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ASSESSMENT OF PESTICIDE RESIDUES IN HONEY SAMPLES FROM ONION FIELDS AND MARKETS OF MAHARASHTRA

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Abstract: A roving survey was conducted to monitor the pesticide residues in honey samples collected from the honey bee colonies kept in onion fields and the markets of Nashik, Pune and Ahmednagar districts of Maharashtra, India during 2023. A total of 30 field samples and 9 market samples were collected and analysed for a total of 201 pesticide residues using MRM LC-MS MS. The result indicated that five samples among 39 were detected with the pesticides above quantification limits among which two were market samples. No pesticide residues were found in the samples gathered from Nashik district. However, samples collected from Pune district revealed the presence of spinosad (0.013mg/kg), fipronil (0.275 mg/kg) and carbendazim (0.012mg//kg). In Ahmednagar district, the samples showed the presence of imidacloprid (0.016 mg/kg), IN-J9Z38 (a metabolite of cyantraniliprole) (0.060), and carbendazim (0.018mg/kg). Given the escalating concern over food contaminants, including pesticide residues in honey, farmers may encourage to follow good agricultural practices (GAP) for ensuring food safety and safeguarding public health.

Index Terms - Residue, Onion, Honey, Seed production, Insecticides, Pesticides.

I. INTRODUCTION

India is the second-largest producer of onion in the world. Indian onions are famous for their pungency with worldwide demand. The country has exported 2.53 million MT of fresh onion to the world, worth Rs. 4,522.79 crores/ 561.38 USD millions during the year 2022-23 (APEDA, 2023). Maharashtra state contributes 43.31 % share in countries total onion production with 11.36 million tonnes (Anon., 2020). Nashik, Pune and Ahmednagar districts of Maharashtra put up a major onion production of state for domestic consumption as well as for export purposes. Onion being a highly cross-pollinated crop relies on insect pollinators for its reproductive success and seed set and honey bees are the dominant pollinators aiding pollination services in onion crop (Pushpalatha *et al*., 2023).

Onion crop on other hand is prone to attack of various insect pests and diseases causing major reduction in yield and triggering economic losses. Therefore, the application of pesticides is inevitable for their management which bring about various environmental concerns including the resurgence of insect pests (Begum *et al*., 2017), toxicity to non-target and beneficial organisms (Bommarco *et al*., 2011) and residues in the produce (Donkor *et al*., 2016). As honey bees regularly visit the flowers during the bloom to collect nectar and pollen, the residue of pesticides sprayed get transferred via nectar to honey, which further possess serious concern to human health as well as bees. Usually, the presence of pesticide residues in honey occurs when bees forage on crops treated with different agricultural chemicals or when beekeepers use chemicals to control pests or diseases affecting bees (Bogdanov, 2006). Several researchers have already documented the existence of various pesticide residues in honey, with concentrations varying across studies (Bargańska *et al*., 2013). This underscores the importance of continual monitoring to ensure the detection of pesticide residues in honey and

to assess potential health risks for both bees and humans (Blasco *et al*., 2011). Furthermore, ensuring the quality of honey as a food or medicinal product is crucial and should never be compromised (García-Chao *et al*., 2010). Therefore, the present study aims to monitor the pesticide residues which may be present in honey samples collected from honey bee colonies installed at onion fields for pollination services and also from the surrounding markets.

II. MATERIALS AND METHODS

2.1 Sample collection

A survey was conducted to collect honey samples from honey bee colonies placed in onion fields during the flowering season for pollination services during *rabi* 2022- 23. The survey also included the collection of market samples. A total of 39 honey samples were gathered from various locations in three districts of Maharashtra, namely Nashik (19.99745 N and 73.78980 E), Pune (18.516726 N and 73.856255 E), and Ahmednagar (19.101053 N and 74.740677 E). Among these samples, 30 were procured from the fields, with 10 samples from each district. The remaining nine samples were from the market, with three samples each from three different brands, from each district. All honey samples were carefully placed in clean bottles, labeled, and stored in an ice chest at 4°C. They were then transferred to the laboratory and kept at -20°C until analysis. The sample size for each honey collection was at least 200 g. The analysis of the samples for pesticide residues was carried out at the National Referral Laboratory project of Agricultural and Processed Food Products Export Development Authority (APEDA), ICAR-NRCG, Pune, Maharashtra, India.

2.2 Chemicals and Reagents:

Certified pesticide standards with a purity range of 94–99 % were procured from Sigma Aldrich, located in Pune, Maharashtra, India. To prepare individual stock solutions of pesticides, concentrations ranging from 250 to 1500 µg/mL were dissolved in acetone, acetonitrile, methanol, or dimethylformamide, and these solutions were stored in amber screw-capped glass vials at a temperature below −20°C. For validation and calibration purpose, mixed standard solutions were prepared by appropriately diluting the stock standard solutions with acetonitrile. Additionally, an internal standard spiking solution was prepared in acetonitrile with concentrations ranging from 0.5 to 50 µg/ mL. Acetonitrile (HPLC Grade), acetone, and methanol were provided by Avantor Performance Material Ltd. in Mumbai, Maharashtra, India. Dimethyl formamide, ammonium formate, formic acid, and sorbents for the clean-up step, namely PSA (Primary Secondary Amin Sorbent) and anhydrous magnesium sulfate (grit), were obtained from Sigma Aldrich in Pune, Maharashtra, India. Deionized water was sourced using the LabLink system by LABAQUA.

2.3 Standard preparation:

Precisely weighed 10 mg of each individual analytical grade insecticide was dissolved in a 10 ml volumetric flask using an appropriate solvent to create a standard stock solution with a concentration of 1000 mg/kg. Further dilutions of the standard stock solution were made to obtain intermediate concentrations ranging from 0.5 to 50 µg/ mL. These intermediate solutions were stored in a refrigerator at -20°C for preservation. From the intermediate standards, working standards with concentrations of 0.50, 0.20, 0.10, 0.05, 0.002, and 0.001 mg kg^{-1} were prepared by diluting the stock solution suitably in acetonitrile for all 201 pesticides. These working standards served as the standard check in residue determination, linearity, and recovery studies.

2.4 Method validation:

Before conducting the analysis of field samples, method validation was performed to ensure the suitability and reliability of the analytical procedure for the specific test, following the guidelines outlined in SANTE 11312/2021 (EU, 2022). In this validation process, honey samples that had already been analyzed and confirmed to be free from pesticide residues were utilized as blanks. These blank samples were spiked with aliquots to carry out validation studies and prepare the procedural standard calibration. Various validation parameters, including Limit of Detection (LOD), Limit of Quantification (LOQ), specificity, linearity, and recovery, were determined prior to the analysis of the actual samples. For the linearity assessment, all 201 insecticides were analyzed using LC-MS/MS at five different concentration levels ranging from 0.001 to 0.1 mg/kg, and each analysis was duplicated. To ensure accuracy and precision, the percent mean recovery and relative standard deviation were calculated before the actual sample analysis. Additionally, the matrix effect (ME) was examined by comparing the slope obtained from the procedural standard calibration curve with that of the solvent standard calibration curve, following a specified equation.

$$ME\% = \frac{\text{Slope of calibration curve in matrix}}{\text{Slope of calibration curve in matrix}} * 100$$

a. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The tested insecticides demonstrated a limit of detection (LOD) of 0.01 mg kg-1, which was determined based on the signal-to-noise ratio of the compound in comparison to the background noise (3:1) obtained from the blank sample. The limit of quantification (LOQ) was established as the lowest concentration of the compound that can be accurately and precisely quantified. To evaluate the trueness and precision, honey samples were spiked with pesticides at four different levels: 0.001, 0.005, 0.01, and 0.1 mg/kg, with two replications for each level.

b. Specificity

Specificity studies were performed by spiking the honey sample and reagent blank with working standards at the concentration of 0.05 mg kg^{-1}. The area of sample and reagent blank was compared with spiked matrix match area.

c. Recovery Studies

To validate the analytical method for estimating pesticide residues in the collected honey samples, recovery studies were conducted using control samples of honey. In this process, five grams of the control honey sample were taken in 50 ml centrifuge tubes, and two replicates were prepared. These control samples were then spiked with a standard mixture of all 201 pesticides at different fortification levels, including the Limit of Quantification (LOQ), 5 times LOQ, and 10 times LOQ. The required fortification levels were achieved by adding an appropriate volume of the working standard with a concentration of 10 mg kg-1. After spiking the samples, they were thoroughly shaken to ensure proper homogeneity of the insecticides within the honey. The subsequent extraction and cleanup procedures were carried out according to the specified protocols. To assess the accuracy of the method, the percent recovery was calculated using the following formula.

$$\text{Per cent recovery} = \frac{\text{Quantity of insecticide recovered}}{\text{Quantity of insecticide injected}} \times 100$$

2.5 Sample Extraction and clean-up procedures

To prepare the honey sample for analysis, 5 g of the honey sample was combined with 10 mL of deionized water and 10 mL of Acetonitrile with 1% Formic Acid in a 50 mL centrifuge tube. The mixture was vortexed for 3 minutes to achieve a homogenized honey mixture. Next, a buffer salt mixture comprising 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride was added to facilitate partitioning. The sample was vortexed for an additional 2 minutes, followed by centrifugation at 5000 RPM for 5 minutes. After centrifugation, 1 mL of the supernatant was transferred to a 15 mL centrifuge tube containing 150 mg of $MgSO_4$ and 50 mg of PSA for clean-up. The clean-up step involved shaking the sample for 5 minutes in a micro-centrifuge, and then 500 µl of the final extract was transferred to another Eppendorf tube. To this, 500 µl of deionized water was added, and the mixture was filtered into an injection vial through a 0.22 µm PTFE filter for LC–MS/MS analysis. For samples showing residues above the highest calibration standard, dilutions were performed, and the samples were injected again to ensure they fell within the linear dynamic range. The residues in such samples were quantified using an appropriate dilution factor during analysis.

2.6 LC–MS/MS analytical conditions:

The chromatographic separation was carried out using a Luna 3 µm Phenyl-Hexyl 150x2.0 mm column, employing water with 5 mM ammonium formate (pH = 6.0, adjusted with formic acid) and acetonitrile as mobile phases. The flow rate was set at 400 µL/min, and the column temperature was maintained at 50 °C. A gradient elution method was applied, beginning with 95% water mobile phase, held for 1 minute, followed by a decrease to 5% over 25 minutes, and then held at 5% for 6.5 minutes. Subsequently, the mobile phase was increased back to 95% and held constant until the end of the 40-minute analysis. The injection volume used was 2 µL.

For mass spectrometric analysis, the AB Sciex QTRAP® 6500 LC–MS/MS system equipped with Turbo Spray Ion Drive was utilized, allowing for both positive and negative ionization. The scheduled Multiple Reaction Monitoring (MRM) advanced mode was employed, with time windows generally set at 60

s (±30 s) from the retention time. The ion spray voltage was set at 5500 V for positive ionization and −4500 V for negative ionization. The source temperature was maintained at 550 °C. Nitrogen was used as the curtain gas (30 psi), collision gas (medium), and for the ion source gases, including nebulizer gas (50 psi) and heater gas (55 psi).

2.7 Residue Determination

Quantitative and qualitative analysis were conducted using MultiQuant software version 3.0, which relied on two highly intense precursor ion-product ion MRM transitions. The calculated analyte concentrations were obtained using Analyst® 1.7.1 software. To account for the sample weight and dilution volume, the software applied the appropriate dilution factor. Linear calibration curves were utilized for both quantification and data analysis purposes.

III. RESULTS AND DISCUSSION

Thirty samples of honey collected from onion seed production fields and 9 samples from the markets were analyzed for the presence of 201 pesticides using the initially validated method. Information on concentration levels of the pesticide residues found in honey samples are depicted in Table 1. The pesticide residues were found in 10 per cent of the field collected honey samples and 22.2 per cent of the market samples. The insecticides fipronil and spinosad were found in the samples collected from Malthan and Kanhar Mesai villages of Pune district respectively. Imidacloprid and IN-J9Z38 (metabolite of cyantraniliprole) were found in a sample collected from Baburdi village of Ahmednagar district. The fungicide carbendazim was reported from the samples of same brand collected from Pune and Ahmednagar districts.

Fipronil and imidacloprid residues in the honey might be the result of usage of these pesticides against thrips and other sucking pest's management in onion crop. Spinosad and cyantiniliprole might had used to manage the *Helicoverpa armigera*, *Spodoptera litura* and *Agrotis* sp. the pests which reported to be the major pests of onion. Nonetheless, the presence of residues in the honey could also be attributed to the use of pesticidal sprays in nearby crop fields, as bees often travel over a distance of 1.5 kilometers or more to gather nectar and pollen (Koetz, 2013). The carbendazim found in the market samples could potentially be linked to disease management practices in the apiaries and their vicinity, given that there were no indications of carbendazim being sprayed on onion crops during the survey.

The results are in line with Gaweł *et al*. (2019) who reported a total of 21 pesticides *viz*., acetamiprid, thiacloprid, DMF, DMPF, azoxystrobin, tebuconazole, carbendazim, dimethoate, coumaphos, cyproconazole, boscalid, flutriafol, tau-fluvalinate, tetraconazole, propiconazole, difenoconazole, diazinon, dimoxystrobin, p,p′- DDD, lindane, and prothioconazole-desthio in analyzed honey samples among which carbendazim was reported from 38% of samples. Bargańska *et al*. (2013) reported that among the 45 samples of honey collected from the apiaries located across the Pomerania (Poland), 29 per cent of the samples were detected with pesticide residues and the concentration of bifenthrin, fenpyroximate, methidathion, spinosad, thiamethoxam, and triazophos exceeded maximum residue levels (MRL) in five samples (11%) (14.5, 16.3, 25.7, 20.6, 20.2, and 20.3 ng/ g respectively).

The samples analyzed in the present study were found to be less contaminated by pesticides than the previous studies of residue analysis in various vegetables (Gaweł *et al*. 2019; Bargańska *et al*. 2013; Nishant & Upadhyay, 2016). However, it is difficult to compare our result with those of other monitoring programs from other countries due to several reasons including change in the geographical area, cropping pattern, pesticides usage patter and range of pesticides considered for monitoring are different.

Bees show greater susceptibility to the pesticides even at a lower concentration due to their smaller size and larger surface area (Arena and Sgolastra, 2014). As they are social insects, the chemical poison has all the possibilities to get shared among the colony members which could cause ill effects on the whole colony including decreased foraging activity, aggression, impairing chemosensory responses, weakening the immunity, reproductive failure further in severe cases the whole colony may collapse which is usually referred as colony collapse disorder (CCD) (Tan *et al*., 2015; Wright *et al*., 2015; Christen *et al*., 2016; Pushpalatha *et al*., 2023). Several reports indicate that neonicotinoids usage on the crops where bees visit is one of the main reasons for CCD (Bekić *et al*., 2014).

When it is concerned about humans, these pesticides may induce reproductive toxicology, hepatotoxicity, neurotoxicity, genotoxicity, endocrine-disrupting effects, fat accumulation, oxidative stress and renal toxicity (Kapoor et al., 2010; Park et al., 2013; Ayse Dilek Ozsahin 2014; Han et al., 2017). Therefore, there is an urgent call to monitor and manage these pesticidal sprays on crop plants during flowering and residues in honey. Given the escalating concern over food contaminants, including pesticide

residues in honey, farmers may encouraged to follow good agricultural practices (GAP) for ensuring food safety and safeguarding public health.

IV. CONCLUSION

The developed analytical procedure for pesticide residue analysis was applied to determine the pesticide residues in collected honey samples. Five samples out of thirty-nine samples were detected with the pesticide residues. Fipronil, spinosad, imidacloprid, IN-J9Z38 (metabolite of cyantraniliprole) and carbendazim were the detected pesticides. As the reports on the problem of residues and other contaminants in honey is increasing day by day and have direct effect on health who are consuming it, there is an urgent need to monitor the pesticidal spray and residues and also to standardize the MRLs of pesticides in honey at Indian level.

V. ACKNOWLEDGMENT

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VI. CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript

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