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Determination and uptake of abamectin and difenoconazole in the stingless bee *Melipona scutellaris* Latreille, 1811 via oral and topic acute exposure☆

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ABSTRACT

Bees are considered as important providers of ecosystem services, acting via pollination process in crops and native plants, and contributing significantly to the maintenance of biodiversity. However, the decrease of bee's population has been observed worldwide and besides other factors, this collapse is also related to the extensive use of pesticides. In this sense, studies involving the assessment of adverse effects and the uptake of pesticides by bees are of great concern. This work presents an analytical method for the determination of the insecticide abamectin and the fungicide difenoconazole in the stingless bee *Melipona scutellaris* exposed via oral and topic to endpoints concentrations of active ingredients (a.i.) alone and in commercial formulations and the discussion about its mortality and uptake. For this purpose, QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) acetate modified method was used for extraction and pesticides were determined by LC-MS/MS. The validation parameters have included: a linear range between 0.01 and 1.00 $\mu g\,mL^{-1}$; and LOD and LOQ of 0.038 and 0.076 $\mu g\,g^{-1}$ for abamectin and difenoconazole, respectively. The uptake of tested pesticides via oral and topic was verified by the accumulation in adult forager bees, mainly when the commercial product was tested. Mortality was observed to be higher in oral exposure than in topic tests for both pesticides. For abamectin in a commercial formulation (a.i.) no differences were observed for oral or topic exposure. On the other hand, for difenoconazole, topic exposure had demonstrated higher accumulation in bees, according to the increase of received dose. Through the results, uptake and the possible consequences of bioaccumulated pesticides are also discussed and can contribute to the knowledge about the risks involving the exposure of bees to these compounds.

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1. Introduction

The pollination services are essential for plants and plays an important role in the maintenance of biodiversity in natural systems; for humans, the pollination can be also fundamental for the crop production or even their increment in some cultures (da Silva et al., 2014; Goulson et al., 2015; Potts et al., 2010). In these crops, bees are responsible for 70% of production (Bradbear, 2009) and, considering their ecological and economical relevance and their role as bioindicators, there is a concern about the decrease of bee's populations worldwide (Goulson et al., 2018).

The evidence of bee's colony collapse has been reported and, besides other facts as natural diseases and deforestation, the decrease and impact in populations are also associated with the pesticide's application in crops, where pollination by these insects occurs (Frazier et al., 2008; Goulson et al., 2015; Pires et al., 2016). In this sense, several studies are carried out focusing on the development of useful tools to predict the environmental risk assessment of those exposures and to avoid the irreversible decline of pollinators, biodiversity, and production. In this scenario, most studies involve the risk assessment related to the European honey

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bee *Apis mellifera* Linnaeus, 1758 (Devillers and Pham-Delegue, 2002; Goretti et al., 2020), while few studies are carried involving stingless bee species, also considered as important pollinators and exposed to pesticides as non-target organisms.

The exposure of bees to different pesticides mainly occurs via foraging activities, when the forager's bees are in contact with the nectar and pollen of contaminated plants, carrying the contamination back to hives (Azpiazu et al., 2019; Christen et al., 2018; Jay, 1986; Rortais et al., 2005). Once in colonies, pesticides can be distributed to different compartments, including the queen and larvae food, bringing deleterious effects on all social scales; this commitment of health and social features can start in larval stages (dos Santos et al., 2016; Rosa et al., 2016; Tomé et al., 2020), once the mortality can decrease the number of workers and, consequently reduces the provided food to future queens (dos Santos et al., 2016). The risk associated with pesticides includes the exposure to different classes of compounds, where synergistic (combined actions) and antagonistic (contrasting actions) effects can be observed. Thus, the development of tools for monitoring different pesticides through the prediction of risks to individuals and colonies is expected.

Stingless bees are represented by more than 400 species distributed in the world; in Brazil, there are more than 200 species, where these stingless bees are considered not only important for ecosystem equilibrium and biodiversity but have also played an important role in the pollination of crops (Bradbear, 2009; De Menezes Pedro, 2014). Besides pollination services, stingless bees are also responsible for valuable bee products as honey, propolis, and beebread (Kwapong et al., 2010). As observed for *A. mellifera*, the stingless bees collect the pollen and, after being processed by the nurse bees, this is the food provided for consumption of larvae individuals (Dorigo et al., 2019; Rosa et al., 2015); however, the transference of pesticides to this food still unclear and under discussion (Rosa et al., 2016). The feature of pollination vibration is an aspect of Melipona stingless bees that is not observed for A. mellifera and helps in pollination efficiency (Slaa et al., 2006). The stingless bee *Melipona scutellaris* Latreille, 1811 (Hymenoptera, Apidae) belongs to the Melliponini tribe and is native from the Brazilian northeast region (Nogueira-neto, 1997), however very well adapted also in southeast region. The sensitivity of genus *Melipona* to pesticides have been previously reported (Costa et al., 2015; Del Sarto et al., 2014; Lourenço et al., 2012; Seide et al., 2018) and can demonstrate the relevance of studies involving this species.

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Difenoconazole is a fungicide belonging to the group of triazoles and commercially introduced at the beginning of the '80s (Fishel, 2017). This compound is also considered as extremely toxic for humans (0.6 mg kg^{-1}) (ANVISA, 2020b) and environment (Syngenta Proteção de Cultivos Ltda, 2018). Besides its toxicity, for bees, according to the PPDB (Pesticide Properties DataBase, 2019), this fungicide is not considered as toxic. The mode of action of this compound includes the inhibition of sterol, essential for fungi cellular membranes (Fishel, 2017).

The application of single abamectin and difenoconazole in Brazil has included a wide range of crops that depends on pollination (ANVISA, 2020a, 2020b). The broad range application can also lead to the detection in the environment. In Brazil, difenoconazole was detected in surface water samples in a concentration of 15 ng L^{-1} (Caldas et al., 2019); abamectin was detected in soil samples from orange fields in concentrations between 1.7 and 18 ng g^{-1} (Ferreira et al., 2016). In combination, these pesticides can be applied in nine different cultures as potato, coffee, citrus and papaya, strawberry, soy and tomato (ANVISA, 2020a, 2020b).

Considering the relevance of access and monitoring the impact of those pesticides in native bees, the development of appropriated analytical methods is imperative. The analysis of pesticides in environmental matrices has been mainly carried using the QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) method (Anastassiades et al., 2003; de Oliveira et al., 2016; Tanner and Czerwenka, 2011). For difenoconazole, determination using this extraction method in honey samples by LC-ESI-MS/MS was observed in the concentration of 1 μg kg^{-1} (Herrera López et al., 2016). For abamectin, other methods were used as pollen extraction using acetonitrile with further analysis by HPLC-FLD (de Oliveira et al., 2016), or the use of solid-phase extraction (SPE) followed by analysis by UHPLC-MS/MS in soil samples, with a detection range of 1.7–18 ng g^{-1} (Ferreira et al., 2016).

As observed in the literature, the determination of these pesticides commonly involves matrices as honey and pollen or even bee tissues. However, the determination in exposed bees can bring some answers about the assimilation and uptake rate. The exposure route of pesticides can also differ the uptake rates in bees, its bioaccumulation, and depuration, where the concentration in bees can help to understand the observed lethal and sublethal effects. In this sense, the present study aims the development of an analytical method for the determination of abamectin and difenoconazole in the stingless bee *Melipona scutellaris* exposed via topic and oral to commercial products and active ingredients (in case of abamectin) as well as the assessment of the uptake, mortality and bioaccumulation rates of those pesticides in this bee's population.

2. Materials and methods

2.1. Reagents and standards

Standards with a high purity level (96.9% and 99.7% for

abamectin and difenoconazole, respectively) were purchased from Sigma Aldrich (São Paulo, Brazil). The commercial products KRAFT 36 EC® (36 g L^{-1} of abamectin as the active ingredient) and SCORE 250 EC® (250 g L^{-1} of difenoconazole as the active ingredient) were purchased from Cheminova and Bayer CropScience and Syngenta S.A. Other reagents were purchased as follow: ethyl acetate (UV-IR-HPLC-HPLC preparative) PAI-ACS and hexane (HPLC) PAI from Panreac (Barcelona, Spain), acetonitrile (HPLC/ACS grade) from Tedia (Fairfield, USA), acetone and dichloromethane from Synth (São Paulo, Brazil); sodium acetate and magnesium sulfate from J.T Baker (Xalostoc, Mexico); acetic acid from Synth (São Paulo, Brazil), formic acid from Honeyweel (Steinheim, Germany) and sodium chloride from Synth (São Paulo, Brazil); the dispersive salts primary and secondary amine (PSA) and C18 were purchased from Agilent Technologies (USA).

For exposures bioassays, the active ingredients were diluted in acetone (PA) for a stock solution (1 $\mu g\,\mu L^{-1}$) and dilutions were prepared using acetone: sucrose (1:1 v/v) in oral exposure and acetone for topic exposure. For commercial products, dilutions were prepared using Triton X-100 0.1% in topic exposure and sucrose solution for oral tests.

2.2. M. scutellaris collection

For method optimization and validation as well as for the exposure tests, adult forager of *M. scutellaris* bees were sampled from 14 healthy hives (high forage activity, absence of predators and pests, good posture of the queen) maintained in an experimental "meliponary" located at Center for Water and Applied Ecology (CHREA) in Itirapina-SP (22º9′56,32″S 47º54′7,00″O).

Bees were sampled during the major activity period (first hours of the morning) directly from the hive entrance using a plastic container. Samples were then transported to an experimental laboratory with the sucrose solution as food supply introduced on a side of each container and kept in the dark. Bees used for the QuEChERS validation method were kept frozen at -2 °C. For exposure experiments, bees (10 individuals per container, three containers per treatment, n = 30) were kept in the dark in a temperature-controlled chamber (28 ± 1 °C) with humidity between 50 and 70% for an adaption period of 2 h before the beginning of the experiment.

2.3. Extraction

For the determination of abamectin and difenoconazole in bees (the whole bee was considered: head + thorax + abdomen), QuEChERS method (Anastassiades et al., 2003) was tested in three compositions: traditional, using sodium chloride; and modified, using sodium acetate and sodium citrate.

To achieve the final method used for the determination of abamectin and difenoconazole in exposed bees, method optimizations were performed and includes extraction solvents as acetonitrile (MeCN); ethyl acetate; dichloromethane; hexane; MeCN + 0.1% acetic acid: ethyl acetate (70:30); MeCN + 0.1% acetic acid; MeCN + 1% acetic acid. For clean-up, the combination of dispersive salts has included PSA, C18 and activated carbon in different amounts and combinations.

Extraction has proceeded as follow: approximately 0.14 g of bees (corresponding to 2 individuals) were placed in a 50 mL polypropylene tube and macerated with a glass stick; then, 15 mL of mixture MeCN containing 1% of acetic acid was added in tubes, homogenized in vortex for 1 min; for spiked samples, a 2 h of interaction time was applied; after, 6 g of magnesium sulfate and 1.5 g of sodium acetate were added and placed in vortex for more 1 min. The mixture was then centrifuged at 2168 g by 15 min and the supernatant removed and placed in a new test tube containing clean-up salts: 150 mg magnesium sulfate and 50 mg PSA. The mixture was vortexed for 1 min and then centrifuged by 5 min. All supernatant was removed and dried under N_2 moderated flow. Finally, 1 mL of the mobile phase was used to transfer the final extract to a vial and was filtered with regenerated cellulose (RC) filter 0.45 μm.

2.4. LC-MS/MS analysis

The analysis of abamectin and difenoconazole in bees was performed using liquid chromatography (Agilent technologies, 1200 series) coupled to a mass spectrometer QTRAP (3200 triple quadrupoles, SCIEX). The ionization was carried by electrospray (ESI) operating in a positive mode using a voltage of 5500 V. The column used for analysis was a C8 (250 × 4.6 mm, 5 μm, Macherey-Nagel) with a temperature of 25 °C. The mobile phase was composed by Milli-Q water +0.1% formic acid (A) and acetonitrile + 0,1% formic acid (B) in a gradient mode: 0–0.5 min 40% B, 0.5–12.5 min 100% B, 12.5–18 min 40% B, in a flow rate of 1 mL min^{-1}. Injection volume was 20 μL and retention times (RT) were 9.4 and 10.2 min for difenoconazole and abamectin respectively. Other LC-MS/MS parameters as MRM precursor/product ion and ionization parameters for target compounds are described in Table 1. The peak area of corresponding ions was processed using the Analyst® 1.6. Reporter AB SCIEX.

The validation of the system and the optimized method was carried considering the calibration curves in solvent and matrix (spiked before and after extraction), linearity of the analytes (linear range - 8 points), limits of detection (LOD) and quantification (LOQ), accuracy and precision (3 spiked levels and inter and intra-day injections) and matrix effect. Quality control was checked using procedural blanks and the injection of quality control (QC) standards in the sample list. For linearity data were checked using Minitab®19 Software. Other figures of merit, as well as the graphs, were estimated using Origin® 9 Software.

2.5. Exposure bioassay

The exposure bioassays were carried based in endpoint levels (48 h) as no observed effect concentration (NOEC); mean lethal

Table 1
MRM Precursor/Product Ion transitions and ionization parameters for the analysis of abamectin and difenoconazole by LC-MS/MS.

	Ion transitions	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Abamectin	895.5 > 751.4	60	70	11	30	41	8
	895.5 > 449.3					51	6
	895.5 > 327.4					55	4
Difenoconazole	406.1 > 251	60	51	9	16	27	4
	406.1 > 111.1					75	
	406.1 > 75.1					105	

DP = desolation potential, EP = entrance potential, CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential.

Table 2
Treatments (pesticides, concentration/dose and endpoints) used for the acute exposure of *M. scutellaris*.

	Exposure	Concentration/Dose	Reference Endpoints	Sample ID	Number of samples
Abamectin (analytical standard)	Oral (μg μL^{-1})	0.001	NOEC	1	30
		0.006	LC_{50}	2	30
		0.01	2× LC_{50}	3	30
	Topic (μg bee^{-1})	0.007	NOEC	1	30
		0.028	LD_{50}	2	30
		0.050	2× LD_{50}	3	30
Kraft (abamectin a.i.)	Oral (μg μL^{-1})	0.001	NOEC	1	30
		0.006	LC_{50}	2	30
		0.01	2× LC_{50}	3	30
	Topic (μg bee^{-1})	0.007	NOEC	1	30
		0.028	LD_{50}	2	30
		0.050	2× LD_{50}	3	30
Score (difenoconazole a.i.)	Oral (μg μL^{-1})	2.5	NOEC	1	30
		5.0	LC_{50}	2	30
		10	2× LC_{50}	3	30
	Topic (μg bee^{-1})	10	NOEC	1	30
		67	LD_{50}	2	30
		100	2× LD_{50}	3	30

NOEC (No Observed Effect Concentration); LC_{50} (Median Lethal Concentration); LD_{50} (Median Lethal Dose).

dose (LD_{50}); and mean lethal concentrations (LC_{50}) obtained from previous studies (Brigante et al. 2020 (Unpublished Work)) involving *M. scutellaris*. These amounts are also in accordance with the MRL (maximum residual level) values in Brazilian crops, where abamectin is in the range of 2–40 ng g^{-1}; for difenoconazole, the recommended MRL is between 0.01 to 2 μg g^{-1} (ANVISA, 2020a, 2020b). The standard of abamectin with high purity level was tested for oral and topic exposure and the concentrations were described in Table 2. Tests were conducted considering NOEC as treatment 1; LD_{50} as treatment 2; and 2× LD_{50} as treatment 3. All these treatments were observed for 24, 48 and 72 h of exposure and samples were taken according to the availability of at least 2 bees (dead and/or alive) for chemical analysis. For Kraft (abamectin a.i.) the same concentrations of the abamectin (analytical standard) were used for oral and topic exposure. A binary mixture of commercial products was also investigated, using abamectin (a.i.) oral doses and keeping the difenoconazole concentration of 2.5 μg a.i. μL^{-1}. For mixture in topic exposure, abamectin used for topic doses were combined with the difenoconazole concentration of 10 μg a.i. μL^{-1}. The exposure procedure has followed the OECD protocol (OECD/OCDE, 1998a, 1998b) developed for *Apis mellifera* as described below. For *M. scutellaris* changes in the original protocol includes only the temperature (28 °C in this work, instead of 25 °C).

Oral acute exposure was carried following the OECD protocol 213 (OECD/OCDE, 1998a) and dilutions of stock solution were prepared using sucrose 50% (w/v). The control group has received just sucrose solution during the experiment. Then, 1 mL of contaminated food was provided *ad libitum* until the end of the exposure test (72 h). Experiments were carried in a temperature-controlled chamber (28 ± 1 °C). After exposure, mortality was verified in 24, 48 and 72 h and dead bees removed and stored frozen (-2 °C) until analysis.

For topic exposure, the concentrations applied in *M. scutellaris* (Table 2) were prepared in Triton X-100 following the NOEC and LD_{50} previously determined in literature and described in Table 1. Following the OECD protocol 214 (OECD/OCDE, 1998b), a total of 10 adult foraging bees were used for each treatment and feed *ad libitum*. The pesticide applications were made in the dorsal region after bees reach the torpid stage by exposure to CO_2 for 10 s. Then a dose of 1 μL of each concentration was applied in tested bees. Control groups were performed and received a topic dose of Triton X-100 diluted in water.

3. Results and discussions

3.1. Method performance

The analysis of abamectin and difenoconazole using LC-MS/MS have been demonstrated a good selectivity and linearity (0.01–1 μg mL^{-1}) (SM 1) using the monitored ions. The analytical performance for a curve prepared in the matrix (bee), as well as the detection and quantification limits for the system and method, are described in Table 3. At the same time, no interferences were observed in the same retention time of analytes, indicating a good method selectivity as well as good sensibility, considering the LOD and LOQ. No detection of abamectin and difenoconazole was observed in blank samples of *M. scutellaris* obtained from the meliponary. The matrix effect was observed for abamectin, with an increase of 20% in angular coefficient from the curve prepared in matrix compared with that prepared in the solvent. For difenoconazole, this effect was not significant (1.8%). The increase of analytical signal can indicate an increase of ionization when analytes are prepared in the matrix (Matuszewski et al., 2003).

Method optimization using different extraction solvents (SM2)

Table 3
Analytical parameters for LC-MS/MS analysis of abamectin (ABA) and difenoconazole (DIF) in the bee *M. scutellaris*.

	Linear equation	(r^2)	Linearity	ME (%)	LC-MS/MS[a]		Method[b]	
					LOD	LOQ	LOD	LOQ
ABA	y = 692.5 + 38142.18×	0.992	0.01 to 1.0	20	0.005	0.01	0.038	0.076
DIF	y = −8210.2 + 6.6 × 10^{6}x	0.999	0.01 to 1.0	1.8	0.005	0.01	0.038	0.076

ME = matrix effect.
[a] μg mL^{-1}.
[b] μg g^{-1}.

Table 4
Accuracy and precision of the validated method for abamectin and difenoconazole determination in *M. scutellaris* using the QuEChERS extraction method and LC-MS/MS analysis.

	Level	Accuracy (%)[a]	Precision (RSD %)	
			Intra-day[a]	Inter-day[b]
Abamectin	Low	114	2.69	11.91
	Medium	115	3.13	6.81
	High	114	5.36	10.27
Difenoconazole	Low	112	7.55	10.61
	Medium	106	3.19	4.90
	High	102	5.25	3.40

[a] n = 3.
[b] n = 6.

had investigated the use of sodium acetate, citrate, and chloride QuEChERS extraction salts as well as the PSA, C18 and activated carbon dSPE salts. Recoveries of tested methods (%) were calculated by the difference of blank samples fortified with 1 $\mu g\,mL^{-1}$ at the end of the extraction process. The better performance was observed for the method using 10 mL of MeCN and the addition of sodium chloride (original QuEChERs method), where recoveries were 89.4% and 95.5% for abamectin and difenoconazole, respectively. However, this result has no repeatability. Thus, the method using 15 mL of MeCN +1% acetic acid and extraction using sodium acetate, combined with magnesium sulfate and PSA as dSPE clean-up salts were selected. For the optimization of the clean-up step, the addition of C18 did not increase the recoveries rate while the use of activated carbon has removed all analytes from the matrix.

After selecting the best QuEChERs performance, accuracy and precision were determined using three levels: 0.38 (low); 1.9 (medium); and 5.8 $\mu g\,g^{-1}$ (high), considering the system linearity. The results can be observed in Table 4 and include inter and intra-day precision. Previous studies involving the determination of other pesticides in bees using QuEChERS method have been also described, demonstrating the efficiency of this extraction technique for bees and related matrices (Barganska et al., 2014; Codling et al., 2016; David et al., 2015; Kasiotis et al., 2014; Wiest et al., 2011). Studies involving the determination of abamectin and difenoconazole in bees have been developed using different QuEChERS extraction salts tested in this study. Kiljanek et al. (2016), using acetate QuEChERS method, present abamectin recovery of 111% in the high-level spiking (100 $ng\,g^{-1}$) and for difenoconazole between 70 and 98% using spiking levels from 1 to 100 $ng\,g^{-1}$) and LOQ of 1 $ng\,g^{-1}$; this method was applied in poisoned bees and difenoconazole was detected in 7 samples with average concentration of 26.1 $ng\,g^{-1}$ with abamectin < LOQ. Other studies using the QuEChERS modified method also present a good recovery (68–90%) for difenoconazole with LOQ of 1.1 $ng\,g^{-1}$, but this fungicide was not detected in bee samples (Kasiotis et al., 2014). Considering other organisms, in odonate nymphs (Jesús et al., 2018) have been also developed a miniaturized QuEChERS for pesticides multiresidue determination, including difenoconazole, with similar recoveries (%) obtained for *M. scutellaris* (96–107%), with a LOQ of 1 $ng\,g^{-1}$. For abamectin, using citrate method (Wiest et al., 2011), present 81–112% recovery and LOQ of 30.6 $ng\,g^{-1}$, with no detection in real samples (<LOQ).

In the present work, method performance has presented good linearity, quantitation limits as well as the accuracy inside the recommended range of 80–120% and the precision below 20%, indicating that the method can be considered satisfactory for the determination of abamectin and difenoconazole in *M scutellaris*. Thus, despite determination in laboratory studies, the developed method can also be applied for environmental samples with reliability.

3.2. Exposure and determination in bees

Exposure bioassays via oral and topic routes were used for the determination of abamectin and difenoconazole in *M. scutellaris* using the validated method. Analyzed samples have included death bees sampled in different exposure times; bees that remained alive until the end of the experiment; and bees from control groups, in a total of 30 bees per treatment. Described data includes the exposure to single commercial products Kraft (abamectin a.i.) and Score (difenoconazole a.i.), their mixture, as well as abamectin (analytical standard) with a high purity level.

Mortality during the oral and topic exposure can be observed in Table 5. In this table, is possible to verify that control groups have no significant mortality during the 72 h exposure, validating the test according to with OECD protocol. For other groups, 100% mortality was observed only for oral exposure, mainly for treatment 2 (LC_{50}) and 3 (2 × LC_{50}). For treatment 1 (NOEC) in oral exposure, the highest mortality (94%) was observed for the mixture of commercial products (Kraft + Score). Oppositely, topic exposure has the highest mortality percentage for abamectin (analytical standard) (78%).

Considering the observed mortality, samples were selected for the determination in bees. For oral exposure, results of food consumption per treatment and the quantification in bees are depicted in Fig. 1. As observed in this figure, for abamectin (1 A), pesticides were not detected (n.d.) for the control group and below the detection limit (<LOD) for other samples. However, for the commercial product (Kraft) (1 B), abamectin was detected for exposure

Table 5
Average mortality (%) of bees during oral and topic exposure: NOEC (1); $LC(D)_{50}$ (2); 2 × $LC(D)_{50}$ (3); and control. n = 30.

Treatments		Mortality (%)									Control		
		1			2			3					
Exposure time (h)		24	48	72	24	48	72	24	48	72	24	48	72
Oral	Abamectin[a]	0	0	25	0	45	97	0	59	85	0	0	3
	Kraft[b]	0	0	16	3	57	100	3	63	100	0	5	5
	Score[c]	31	49	73	70	97	100	63	100	100	0	0	6
	Kraft + Score	6	69	94	4	89	100	34	97	97	0	0	7
Topic	Abamectin[a]	15	22	30	17	27	37	15	50	78	0	0	0
	Kraft[b]	6	11	11	3	19	32	3	13	19	0	1	1
	Score[c]	19	23	26	54	54	54	59	59	62	0	7	11
	Kraft + Score	12	15	18	15	19	22	8	12	15	2	4	7

[a] (analytical standard).
[b] (abamectin a.i.).
[c] (difenoconazole a.i.).

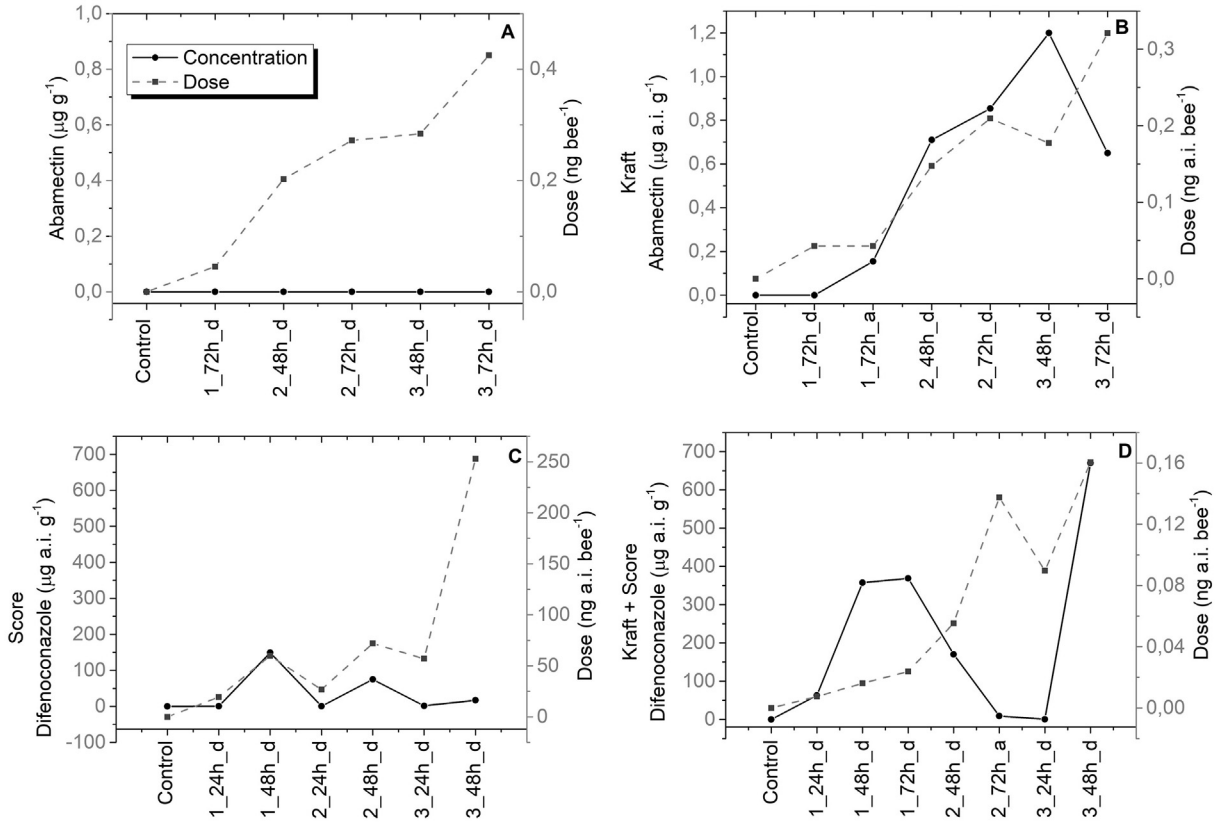

Fig. 1. Concentration of abamectin and difenoconazole in *M. scutellaris* (black line) and total dose from oral exposure (dashed line) for Abamectin (analytical standard) (A); Kraft (abamectin a.i) (B); Score (difenoconazole a.i) (C); Kraft + Score (D) in samples: NOEC (1); LC_{50} (2); and 2 × LC_{50} (3); alive bees (a); dead bees (d).

in LC_{50}: 0.71 and 0.85 μg g^{-1}; and 2× LC_{50}: 1.2 and 0.65 μg g^{-1} levels for samples in 48 and 72 h, respectively. Besides the detection in bees, the food consumption for Kraft (abamectin a.i.) exposure was like those exposed to abamectin (analytical standard).

Based on our results, the detection of abamectin in *M. scutellaris* exposed to the commercial product can indicate inhibition of detoxification metabolism by other inactive ingredients of commercial product formulation, and consequent accumulation of this insecticide in bees. This fact must be deeply investigated in further studies, mainly in concern of the inactive ingredients of the commercial formulation and that can affect the detoxification metabolism of bees and allow the accumulation of that pesticide. The toxicity of inactive ingredients in bees was already discussed, once active ingredients are not introduced alone in field applications, resulting in prochloraz, the composition of commercial formulations can include surfactants, penetrant enhancers, spreaders, stickers, and co-solvents. In past studies involving the exposure of the stingless bee *Melipona quadrifasciata* Lepeletier, 1836 to the commercial product, Vertimec 18 EC had demonstrated toxicity 8970 times greater than the abamectin (Del Sarto et al., 2014).

For single Score exposure the accumulation of difenoconazole in *M. scutellaris* was observed for all tested concentrations (Fig. 1C) and follow the order: 24 < 48 h, according to the increase of exposure dose. These continuous contact with contaminated food can lead to an accumulation in the bee body, where the depuration rate is suppressed by the uptake. The food consumption observed for Score, besides in lower amounts of that observed for abamectin and Kraft exposures were provided in higher doses and were enough to bioaccumulate and be detected in the bee. However, this lower consumption can be indicative that other ingredients of a commercial product, including the strong odor of this formulation, could contribute to this food refusal. The same behavior was observed for the mixture (Kraft + Score). This effect of bees' repellence against pesticides have been described by Rieth and Levin (1988) for pyrethroids and appears to be transitory after 24 h exposure; however, Delabie et al. (1985) have discussed that the repellence of the pyrethroid cypermethrin was associated only with the ingredients from commercial formulation, once this aspect was not observed using only the active ingredient in the exposure.

In the mixture (Kraft + Score), abamectin (not demonstrated in graphs) was detected only in one sample (3_48 h) in the concentration of 0.336 μg g^{-1}, the same treatment with maximum concentration observed for single Kraft oral exposure. However, for the mixture with Score (difenoconazole a.i.) (Fig. 1D), as well as for the single Score (difenoconazole a.i.), all samples presented this fungicide in bees. In the commercial mixture, the difenoconazole concentration range was 0.45–680 μg g^{-1}, with the highest concentration observed for in treatment 3_48 h, almost 40 times the concentration observed in single Score exposure (17.18 μg g^{-1}) for the same exposure concentration. As observed above, the presence of the inactive ingredients in both commercial products can lead to a higher accumulation of difenoconazole in *M. scutellaris* due to the possible inhibition of the bee's metabolism.

For topic exposure (Fig. 2), bees have received only one dose with concentrations described in Table 2. The standard abamectin was not detected (Fig. 2A). The concentration of abamectin (a.i.) in bees was only detected in Kraft exposure in treatment 2 (LD_{50}) for 48 h (0.83 μg g^{-1}) and 72 h (0.78 μg g^{-1}) and Kraft treatment 3 (2 × LD_{50}) in 48 h (1.25 μg g^{-1}) and 72 h (0.48 μg g^{-1}) (Fig. 2B). In the mixture Kraft + Score, no abamectin (a.i.) was detected.

As observed in Fig. 3, abamectin (a.i.) concentrations from the single topic exposure of the commercial product, are very similar to

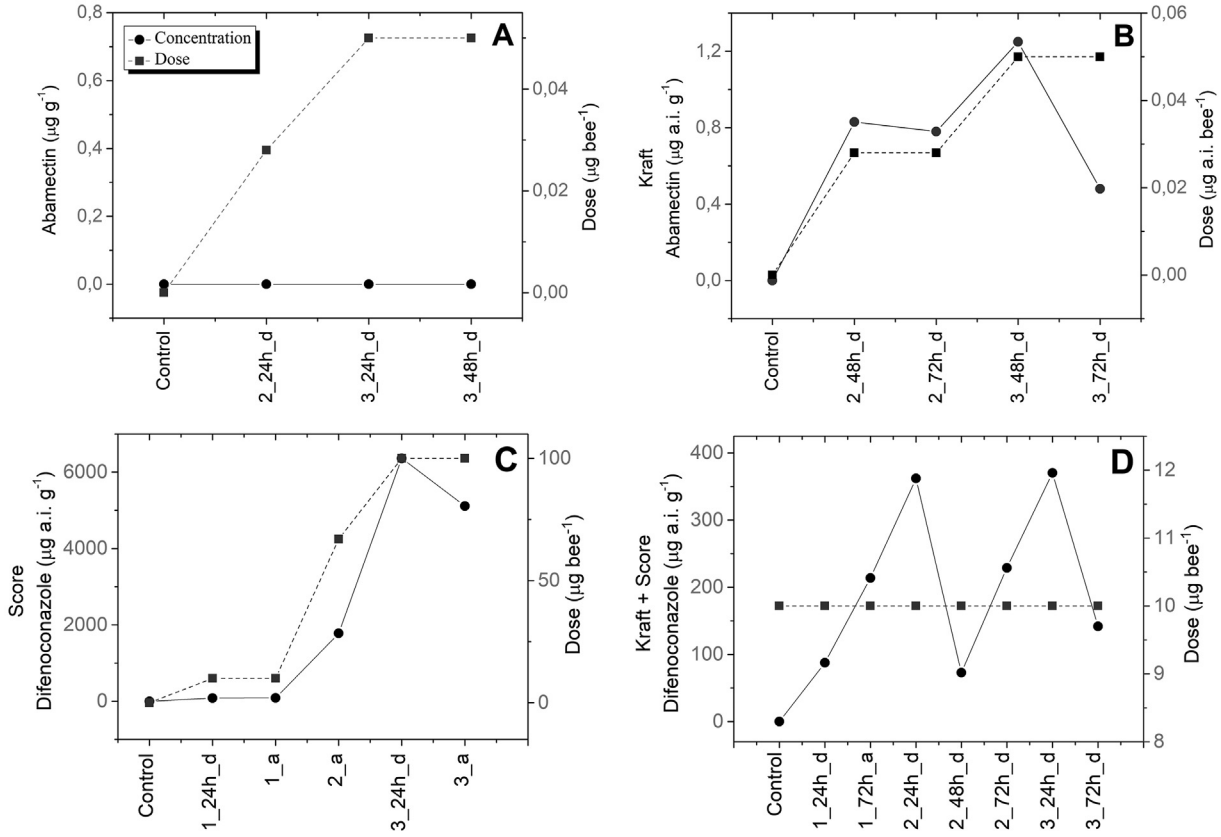

Fig. 2. Concentration of abamectin and difenoconazole in *M. scutellaris* (black line) and total dose from topic exposure (dashed line) for Abamectin (analytical standard) (A); Kraft (abamectin a.i) (B); Score (difenoconazole a.i) (C); Kraft + Score (D) in samples: NOEC (1); LC_{50} (2); and 2 × LC_{50} (3); alive bees (a); dead bees (d).

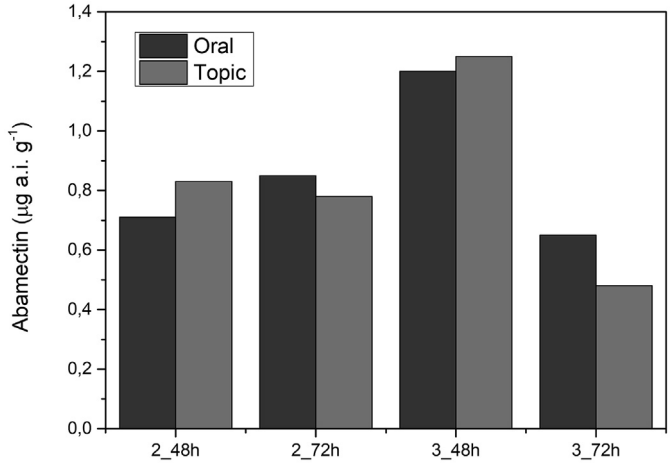

Fig. 3. The concentration of abamectin from Kraft (abamectin a.i) exposure in *M. scutellaris* via oral and topic. Samples detected for treatment 2 (LC_{50} and LD_{50}) and 3 (2 × LC_{50} and LD_{50}) at 48 and 72 h.

the concentrations observed for oral exposure. This aspect can indicate that independent of exposure route, by spray drift or even by contaminated food (in this case, related with nectar consumption), this insecticide present in the commercial product (Kraft® 36 EC) can bioaccumulate in *M. scutellaris*. However, besides the accumulation in investigated bees was similar, mortality was up to 5 times higher in bees orally exposed. This fact can be related with the direct ingestion of tested pesticide via oral, where the metabolization will occur in bee midgut; however, in the topic exposure, the transference of pesticides and the effect of them in metabolism will depend first on the permeation through the cuticle (Thompson et al., 2014). In the environment, topic exposure is associated with the application of those pesticides by spray drift (Thompson, 2001). With the detection of abamectin in bees exposed via topic, we can observe that part of this pesticide is bioaccumulated being an important exposure route.

For difenoconazole, uptake via topic exposure was observed in a single commercial product (Score) (Fig. 2C) and the mixture (Fig. 2D), and bees' mortality was strongly observed on the first 24 h of exposure, with a small increase in 48 and 72 h. As observed in results, the concentration of difenoconazole (a.i.) in *M. scutellaris* has increased with applied dose in the exposure alone, with the highest concentration observed for dead bees of treatment 3_24 h (6361 μg g^{-1}). Due to low mortality (%), alive bees could be sampled from all three treatments in Score exposure, and the increase of concentration in the bees' body was observed following the increase of the exposure levels (Fig. 2A). Differently of Kraft exposure, where concentrations in oral and topic tests were very similar, difenoconazole (a.i.) accumulation in the body via topic exposure was higher than the exposure via oral. This result can be associated with the highest exposure levels used for topic exposure (almost 10 times the oral test). In our results, as well as observed for abamectin, *M. scutellaris* mortality was higher in oral exposure. In this sense, the fact of bees receives a continuous intake of pesticides may affect more severely than a single dose as well as the permeation aspects mentioned above.

In commercial mixture (Fig. 2D), lower difenoconazole concentrations were observed, where the maximum observed

concentration was 370 μg g^{-1} for the same treatment 3_24 h. In this mixture, the same difenoconazole dose was applied for all treatments (10 μg g^{-1}), varying just the concentration of abamectin (a.i.). Because of this exposure, concentrations of difenoconazole (a.i.) have shown similar variations in concentrations during tested exposure times, with higher amounts observed for 2_24 h and 3_24 h, decreasing in the next exposure times 2_48 h and 3_72 h. The effect of metabolization of difenoconazole after 24 h can be investigated in further studies.

A wide range of factors can act over bee's susceptibility to pesticides and includes: (a) different mode of action of the active ingredient (Sanchez-Bayo and Goka, 2016); (b) morphological and physiological differences between species (Li et al., 2017); (c) exposure way and rate (Azpiazu et al., 2019), where the distribution in plant tissues and the degradation rate is considered as relevant for this exposure (Juraske et al., 2009). Tested LC_{50} endpoint (48 h) used in this study for *M. scutellaris* (0.006 μg a.i. μL^{-1}) with a LD_{50} (48 h) of 0.0002 μg a.i. bee^{-1} was lower than endpoints found for LD_{50} (24 h) abamectin exposure for *M. quadrifasciata* (0.015 μg a.i. bee^{-1}) or *A. mellifera* (0.011 μg a.i. bee^{-1}) (Del Sarto et al., 2014), demonstrating the sensibility of this species. *M. scutellaris* was also exposed to other pesticides as imidacloprid with a LC_{50} (24 h) of 0.002 μg a.i. μL^{-1} and LC_{50} (48 h) of 0.00081 μg a.i.μL^{-1} (Costa et al., 2015) and fipronil with LC_{50} (48 h) of 0.000011 μg a.i. μL^{-1} (Lourenço et al., 2012).

Besides lethal effects, the concentration of abamectin and difenoconazole detected in *M. scutellaris* can also lead to sublethal effects as observed for other pesticides. Tomé et al. (2020) have also investigated the impact of different pesticides on the development of *Apis mellifera* larvae fed with these compounds in a concentration range of 0.8–28703 μg mL^{-1}, affecting the survival and health of immature bees, contributing to the general stress or loss of the colony and altering the gene expression of detoxification enzymes. For the neonicotinoid thiamethoxam, the chronic exposure to 2.4 μg L^{-1} has been affected the short-term memory in bumblebees (Stanley et al., 2015), while a dose of 1.96–2.90 ng bee day^{-1} can affect the foraging activity and the return back to the hives (Tosi et al., 2017).

For pesticides, synergistic interactions may occur when different substances have the same mode of action and antagonist interactions when the substances have a different mode of action (Johnson et al., 2013). Considering the different modes of action of those pesticides, where abamectin can affect directly the nervous system and difenoconazole, as a fungicide, affects the synthesis of sterol in fungi, an independent response is expected. Moreira et al. (2017) performed acute toxicity tests with Kraft® 36 EC and Score®250 EC mixtures in neotropical cladocerans and observed that antagonism was observed at low concentrations of pesticide mixtures and high concentrations synergism occurred. For fishes, Sanches et al. (2017) also performed acute toxicity tests with the Kraft® 36 EC and Score®250 EC mixtures on adult zebrafish (*Danio rerio* Hamilton, 1822) and the results showed that mixing the two pesticides caused greater fish toxicity (synergism) than when tested individually. In the same way, this synergistic effect could be observed for *M. scutellaris,* where mortality has been increased for all treatments tested in the presence of commercial products mixture.

In bees, Schmuck et al. (2003) also observed a synergistic effect of the combination between ergosterol biosynthesis inhibiting fungicides and the thiacloprid insecticide in *Apis mellifera*. In laboratory tests prochloraz and tebuconazole fungicides strongly increased insecticide toxicity, with mortality of 87 and 70% of treated bees, respectively; Johnson et al. (2013) tested combinations between the insecticide/acaricide tau-fluvalinate and sterol biosynthesis inhibiting fungicides (prochloraz, propiconazole, fenbuconazole, metconazole, and myclobutanil) in *Apis mellifera* bees. Of the fungicides tested, four increased the dose-dependent toxicity of tau-fluvalinate and the authors attributed this synergism to inhibition of P450 enzymes. However, this synergism considering a binary exposure of pesticides from different classes and modes of action can differ. For the solitary bee, *Osmia bicornis* Linnaeus, 1758. Azpiazu et al. (2019) did not find synergistic effects in the exposure of the insecticide imidacloprid and the fungicide myclobutanil orally exposed via nectar and pollen in environmental levels, indicating that this synergism can be related with the exposure concentration. The same results were observed by Thompson et al. (2014), for *A. mellifera*, where the results showed, in general, a low synergism between fungicides and neonicotinoids. However, besides concentration and chemical family, synergism between pesticides can also be related to other factors acting over the metabolic pathway, where interactions and effects cannot be predicted (Hernández et al., 2017).

For abamectin, the inhibition of detoxification metabolism is not reported. Metabolism is identified in some species and related to the P450 monooxygenase system (Chandor-Proust et al., 2013; Riga et al., 2014; Zeng et al., 1996). In this sense, as discussed above, this compound could be eliminated from the organism in the tested concentrations without accumulation; however, the inactive ingredients can act in this inhibition and allow the concentration of the active ingredient, increasing the toxicity of commercial formulations applied in the field. Differently, azole fungicides, as difenoconazole, can be considered as inhibitors of P450 monooxygenase enzymes, responsible for the metabolization of xenobiotics (Feyereisen, 2015; Gottardi et al., 2018). This possible inhibition of P450 detoxification metabolism observed for a commercial product containing abamectin, difenoconazole, and their binary mixture could result in the accumulation of investigated pesticides in bees. Through the determination of abamectin and difenoconazole in exposed bees was possible to verify the relationship between accumulation and mortality effect. In this sense, further studies must be carried for the investigation of the direct effects of these detected concentrations over bee's metabolism, where sublethal effects must be also considered, once the toxicity over the organism's health can appear further in the lifetime, affecting the hive survivor.

4. Conclusions

The determination of abamectin and difenoconazole in the stingless bee *Melipona scutellaris* was successfully proceeded with good accuracy and precision and was applied in exposed bees. The exposure of those pesticides via topic and oral to commercial products as well as standard with a high purity level, in case of abamectin, had demonstrated the sensibility of this species to concentrations already described in the literature as NOEC, $LC(D)_{50}$ and 2 times $LC(D)_{50}$. Mortality was higher through oral intake, however, the concentrations in the bee's body were observed in both ways of exposure, demonstrating the uptake of those pesticides and accumulation in *M. scutellaris*.

Results had also demonstrated that with the single exposure to standard with a high purity level of abamectin is not possible the detection of this pesticide in bees, while in the commercial product (abamectin a.i) this accumulation is observed. The exposure of abamectin (a.i.) via oral or topic presents the same concentrations in investigated bees and can indicate that independently of the way of exposure bees can be seriously affected by the accumulation of this contaminant in its body. For difenoconazole (a.i.), in the commercial product mixture, it could be observed that the

accumulation of this pesticide can be increased compared with single difenoconazole (a.i).

All these results can contribute significantly to the comprehension of the impact of pesticides in bees and the capacity of accumulation in *M. scutellaris*, as a result of different routes of exposure. Further studies must be carried for the investigation of the effects of these concentrations in metabolism and the health of bees, effects that can contribute to the increase of bee's mortality and colony collapse. At the same time, the developed method can also be applied for environmental samples, where the environmental risk assessment can be accessed.

Declaration of competing 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 paper.

CRediT authorship contribution statement

Fernanda Scavassa Ribeiro do Prado: Conceptualization, Methodology, Investigation, Writing - original draft. **Dayana Moscardi dos Santos:** Investigation, Writing - review & editing. **Thiessa Maramaldo de Almeida Oliveira:** Methodology. **José Augusto Micheletti Burgarelli:** Methodology. **Janete Brigante Castele:** Conceptualization, Methodology, Investigation. **Eny Maria Vieira:** Conceptualization, Supervision.

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Appendix A. Supplementary data

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