CHAPTER III
MATERIALS AND METHODS
III. MATERIALS AND METHODS

The following methods were practiced and the materials involved therein were utilized for carrying out the present research work on the topic entitled “Pesticide Residues in Bovine and Human Milk in Dhanbad City, Dhanbad, Jharkhand”.

PROCUREMENT OF RAW BOVINE MILK AND HUMAN MILK

Procurement of raw bovine milk

The area of this study comprised of Dhanbad city, Dhanbad, Jharkhand. Taking a reference of the Dhanbad city map, a survey of the entire city was carried out systematically. Ten (10) major locations of Dhanbad city i.e. Saraidhela, Bhuipore, Bartand, Jharudih, Rangatand, Hirapur, Bank More, Wasseypur, Matkuria and Dhaunsar were chosen for collection of milk samples. The above mentioned locations were chosen on the basis that they covered the geographical area (2052 km²) of the city and hence the maximum population of the city.

From each of the 10 location 6 dairy farms were selected randomly for sample collection. The detailed names of these places are summarized in figure A. From each of the selected 6 dairy farms, milk samples were collected 3 times in each season. The three seasons were rainy (July to October), winter (November to February) and summer (March to June).
This procedure was carried out for the collection of raw milk samples of cow and buffalo for estimation of organochlorine pesticide residues.

Thus,

The total number of cow milk samples collected = \(10 \times 6 \times 3 \times 3\)

\[= 540\] and

The total number of buffalo milk samples collected = \(10 \times 6 \times 3 \times 3\)

\[= 540\]

Where, 10 – No. of major areas selected

6 – No. of dairy farms selected from each of the 10 major areas

3 – No. of times milk sample was collected from each of the 6 milk farms

3 – No. of seasons for milk sample collection
Figure A. Flow chart of experimental site for bovine milk collection
Figure B. Flow chart of experimental site for human milk collection
**Procurement of human milk**

For the analysis of organochlorine pesticides in human milk, another survey was carried out systematically to make a list of total number of maternity hospitals and nursing homes within Dhanbad city. From the above survey, 3 maternity hospitals and 1 nursing home were selected for human milk sample collection. The respective maternity hospitals were Central Hospital, Dwarka Das Jalan Memorial Hospital, R.C. Hazra Memorial Hospital and the nursing home was Park Clinic. The detailed names of these hospitals are summarized in figure B. The above mentioned maternity hospitals and nursing home was chose on the basis that they covered the 4 broad areas of Dhanbad city i.e. Saraidhela area, Hirapur area, Bartand area and Bank More area and its population.

From each maternity hospital and nursing home (total 4), milk samples were collected randomly from 5 different lactating women in all the three seasons i.e. rainy, winter and summer.

Thus,

\[ \text{The total number of human milk samples collected} = 4 \times 5 \times 3 \]

\[ = 60 \]

Where, 4 – Total no. of maternity hospitals and nursing home.

5 – No. of lactating women from each of the 4 maternity hospitals and nursing home.

3 – No. of seasons for milk sample collection.
Sampling of raw bovine and human milk

Collection of sample is a highly technical process. Samples were collected in thoroughly washed, wide mouthed glass bottles with glass stopper. The amount of raw milk sample collected was 500 ml.

The sample size of human milk varied from 20-50 ml according to availability. Immediately after sampling, the mouth of bottles was sealed through bottle stoppers. Precautions were taken to avoid any air incorporation in the samples. The collected samples were labeled with pertinent information such as code of sample, place of sampling, date of sampling etc. for proper identification. Plastic or metallic containers were not used for the collection of samples to be analyzed by ECD because minute traces of such materials produce spurious response in gas chromatography.

Preservation and storage of samples

After sampling, samples were immediately transferred into the ice-box to prevent microbial contamination by checking microbial growth during transportation as well as storage. Samples were kept in between the ice-cube of ice-box. In laboratory, these samples were kept in deep-freezers (below 4°C) till the extraction of the samples. All the samples were extracted within a period of 24-36 hours.

ANALYTICAL PROCEDURE

The samples were extracted in the laboratory of the Department of Environmental Science and Engineering of Indian School of Mines (ISM), Dhanbad,
Jharkhand. The GC analysis of the extracted samples was also done in the same laboratory.

**Chemical Reagents**

All the chemicals used in the study were of analytical reagent (AR) grade and high-grade purity especially supplied for HPLC or GLC work. Most of the solvents were distilled and analyzed for purity and contamination of pesticide, before using it for residue analysis.

The chemicals used during extraction, clean-up and estimation of the pesticide residues were:

- **Acetone**: Distilled over glass beads and collected at 56°C
- **n- Hexane**: GLC grade
- **Concentrated H₂SO₄**: Extra pure with specific gravity 1.84
- **Anhydrous Na₂SO₄**: 10 LAR- 1 grade
- **Standard pesticide**: GLC grade

The standard pesticide was purchased from Sigma Aldrich.

**Apparatus**

The apparatus used during collection of sample, extraction, clean-up and estimation of the pesticide residues were:

**Glass bottles**: 500 ml capacity, wide mouthed with glass stopper
Ice - Box: 10 litres capacity

Separating funnel: 500 ml and 100 ml capacity

Vacuum pipette: 50 ml

Analytical parameters of Gas Chromatograph used for the GLC analysis

Model of GC: Chemito series 2865, micro-processor controlled

Detector: Electron capture detector (ECD) with Ni (63) foil as the electron source.

Column: 2 m glass, 0.25 in internal diameter (I.D.) packed with OV-17/1.95% QF- 1 on Gas Chrom Q (100-120 mesh)

Injection port temperature: 220°C

Column oven temperature: 200°C (6 min), 215°C (5 min) and 230°C (5 min)

Detector temperature: 280°C

Carrier gas: 10 LAR-1Nitrogen (N₂)

Flow rate: 50 ml / minute

Attenuation: 1

Current: 10⁻⁹mA

Voltage: 90 mV

Limitation of detection: 0.01 PPM
**Extraction of samples**

A method described by Faubert Maunder et al (1964), with certain modification by Dhaliwal and Kalra (1978) as described was used for extraction of samples. This particular method was chosen on the basis of its maximum efficiency of extraction of pesticide residues.

Extractions of the samples were done by the following method:

1. 20 ml of thoroughly mixed bovine/human milk sample was taken in 100 ml stoppered separating funnel.
2. 40 ml each of n-hexane and acetone (GC grade) was added in the sample.
3. The separating funnel was stoppered and thoroughly shaken for two minutes.
4. It was allowed to stand for twenty minutes or till there was a clear separation of phases obtained.
5. The upper n-hexane layer was drawn out with the help of a vacuum pipette and dried by passing through 5g of anhydrous sodium sulphate (Na$_2$SO$_4$) taken in a funnel.
6. The lower aqueous acetone phase was re-extracted twice with 40 ml n-hexane. All the three batches of n-hexane layers were combined and dried over anhydrous Na$_2$SO$_4$.
7. Again the combined n-hexane extract was concentrated to about 1 mg (1 ml) on a rotary vacuum evaporator (with n-hexane).
8. The concentrated residue was dissolved in 40 ml n-hexane.
Clean up of samples

Pesticide residues in n-hexane layer were cleaned from fat and co-extractives by acid digestion method as mentioned by Veirov and Ahaaronson (1978), Kapoor et al (1980), Kapoor and Kalra (1988).

Clean up of samples was done by the following method:

1. The concentrated n-hexane phase was transferred in a 250 ml separatory funnel and about 40 ml concentrated sulphuric acid (H₂SO₄), sp.gravity 1.84 was added in it dropwise slowly to allow the contact time of the extraction (1 hour).

2. The lower dark reddish brown/ dark yellowish brown/ dark yellowish layer of digested lipids and H₂SO₄ was discarded.

3. The organic solvent layer (upper n-hexane layer) was washed with lukewarm distilled water (6 or more times using 50 ml each time) and ensured that extract was free from acid with the help of neutral litmus paper.

4. The n-hexane extract was dried by passing it through 5-10 gms of anhydrous sodium sulphate (Na₂SO₄) taken in the funnel.

5. The contents were finally transferred to graduated glass tube upto 5 ml for GLC estimation.

6. Clean up sample were labelled and stored in deep freeze of refrigerator till analysis of pesticide residues in the samples were done.
**Pesticide Standard**

Pesticide reference standards of the following organochlorine compounds were obtained from Sigma Aldrich:

1. Alpha BHC
2. Gamma BHC (lindane)
3. Beta BHC
4. Heptachlor
5. Delta BHC
6. Gamma HCH
7. Heptachlorepoxide Isomer B
8. Aldrin
9. Delta HCH
10. Endosulfan 1 (alpha)
11. 4,4′-DDE (p,p′-DDE)
12. Dieldrin
13. Endrin
14. 4,4′-DDD (p,p′-DDD)
15. Endosulfan 2 (beta isomer)
16. 4,4′-DDT (p,p′-DDT)
17. o,p′-DDT
18. Endrin Aldehyde
19. Endosulfan sulfate
20. Methoxychlor

The standards were prepared according to method of Association of Official Analytical Chemist (AOAC).

**Pesticide stock concentrate**

Stock standards or secondary standards were prepared from the primary standards. 50 mg of the standard was dissolved in 50 ml of n-hexane.

**Intermediate stock standards**

Concentrated stock standard were diluted in n-hexane to desired work concentration level for injection in GLC. The different working standards prepared were 0.00001 mg/l (ppm), 0.0001 mg/l (ppm), 0.001 mg/l (ppm), 0.01 mg/l (ppm), 0.1 mg/l (ppm), 0.5 mg/l (ppm) and 1 mg/l (ppm).

**Estimation of pesticide residues**

Gas liquid chromatograph was standardized by giving several injection of standard (working standards prepared) simultaneously. Standard mixture were injected at all the different concentration levels of standard prepared to obtain 30 to 40% and 60 to 80% full scale deflection (FSD) for various compounds and checked variation due to non-linearity of electron capture detector. 2 to 8 ml of aliquot of the clean-up extracts was injected so that the injection represented about 5 mg sample equivalent. If required, the extract was diluted to get peak height within the scale. For identification of organochlorine pesticides (OCPs), the peaks were identified by comparison of retention time with those in corresponding standards.
Quantification of peak representing various organochlorine pesticides in samples was carried out by comparing the peak height of the pesticides in the chromatogram of samples (taking the average of the duplicate analysis) with the peak height of the corresponding pesticides in the chromatogram of the pesticide standard solution containing known amount of various pesticide injection under the identical GLC condition.

The amount of pesticide residues in the bovine milk and human milk sample was calculated using the following formula:

\[
\text{Residue level (mg/L or mg/kg or ppm)} = \frac{H_s}{H_{std}} \times \frac{M}{M_1} \times \frac{V}{V_1} \times F
\]

Where,

\(H_s\) = Peak height of the sample

\(H_{std}\) = Peak height of the standard

\(M\) = ng of standard injected

\(M_1\) = microl of sample extract injected

\(V\) = Volume of final extract in ml

\(V_1\) = Weight of sample in gm

\(F\) = Recovery factor

\[
\text{Recovery factor} = \frac{100}{\% \text{ Mean recovery}}
\]
Determination of recovery percentage of pesticide residues

An attempt was made to find out the efficiency of extraction of OCP residues from milk by determining the recovery percentage of various OCP residues by this method.

20 g of two accurately weighed milk samples (bovine) were taken and mixed with known amount OCP standard solution containing various pesticides, shaken gently and kept for an hour for the pesticide standard solution to get mixed with the sample. The samples were transferred to 250 ml separating funnel. Samples were extracted as described earlier. This was designated as Step I (control + standard).

Similarly, 20 g of accurately weighed milk samples (bovine) were taken but without the addition of pesticide standard and processed. This was designated as Step II (control).

Likewise, another sample was run without milk samples and large concentration of pesticide standard solution used in Step I was added in this step. This was designated as Step III (blank).

The quantity of each pesticide in control (Step II) was subtracted from the respective quantity of control + standard (Step I). The subtracted value was compared with the value of blank (Step III), which when multiplied by 100 gave the percentage recovery of each pesticide by the given method.
Table. A. Recovery percentage of pesticide residues in bovine milk samples

<table>
<thead>
<tr>
<th>Serial No</th>
<th>Name of Pesticide</th>
<th>Percentage recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aldrin</td>
<td>89.70</td>
</tr>
<tr>
<td>2</td>
<td>α HCH</td>
<td>89.00</td>
</tr>
<tr>
<td>3</td>
<td>β HCH</td>
<td>87.40</td>
</tr>
<tr>
<td>4</td>
<td>γ HCH</td>
<td>91.00</td>
</tr>
<tr>
<td>5</td>
<td>p,p’-DDE</td>
<td>87.90</td>
</tr>
<tr>
<td>6</td>
<td>p,p’-DDD</td>
<td>90.00</td>
</tr>
<tr>
<td>7</td>
<td>o,p’-DDT</td>
<td>90.00</td>
</tr>
<tr>
<td>8</td>
<td>p,p’-DDT</td>
<td>82.60</td>
</tr>
<tr>
<td>9</td>
<td>α-Endosulfan</td>
<td>85.50</td>
</tr>
<tr>
<td>10</td>
<td>β-Endosulfan</td>
<td>85.50</td>
</tr>
<tr>
<td>11</td>
<td>Endosulfan Sulfate</td>
<td>88.40</td>
</tr>
</tbody>
</table>

Confirmation of the residues

In this study, in order to confirm the identity of the various OCP residues in the samples of bovine and human milk through GLC analysis, additional one sample out of five samples was analyzed for confirmation.

Statistical Analysis

The present research was designed by Completely Randomized Design (CRD) and Factorial Randomized Block Design (FRBD).

All sets of data were statistically analyzed by using SPSS Version 20 software (SPSS.Inc., Chicago, IL, USA) following statistical designs CRD and FRBD as suggested by Gomez and Gomez (1984) and Panse and Sukhatme (1954) respectively.
The data taken in the three seasons were pooled. The pooled data represents the data of the whole year. The pooled data represents the data of the whole year

The pooled data was then used to generate the mean table for individual isomers of the OCP residues and thereby analyze the pesticide residues in different seasons and locations.

The Analysis of Variance (ANOVA) table was used to generate the F value and the paired t-test for determining whether significant difference exists between means depending on calculated and table F values at 0.01 and 0.05 levels.

Seasonal variation was studied by comparing the data of each season with the other for each OCP. Here the ANOVA was performed to see whether any significant level of difference exists among the OCPs for the seasons and locations. Then the paired t-test was performed to see if the data for each season and location was significantly different from the other season and location.

Correlation was also performed in order to see whether any significant relation exists among the level of OCPs in the different seasons.

All the data of the pesticide concentration in bovine and human milk were subjected to Analysis of Variance (ANOVA) for determining whether significant difference exists between means, depending on calculated and table F values at 0.01 and 0.05 levels.

Several correlation between the various experimentally determined pesticide residues, were calculated using SPSS Version 20 software (SPSS.Inc., Chicago, IL,
USA). The different correlation studies were done for determining the seasonal variation of pesticide residue levels in milk samples.

**Comparison with Maximum Residue Limit (MRL)**

The pesticide residue levels obtained experimentally for milk of both bovine and human milk by Gas Chromatography was compared with the Maximum Residue Limit (MRL) of the respective pesticides as recommended in the database by World Health Organization (WHO)/ Food and Agricultural Organization (FAO). This database contains Codex Maximum Residue Limits for pesticides and Extraneous Maximum Residue Limits adopted by the Codex Alimentarius Commission upto and including its 36th session (July 2013). The above comparison was done in order to study the possible health hazards of the particular pesticides obtained experimentally.

**Table B. Maximum Residue Limit (MRL) of OCPs by WHO/FAO/FSSAI (2011)**

<table>
<thead>
<tr>
<th>Organochlorine Pesticide</th>
<th>WHO/FAO MRL (Fat basis in mg/l or ppm)</th>
<th>FSSAI MRL (Fat basis in mg/l or ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>HCH</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>DDT</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>