Original Article

PESTICIDE RESIDUES IN HONEY FROM STINGLESS BEE *MELIPONA SUBNITIDA* (MELIPONINI, APIDAE)

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Abstract

The present study aimed to detect pesticide residues in the honey produced by the stingless bee *Melipona subnitida*. A total of thirty-five samples of honey from *M. subnitida* were collected from twelve municipalities of the semiarid region of Rio Grande do Norte state, northeastern Brazil. Of these thirty-five samples, fourteen were from colonies raised in an urban area, while the other twenty-one were from the countryside. The pesticides in the samples were extracted using a modified QuEChERS method. The simultaneous analysis of 116 analytes in the honey samples was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Twenty-five samples (71.4% of samples) were contaminated by some amount of pesticide, and of them twenty-four had just one pesticide and one had three. The detected compounds included organophosphate pesticides (OPP) monocrotophos (24 samples), trichlorfon (6 samples) and chlorpyrifos-methyl (2 samples). In conclusion, the honey produced by the stingless bee *M. subnitida* may be contaminated by pesticides, which emphasizes the need for food monitoring before commercialization.

Keywords: honey, LC-MS/MS, *Melipona subnitida,* multiresidue analysis, pesticides residues, stingless bees

INTRODUCTION

Pesticides are widely used to protect agricultural plantations from pests, and thus improve crop production. However, inappropriate use may result in residues in human foods such as vegetables (Madureira et al., 2012), fruits (Oliveira et al., 2012), meat (Oliveira et al., 2018), fish (Oliveira et al., 2015), eggs (Hildmann et al., 2015), milk (Oliveira et al., 2014) and honey from the honeybee *Apis mellifera* (Pacífico da Silva et al., 2015). People consuming these contaminated foods are thus inadvertently exposed to residual pesticides, which increase the occurrence of such toxic effects as carcinogenesis, immunologic disorders and neurological disturbances (Kotsonis & Burdock, 2013).

Beyond the risk for consumers of contaminated honey, environmental contamination by pesticides affects the health of the bees, impairing their populations. In fact, pesticides including OPP may interfere with their cognitive abilities, behavior and immune responses against pathogens, and at high concentrations, OPP may

Fig. 1. Locations in Rio Grande do Norte state, northeastern Brazil, of the collection of honey samples from the stingless bee *Melipona subnitida*.

even prove fatal (Rortais et al., 2005; Pacífico da Silva et al., 2015; Pacífico da Silva et al., 2016). The reduction in bee population may affect agricultural production, because about 35% of crops depend on flower pollination by animals (Klein et al., 2007), and this dependence is increasing worldwide (Aizen et al., 2008; Aizen & Harder, 2009). A number of bee species are known to act as pollinators but considerably vary in the plants they pollinate. Some pollinating bee species include the honeybee *A. mellifera* and bees from the Meliponini tribe (Maia-Silva et al., 2012).

The insects from the Meliponini tribe, Apidae family, are a large group of highly eusocial stingless bees (Camargo & Pedro, 1992; Pedro, 2014). Several species, mostly from the *Melipona* genus, are used for beekeeping i.e., meliponicultur). One such species kept in Brazil is the *Melipona subnitida* Ducke, popularly known as "Jandaíra," with an average annual production of 2.5 L of honey per colony and maximum production of 5.6 L per colony (Cortopassi-Laurino et al., 2006). The honey from the *Melipona* species is commercialized as a natural medicine for children, the elderly and the sick (Jones, 2013; Carvalho, Martins, & Mourão, 2014). Thus, this honey must be inspected for the possible presence of residual pesticides that might affect consumers.

The present study aimed to determine the occurrence of pesticide residues in the honey of *M. subnitida*. For this, the simultaneous detection of 116 analytes in the honey samples was performed using a sensitive chromatographic method by liquid chromatography-tandem mass spectrometry (LC/MS-MS) (Souza Tette et al., 2016).

MATERIAL AND METHODS

Samples

A total of thirty samples of honey produced by *M. subnitida* bees were collected from different colonies in twelve municipalities of the semiarid region of Rio Grande do Norte state, northeastern Brazil: Alto do Rodrigues (one sample), Barcelona (one sample), Galinhos (one sample), Governador Dix-Sept Rosado (one sample), Jandaíra (seven samples), Mossoró (eight samples), Pau dos Ferros (one sample), Riachuelo (two samples), São Francisco do Oeste (five samples), São Paulo do Potengi (two samples), Serra do Mel (five samples) and Tibau (one sample) (Fig. 1). From the thirty-five samples, fourteen were from colonies raised in an urban area (peripheral regions), and the other twenty-one were from the countryside. Collection was done from August to October 2013.

Sample preparation

All analyses were performed in triplicate. The extraction procedure was adapted from the QuEChERS method (Souza Tette et al., 2016). Honey samples (5.0 g) were transferred to polypropylene tubes (50 mL) containing ultra-pure water (10.0 mL) and then the tubes were agitated at 3,000 rpm for 1 min. A total of 10.0 mL acetonitrile:ethyl acetate solution (70:30) was added to each tube before being agitated again at 3,000 rpm for 1 min. After that, 4.0 g magnesium sulfate and 1.0 g sodium acetate were added. The tubes were agitated once more at 3,000 rpm for 1 minute and then centrifuged at 4,000 rpm for 9 min. The supernatant (1.0 mL) was transferred to a polypropylene microtube (2 mL) containing 150 mg anhydrous magnesium sulfate, 50 mg Florisil and 50 mg PSA (primary-secondary amine), followed by agitation at 3,000 rpm for 1 min and then centrifugation at 9,000 rpm for 9 min. The supernatant (500 µL) was transferred to a vial for injection into the LC-MS/MS system.

Chromatographic and mass spectrometric conditions

The chromatographic and mass spectrometric conditions were based on an earlier study by Souza Tette et al. (2016). A UFLC system (Prominence UFLC, Shimadzu, Kyoto, Japan) composed of a Parallel-type double plunger (LC20ADXR), an auto-sampler (SIL-20AC) and a column oven (CTO-20AC), coupled to mass spectrometer with an electrospray ionization source (ESI) (5500 Triple Quad, AB SCIEX, Ontario, Canada), was used. The injection volume was 5 µl. The chromatographic separations were performed on a Shim-pack XR-ODSII column (2.0 x 100 mm, 2.2 µm), at a column temperature of 60°C. The mobile phase was 10 mmol/L ammonium acetate with 0.1% formic acid (phase A) and methanol (phase B), as a gradient elution at flow rate of 0.5 mL/min. The gradient elution program was as follows: A, 50% and B, 50% for 7.0 min; A, 20% and B, 80% for 4.0 min; A, 10% and B, 90% for 0.5 min; A, 50% and B, 50% for 1.5 min; total chromatographic run time, 13 min. For the mass spectrometry analysis, an electrospray ionization source (ESI) was used in both negative (ESI-) and positive (ESI+) modes. Source parameters were set as follows: ion spray voltage, 5.5 kV for ESI+ and 4.5 kV for ESI-; ion source temperature, 500°C; curtain gas, 20 psi; nebulizer gas, 35 psi; auxiliary gas, 35 psi; collision gas, 8 psi. The tested analytes and their mass spectrometric (MS/MS) conditions, limit of detection (LOD) and limit of quantification (LOQ) are described by an earlier study (Souza Tette et al., 2016).

Statistical analyses

The statistical analyses were performed using R software version 3.1.3 (R Development Core Team, 2018). The Fisher's Exact Test from count data was used to compare the frequencies of contaminated samples from the countryside versus urban areas. The level of statistical significance was set at $P < 0.05$.

RESULTS

The evaluation of the presence of pesticides in *M. subnitida* honey revealed that twenty-five samples (71.4%) were contaminated by some amount of pesticide (Tab. 1). From these twenty-five positive samples, twenty-four (96.0%) had just one pesticide detected, and just one (4.0%) had three pesticides. The detected compounds were the organophosphate pesticides (OPP) chlorpyrifos-methyl (2 samples, 5.71% of all tested samples), monocrotophos (24 samples, 68.6%) and trichlorfon (6 samples, 17.1%). Residual levels exceeding the maximum residue levels (MRL; EC, 2008; EC, 2012; EC, 2018) was found for monocrotophos in 68.6% of tested samples and trichlorfon in 5.71%. On the other hand, none of the tested samples showed chlorpyrifos-methyl levels over the MRL.

Of the twenty-one samples collected in the countryside, fifteen (71.4%) were positive for some amount of pesticide, one (4.76%) for chlorpyrifos-methyl, fourteen (66.7%) for monocrotophos, and two (9.52%) for trichlorfon. Within the fourteen samples from urban areas, ten (71.4%) showed some amount of pesticide, one (7.14%) for chlorpyrifos-methyl, ten (71.4%) for

Table 1.

Detected pesticide levels in 35 tested samples of honey from the stingless bee *Melipona subnitida*

Sample number	Municipality	Collection site	Chlorpyrifos-methyl (mg/kg)	Monocrotophos (mg/kg)	Trichlorfon (mg/kg)
1	Alto do Rodrigues	Countryside	n.d. [a]	0.055	n.d.
2	Barcelona	Countryside	n.d.	n.d.	n.d.
3	Galinhos	Countryside	n.d.	n.d.	n.d.
4	Governador Dix-Sept Rosado	Countryside	n.d.	0.072	n.d.
5	Jandaíra	Urban	n.d.	n.d.	n.d.
6	Jandaíra	Urban	n.d.	0.038	n.d.
7	Jandaíra	Countryside	n.d.	0.057	n.d.
8	Jandaíra	Countryside	n.d.	n.d.	n.d.
9	Jandaíra	Countryside	n.d.	0.015	n.d.
10	Jandaíra	Countryside	n.d.	0.021	n.d.
11	Jandaíra	Urban	< LOQ [b]	0.049	< LOQ
12	Mossoró	Urban	n.d.	0.081	n.d.
13	Mossoró	Urban	n.d.	0.018	n.d.
14	Mossoró	Urban	n.d.	0.057	n.d.
15	Mossoró	Urban	n.d.	0.033	< LOQ
16	Mossoró	Urban	n.d.	n.d.	n.d.
17	Mossoró	Urban	n.d.	n.d.	n.d.
18	Mossoró	Urban	n.d.	0.062	< LOQ
19	Mossoró	Urban	n.d.	0.050	0.023
20	Pau dos Ferros	Countryside	n.d.	n.d.	n.d.
21	Riachuelo	Countryside	n.d.	0.037	n.d.
22	Riachuelo	Countryside	< LOQ	n.d.	n.d.
23	São Francisco do Oeste	Urban	n.d.	0.061	n.d.
24	São Francisco do Oeste	Urban	n.d.	0.052	n.d.
25	São Francisco do Oeste	Countryside	n.d.	0.074	n.d.
26	São Francisco do Oeste	Countryside	n.d.	0.011	n.d.
27	São Francisco do Oeste	Countryside	n.d.	n.d.	n.d.
28	São Paulo do Potengi	Countryside	n.d.	n.d.	n.d.
29	São Paulo do Potengi	Countryside	n.d.	n.d.	n.d.
30	Serra do Mel	Countryside	n.d.	0.043	0.074
31	Serra do Mel	Urban	n.d.	0.033	0.059
32	Serra do Mel	Countryside	n.d.	0.046	n.d.
33	Serra do Mel	Countryside	n.d.	0.056	n.d.
34	Serra do Mel	Countryside	n.d.	0.024	n.d.
35	Tibau	Countryside	n.d.	0.101	n.d.
	Median (mg/kg)		0	0.049	0
	Minimum (mg/kg)		0	0	0
	Maximum (mg/kg)		0.005	0.101	0.074
	MRL [c] (mg/kg)		0.050	0.010	0.010
	Samples >MRL		0	24	2
	% samples >MRL		0	68.6	5.71

[a] n.d.: not detected

[b] < LOQ: lower than limit of quantification (0.010 mg/kg)

[c] MRL: maximum residue levels (EC, 2008; EC, 2012; EC, 2018)

monocrotophos and four (28.6%) for trichlorfon. A comparison of the frequencies of pesticides between both areas showed no significant difference ($P > 0.05$, Fisher's Exact Test from count data).

DISCUSSION

In the present study, *M. subnitida* honey showed three OPP: monocrotophos, trichlorfon, and chlorpyrifos-methyl. The residual levels exceeded the maximum residue levels (MRL; EC, 2008; EC, 2012; EC, 2018) for monocrotophos (68.6% samples) and trichlorfon (5.71%). This contamination of the honey might be attributed to environmental contamination, due to inappropriate agricultural utilization of pesticides. In fact, in a region near where our study was conducted, the water was found to contain residual levels of pesticides including chlorpyrifos (Gama, Oliveira, & Cavalcante, 2013; Pinheiro et al., 2017) and monocrotophos (Pinheiro et al., 2017).

Earlier studies also aimed to search residual pesticide levels in honey in Brazil and found OPP. Pacifico da Silva et al. (2015) found nineteen different pesticides after testing fifty-three honey samples form the Rio Grande do Norte state, Northeastern Brazil. Seven of these pesticides were OPP: chlorpyrifos, ethion, paraoxon, parathion, phosalone, and sulfotep. On the other hand, Souza Tette et al. (2016) tested one hundred honey samples from five states of Brazil, but just the organophosphorus trichlorfon was found in one sample.

The frequencies of pesticides from colonies raised in an urban area and from the countryside were similar (71.4%). This finding indicates that both colonies had similar foraging patterns, which was reinforced by a pollen analysis of all tested honey samples showing about 52% of the amount of pollen from *Mimosa tenuiflora* (data not shown). In fact, *M. subnitida* bees can fly distances of up to 4,000 m to forage (Silva et al., 2014).

Due to their high toxicity, monocrotophos and trichlorfon as insecticides have not been not allowed to be used in Brazil since 2006 and 2010, respectively (ANVISA, 2006; ANVISA, 2010). However, the use of such illegal compounds as pesticides is frequent in Brazil due to the high difference in market price (Lemos, Carvalho, & Ortiz, 2018). Illegal commerce has health and environmental concerns, and in fact both monocrotophos and trichlorfon were found in honey in the present study in levels higher than MRL. Thus, this finding reinforces the risk to human health due to use of illegal compounds that might contaminate food.

OPP are a commonly used class of insecticides with hundreds of commercial compounds available worldwide. Its primary toxic mechanism is the inhibition of acetylcholinesterase (AChE), an esterase that hydrolyzes the neurotransmitter acetylcholine. Fatal acute poisoning is due to the over-stimulation of cholinergic receptors in the peripheral and central nervous systems. Some OPP may induce delayed polyneuropathy days after a single exposure (Costa, 2006). The residual levels of OPP found in honey in our study were too low to produce either of these two toxic effects, but the consumption of contaminated honey might contribute to chronic OPP exposure.

Some epidemiological studies in humans connected long-term exposure to low doses of OPP with the development of neuropsychological disturbances. Furthermore, studies using laboratory animals have shown neurobehavioral interferences by doses lower than those that induce cholinergic signs of poisoning (Ray & Richards, 2001). One possible mechanism is the induction of neuroinflammation by OPP that potentially results in neurodegeneration and neurodegenerative disorders (Banks & Lein, 2012; Nurulain et al., 2014). Monocrotophos was found to inhibit AChE activity and induce oxidative stress in the brain of rats exposed to doses lower than No Observed Effect Level (Karumuri et al., 2019). Thus, we hypothesize that people who continuously consume this contaminated honey might be at risk of developing these neuropsychological disturbances.

Another risk associated with chronic OPP exposure is the development of cancer (Mostafalou & Abdollahi, 2013). Several epide-

miological studies conducted in farmers and commercial pesticide applicators have shown an increased risk for several types of cancer due to OPP exposure. Among the OPP compounds found in the honey samples in our study, an increased risk for development of non-Hodgkin lymphoma by exposure to chlorpyrifos, and trichlorfon (Waddell et al., 2001) and lung cancer by chlorpyrifos (Lee et al., 2004) were found.

In conclusion, the honey produced by the stingless bee *M. subnitida* may be contaminated by pesticides, which emphasizes the need for food monitoring before commercialization. The high frequency of positive samples (71.4%) found in our study is indicative of environmental contamination, probably due to the inappropriate agricultural utilization of pesticides.

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carbosulfan, chlorfenapyr, chlorthal-dimethyl, chlorthiamid, cyhexatin, diazinon, dichlobenil, dicofol, dimethipin, , diniconazole, disulfoton, fenitrothion, flufenzin, furathiocarb, hexaconazole, lactofen, mepronil, methamidophos, methoprene, monocrotophos, monuron, oxycarboxin, oxydemeton-methyl, parathion-methyl, phorate, phosalone, procymidone, profenofos, propachlor, quinclorac, quintozene, tolylfluanid, trichlorfon, tridemorph and trifluralin in or on certain products and amending that Regulation by establishing Annex V listing default values Text with EEA relevance. *Official Journal of the European Union*, *L 273*, 1-75. 6.10.2012

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