INTRODUCTION

In the modern agricultural practice, large numbers of pesticides are being used to protect plants from pests, weeds, fungi and insects. Indiscriminate and injudicious use of these pesticides has led to the accumulation of their residues in the environment in general and in foods of animal origin in particular. Persistent exposure to man and animals with the residues of pesticides results into a variety of health problems (Ritter, 1997).

Synthetic pesticides are fat soluble, rapidly absorbed, stored in fatty tissues and slowly excreted (Hansen, 1987). The organophosphate pesticides are less persistent; but are highly toxic to higher mammals (Coulibaly and Smith, 1994). About 30 % of pesticides sold in the developing countries do not conform to the international quality standards thereby entailing potential risk to human, animal and the environment health. Biological magnification of pesticide residues in the food chain further compounds the problem (Gupta et al. 2000). Though organophosphate pesticides are less persistent and non-lipophilic but are highly toxic and their effects are irreversible (Coulibaly and Smith, 1994). Chlorpyriphos is a non-systemic organophosphorous insecticide acting as a cholinesterase inhibitor with contact, stomach and respiratory actions; it is used in agriculture, construction (as termicide), in animal practice as ectoparasitic (pet collars, cattle ear tags), in the treatment of lawns / ornamentals pasture and farmsteads, indoor crack and spot treatment, etc.

In the year 1854, Clermount synthesized the first organophosphate pesticide named tetra ethyl pyrophosphate (TEPP) which was a by-product of nerve gas used during World War II (Kanekar et al. 2004). Today, hundreds of organophosphates such as malathion, parathioan, phorate, fenthion, phosphamidos, monocrotophos, etc are synthesized for their extensive applications viz. agriculture, public health, animal husbandry and other allied sectors. However, the synthesis of chlorpyrifos was described by Regeterimk and Kenaga in 1966 by Daw chemicals (now known as Dow Elanco). There are more than 164 registered products containing chlorpyrifos and some of the common products available in the

Keywords: Pesticide, residue, chlorpyrifos, HPLC, MRL

Occurrence of Chlorpyrifos Residues in Milk of Tarai Region of Uttarakhand, India

Nagappa S. Karabasanavar1 and Suresh P. Singh2

Department of Veterinary Public Health, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar-263 145 (U. S. Nagar), Uttarakhand, India

Pesticides are used in plant protection and public health to defend against pests. But, the harmful residues that enter the food chain are of great health concerns. Keeping in view application of chlorpyrifos in agriculture and allied fields, the present study was aimed at the determination of its residues in milk. Residual concentrations of chlorpyrifos were determined in milk collected from different locations of Tarai and Kumaon regions of Uttarakhand state. Sample extraction was carried out using liquid-liquid partition followed by clean up using alumina column chromatographic and quantification of the residues was undertaken with high performance liquid chromatography (HPLC). Of the total 170 milk samples collected, 4.7% samples showed chlorpyrifos residues with the mean residual concentration of 0.092 μg mL⁻¹; of which most of them were found to contain residues above the prescribed maximum permissible limit (MRL) of 0.02 mg kg⁻¹. Since chlorpyrifos residues have health and environmental issues, hence farmers must be educated about their judicious use.

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1 Present Address: Assistant Professor, Dept. of Veterinary Public Health and Epidemiology, Bombay Veterinary College, Parel, Mumbai 400012.
2 Professor and Head, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar-263 145 (U. S. Nagar), Uttarakhand, India

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market are Tricel, Mukka, Durban, Lorsban, Agromil, Chlophos, Destroyer, Dhanwan, Dorson, Omexan, Panda and Bullet.

Keeping in view the lack of appropriate data on occurrence of residues in food and methodologies used for determination of pesticide residues in foods of animal such as milk and also considering hazardous effects of chlorpyrifos on public health, the present study was undertaken with objective of screening milk samples for chlorpyrifos residues collected from Tarai and Kumaon regions of Uttarakhand.

**MATERIALS AND METHODS**

**Collection of samples**

Milk samples (n=170) of cow, buffalo, doe and ewe were collected from the individual farmers, local milk distributing agencies, co-operative milk societies and retail outlets from Kashipur (18), Jawahar Nagar (43), Rudrapur (20), Pannagar (58), Kichha (15), Shantipuri (3) and Lakkuan (13). Samples were collected in clean, dry and neatly labeled sample containers and transported to laboratory in cold chain.

**Analysis of samples**

Samples were subjected to liquid-liquid partition as per the method detailed by Bottomley and Baker (1984) for the extraction and clean-up with suitable modifications.

**Extraction**

Ten milliliters of milk sample was added with 30 ml of acetone: methanol (1:1) mixture in a clean spout beaker of 100 mL capacity. The contents were homogenized with a high-speed blender (Polytron®) for 5 minutes and centrifuged at 9,744 g for 10 minutes at 4°C. The contents were filtered through Whatman # 42 filter paper and the filtrate was collected. The residues left on the filter paper were suspended in 10 mL of acetone:methanol mixture and once again homogenized and passed through the filter paper. The filtrates were combined and transferred to a 250 mL capacity separatory funnel. A volume of 50 mL of sodium sulphate (2.5%) and 30 mL of dichloromethane were added to the filtrates. The contents were then agitated vigorously for 2-5 minutes and kept undisturbed for 10 minutes. After layer separation, the lower organic phase was collected into a clean beaker. The upper aqueous phase was again partitioned with 20 mL of dichloromethane and lower organic layer collected.

The sodium sulphate columns were prepared in dichloromethane keeping the internal diameter and length of the column approximately 1 and 8 cms, respectively. The column was prepared carefully so that it does not contain any air bubbles. Before use, the columns were washed with 10 mL dichloromethane. Combined lower organic phases were dehydrated on sodium sulphate columns. The dehydrated extract was evaporated at room temperature under gentle stream of air to get about approximately 10 mL of the final concentrate. The contents so obtained were further cleaned up by alumina column chromatography.

**Clean up by Alumina column chromatography**

In order to eliminate the co-extracts, the alumina chromatography was performed. The columns were prepared by packing the slurry consisting of aluminium oxide (10 g) and dichloromethane (20 mL) in the burettes (dia x length). All possible care was taken to avoid trapping of air bubbles in the columns. The extracts containing pesticide residues were passed through alumina columns until the liquid level reaches the top of the column. Finally, the columns were eluted with 10 mL of dichloromethane and the contents were subjected to complete evaporation under the gentle stream of air. After evaporation the contents were reconstituted in 1 ml acetonitrile and filtered through 0.22 μm millipore filter. A total volume of 20 μL from each sample was injected to the HPLC system for the detection and quantification of pesticide residues.

**Detection and quantification of chlorpyrifos residues by HPLC**

The HPLC system (Perkin Elmer® Model Series 200) comprising of Quaternary LC Pump 200 Q, auto sampler, Diode Array Detector (DAD) and Peltier Column Oven along with reverse phase column (LichroCART LiChrospher 100 RP-18e) end capped 5 m (250 mm × 4 mm) was used for the analysis of the pesticide residues.

**HPLC conditions: An isocratic mobile phase of acetonitrile**

Water (65: 35, v/v) was used. The flow rate was kept at 1 ml min⁻¹. Chromatography was performed at 40°C using Diode Array Detector (DAD) at 202 and 220 nm and the reference wavelength was kept at 360 nm. The
chromatograms were analyzed by ‘Total Chrom’ software.

**Quantification of chlorpyrifos residues**

Standard calibration curve: In order to prepare a stock solution of 100 μg mL⁻¹ concentration, 5.0 mg of pure chlorpyrifos (98.5%) was dissolved in 50 mL of acetonitrile. One ml of stock solution containing 100 μg chlorpyrifos was further diluted in 9 mL acetonitrile so as to get 10 μg mL⁻¹ concentration. Two fold dilutions of this solution were made to obtain 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 μg mL⁻¹ concentrations. An aliquot of 20 μL of these concentrations were injected into the HPLC system and a standard curve was obtained by plotting concentrations versus the peak areas.

**Recovery Analysis**

Method of Ioeger and Smith, (1993) was followed with slight changes for the estimation of percent recovery of pesticide residues in the samples of milk, feed, fodder and water. The samples free from the pesticide residues were fortified with different known concentrations of standards to calculate the percent recovery. This recovery percentage was used for the estimation of actual concentration of the pesticide residues in the samples. The values of the percent recovery and the correction factor were derived for each specimen. The final concentration of the pesticide was expressed after multiplying the concentration found by the proposed method with the suitable correction factors.

Calculation of percent recovery: Recovery of pesticide residues were estimated by employing the following formula:

\[
\text{Recovery} \% = \left( \frac{N \sum xy - (\sum x)(\sum y)}{N \sum x^2 - (\sum x)^2} \right) \times 100
\]

Where,

- \(x\) = amount of standard pesticide
- \(y\) = amount of pesticide found by the proposed method
- \(N\) = number of observations

**Correction Factor**

Correction factor (C.f.) for a particular residue was calculated by the following formula for each of the residual concentration (Leoni et al. 1992).

\[
\text{C. f.} = \frac{100}{\text{Percent recovery}}
\]

**Statistical analysis**

In the present study, mean as a measure of central tendency and range as a measure of dispersion was employed for the statistical analysis of data as described by Das, (2000). Values of range and means for the residues of chlorpyrifos in test samples were calculated.

**RESULTS AND DISCUSSION**

Based on the survey conducted on usage of pesticide in Pantnagar and adjoining areas (Simon, 2003), chlorpyrifos was selected for the present study. With a view to develop simple and sensitive method for the extraction and clean-up of chlorpyrifos residues from milk the procedure of Bottomley and Baker (1984) was used with suitable modifications. The modified procedure reduced volume of the extracting solvents without affecting the recovery of the pesticides; thereby reducing sample processing cost and time.

Milk samples were superlatively screened for the presence of chlorpyrifos residues and only negative samples were spiked with known concentrations of standard chlorpyrifos. Fortified samples (containing different concentrations of standard chlorpyrifos) were subjected to extraction, clean-up, and quantitation of chlorpyrifos residues. The retention time, recovery percentage and correction factors were calculated using standard chlorpyrifos. Chlorpyrifos appeared as a clear cut symmetrical peak at 16.4 minutes in the chromatogram. The recovery percentage of 90.77% was attained during extraction and clean-up. Hence, in order to compensate the residual loss during sample preparation a correction factor of 1.101 was applied so as to express the final residual concentration.

Of the total of 170 milk samples analyzed 8 (4.7 %) samples were detected positive for chlorpyrifos residues with the mean residual concentration of 0.092 μg mL⁻¹ in samples collected from cow, buffalo, doe and ewe. Further, most of positive samples had residual concentration above the prescribed MRL of 0.02 mg kg⁻¹ (Table 1-2).

The prevalence of 4.7% seen this study is in accordance with the result of Misra (2001); who
recorded the mean residual concentration of 0.286 μg mL⁻¹ which is higher than the present study (0.092 μg mL⁻¹). Species-wise distribution of chlorpyrifos revealed chlorpyrifos residues in 2.88% of cow milk and 8.89% of buffalo milk; similar observations have been made by Misra (2001). Chlorpyrifos was detected as a major contaminant in milk (Gazzotti et al. 2008); it was detected up to a very high level of 5 to 18 mg kg⁻¹.

The residues in the milk are consequent to the presence of pesticides in the soil (Katpal et al. 1992), water (Kumari et al. 1996), feed and fodder (Battu et al. 1980; Kannathasan and Regupathy, 1992 and Ahuja and Awasthi 1993); that get entry directly or indirectly animal. Their lipophilic nature makes partition into lipid rich milk (John et al. 2001). Further, chlorpyrifos is known to be excreted in the milk if animals consume contaminated feed or fodder (Claborn et al. 1968; Ivey et al. 1968; Johnson et al. 1969; Leshchev et al. 1972). Pesticide residues accumulate in food chain linked by animal derived foods such as milk and meat thereby reaching humans leading to health hazards (Pagliuca et al. 2005; Muhammad et al. 2010).

Results of this study indicate need for creation of awareness among farmers about the spurious use of pesticides. The injudicious and indiscriminate of use of such agrochemicals would essentially lead to residues in food chain thereby jeopardizing the public health. A strong commitment both ideologically and at policy level on this issue is the need of the hour. Alternatively, possibility of natural or bio-pesticides needs to explored and encouraged in plant protection and public health programmes.

**CONCLUSIONS**

Present study was undertaken to determine residual concentrations of chlorpyrifos in milk (n=170) samples collected from various locations of Tarai and Kumaon regions of Uttarakhand. About 4.7% samples were detected positive for the chlorpyrifos residues using HPLC, with mean residual concentration of 0.092 μg mL⁻¹ and most of the positive samples contained residues above the prescribed MRL. However, results of HPLC analysis require to be further confirmed by GC-MS/MS so as to establish residues in sample matrix. Owing to effects on human, animal and the environmental health of pesticide residues need for education and awareness among farmers about extensive use of pesticide was envisaged.

**ACKNOWLEDGEMENT**

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<table>
<thead>
<tr>
<th>Place</th>
<th>Number of Samples analyzed</th>
<th>Number of positive samples (Percentage)</th>
<th>Residual concentration (μg ml⁻¹)</th>
</tr>
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<tr>
<td>Pantnagar</td>
<td>58</td>
<td>1 (1.72)</td>
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</tr>
<tr>
<td>Kichha</td>
<td>15</td>
<td>1 (6.66)</td>
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<td>Jawahar Nagar</td>
<td>43</td>
<td>1 (2.32)</td>
<td>0.063</td>
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<td>Kashipur</td>
<td>18</td>
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<td>Shantipuri</td>
<td>3</td>
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<td>ND</td>
</tr>
<tr>
<td>Lalkuan</td>
<td>13</td>
<td>1 (7.69)</td>
<td>0.268</td>
</tr>
<tr>
<td>Rudrapur</td>
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<td>3 (15)</td>
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<tr>
<td>Total</td>
<td>170</td>
<td>8 (4.70)</td>
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</tr>
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ND = not detected; * MRL of Chlorpyrifos = 0.02 mg kg⁻¹

<table>
<thead>
<tr>
<th>Species of animal</th>
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<th>Number of positive samples (Percentage)</th>
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<tr>
<td>Cow</td>
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<td>Buffalo</td>
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<td>Doe</td>
<td>16</td>
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<tr>
<td>Ewe</td>
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<tr>
<td>Total</td>
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<td>8 (4.7)</td>
<td>0.091</td>
</tr>
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</table>

ND = not detected; * MRL of Chlorpyrifos = 0.02 mg kg⁻¹
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REFERENCES


