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European Journal of Zoological Research, 2013, 2 (3):22-28
(http://scholarsresearchlibrary.com/archive.html)

ISSN: 2278–7356

Analysis of multifloral honey of the giant honeybee, *Apis Dorsata* F., for pesticide residues in Southern Karnataka, India

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ABSTRACT

Multifloral honey samples were collected from the colonies of giant honeybee, Apis dorsata Fabricius (Hymenoptera: Apidae) in the wild, located at different districts of southern Karnataka. The samples were analyzed based on solid-phase extraction with C18 followed by Gas Chromatography-Mass Spectrometry (GC-MS) for Organochlorine and Pyrethroids and by Liquid Chromatography-Mass Spectrometry (LC-MS) for Organophosphorus, herbicides and other pesticide residues. All the honey samples were screened for 11 Organochlorine, 19 Organophosphorus, four Pyrethroids, three Herbicides and two other pesticides (ex. Carbaryl and Carbofuran). Interestingly, none of the A. dorsata honey samples were contaminated with detectable pesticide limit. However, the residual pesticide detection limit was 0.01ppm and most of the honey samples didn't show higher concentrations of Organochlorine, Organophosphorus, Pyrethroids and Herbicides. Thus, the combs of A. dorsata in the wild are not contaminated by chemical pesticides and their residues level (ppm) in multifloral honey was below detection limit and safe for consumption.

Key words: Pesticides, multifloral honey, *Apis dorsata*, southern Karnataka

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INTRODUCTION

In several developing countries, beekeeping has been sustaining heavy loss since the advent of synthetic pesticides several decades ago. The widespread and careless use of toxic pesticides during the blooming periods of agricultural and horticultural crops not only kills honeybees but also contaminates hive products [1]. Pesticides might be introduced into honey by bees, which feed on nectar or pollen from contaminated blossoms [2]. In recent years, honey contamination became predominant in several countries of the world [3] and finally it may reach to honey consumers. In India, the most widely used pesticides are Organophosphorus pesticides, synthetic pyrethroids and carbamates which have almost completely replaced Organochlorine pesticides [4] [5]. The accumulated pesticide causes a potential risk for human health, because of their sub acute and chronic toxicity.

Several researchers [6] [7] [8] have reported the relative toxicities of commonly used pesticides on *A. cerana* in India (Table 1). These pesticides have been classified into highly toxic (ex**.** Carbaryl 50% WP, Carbofuran 3% WP, Carbophenothion 20 EC, Cypermethrin 10 EC, Decamethrin 20 EC, Dichlorovos 100 EC, Dimethoate 30 EC, DDVP 100 EC, Monocrotophos 36WSC, Parathion and Phosphamidon 100 EC, Phorate, Permethrin 25 EC, Quinalphos 25 EC, Sumithion 50 EC and Thiometon 25 EC), moderately toxic (ex. BHC 50%, Carbyl 50 WP, DDT 50%, Dieldrin, Endrin, Heptachlor 10 WP, Malathion 50 EC, Methyl Demeton, Monocrotophos 40 EC, Diazinon 20 EC, Ethyl parathion 46%, Fenitrothion 100 EC & 50 EC, Lindane, Metacid 50 EC and Methyl parathion 50 EC), moderately toxic fungicides (ex**.** Dithane M-45, 75 WP and Bavistin 50 WP) and relatively non-toxic insecticides (ex. Endosulfan 35 EC, Menazon 70 DP and Phosalone 35 EC). [9] [10] [3] [4] [5] have reported

the various types of pesticides contamination in food products including fruit juices, honey and vegetables. [11] have identified the contamination of honey from different pesticides including fungicides. [12] have recorded the pesticide residues in honey collected from Himachal Pradesh. [2] [13] have detected certain pesticides in hive products namely pollen, honeycomb walls and developing brood as a result large number of bees are killed due to contamination and finally the honey quality decreases considerably [14].

Because of the increasing attention of public to the quality of honey, the control of pesticides in honey is a vital issue for primary health around the world. Because the chemical contents in honey are increasing considerably in the recent past [15]. Moreover, due to pesticide contamination in hive products bee's population is decreasing drastically in various ecosystems [16]. For this reason the analysis of residues in honey has received a special attention [2] at honey producing countries. The presence of pesticide residues in honey has impelled the need for analysis. Therefore, in the present study, *A. dorsata* honey collected from their colonies was used and results are presented in this paper.

MATERIALS AND METHODS

Preparation of samples: The freshly harvested honey from the wild colonies of *A. dorsata* was collected from different districts of southern Karnataka. The collected honey samples (500 to 1000g) were stored in sterilized containers at dark place until their analysis as per [1]. The honey samples were centrifuged at 4000 rpm for 15min to separate the extraneous matter including beeswax. After centrifugation, the honey was filtered through a glass plate as per [2]. The uncontaminated honey was used as control to optimize and validate the samples.

Organic solvents and reagents: The analytical grade acetone, *n*-hexane, acetonitrile, ethyl acetate and dichloromethane were procured from Merck Co. for pesticide residue. The Baker bond octadecyl (C18), Florisil (3ml) and Alumina (500 mg) were used during the analysis.

Extraction: In order to analyze a number of pesticides, modified method of [1] was followed so as to detect multiresidue pesticides in honey samples. A 25g of honey sample was weighed in an Erlenmeyer flask and spiked when required with the pesticide standard solution and mixed with 5ml of water and homogenized by shaking to reduce its viscosity and facilitate its handling. The sample was mixed with 50ml of acetonitrile or acetone or ethyl acetate or dichloromethane solvents tested and submitted to extraction by agitating for 20min. Then, the organic phase was separated by centrifugation at 2500 rpm for 10 min. The supernatant was collected and the residue was re-extracted with 40ml of solvent. The two portions namely mobile phase and organic phase was collected, combined and the solvent was evaporated in a rotary evaporator under reduced pressure at 65^0 C and dried under a gentle stream of pure nitrogen. Finally the residue was dissolved in 5 ml of ethyl acetate and passed through 0.50µm sized pore PTFE filter. For honey fortification 10g of the control (uncontaminated) sample was heated in a water bath at 40^0 C for 20 min and spiked by adding an appropriate volume of standard working solution to reach the concentrations 0.02 and 0.20 mg/kg. The mixture was mechanically stirred in a blender to ensure homogenization and then submitted to the extraction step (Rissato et al., 2004).

Clean-up: The clean-up of the samples was performed by means of a Supelco VISIPREP-12 manifold using Alumina, Florisil and C18 cartridges which were conditioned with approximate 5 ml of acetone. The extract was added to the column and eluted under gravity with two portions of 10ml for each of the tested mixtures of hexane/ethyl acetate at several ratios (80:20, 70:30, 60:40 and 50:50 v/v). Once elution was completed the collected extracts were concentrated under a gentle Nitrogen stream and the residue was dissolved in 1 ml ethyl acetate and submitted to analysis by GC-MS.

GC-MS/ECD: Confirmatory run analysis was done on a Hewlett-Packard Model 5890 series II gas chromatograph with a HP 5972 mass selective ion detector (quadrupole) and a fused silica capillary column LM-5-5% phenyl 95% dimethlypolysiloxane (35m x 0.25 mm i.d., film thickness 0.25 µm). The GC was operated under following conditions:

Initial temperature: The initial temperature was 60^0 C, gradually increased at 25^0 C/min to up to 150^0 C held for 1 min, then increased at 3^0 C/min to 200^0 C held for 1 min and 8^0 C/min to 290^0 C and finally held for 8 min. The rate of carrier gas (helium) constant flow mode at 1.0 ml/min. Splitless injection of a 1µl volume was carried out at 25^0 C with the purge valve at 2min. The liner used was amino deactivated single gooseneck from Restek (Bellefonte,

USA). The mass spectrometer was operated in electron ionization mode with impact ionization voltage 70 eV, a transfer line temperature of 290^0 C, ion source 230^0 C, electron multiplier voltage 1200 V, solvent delay 2.9 min and selected ion monitoring (SIM) mode. Dwell time was adjusted so that the number of cycles per second was 1.4 throughout the chromatographic run, providing a sufficient number of chromatographic points for all compounds.

Limits of detection: Detection limits (LOD) of the GC/MS were determined for each pesticide by the successive dilution of the standard mixed pesticide solution followed by injection into the GC-volume several times. Serial dilution experiments provided the necessary information to calculate the detection limits [17] [18].

Quality Control: The quality control for the analysis of pesticides in honey consisted of five honey samples, one honey spike, one water blank, one water spike, eight calibration standards (ranging from 0.010 to 2.00mg/l of mixed pesticide solution standards), a calibration check standard and ethyl acetate rinses. The honey spike was selected from a set of several free pesticide samples and consisted in fortifying the honey with a mixed pesticide spike standard. The honey and water samples were fortified at 0.020mg/l and analyzed from 60% to 130%. The positive results in the honey samples were confirmed by comparing the retention time and identifying the main ions in relation to those of a pesticide standard. Retention times were within ±0.20 min of the expected retention times. The water blanks and spikes were analyzed in order to account for any residual interference or possible contamination sources such as glassware, handling and others. The presence and confirmation of pesticides or pesticide residues in the water blanks resulted in the extraction and analysis of the entire batch. After completion of the standards, blanks, spikes, sample extracts and rinses, a 0.200mg/l calibration standard was analyzed to account for any difference or variations during the entire batch analysis. Any deviation beyond 15% required a new injection or analysis of the entire batch to be repeated.

Liquid Chromatography Mass Spectrometry (LC-MS/MS)

Pesticide standards were obtained from Sigma-Aldrich (Madrid, Spain). HPLC – grade methanol was purchased form Merck (Darmstadt, Germany) and Sodium chloride (Analytical grade) was supplied by Scharlau (Barcelonia, Spain). The individual stock solution were prepared in methanol at a concentration of 1000mg/l and stored at 4^0 C standard working solutions at various concentrations were daily prepared in ultra pure water obtained from Milli-Q SP reagent water system (Millipore, Bedford, MA, USA).

LC-MS: The LC-MS was performed in a *Hewlett- Packard* (Palo Alto, CA, USA) Hp-1100 series LC-MSD system consisting of an LC connected to a single quadrupole. The MS analyzer with an APCI interface was usable in either positive ionization (PI) or negative ionization (NI) modes. An HP chemstation software version A.06.01 was used for LC-MS control and signal acquisition. The LC separation was carried out on a Luna C18 column (4mm X 2mm inner dia.) both from Phenomenex (Madrid, Spain). For the separation of Organophosphorus pesticides the mobile phase was a methanol/water gradient at a flow rate of 0.7ml/min. The gradient was 80% methanol from 0-15min, followed by a linear gradient to 90% from 15-20min then increased again linearly to 95% from 20-25min and finally maintained at 95% methanol from 25-30min and reequilibrates to the initial conditions 10min. Optimum operating parameters of the APCI interface in Negative ion mode were Vaporizer temperature 450^0 C, Nebulizer gas-nitrogen at a pressure of 60psi (1psi = 6894.76 pa), drying gas also nitrogen at a flow rate of 41 min and temperature of 350^0 C, Capillary voltage 3500V and corona current 25µA. The chromatograms were recorded in full-scan and selected ion monitoring (SIM) modes. Full scan conditions were: m/z ranged from 50-400, with a scan time of 0.75S. Time scheduled SIM using four windows was developed. The most intense ion was used for quantification and the second and third ion for confirmation [19].

RESULTS

The multifloral honey samples collected from Chamarajanagar, Kodagu and Mysore districts revealed that, the different class of pesticide compounds namely Organochlorine, Organophosphorus, Pyrethroids and Others (ex. Carbofuran and Carbaryl) are within the limit of detection level i.e., 0.01 ppm and showed below detection limit (BDL) (Table 2). Further, the GC-MS analysis revealed the significant peak curve responses (mV) in the estimated time intervals (0-35 min) (Fig 1 a, b & c) and that indicated the very low concentration of various pesticides, which unable to appear in chromatograms. A complex series of peaks of not very high intensity (Fig. 1 a, b & c) obtained by GC analysis. Several pesticide compounds were present at trace levels, so a quantitative data evaluation was not applicable and thus it was stated as BDL. The value was 0.01 ppm and all the tested *A. dorsata* honey samples

responded to peak curve responses within the range, but not above the detection level. Thus, it is concluded that the screened *A. dorsata* honey samples are free from pesticides and safe for human consumption.

DISCUSSION

Honeybees have close relationship with the environment [20], where they often exposed to the pesticides or pollutants during their foraging. The harmful pesticides enter into beehive through nectar or pollen when offered by honeybees. When once those pesticides introduced into the honey comb, the honey gets contaminated and becomes unfit for human consumption. Pesticides contamination most often affects the physico-chemical properties of honey. It may alter the inorganic and organic constituents and alter the property of honey. Maintaining pesticide free nest or hive either at apiary or arboreal conditions has still remained challenge to mankind even after having so many advanced techniques. Despite systematic innovative methods put in use, pesticide contamination is completely not avoided under wild conditions. Therefore, maintenance of pesticide free hive/nest depends on contamination free nectar and pollen. This could be achieved only by legitimate use of pesticides by farmers at their croplands. Because, honeybees are voracious foragers, travel to wider area, visit variety of flowers for nectar and pollen. It is in this regard, farmers shouldn't apply on bee forage.

Table 1: Analysis of residual pesticides in honey samples collected from different parts of the world

Sl. No.	Pesticides	Country	Reference
I.	**ORGANOCHLORINE:** Heptachlor hydrazine (HCH), Lindane, Hepatachlor, Aldrin, Hepatchlor epoxide, Dieldrin, Endrin, Dicafol, γ HCH, isomers of HCH (α & β)	Turkey Spain Poland Brazil Portugal India	[20] [25] [11] [1] [26] [5][27] [28]
II.	**ORGANOPHOSPHORUS:** Chlorpyrifos, Diazion, Dichlorvos, Ethion, Fenitrothion, Fenthion, Malathion, Methidathion, Parathionmethyl, Phenotate, Pirimphos-methyl, Profenophos, Pyrazophos, Heptenophos, Methidathion, Quinolphos.	Portugal Brazil India	[26] [1] [5] [27] [28]
III.	**PYRETHROIDS :** Cypermethrin, λ – Cyalothrin, Cyfluthrin, Fluviline, Fenvalerate, Deltamethrine	Brazil India	[1] [5] [28]
IV.	**OTHERS** Carbofuran and Carbaryl	Portugal India	[26] [27]

Several researchers have studied the pesticides contamination in various honey samples collected from different regions of the world (Table 1). Different pesticide residues were found in honey produced from France, Jordan, Italy, Portugal, Spain and Switzerland [21]. The Organochlorine, Organophosphorus, acaricides, fluvalinate, coumaphos and bromopropylate are the most common pesticides detected in honey samples. Moreover, Methidathion and Methiocarb were also detected along with Organophosphorus pesticides in honey samples collected from Spain [19]. Further, [21] reviewed the contamination of Organochlorine pesticide residues in honey. The pesticide levels found in honey samples collected from different countries varied considerably, but it was below 0.5mg/kg. [22] have reported the pesticides contamination in 27 honey samples collected from different parts of India. Among them, majority (55%) of the honey samples were contaminated with Organophosphorus (ex. DDVP, Chlorpyriphos, Monocrotophos, Dimethoate and Fentirothion), Carbofuran and Carbaryl compounds. However, the Organochlorine contamination was little more than that of Organophosphorus and carbamates, but those were recorded below detection limits. Furthermore, [21] have reported the Organochlorine, Organophosphorus and fungicides contamination in 27 honey samples from Switzerland and these contaminants didn't show detectable level of pesticides in all these honey samples. Identification and quantification of pesticide residues in honey is

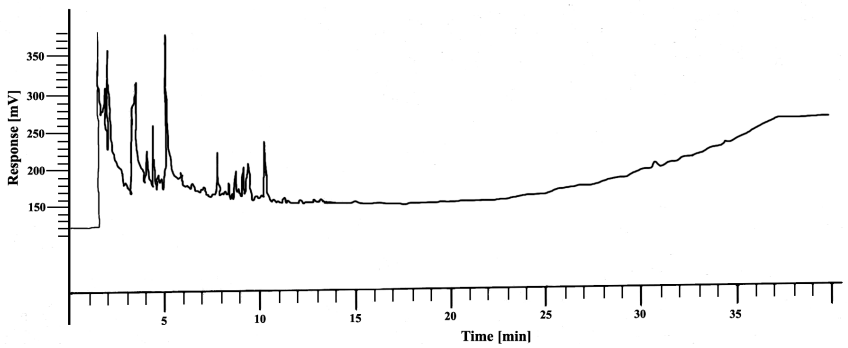
routinely carried out by Gas Chromatography (GC) [14]. However, for either thermal unstable compounds or compounds with low volatility, it was advised to use liquid chromatography (LC) also [14]. Thus, Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) allowed the separation and quantification of various pesticides in multifloral honey collected from *A. dorsata* colonies. The multifloral honey samples collected from various geographical regions of southern Karnataka have shown that, different pesticides namely Organochlorine, Organophosphorus, Cypermethrin and Pyrethroids residues were less than 0.05ppb. Since, 0.01 to 0.05mg/kg is considered as below detection limits (BDL) [23] that is not considered as contamination and which is far from being hazardous for humans. As the multifloral honey samples were obtained from the natural hives of *A. dorsata*, located on road side tall trees nearby forest ecosystem, chances of pesticide spray to the blooming plants is meager [24].

Table 2: Analysis of residual pesticides in multifloral honey samples collected from the colonies of *A. dorsata* from southern Karnataka

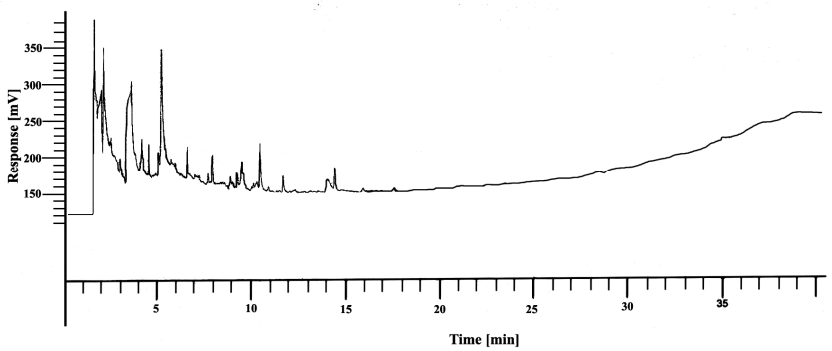
Sl. No.	Pesticide	Honey sample from		
		C. Nagar	Kodagu	Mysore
I.	**ORGANOCHLORINE**: 1. Lindane & its isomers (α, β & ð), 2. DDT & its analogous of OP & PP(DDE), 3. Aldrin, Endrin & its analogous (Ketone and aldehyde), 4. Dieldrin, 5. Heptachlor and its Epoxide, 6. Endosulfan isomers & its analogous (α, β & sulfate), 7. Methoxylchlor, 8. Chlordane and 9. Dicofol.	BDL	BDL	BDL
II.	**ORGANO PHOSPHORUS:** 1. Chlorpyrifos, 2. Malathion, 3. Ethion, 4. Quinolphos, 5. Fentirothion, 6. Phorate, 7. Chlorfenvinfos, 8. Methyl Parathion, 9. Chloropyrifos, 10. Methyl, 11. Phosphomidon, 12. Acephate, 13. Phorate sulfoxide, 14. Fenthion, 15. Dimethoate, 16. Methyl Paraoxan, 17. Phosalone, 18. Diazinon and 19. Dichlorvos (DDVP).	BDL	BDL	BDL
III.	**HERBICIDES:** 1. Alachlor, 2. Atrazone and 3. Butachlor,	BDL	BDL	BDL
IV.	**PYRETHROID:** 1. Cypermethrin, 2. Deltamethrin, 3. Fenvalerate and 4. Permethrin	BDL	BDL	BDL
V.	**OTHER PESTICIDES:** 1. Carbofuran and 2. Carbaryl	BDL	BDL	BDL

Note: *C. Nagar = Chamarajanagar; BDL = Below Detection Limit.*

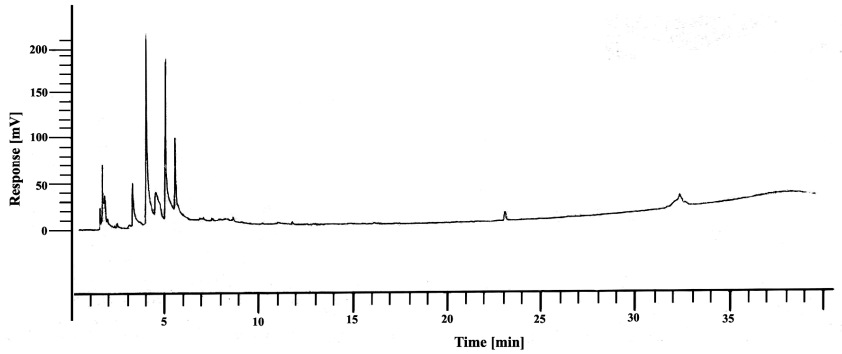
Moreover, *A. dorsata* population might have more depended on forest vegetation where, pesticides application is scanty. Further, *A. dorsata* might have restricted its foraging range near by forest vegetation, where there were no cultivated crops within ten kilometer area amidst forest ecosystem. Perhaps, this might be the reason for uncontamination of pesticides in the honey collected from southern Karnataka.



a. Chamarajanagar district honey sample



b. Kodagu district honey sample



c. Mysore District honey sample

Figure 1: Chromatographs of GC-MS showing pesticides levels in Wild honey samples from southern Karnataka

CONCLUSION

The study signifies the importance of pesticide analysis to know about the level of contamination, to safeguard the consumer's health and to maintain honey as a natural product that is devoid of any contaminants. Thus, results from the present investigation clearly indicated that, there is no significant contamination of pesticides in multifloral honey produced from *A. dorsata* colonies at southern Karnataka. Although, reports of this kind are first to southern Karnataka, presently the multifloral honey from the hives of *A. dorsata* is free from pesticide residues.

Acknowledgements
Authors thank to the University Grants Commission (UGC) and UGC-BSR-RFSMS, New Delhi, for extending financial assistance to conduct this work. Thanks are also due to Shiva Analyticals (India) Limited, Bangalore for their help and co-operation extended during the analysis of pesticides in honey samples and thanks to the Chairman, Department of Studies in Zoology, University of Mysore, Mysore.

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