

Research Article



Analysis of Pesticide Residues in Pollen and Nectar Samples from Various Agricultural Areas of Pakistan through High Performance Liquid Chromatography

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Abstract | The present study was devised with an aim to assess pesticide risk toward honeybees (*Apis mellifera* L.) by detecting pesticide residues in pollen and nectar samples collected from various agricultural areas of Pakistan through high performance liquid chromatography analysis. Pollen and nectar were collected from 2016 to 2017 from different agricultural areas of Pakistan during a field survey. Pollen was collected by installing traps and nectar was collected through capillary method allied with centrifugation. The HPLC technique was used for fast and simple detection of pesticide residues of commonly used pesticides (imidacloprid, deltamethrin, chlorpyrifos, fipronil, thiamethoxam, carbaryl, profenophos and bifenthrin). Among a total of 100 pollen samples, 47% were found to be positive. The most frequently found residues were imidacloprid (11%), thiamethoxam (7%) and carbaryl (6%). Among all 100 nectar samples, 36% samples were positive and most abundant pesticides in nectar were imidacloprid (8%), thiamethoxam (6%) and fipronil (5%). In pollen samples, the residual level of imidacloprid and thiamethoxam was detected at a level of 0.032 - 0.76 ng/g and 0.055 - 0.78 ng/g, respectively while in nectar samples imidacloprid and thiamethoxam were detected at a level of 0.009 - 0.53 ng/g and 0.045 - 0.76 ng/g, respectively. The results obtained suggest careful monitoring of pesticide used in order to save honeybee population.

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Keywords | Pollen, Nectar, Honeybees (*A. mellifera*), HPLC, Pesticide residues

Introduction

The long term security of insect pollination for maximum food crops is a major concern around the world (Aizen et al., 2008; Potts et al., 2010). Beekeepers have suffered major decline of honeybee population (Engelsdorp and Meixner, 2010; Engelsdorp et al., 2011). The decline in their population is attributed to extensive use of various pesticides (Williamson et al., 2013). Pesticides used on crops can drift by wind on surrounding areas (Porrini

et al., 2003). Organochlorine pesticides persist in the environment and are bio-accumulated in pollens and plant tissues (Ruiz et al., 2018). Systematic pesticides are applied on seed coating. (Schmuck et al., 2001; Tapparo et al., 2011; Jeschke et al., 2011; Bonmatin et al., 2015). These pesticides travel through treated seeds to entire plant parts including flowers. Contaminated pollens and nectars taken up by foraging honeybees during foraging activities expose the hive bees to pesticides (Ornates et al., 2010). These residues are fed to the developing larvae and queen thus, possess

greater risk to the colony and make it vulnerable to colony collapse (Tapparo et al., 2012). Exposure of honeybees to pesticides through contaminated pollen and nectar consumption impairs their natural foraging behavior. Lately, owing to climatic vulnerability, the honeybee population in Pakistan is at a sharp decline. This has alarmed all the stakeholders equally (Irshad and Stephen, 2014; Nafees et al., 2008). To the best of our knowledge, no work has been reported regarding assessment of pollen and nectar pesticide residues effects on honeybees.

The present study was hence, devised with the main objective of assessing pesticide risk toward honeybees through detection of pesticide residues in their pollen and nectar samples collected from various agricultural areas of two provinces Punjab and Khyber Pakhtunkhwa, Pakistan using HPLC multi-residual analysis.

Materials and Methods

Study area

Pollen and nectar samples were collected from different agricultural areas of Pakistan viz. Khyber Pakhtunkhwa province (Peshawar and Swat Districts) and Punjab (Sargodha, Bhalwal, Sahiwal, Faisalabad, Multan and Lahore Districts). In selected areas, different pesticides were used for the control of pests. In Punjab, the selected apiary sites had wheat (*Triticum aestivum*), corn (*Zea mays*), Sarso (*Brassica compestris*) (crops), mango (*Mangifera indica*), orange (*Citrus sinensis*), ziziphus (*Ziziphus jujube*) and mulberry (*Morus alba*) orchards while in Khyber Pakhtunkhwa corn crops and orchards of apple (*Malus domestica*), almond (*Prunus dulcis*), cherries (*Prunus avium*) and peaches (*Prunus persica*) were present.

Pollen collection

At each studied site bee hives were placed. Pollen traps were installed in front of the hive. As foraging worker bees enter in the hive pollen grains dislodge from their body and fall in the trap draw. Pollen loads were gathered and stored in the glass vials at -20°C until analysis.

Nectar collection

Nectar was collected from flowers through capillary action (Stoner and Eitzer, 2012) allied with centrifugation. Sepals and petals were removed to exposed flower bases where nectarines were present.

Nectar drop was collected through capillary action of micropipette. In the same way, for small-sized flowers, nectar was collected through centrifugation. A small hole was made in the bottom of centrifuge tube with a needle. Flowers were placed in this tube and this tube was inserted into another centrifuge tube. Tubes were centrifuged at 12,000 rpm for 20 minutes until all nectar was collected at the bottom of the second tube. Nectar was collected with the help of micropipette and stored in PTFE line tubes at -20°C until analysis.

Extraction

Neonicotinoid pesticide residues were extracted by protocol of Chauzat et al. (2006). Pollen grains (10gram) were ground with water and few drops of acetonitrile were added to make it slurry. For imidacloprid and thiamethoxam analysis, samples were acidified by adding few drops of sulphuric acid (H_2SO_4). The slurry was centrifuged at 12,000×g for about 20 minutes. Supernatant was taken and filtered by passing through microfilter paper (0.45µm pore size). After filtration the sample was evaporated to dryness and dissolved dry sample residues in acetonitrile. They were re-filtered by passing through microfilters (0.45µm pore size). Resultant samples were stored in freezer at -20°C until analysis. Acetonitrile was used as extracting solvent for neonicotinoid residues extraction.

Similarly, for detection of other pesticide residues, similar procedure was performed except that the samples were not acidified by H_2SO_4 . For bifenthrin, deltamethrin and fipronil acetonitrile: water (76:24) was used as extractant solvent. Profenophos residues were detected by methanol: water (70:30) extractant. For chlorpyrifos and carbamate residue analysis, method of Manzoor et al. (2012) was used. For chlorpyrifos and carbamate, 10 grams of pollens were taken and slurry was made by adding water and acetone. Acetone: Water (70: 30) was used as extracting solvent for chlorpyrifos and carbaryl. Slurry was added to Erlenmeyer flasks (250ml) along with 5ml of acetone: water (70:30 v/v). All flasks were placed into the horizontal shaker incubator set at 170rpm and 28 °C for 2 hours for agitation. The extractant solvent (acetone: water) was evaporated by placing samples in rotary evaporator. To remove fat content and other organic debris, extracts were cleaned by passing through the separatory funnel. Aqueous phase eluted from separatory funnel collected and stored in

PTFE lined screw cap glass vials after filtration at -5 °C (to avoid degradation) until analysis.

HPLC analysis

All samples and mobile phase were filtered by passing through filtration assembly (microfilters 0.45 µm pore size) apparatus. Samples were sonicated at 31°C for 15 minutes. All extracted pollen and nectar samples were analyzed separately using HPLC (Varian 9012 pump) system. Validate HPLC instrument by using different combination of HPLC graded acetonitrile and water solvents. Acetonitrile/water (70:30 v/v) showed best extraction efficiency, giving straight base line without noise peaks. Finally, acetonitrile/water (70:30 v/v) was used as mobile phase for validation of HPLC instrument as well as pesticide residues in unknown samples. A reverse phase C18 column with 250 × 4.6mm internal diameter and 5µm particle size was used for pesticide analysis. Analysis was carried out under isocratic conditions at a flow rate of 1.0 ml/min, temperature 28°C and high pressure about 20Mpa. Injection volume for each sample was 10µl. The running time for each standard was 20 minutes. Peak analysis was carried out by using UV detector set at 270nm and 204nm.

Standard preparation

Technical grade of powder form of fipronil (Termadore from BASF), bifenthrin and deltamethrin (FMC Corporation, Pakistan), imidacloprid, thiamethoxam (Sigma-Aldrich), carbaryl, prophenophos, chlorpyrifos with (99.9% purity) were purchased from Ali Akbar Group of Industry. Standard stock solutions were prepared by dissolving standard in acetonitrile (0.5, 1, 1.5, 2 ng/ml).

Statistical analysis

Data collection and peak analysis were performed using Breeze chromatography workstation connected to a computer. Standard curve was plotted between absorbance and known concentrations (0.5, 1, 1.5 and 2 ng/ml) of pesticide standards as shown in Figure 1. Standard curve values were used as reference values to calculate the unknown pesticide concentrations in collected pollen and nectar samples. Percentages of contaminated pollen and nectar samples were calculated by dividing the number of positive samples by the total number of samples. Descriptive statistic was used to calculate minimum and maximum residual level in particular samples by using Graph pad prism (version 4). Limit of detection (LOD) and

limit of quantification (LOQ) values were calculated for all pesticides by using following formulas.

$$LOD = 3.3 (STD/slope) \text{ and } LOQ = 10 (STD/slope)$$

Amount of the pesticides was calculated from the peak area by using Equation 1, 2 and 3.

$$\text{Amount of analyte} = \frac{\text{Peak area}}{\text{Response factor}} = \text{sample amount} \dots (3)$$

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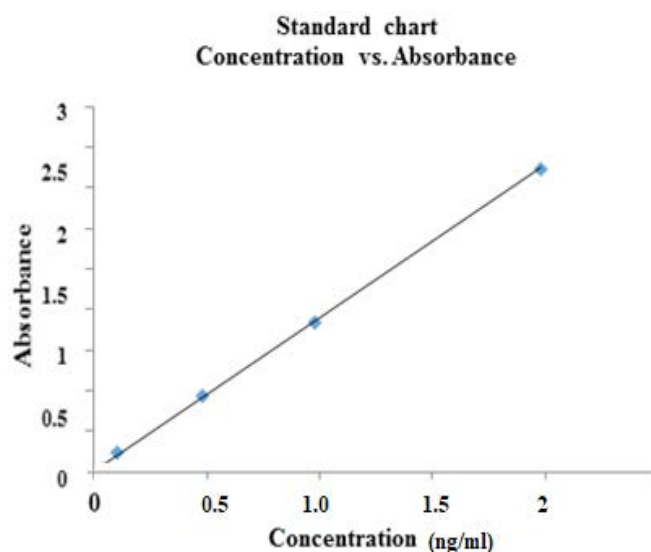


Figure 1: Standard curve between concentrations of pesticide standard and absorbance.

Table 1: Percentage frequency of pesticide residues detection in pollen grains and nectar samples.

Pesticide detected	Pollen samples N= 100	Nectar samples N= 100
Fipronil	3.0%	5.0%
Deltamethrin	2.0%	3.0 %
Chlorpyrifos	2.0%	4.0 %
Imidacloprid	11.0%	8.0%
Thiamethoxam	7.0%	6.0%
Profenophos	3.0%	ND
Carbaryl	6.0%	4.0%
Bifenthrine	2.0%	4.0%
Carbaryl + Thiamethoxam	4.0%	ND
Bifenthrin+ Imidacloprid	3.0%	ND
Bifenthrin+ Fipronil	2.0%	ND
Carbaryl+ Imidacloprid	2.0%	2.0%
Total	47%	36%

ND*: Not detected.

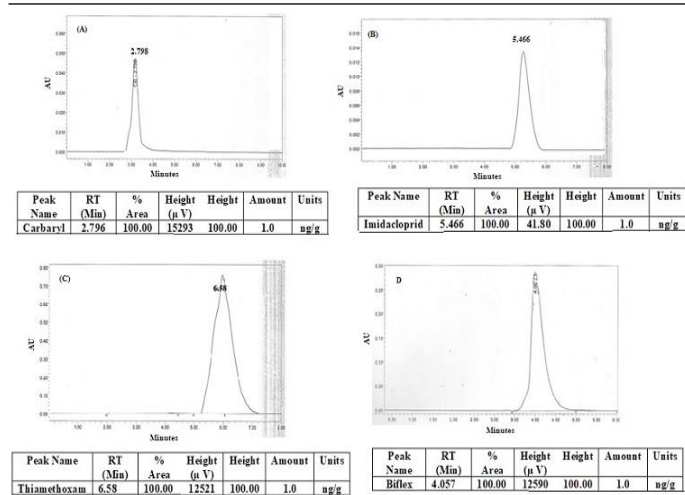


Figure 2A: Chromatograms of technical grade standard carbaryl (A), imidacloprid (B), thiamethoxam (C) and bifenthrin (D).

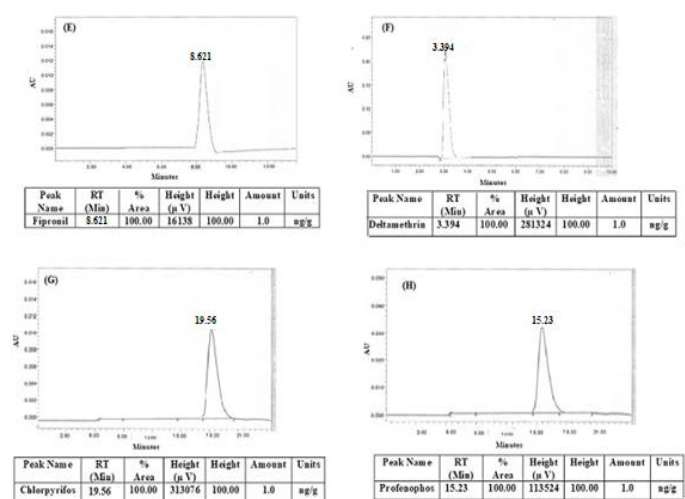


Figure 2B: Chromatograms of technical grade standard fipronil (E), deltamethrin (F), chlorpyrifos (G) and profenophos (H).

Results and Discussion

Total 100 pollen and 100 nectar samples were analyzed. Acetonitrile/water (70:30) was used as mobile phase for validation of HPLC instrument as well as pesticide residues in unknown samples. Chromatogram of technical grade standards obtained at optimized conditions were shown in Figure 2A and 2B. Among a total of 100 pollen samples, 47% were found to be positive. The most frequent residues were imidacloprid (11.0%), thiamethoxam (7.0%) and carbaryl (6.0%) as shown in Table 1. About 11% Pollen samples polluted with multiple pesticides residues. Mean level of pesticide residues in pollen samples is shown in Table 2. Pollen sample chromatograms were shown in Figure 3A and 3B. Retention time (RT) of unknown pollen sample peaks was compared with the RT of standard peaks. Chromatogram peak of unknown pollen samples appeared near to standard peak retention time (RT) were consider as respective

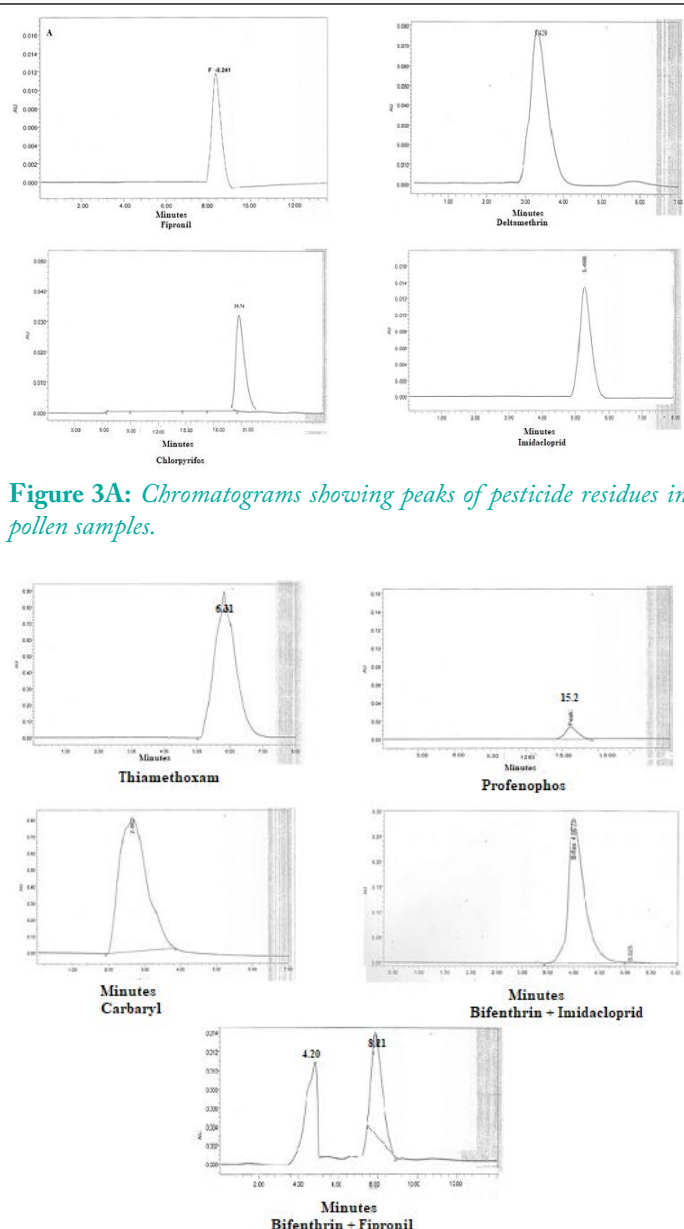


Figure 3A: Chromatograms showing peaks of pesticide residues in pollen samples.

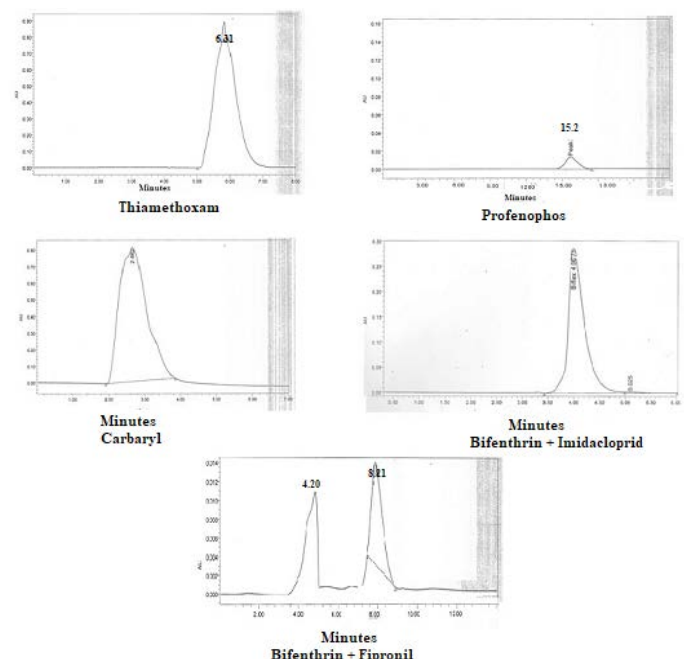


Figure 3B: Chromatograms showing peaks of pesticide residues in pollen samples.

pesticide residue. HPLC analysis showed that as carbaryl standard run, a peak appeared at 2.796 min retention time (RT). Any peak in unknown pollen and nectar samples appeared near to it was considered as carbaryl peak. Based on pollen HPLC analysis, carbaryl was detected at a level 0.032 - 0.60 ng/g. For imidacloprid and thiamethoxam, standard peaks appeared at 5.46 min and 6.58 min RT respectively. Residual level of imidacloprid and thiamethoxam was detected at a level 0.032 - 0.76 ng/g and 0.055 - 0.78 ng/g, respectively. Similarly, for bifenthrin standard peak seemed at 3.394 min RT. Analysis showed that bifenthrin and deltamethrin residues were detected at a level 0.001-0.20 ng/g and 0.007-0.54 ng/g, respectively. In the same way, fipronil

standard run a sharp peak appeared at 8.621min RT. The fipronil residue detected at a level 0.026-0.65 ng/g. Chlorpyrifos showed peak at 19.56 min and profenophos standard showed peak at 15.25 min RT. Analysis showed that chlorpyrifos and profenophos residues were detected at a level 0.025 - 0.56 ng/g and 0.02 - 0.40 ng/g respectively. LOD and LOQ values of insecticides residues detected in pollens were shown in Table 2. Box plot shows graphical representation of pesticide residues in pollen samples (Figure 5).

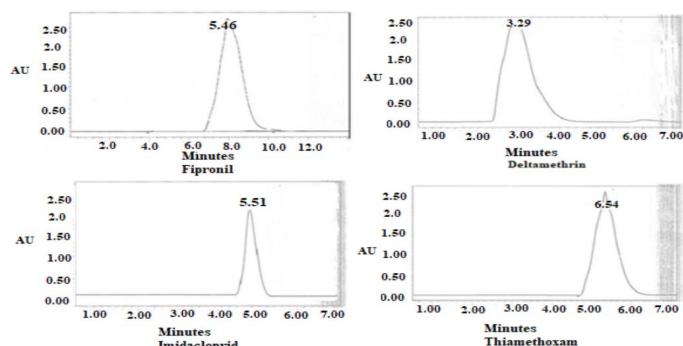


Figure 4A: Chromatograms showing peaks of pesticide residues in nectar samples.

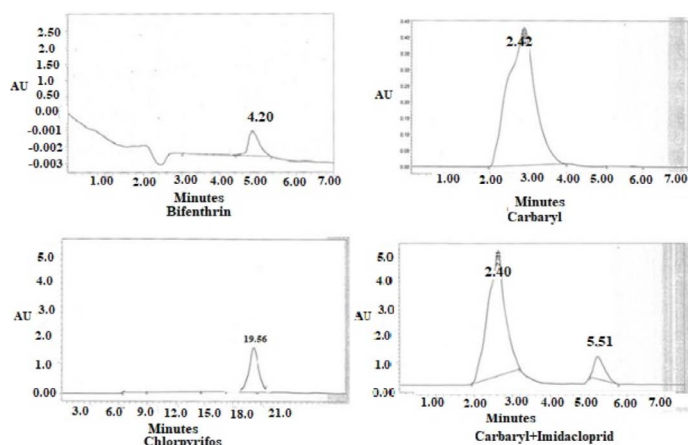


Figure 4B: Chromatograms showing peaks of pesticide residues in nectar samples.

About 100 nectar samples were collected and analyzed. Among all nectars, 36% samples were positive. The most abundant pesticide in nectar was imidacloprid (8.0%), thiamethoxam (6.0%) and fipronil (5.0%). Mean LOD and LOQ values of pesticides detected in nectar samples were shown in Table 3. For each pesticide residue analysis in nectar pesticide standard were run. Chromatogram peaks of unknown nectar samples were shown in Figure 4A and 4B. Retention time (RT) of standard peaks compared with RT of unknown sample peak. Peak of unknown nectar samples appeared near to standard peak retention time (RT) were consider as respective pesticide residue. HPLC results showed that in

nectar samples carbaryl residue was detected at a level (0.032-0.035 ng/g), imidacloprid (0.009-0.53 ng/g), deltamethrin (0.008-0.32ng/g), thiamethoxam (0.045-0.76 ng/g), bifenthrin (0.038-0.19 ng/g) and fipronil (0.056-0.19 ng/g). Box plot shows graphical representation of pesticide residues in nectar samples (Figure 6). Systematic pesticides (thiamethoxam and imidacloprid) were detected at maximum level in both type samples. While deltamethrin and bifenthrin were also detected at considerable amount.

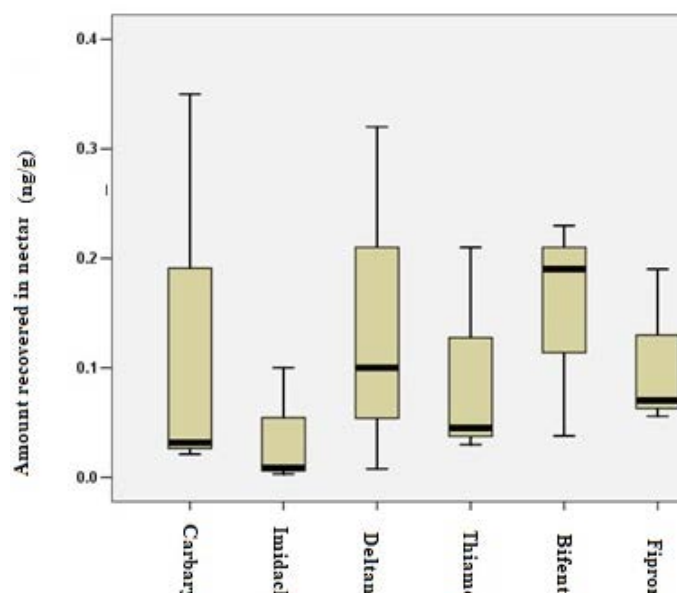


Figure 5: Box-plot comparison of pesticide residues detected in pollen samples through HPLC.

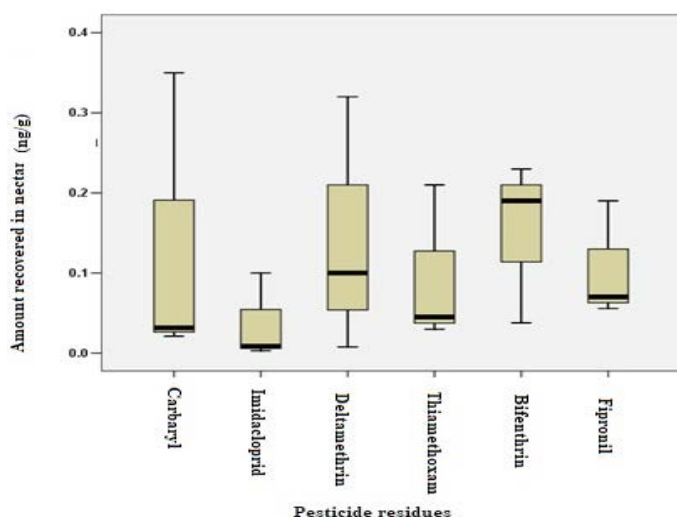


Figure 6: Box-plot comparison of pesticide residues detected in nectar samples through HPLC.

Pesticide usage for insect management has come a long way. Pakistan is an agricultural area. Various chemicals are used to manage pests and insects. Presence of pesticide residues in pollen and nectar samples is a serious concern as they directly affect pollinator. Pesticide residues in pollen and nectar are

Table 2: Mean, limit of detection (LOD) and limit of quantification (LOQ) values of pesticides residues detected in pollen samples.

pesticides detected	Mean (ng/g)	Min-max (ng/g)	Sum	Std. Error mean	Std. Deviation	LOD (ng/g)	LOQ(ng/g)
Carbaryl	0.668	0.032-0.60	4.161	0.070	0.252	0.0026	0.0020
Profenophos	0.377	0.02-0.40	0.723	0.085	0.170	0.0013	0.0020
Imidacloprid	0.751	0.032-0.76	3.876	0.075	0.261	0.0051	0.0016
Deltamethrin	0.933	0.007-0.54	2.091	0.135	0.359	0.0011	0.0013
Thiamethoxam	0.632	0.055-0.78	1.915	0.156	0.313	0.0018	0.0022
Bifenthrin	0.638	0.001-0.20	0.903	0.1005	0.2463	0.0012	0.0023
Fipronil	0.174	0.026-0.65	0.509	0.029	0.870	0.0015	0.0001
Chlorpyrifos	0.735	0.025-0.56	1.12	0.1709	0.3919	0.0018	0.0011

Table 3: Mean, limit of detection (LOD) and limit of quantification (LOQ) values of pesticides residues detected in nectar samples.

Pesticides detected	Mean (ng/g)	Min-Max (ng/g)	Sum	Std. Error mean	Std. Deviation	LOD (ng/g)	LOQ(ng/g)
Carbaryl	0.31	0.032-0.35	1.047	0.042	0.111	0.0012	0.0023
Imidacloprid	0.521	0.009-0.53	1.819	0.079	0.1941	0.0013	0.0023
Deltamethrin	0.321	0.008-0.32	1.210	0.067	0.231	0.0011	0.0027
Thiamethoxam	0.725	0.045-0.76	2.525	0.1259	0.2816	0.0045	0.0012
Bifenthrin	0.152	0.038-0.19	0.444	0.0376	0.0752	0.0030	0.0009
Fipronil	0.425	0.056-0.19	0.465	0.064	0.153	0.0048	0.0015

taken by forager bees to their colonies and remain in the hive food for quite some time. These residues are then fed to the larvae and queen which are affected in similar ways as the forager bees (Sanchez and Goka, 2014). This seems evident through the results harvested from the present study.

In the present study, systematic pesticide residues were detected at a highest level. Pollen and nectar samples collected from areas of Punjab province contained more pesticide residues as compared to those from KPK province. In Punjab cotton and wheat are major crops, on which various chemicals are used to control pests at different growing stages, while in KPK mostly selected areas had fruit orchards which were sprayed before flower blooming. Previous study also supported these results. Hayat et al. (2018) stated that higher proportion of pesticides being used in Punjab Province (88.3%) followed by Sindh (8.2%), Khyber Pakhtunkhwa (2.8%) and Balochistan (0.76%). These results correlated with those of Blacquiere et al. (2012). They demonstrated that bees exposed to neonicotinoid pesticides toxicity via contaminated pollen and nectar ranged between 0.9-3.1 ng/g. Similarly, in another study, imidaclopid residues ranging from 2 to 5 ng/g in pollen and >1.5 ng/g in nectar of treated seed of corn, sunflower and rape (Maus et

al., 2003; Bonmatin et al., 2005) poses sublethal and lethal effects toward forging honeybees. In present study imidacloprid (0.751 ng/g) and thiamethoxam (0.632 ng/g) residue level in pollen level were close to reported values and showed potential threat of sublethal toxicity toward foraging and nurse bees. In another study Pohorecka et al. (2012) studied sublethal toxicity of thiamethoxam treated seeds toward bees. Consumption of thiamethoxam pesticide residues intake (0.4- 3.3 ng/bee/day) were lead to disruption of learning and memory. Similarly, in another study residue of chlorpyrifos at a level of 0.072 µg were meet oral LD₅₀ (Sanchez and Goka, 2014). Similarly, consumption of fipronil active ingredient in the range of 0.07- 0.15 ng were had deleterious effects toward honeybee learning performance. Literature showed that consumption of 33 grams of contaminated pollen by one individual would be needed to meet the oral LD₅₀ (Chauzat et al., 2006). Brood and adult bees fed with pollen or bee bread and are directly and indirectly exposed to pesticide residues. Different researchers have been focused on quantifying the amount of pollen needed to rear a larvae. It had been shown that worker honeybee larvae required average 86 mg of maize pollen for complete development (Babendreier et al., 2004). So, bees consumed contaminated pollen and nectar in such quantity would be enhanced

possible risk of pesticide poisoning in forging, nurse and larvae honeybees. Similarly, [Chauzat et al. \(2006\)](#) conducted pesticide residue analysis in pesticides. They were conducted a field survey to monitor honeybee (*A. mellifera*) population decline in France, multiple residual analysis was done on collected pollen grains. About 19 pesticide residues were found in pollen samples. [Boily et al. \(2013\)](#) evaluated that in Quebec rapid decline of honeybee (*A. mellifera*) population attributed to pesticide exposure. Another study conducted by [Bonmatin et al. \(2005\)](#) for the quantification of pesticide residues in maize crops. It was concluded that maize crops and flowers contained pesticide residues at a level sufficient to cause bee mortality. Pesticide used for treatment of seeds can be transported throughout growing plants and contaminate the nectars and pollens. The presence of imidacloprid residues (3µg/kg) in pollen grains has been reported in Gaucha seed dresses sunflower ([Bonmatin et al., 2003](#)). Similarly, a survey of pesticide residues in pollen loads was conducted in France. Survey report was showing the presence of imidacloprid and its metabolites nicotinic acid (49% of 81 analyzed samples), Fipronil and its metabolites fipronil sulfone and fipronil disulfonil (12% of 81 analyzed samples) ([Chauzat and Faucon, 2007](#)). In present study level of pesticide residues in nectar samples less compared to pollens, as depending upon the treatment regimen and type of crop ([Kyriakopoulou et al., 2017](#)). Pollen grains were collected from corn, wheat and brassica crops, as these crops have large proportion of pollen compared to nectar and frequently sprayed at different developmental stages. While nectar collected from ornamental and fruit flowers which were less likely sprayed with pesticides after blooming. These results correlated with [Dively and Kamel \(2012\)](#). They measured neonicotinoid residues in pollen and nectar from pumpkin crops. Results showed that in nectar pesticide residues were 73.5 to 88.8% less than pollen residues. In present study deltamethrin and bifenthrin detected in considerable amount. These pesticides are lipophilic in nature and easily absorbed in plant body. The presence of pesticide residues in pollen and nectar not only caused mortality but also lead to sublethal toxicity such as disrupt normal flight, learning and memory ([Decourtye et al., 2005](#); [Aliouane et al., 2009](#)). Exposure of bees to low levels of pesticide residues can elicit sublethal toxicity, not killing them outright but affecting their behavior and immune system ([Desneux et al., 2007](#)). So, it can be infer from present study pesticides which were detected even at

low level can induce sublethal toxicity toward bees.

Conclusions and Recommendations

In conclusion, this study has been demonstrated the presence of wide range of pesticides in nectar and pollen grains collected by honeybees. These pesticides were found at various concentrations and provide possible route of exposure. Systematic insecticides were detected at highest residual level and imidacloprid was most persistent pesticide in all collected samples. Imidacloprid and thiamethoxam residues level in pollen and nectars were close to previously known sublethal values. Other pesticide residues were also detected in considerable amount. Thus, represent a serious concern in agriculture sector of Pakistan as they possess greater risk to honeybee colony development.

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Novelty Statement

This is a novel approach to monitor the level of pesticide residues in pollen and nectar because contaminated nectars and pollen grains are used as food source by hive bees, therefor have deleterious effects on brood and colony development. The developed analytical procedure (HPLC) was suitable for the detection of pesticides in pollen and nectar.

Author's Contribution

Mahnoor Pervez: Collected the samples, designed methodology, analysed the data and wrote the article.
Farkhanda Manzoor: Supervised the research, facilitated in sample collection, helped in article writing.

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