*Pakistan J. Zool., vol. 49(5), pp 1761-1766, 2017.* **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.5.1761.1766

# Detection of Insecticide Residues in Honey of *Apis dorsata* F. from Southern Punjab, Pakistan

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**Article Information**
**Received 23 October 2016**
**Revised 11 January 2017**
**Accepted 25 March 2017**
**Available online 07 September 2017**

**Authors' Contribution**
**MAF performed the experiments and wrote the article, MH and MA conceived and designed the study and SA, MNA and MA help in data analysis**

**Key words**
**Insecticides, Residues, Raw honey, HPLC-UV.**

## ABSTRACT

A simple and fast analytical method using High Performance Liquid Chromatography with ultraviolet detection (HPLC-UV) was used to determine residues of commonly used agricultural insecticides (imidacloprid, thiametoxam, profenofos, endosulfan, spinosad and deltamethrin) in multi-floral raw honey of *Apis dorsata* F. collected from the cotton belt area of Punjab, Pakistan. The residues of these insecticides were extracted using ethyl acetate. Honey samples were spiked at 0.1 and 0.01 mg/kg. The mean recoveries of these insecticides in the spiked samples were 74-92% with relative standard deviation less than 20%. The residues of imidacloprid, endosulfan and deltamethrin were detected with a range of 0.005-0.055 mg/kg while other insecticides were not detected in any samples. The results obtained, point the urgent need to establish reliable monitoring programs in honey.

## INTRODUCTION

Application of insecticides on different crops has resulted in serious threat to non-target organisms and human health (Rambabu and Roa, 1994). Honeybees are good bio-indicators of different contaminants in environment due to close contact with insecticides and other chemical substances during their foraging on different flowering plants (Bozena, 2002). Insecticides are transferred to the honey by the bees, as they collect pollens and nectars from different plants, where these have been applied (Devillers and Pham-Delegue, 2002; Bogdanov, 2006). The widespread use of insecticides has created a need for their monitoring in different food products (Hussain *et al*., 2001).

Insecticide residues determination in bee products is necessary to monitor contamination for safe consumer health (Fernandez *et al*., 2002; Rissato *et al*., 2007), to assess the potential risk of this product to consumers. Maximum residue limit (MRLs) of different insecticides in different foodstuffs including honey has been set by European Union Commission (EU) for the safety of consumers and to regulate international trade (European Commission, 2006). The main objective of this study was to determine the residues of commonly used insecticides in honey samples of giant honeybee (*A. dorsata*) which were being used in cotton belt area of Punjab, Pakistan against different insect pests of field crops.

## MATERIALS AND METHODS

### *Chemical standards, reagents and solvents*

Certified analytical standards of profenofos, deltamethrin, imidacloprid thiamethoxam, spinosad and endosulfan (Table I) were purchased from their respective manufacturing companies. Their purity was > 98% except spinosad which was 88.4% pure. Distal water was obtained with the help of glass-distilled and further purified with the help of a Millipore Milli-Q water purifier. HPLC grade acetonitrile, ethyl acetate, Sodium chloride and anhydrous sodium sulphate. C18-bonded silica (50 µm), florisil (60-100 mesh) was purchased from Merck limited.

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0030-9923/2017/0005-1761 $ 9.00/0

**Table I.- List of insecticides with formulations, chemical names, activity, chemical groups and toxicity.**

| Trade name | Common name | Chemical group | Toxicity |
|---|---|---|---|
| Confidor @200 SL | Imidacloprid | Nicotinoid | II/WHO |
| Actara @25 WG | Thiamethaxam | Nicotinoid | III/WHO |
| Decis @2.5 EC | Deltamethrin | Pyrethyroid | II/WHO |
| Curacron @500 EC | Profenofos | Organophosphate | II/WHO |
| Thiodan @35 EC | Endosulfan | Organochlorin | II/WHO |
| Tracer @240 SC | Spinosad | Bio Insecticide | IV/WHO |

WHO (2000) and Fishel (2010).

*Collection of honey Samples*

For residues determination of insecticides, 16 samples of multi-floral raw honey of *Apis dorsata* F. were collected from four Districts including Multan, D.G. Khan, Layyah and Bahawalpur during the harvesting season of honey in 2013-14. Four samples were collected from each District, and then stored in dark at 10 °C until analysis.

*Standard stock solutions*

The insecticides standard stock solutions were individually prepared in acetone by dissolving 20 mg in 25 ml of solvent and were stored in a freezer at -18 °C. The stock standard solutions were used up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

*Residues extraction*

Rissato *et al*. (2004) method was used with some modifications for insecticide residues extraction with ethyl acetate.

*Cleaning of samples*

The samples were cleaned up by adding 0.5 g silica gel, 1g anhydrous sodium sulphate, 5g mixture of activated carbon and silica gel or florisil. The samples were passed through a chromatographic column and then the filtered eluate was received, the collected extracts were dried under a gentle stream of pure nitrogen ($N_2$). Then 1 ml of ethyl acetate was added to this eluate and was submitted to analysis by High Performance Liquid Chromatography equipped with ultraviolet (HPLC-UV).

*Method validation*

For validation, the parameters accuracy, precision, linearity and limits of detection (LOD) and limit of quantification (LOQ) were considered. The accuracy of the method was determined by recovery tests; using samples spiked at two different levels of 0.1 and 0.01 mg/kg with known concentration of the pure insecticides standard solution of each type and extraction and cleaning were performed as described earlier. The concentration of each insecticide in the final extracts was calculated. Recovery studies were performed to examine the efficacy of extraction and cleaned up. These recovery tests of insecticides were necessary to meet the requirements of the European Commission 31, which indicate that a method can be considered accurate and precise when the accuracy of data is between 70 and 110%, with Relative Standard Deviations (RSDs) not higher than 20%. Linearity was determined by different known concentrations which were prepared by diluting the stock solutions.

*Chromatographic separation parameters*

The HPLC-UV system was used for determination of insecticide residues (Alyaseri *et al*., 2012; Rao *et al*., 2012) in honey, consisting Shimadzu High Performance Liquid Chromatography with LC-20AT pump and SPD-20A and was interfaced with LC solution software, was equipped with a reversed Phase C-18 analytical column of 250 mm×4.6 mm and particle size 5.0 µm (Phenomenex). Column temperature was maintained at 30 °C. The injected sample volume was 20 µL. Mobile Phases A and B were acetonitrile and Milli -Q water (75:25(v/v)). The flow- rate used was kept at 1.2 mL/min. The detector wavelength was 230 nm. The external standard method was used for these analyses.

*Identification and calculation*

The compounds were identified by comparing the retention times of the samples peaks with that of the standard peaks and the amount of residues (mg/kg) was recorded in the integrator chart. The amount of residues in mg/kg is calculated as follows (Kumari *et al*., 2003):

$$\text{Residues (mg)} = \frac{\text{A1 x V1 x C}}{\text{A2 x W}}$$

Where, A1 is area of the sample in chromatogram, A2 is area of the standard in chromatogram, V1 is total volume of the sample in mL, C is concentration of analytical standard in ug/mL, W is weight of the sample in g and RF is recovery/response factor.

**Table II.- Recoveries (%) of insecticide residues in spiked samples of honey at 0.1 and 0.01 mg/kg with RSD %.**

| Insecticides | H.S. Rec. % (RSD) | L.S. Rec. % (RSD) |
|---|---|---|
| Imidacloprid | 92 (5.6) | 86 (5.8) |
| Thiamethaxam | 81 (6.1) | 82 (3.6) |
| Deltamethrin | 84 (11.3) | 77 (6.1) |
| Profenofos | 89 (6.7) | 83 (4.8) |
| Endosulfan | 81 (7.6) | 77 (3.8) |
| Spinosad | 78 (7.6) | 74 (4.3) |

Values are converted into percentage and RSD stands for relative standard deviation (if RSD is less than 20% the method is considered well for residue determination).

## RESULTS AND DISCUSSION

*Recoveries of insecticide residues*

The recovery tests of different insecticides were performed by the analysis of honey samples through method validation because validation is a prerequisite of any reliable chromatographic analysis (Levison *et al*., 1995). In this method linearity of calibration curve, sensitivity and selectivity of the solute detection, reproducibility, instrument precision, detection limit, quantification limit, and recovery of insecticide residues were performed (Lee *et al*., 1995). Honey samples were spiked with two different concentrations of pure insecticide standards at 0.1 mg and 0.01 mg/kg. The range of recoveries of these insecticides in the spiked samples was 74-92% and the range of Relative Standard Deviation was 3.8-11.3%. These values were quite satisfactory and met the requirements of the European Commission Regulations (2000) (Table II).

*Contamination results of real honey samples*

Table III shows residues of imidacloprid, thiamethoxam, deltamethrin, endosulfan, profenofos and spinosad in 10 out of 16 honey samples analyzed. Maximum contamination was observed with imidacloprid detected in 50% of samples, followed by endosulfan and deltamethrin, detected in 37.5% and 31.2% of samples, respectively. The residues concentrations of imidacloprid endosulfan and deltamethrin, ranged from 0.012-0.055, 0.007-0.026 and 0.01-0.023 mg/kg, respectively. Imidacloprid and endosulfan were detected at the highest levels of 0.55 and 0.26 mg/kg, respectively and levels exceeded maximum residue limits (MRLs) from the Districts of Bahawalpur and Multan based on European Commission (EC) Regulation in honey.

**Table III.- Insecticide residues detected in honey (mg/kg) from Southern Punjab.**

| S. No | Sample Code | Imidacloprid | Thiametoxam | Spinosad | Deltamethrin | Endosulfan | Profenofos |
|---|---|---|---|---|---|---|---|
| 1 | $DGK_1$ | 0.012±0.001 | Nd | Nd | Nd | 0.007±0.004 | Nd |
| 2 | $DGK_2$ | 0.033±0.001 | Nd | Nd | Nd | Nd | Nd |
| 3 | $DGK_3$ | Nd | Nd | Nd | Nd | Nd | Nd |
| 4 | $DGK_4$ | Nd | Nd | Nd | 0.023±0.01 | Nd | Nd |
| 5 | $MTN_1$ | Nd | Nd | Nd | Nd | Nd | Nd |
| 6 | $MTN_2$ | 0.018±0.015 | Nd | Nd | 0.019±0.01 | 0.008±0.06 | Nd |
| 7 | $MTN_3$ | Nd | Nd | Nd | Nd | Nd | Nd |
| 8 | $MTN_4$ | 0.013±0.01 | Nd | Nd | 0.015±0.01 | 0.026*±0.01 | Nd |
| 9 | $BWP_1$ | 0.026±0.022 | Nd | Nd | Nd | 0.007±0.002 | Nd |
| 10 | $BWP_2$ | 0.055*±0.03 | Nd | Nd | 0.01±0.003 | 0.007±0.002 | Nd |
| 11 | $BWP_3$ | Nd | Nd | Nd | Nd | Nd | Nd |
| 12 | $BWP_4$ | Nd | Nd | Nd | Nd | Nd | Nd |
| 13 | $LYH_1$ | 0.011±0.002 | Nd | Nd | Nd | Nd | Nd |
| 14 | $LYH_2$ | 0.043±0.024 | Nd | Nd | Nd | 0.009±0.021 | Nd |
| 15 | $LYH_3$ | Nd | Nd | Nd | 0.031±0.012 | Nd | Nd |
| 16 | $LYH_4$ | Nd | Nd | Nd | Nd | Nd | Nd |

Values are expressed as means ± standard deviation and are means of triplicate samples.*, residue exceeding MRL; Nd, not detected; DGK, D.G. Khan; MTN, Multan; BWP, Bahawalpur; LYH, Layyah.

The MRLs of pesticides, legally permitted in honey, has been established by different countries usually about acaricides. However, the European Union (EU) legislation has regulated the MRLs for different insecticides in honey (European Commission, 2006) which are given in Table IV.

**Table IV.- Maximum residues limits of insecticides in mg/kg, studied in honey.**

| Insecticides | MRL | Insecticides | MRL |
|---|---|---|---|
| Imidacloprid | 0.05 | Profenofos | 0.01 |
| Thiamethaxam | 0.01 | Endosulfan | 0.01 |
| Deltamethrin | 0.03 | Spinosad | 0.05 |

European Commission (2006).

Several researchers have studied the insecticides contamination in various honey samples collected from different regions of the world previously for monitoring of insecticide residues. In a previous study, 50 pesticide residues detected in 26 honeys from Jordan (Al-Rifai and Akeel, 1997). In a study conducted in Indian, 55% samples of honey were contaminated with residues of different class of insecticides (Anju *et al*., 1997) with a detection range of 0.01-9 mg/kg and from Spain, 38% contamination of honey was observed with different pesticides residues (Garcia *et al*., 1995). Previous investigations (Blasco *et al*., 2003) detected organochlorines, organophosphates and carbamates insecticides residues in honey from Portugal and Spain, with residues ranging 0.03-4.31 mg/kg. Contamination of honey with different pesticides has been also reported from France (Chauzat and Faucon, 2007) and Switzerland (Bogdanov *et al*., 2003).

It is difficult to compare our results with those of other monitoring programs from other countries, because the range of pesticides detected varies from many parts of the world (Blasco *et al*., 2004; Jimenez *et al*., 2002; Martel and Zeggane, 2002). The quantification limits of different insecticide residues detected in honey ranged from 0.1 to 0.6 mg/kg for organochlorin pesticides and from 5.0-25.0 ug/kg for organophosphates in a study from Aragon, Spain (Herrera *et al*., 2005). Residues of endosulfan, organophosphates and pyrethroids were detected in honey and were above MRLs previously reported from Brazil (Rissato *et al*., 2007). In developed countries, gas chromatography (GC) and liquid chromatography (LC) equipped with mass spectrometry (MS) are recently being used for the multi-residues detection and quantifications of pesticides in various monitoring programs of honey samples due to their potential to detect and quantify various pesticides relatively in a short time period with minimum extraction of samples. The current results of the contamination of honey with different insecticides residues from Pakistan, indicates that their presence is an alarming situation and there is an urgent need to monitor their contents in honey for consumers safety.

## CONCLUSION

The results obtained from the present investigations, clearly indicated that there is a significant contamination of insecticides in multi-floral honey produced from *Apis dorsata* F. in Southern Punjab. The maximum contamination was observed with imidacloprid.

## ACKNOWLEDGEMENTS

The author pays his special thanks to Prof. Dr. Anjum Suhail (Late) Ex-Chairman, Department of Entomology, University of Agriculture, Faisalabad due to his kind help for the completion of this research work and to Higher Education Commission (HEC) of Pakistan for providing financial support under Indigenous PhD Fellowship.

*Statement of conflict of interest*

Authors have declared no conflict of interest.

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