed and degraded under natural conditions there will not be any practical problems concerning the accumulation of residues or the toxicity of cyhalothrin or lambda-cyhalothrin in aquatic species. (IPCS INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY Health and Safety Guide No. 38)

Studies with permethrin, cypermethrin and fenvalerate have established that rates of metabolism and elimination in rainbow trout are significantly lower than those reported toxicity of cypermethrin was studied by Miyamoto (1976). The residues of Aldicarb pesticide in laying hens was studied by Hicks et al., (1972). The residue in liver and kidney tissue has twice the amount than muscle, 6 hr after treatment. Hunt and Gilbert (1977) reported residues of phenothrin were highest in fat and in the excretory system and moderate amounts of residues were found in adrenal, thyroid and pituitary glands of lactating goats.
### TABLE VI. 1
Pesticide Standard Confirmation and the Tissue Chromatogram

<table>
<thead>
<tr>
<th>Layer</th>
<th>Silica gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>Hexane + Acetone (90+10, 75+25 V/v)</td>
</tr>
<tr>
<td>Front</td>
<td>10 cm</td>
</tr>
<tr>
<td>Impregnated reagent</td>
<td>Silver nitrate</td>
</tr>
<tr>
<td>Time</td>
<td>30 minutes</td>
</tr>
<tr>
<td>UV light exposure</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Hours &amp; Exposure</td>
<td>24, 48, 72 and 96 and 8 days.</td>
</tr>
</tbody>
</table>

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**Rf Values of standard in different solvent systems**

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Ratio (\text{V} / \text{V})</th>
<th>100 x Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane + Acetone</td>
<td>9:1</td>
<td>69.5</td>
</tr>
<tr>
<td>Hexane + Benzene</td>
<td>1:1</td>
<td>86.4</td>
</tr>
<tr>
<td>Hexane + Acetone</td>
<td>1:1</td>
<td>82.6</td>
</tr>
<tr>
<td>Benzene + Hexane</td>
<td>4:6</td>
<td>52.0</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Tissue</th>
<th>100xRf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>88.6</td>
</tr>
<tr>
<td>Gill</td>
<td>90.2</td>
</tr>
<tr>
<td>Liver</td>
<td>88.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>-----</td>
</tr>
<tr>
<td>Muscle</td>
<td>84.6</td>
</tr>
</tbody>
</table>
The residue analysis of permethrin, cypermethrin, deltamethrin and fenvalerate in the fat and brain of treated rats show that residues are much higher (Marei et al., 1982). Saleh et al., (1986) studied the persistence and distribution of cypermethrin in white leghorn hens after a single oral dose of 10 mg/kg, residues were found in the fat, skin, blood, heart, brain, liver kidney, ovary and eggs through 14 days post dose. Veeraiah (2002) reported qualitatively the presence of cypermethrin in brain, liver, gill and kidney of the fish Labeo rohita.

A chromatographic analysis of cypermethrin in the animal material was studied by Jebakumar et al., 1992; Abd El-Salam et al., 1982; Mahmoud Abbas Saleh, 1986. However, their persistence in aquatic ecosystems may be sufficient to cause environmental impact from direct field application.

In support of the present study, Marei et al., 1982 observed high levels of cis-cypermethrin in rat brain at 3 hours of exposure and reported that pyrethroid residue appearance initially attained in fat and brain are probably inversely related to their relative rates of metabolism in liver.

The pyrethroid residue levels decrease less rapidly in fat and seem to be inconsistent in the case of brain (Bradbury and Coats, 1989). The tissue of radio labeled cypermethrin decreased in the order of fat > Kidney > blood > muscle and brain, after 24 hours oral administration to mice (Crawford et al., 1981a; Hutson et al., 1981).

Cyhalothrin and lambda-cyhalothrin are very toxic for fish, aquatic invertebrates, and honey-bees but, because very low exposure levels normally occur, this would only cause a problem in the case of spillage. The toxicity for birds is low and with recommended techniques and rates of application, it is unlikely that cyhalothrin and lambda-cyhalothrin and their degradation products will attain levels of adverse environmental significance.
The results of the present study revealed that prolonged exposure to sublethal concentrations of cyhalothrin *Labeo rohita* leads to increased accumulation of residues. This is in corroboration with the earlier reports. A thorough literature search revealed that repeated or continuous exposure to low concentrations of pesticides can lead to high residue concentrations without mortalities. Thus the uptake and persistence of cyhalothrin depends not only on a number of physical and chemical conditions, but also varies according to the biological conditions. The fish diseases are prevailing, and locally fish farmers are spraying synthetic pyrethroid compounds for control, which has to be monitored and regulated.

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