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Chromatographic analysis of the insecticides thiamethoxam and imidacloprid in the stingless bee *Melipona scutellaris*: determination, toxicity, and evaluation of the effects of biochemical markers

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Thesis submitted to the Institute of Chemistry Sao Carlos (IQSC), University of Sao Paulo as part of the requirements for the award of degree of doctorate in chemistry.

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DEDICATION

This thesis is dedicated to my beloved parents, Abdur Rahman Nasir and Ayesha Rahman, to my lovely daughter Syyeda Hania Ali and especially to my husband Dr. Mian Abdul Ali whose prayers, love, guidance, and cooperation inspired me to the intention.

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RESUMO

O foco deste estudo foi desenvolver um método analítico para a determinação dos inseticidas tiametoxam (TMX) e imidaclopride (IMD) em tecidos de abelhas sem ferrão Melipona scutellaris para avaliar a concentração letal (CL50) e a dose letal (DL50) desses inseticidas em direção a abelha. O método QuEChERS modificado foi usado para preparar as amostras, seguido por cromatografia líquida de alto desempenho com detector de matriz de diodos (HPLC-DAD) e cromatografia líquida - espectrometria de massas sequencial (LC-MS/MS). Os parâmetros de validação do método desenvolvido incluíram: faixa linear entre 0,0005 ng μL^{-1} a 0,5 ng μL^{-1}com R^2 > 0,99. O limite de quantificação (LOQ) para o sistema LC-MS /MS foi 0,0012 ng μL^{-1} e 0,0011 ng μL^{-1} e para HPLC-DAD 3,06 ng μL^{-1}e 3,15 ng μL^{-1} para TMX e IMD, respectivamente. O LOQ do método foi de 2,5 ng $abelha^{-1}$ para ambos os inseticidas. O acúmulo de TMX e IMD nas vias oral e tópica de exposição foi verificado nas abelhas testadas e os resultados obtidos indicaram o que? é adequado para a determinação e quantificação de resíduos de inseticidas neonicotinoides nesta espécie. A mortalidade de abelhas foi maior para a exposição por via oral do que a tópica para ambos os pesticidas. Além disso, métricas analíticas verdes foram calculadas e comparadas com métodos descritos na literatura envolvendo análise de neonicotinoides em abelhas. Como resultado, o presente trabalho apresentou os maiores escores Eco e HPLC-EAT, e a segunda menor quantidade de amostra e de resíduos gerados. Mais longe estudo foi realizado para a investigação dos efeitos diretos das concentrações detectadas sobre o metabolismo das abelhas, sistemas enzimáticos e não enzimáticos. As atividades da glutationa S-transferase (GST) e neurotransmissores, octopamina, dopamina e serotonina foram determinadas em tecidos de abelhas expostas a pesticidas para explorar as estratégias de desintoxicação e tolerância das abelhas. Os resultados mostraram que neurotransmissores, octopamina e dopamina foram detectados em todas as amostras de tecido, enquanto a serotonina foi detectada apenas para exposição oral e tópica de abelhas aos padrões do IMD, em vez de compostos comerciais. Os resultados baseados em GST mostraram que as atividades enzimáticas são altamente afetadas com a exposição oral e tópica desses inseticidas às abelhas.

Palavras-chave: Thiamethoxam, imidacloprid, QuEChERS, HPLC-DAD, LC-MS/MS, métricas verdes, bioquímicos marcadores.

ABSTRACT

The focus of this study was to develop an analytical method for the determination of the insecticides thiamethoxam (TMX) and imidacloprid (IMD) in stingless bee tissues *Melipona scutellaris* to assess the lethal concentration (LC_{50}) and lethal dose (LD_{50}) of these insecticides towards the bee. The modified QuEChERS method was used to prepare the samples, followed by high performance liquid chromatography with diode–array detector (HPLC-DAD) and liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis. The validation parameters of the developed method included: linear range between 0.0005 ng μL^{-1} to 0.5 ng μL^{-1} with $R^2 > 0.99$. The limits of quantification (LOQ) for the LC-MS/MS system was 0.0012 ng μL^{-1} and 0.0011 ng μL^{-1} and for HPLC-DAD were 3.06 ng μL^{-1} and 3.15 ng μL^{-1} for TMX and IMD, respectively. The method LOQ was 2.5 ng bee^{-1} for both insecticides. The accumulation of TMX and IMD in both oral and topical paths of exposure was verified in the tested bees and the obtained results indicated that what is suitable for the determination and quantification of neonicotinoid insecticides residues in this species. The mortality of bees was higher for exposure by oral paths than the topical one for both pesticides. Moreover, green analytical metrics were calculated and compared to methods described in the literature involving neonicotinoids analysis in honeybees. As a result, the present work displayed the highest Eco score and HPLC-EAT score, and the second smaller amount of sample and of waste generated. Further study was carried out for the investigation of the direct effects of the detected concentrations over bee's metabolism, enzymatic and non-enzymatic systems. The activities of the glutathione S-transferase (GST) and neurotransmitters, octopamine, dopamine and serotonin were determined in bee tissues exposed to pesticides to explore bee's strategies for detoxification and tolerance. Results showed that neurotransmitters, octopamine and dopamine were detected in all tissue samples while serotonin was detected only for oral and topical exposure of bees to IMD standards rather than commercial compounds. Results based on GST showed that the enzymatic activities are highly affected with both the oral and topical exposure of these insecticides to bee's.

Keywords: Thiamethoxam, imidacloprid, QuEChERS, HPLC-DAD, LC-MS/MS, toxicity analysis, green metrics, biochemical markers.

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LIST OF ABBREVIATIONS

AChE – Acetylcholinesterase

ACN – Acetonitrile

ANVISA – National Health Surveillance Agency

AOAC – Association of Official Analytical Chemists

BOD – Biochemical Oxygen Demand

CAT – Catalase

CE – Collision energy

CEN – European Committee for Normalization

CEP – Collision cell entrance potential

LC_{50} – Average Lethal Concentration

CXP – Collision cell exit potential

DCC – Colony Collapse Disorder

LD_{50} – Mean Lethal Dose

DP – Declustering potential

d-SPE – Dispersive Solid-Phase Extraction

ME – Matrix Effect

EP – Entrance potential

EPE – Extraction Process Efficiency

ESI – Electrospray Ionization

FD – Fluorescence Detector

GABA – Gamma-Aminobutyric Acid (Gamma-Aminbutyric acid)

GC-MS – Gas Chromatography Coupled with Mass Spectrometry

GPx – Glutathione PeroxidaseGSH Glutathione

GST – Glutathione S-Transferase

HPLC-DAD – High Performance Liquid Chromatography with Diode-Array Detector

LC-MS/MS – Liquid Chromatography – Tandem Mass Spectrometry

a. i. – Active ingredient

IMD – Imidacloprid

INMETRO – National Institute of Metrology, Quality and Technology

LOD – Limit of Detection

LOQ – Limit of Quantification

MAPA – Ministry of Agriculture, Livestock and Supply

MDA – Malondialdehyde

NOEC – No Observed Effect Concentration

OECD – Organization of Economic Co-operation and Development

C. p. – Commercial product

PPDB – Pesticide Properties DataBase

PSA – Primary and Secondary Amine

QuEChERS – Quick, Easy, Cheap, Effective, Rugged, Safe

$\mathbf{r^2}$ – Determination coefficient

RE – Recovery

ROS – Reactive Oxygen Species

RSD – Relative Standard Deviation

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SOD – Superoxide Dimutase

TMA – Thiamethoxam

TBA – Thiobarbituric Acid

TMP – 1,1,3,3-tetramethoxypropane

UV/VIS – Ultraviolet-Visible

SUMMARY

1. INTRODUCTION

The exponential population growth and the resulting increased demand for food in the world led to a model of agricultural production based on the intensive use of pesticides for improving crop yields [1–3]. The use of those chemicals is often a major concern due to potential harm to the environment and suspected toxic effects on the health of bees, which are extremely sensitive to pesticides [4].

The use of pesticides in agriculture is prominent in Brazil and when used improperly, residues of these compounds can persist in food, can be a source of human exposure to toxic products and still contaminate bees that travel miles in search of food such as water, nectar, and pollen. In this path traveled by bees, microorganisms, particles suspended in the air, and chemicals such as pesticides can be intercepted by these pollinators and may be retained in the superficial hairs of their body and/or inhaled and retained in their respiratory tract. If these particles are taken bees to the hive, they can contribute to the extinction of the colony or trigger the phenomena known as Colony Collapse Disorder (CCD). The extinction of hives causes damage to native plants and also for agricultural production in general that depend on the pollination of these insects.

1.1 Ecological and economic importance of bees

Bees are the most important pollinating agents responsible for the pollination of 70% of crops and one-third of all angiosperms; bees are essential for the balance of the ecosystem and also for the maintenance of agricultural production [5]. For the plants to reproduce sexually, especially plant pollination is essential. When this is insufficient or absent, there is the loss of genetic variability, reduction of agricultural production, increase of deformed fruits and in more severe cases plant extinction may occur with consequent reduction of animals dependent on their products, changes in the soil and the regimes of water [6]. Understanding the ecological and economic importance of bees need for the application of conservation actions for these pollinators, mainly through proper management and sustainable use. Such practices involve greater control over the use of agricultural defenders in crops; avoid excessively management of colonies; the non-introduction of exotic species; careful in the preparation of land for planting to maintain nests of social and solitary bees occurring in the soil and maintenance of native vegetation at the edges of plantations that may offer resources to

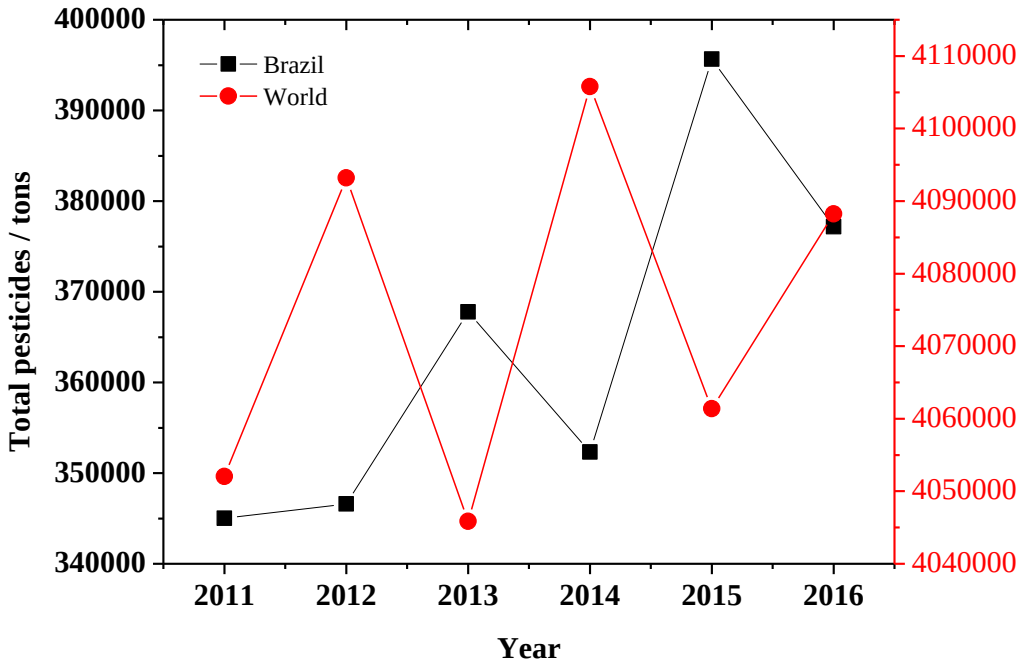
pollinators [7–9]. Bees can be used as indicators of environmental pollution because of their morphological characteristics and foraging activity, and their ability to retain and bioaccumulation in their bodies' substances which they are in close contact with during pollination.

Bees play a vital role on earth. Some even claim that if they go extinct, humanity would be next. So the decline in the bee population, should we be worried? What if the bees all die? Simply put, if a plant produces a flower, you can bet that bees help them reproduce. This long-standing, working relationship evolved with the flower being bright and fragrant to attract bees, and the bee fuzzy, Velcro-like bodies helping them to efficiently transfer pollen from the male part of a plant to the female part, this seemingly simple mechanism is directly responsible for the production of 70% of fruits, vegetables, seeds and nuts that we consume on a daily basis, this translates into almost $200 billion in global agriculture revenue [7,8]. This huge responsibility is accomplished by droves of commercial bees, reared by professional beekeepers for the sole purpose of being transported to farms and orchards to pollinate crops but since 2006, these hard-working, busy bees have been mysteriously disappearing. Some European beekeepers have reported extinction of honey bee colonies located near crops treated with pesticides, even at a low dose [6]. Stingless bees are represented by more than 500 species distributed in the neotropical region of the Earth [10]. In Brazil, there are about 300 eusocial species, in which these stingless bees are considered important not only for the balance and biodiversity of the ecosystem but also plays an important role in crop pollination [11,12]. Stingless bees are responsible for the pollination of 30 to 90% of the native Brazilian flora, depending on the ecosystem in which they are present and the pollination of up to 33% of agricultural crops [11-13]. *Melipona scutellaris* produces significant amounts of honey (compared to other meliponids). The honey of these bees is considered medicinal mainly by regional populations, being used as a fortifier and aphrodisiac, and for the treatment of flu, bronchitis and whooping cough, in addition to other diseases such as fatigue, cancer, scarring, sore throat, asthma, amoebiasis, cataracts, thrombosis, and tuberculosis, among others [13,14].

1.2 Use of pesticides in Brazil and worldwide, linked to decline in the bee population

The use of pesticides in the agricultural production process is responsible for billions of dollars worldwide. In 2016, Brazil ranked third in the world ranking pesticide trading countries, having consumed or sold to the agricultural sector 377,176 tons of pesticides. In the same year, about 5,860,537 tons of pesticides were sold worldwide [15]. Figure 1 shows the consumption of pesticides in the period from 2011 to 2016 in Brazil and worldwide.

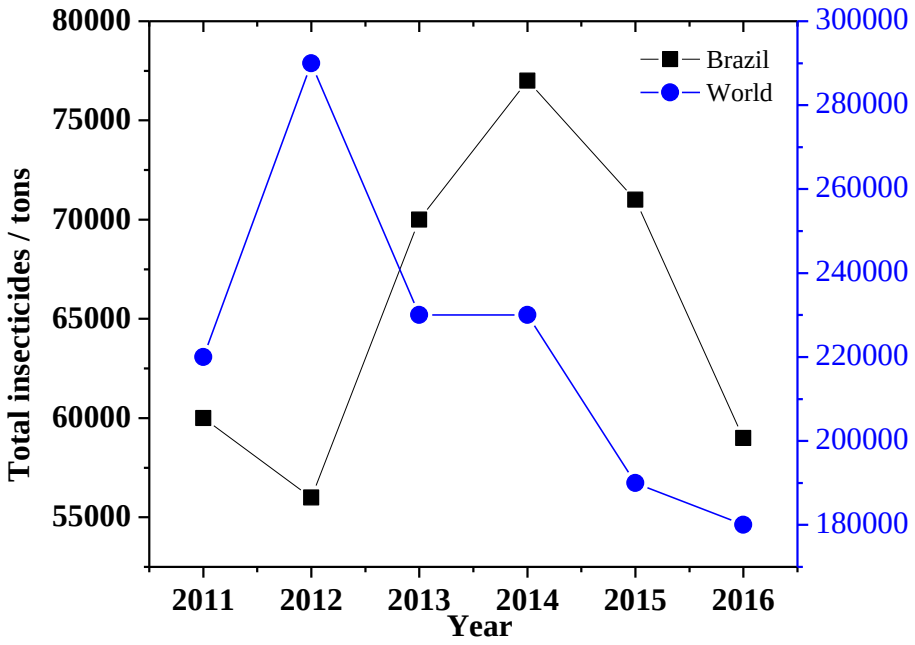
Figure 1. Marketing of pesticides in Brazil and worldwide



Source: Adapted from Ref. [15]

Brazil consumed 60,607 tons of insecticides, while that worldwide consumption was approximately 181,131 tones [15]. Figure 2 shows the commercialization of insecticides in the period from 2011 to 2016 in Brazil and worldwide.

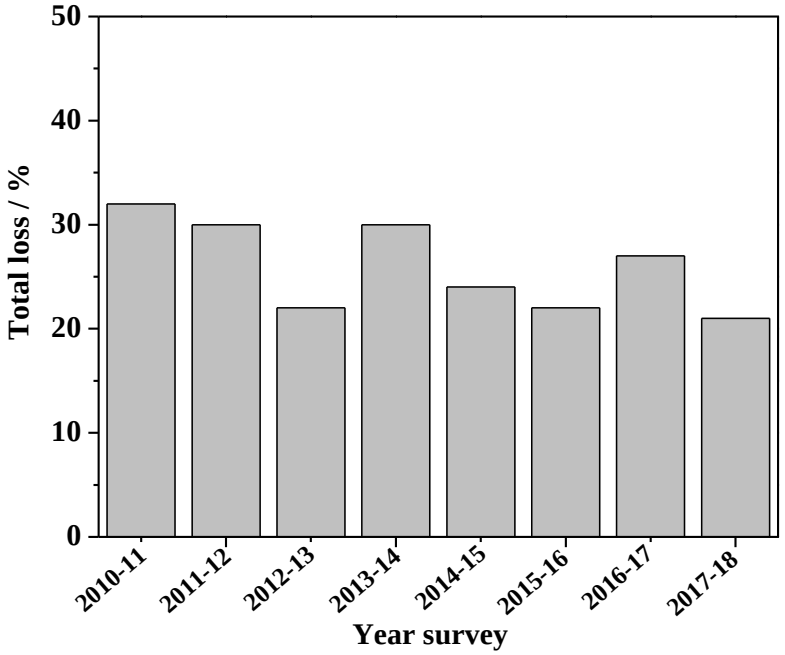
Figure 2. Marketing of insecticides in Brazil and worldwide.



Source: Adapted from Ref. [15]

The use of pesticides, on the one hand, is seen as beneficial due to its action in pest control, on the other hand, is seen as causing the contamination of the soil, water, food, and insects, among others, since these compounds present permanence in the environment and tendency to bioaccumulation [16]. In recent years, due to the global decline in the bee population, mainly those species of the genus *Apis*, the health of the bees has become a matter of public interest. Since 2003, in North America and Europe, the phenomenon is known as Colony Collapse Disorder (CCD). European data revealed that annual colony mortality rates reported between 2012 and 2014 reached 36%. United States data showed that annual colony losses reported by beekeepers reached 45 % [1]. In the United States, the increase in bee colony losses is a major concern for the country. Colony losses are monitored annually since the winter of 2006-2007 [17]. Figure 3 shows the annual colony losses in the United States from 2010 to 2018. High colony losses has also been reported in the Middle East [18] and Japan [19].

Figure 3. Monitoring of annual colony losses in the United States.



Source: Adapted from Ref. [20]

The CCD mechanism remains unknown, but there is a compromise between scientists that there are several factors that can cause colony losses and exposure to pesticides and insecticides may be one of the factors that contribute to the decline of pollinators and increased CCD [21,22].

Losses of large-scale *Apis* hives in Brazil occurred simultaneously with the expansion of crops for agrofuels and the consequent increase in the use of pesticides. Beekeepers attributed these losses to the use of insecticides, especially neonicotinoids [23]. Infestation by the *Varroa* sp. is evidently high in the states of Rio Grande do Sul and Santa Catarina and beekeepers report loss of hives attributed to that mite. It looks like *Varroa* and also the *Nosema* bee parasite spp. are becoming more problematic than they used to be and can be a consequence of the increase in the use of pesticides that cause the weakening of bees' immune or behavioral defenses [23]. For these reasons, *Apis mellifera* bees treated in the laboratory with sublethal doses of insecticides showed reduced movement and mobility, decreased communication and learning capacity, decreased return to the colony, in the foraging behavior, and pollination [24–27]. Examples of these effects are seen when *A. mellifera* bees are exposed to pyrethroid insecticides in concentrations equal to those found in the environment. Pyrethroids impair foraging behavior and increase the mortality of workers causing significant

reductions in the development of the litter and colony. Exposure to low levels of pyrethroids can affect the ability of bees to return to the hive [28]. In one study it was reported that 43% of *A. mellifera* foragers returned to the hive once after exposure to low pyrethroids, only 4% returned twice after treatment and none of the exposed bees were present in the hive at the end of the day or the next morning. Bees still presented behavioral disorders, such as excessive self-cleaning, contracted abdomen, and shaky dance [29]. Pyrethroids are one of the classes of pesticides more related to CCD. Neonicotinoids are another class of pesticides related to the causes of CCD [28]. *A. mellifera* bees exposed in the laboratory to sublethal doses of neonicotinoids showed abnormal foraging activity, reduced olfactory memory, and learning performance, and decreased return to Colony [30]. Low concentrations of neonicotinoids in nectar did not show lethal effects but reduced the expected performance in adult bees in approximately 6 % and 20 % [31]. An additional effect observed on bees *A. mellifera* and *Bombul Terrestris* was the alteration of their foraging activities making them prefer foods mixed with neonicotinoids instead of other sources of nectar [32].

Researchers should disseminate knowledge of the role of pesticides, as the main factors that affects the health of bees, by the development of new, more sensitive and reliable methods to detect very low levels of concentration. May be presence of pesticides and the interaction between different compounds in environmental samples can harm the defense systems of bees that allow parasites or viruses to cause the colony to become extinct [6].

1.3 Stingless bee (*Melipona scutellaris*)

M. scutellaris is a eusocial stingless bee species of the order Hymenoptera and the genus *Melipona* and is found with the largest distribution in the North and Northeast regions of Brazil, with records from the Rio Grande do Norte to Bahia. This species also seems to be well adapted to the climate and ecology of the State of São Paulo [1]. Its common name is Uruçu, comes from the word "eiru su" of the indigenous language “Tupi", means "big bee". It belongs to the group of species with the moderately large body size of Melipona (between 10 and 13 mm of total length) and body mass above 60 mg [33]. *M. scutellaris* nests in cavities of tree trunks in the Atlantic rainforest and is widely distributed in the Northeast of Brazil, where it is commonly kept by regional and traditional beekeepers for honey, pollen, and wax. To construct their nests, *M. scutellaris* use cerumen, a mixture of wax and floral resins. Like honey bees, stingless

bees form perennial, swarm-founded colonies. *M. scutellaris* colonies typically contain a population of approximately 1,500 bees and are headed by a single, once mated queen. The development time from egg to adult is about 40 days [5,7,34,35].

Figure 4. Individuals of the species *M. scutellaris*: From left to right: worker, male and queen.

Source: Adopted from WEBBE (2014).

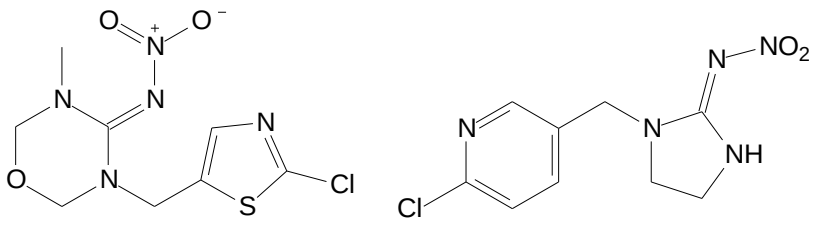
This bee species has been often reported to be more sensitive to insecticides than *Apis mellifera*, which has traditionally been used as standard bee test species [36,37]. The exposure assessment of this species has been recommended to better understand the high sensitivity of insecticides [38].

1.4 Studied pesticides

Nowadays, neonicotinoid insecticides are widely used in agricultural practice to control pests and diseases in crops. The decomposition of these compounds in the environment and extensive or improper use can lead to the contamination of various ecosystems. Widespread distribution of these chemicals is also known to cause problems to the apiculture industry. Bees may be contaminated by these chemicals residues during harvesting and contaminants can be transported on bee bodies or with forages to the hive, from where they can contaminate honey. The presence of such contamination in bee products can decrease their quality and can also devalue their properties [39,40]. Neonicotinoids are a class of pesticides with widespread use in agriculture and they have been indicated as one of the main reasons for the worldwide bee colony collapse disorder phenomenon [41]. Both TMX and IMD are systemic insecticides. TMX is a white, odorless, and amorphous compound while IMD is a colorless crystalline compound, but with a faint, characteristic odor [42]. Those insecticides interfere with the transmission of stimuli throughout the insects nervous systems. They interrupt the nicotinic neuronal pathway by blocking nicotinic

acetylcholine receptors [43]. Therefore, they prevent acetylcholine from transmitting impulses between nerves, resulting in the insect paralysis and eventual death. They are effective both by contact and ingestion [43–45]. TMX hydrolysis is quite slow at room temperature and in neutral or slightly acidic solutions (half-lives: 200-300 days to more than one year at pH 5 and 7, respectively) [46,47]. It is more labile at pH 9 at which the half-life is only a few days [46,47]. It readily undergoes photolysis (half-life: approximately 1 h) [46,47]. Similarly, the half-life of IMD in soil ranges from 40 days in unamended soil to 124 days in recently amended soil with organic fertilizers [46–48]. Figure 5 shows the chemical structures of IMD and TMX. The IMD and TMX both are a broad-spectrum, systemic insecticide, which means it is absorbed instantly by plants and transferred to all of its parts, including pollen, where it acts to prohibits insect feeding. These are potent on both contact and via stomach action. Chronic exposure of honeybees to these pesticides at concentrations that could approximate field-level exposure harms their natural foraging behavior and increases worker bee's mortality leading to a significant decrease in brood development and colony success. Therefore, determination of residues of these compounds is very important.

Figure 5. Chemical structure of (a) TMX and (b) IMD pesticides.



(a) **(b)**

1.5 Sample pre-treatment procedures prior to chromatographic determination

Bees bodies contain large amounts of waxes, proteins, and other substances, which are detrimental to the results of chemical analyses. To accurately determine minute amounts of residual pesticides in complex matrices such as bee bodies, sample pre-treatment procedures must be, therefore, performed before chromatographic separations [49–51]. It is no exaggeration to say that the results of the procedures have a decisive influence on the reliability of the data measured. This also applies to the analysis of neonicotinoid insecticides (TMX and IMD).

Many sample preparation techniques — solid-phase extraction (SPE), liquid-liquid extraction (LLE), solid-phase micro-extraction (SPME), liquid-phase micro-extraction (LPME), etc. — have been used for the quantification of pesticides in water and other matrices [52–57]. However, the most universal technique for extracting a wide range of pesticides from complex matrices is the QuEChERS method. Liquid-liquid partition has been used since the initial phase of the market release of imidacloprid. However, that technique presents problems: e.g. a large amount of organic solvent is used, and an emulsion is formed at the liquid-liquid interface depending on the extraction sample that is used [58–60]. For that reason, it has been increasingly replaced by clean-up mainly by SPE. Watanabe et al. used re-extraction with diatomaceous earth SPE and clean-up with GCB/NH_2 SPE in the development of simultaneous analysis by HPLC-DAD of seven neonicotinoid insecticides released on the market [49]. Matrix solid-phase dispersion (MSPD) or solid-phase extraction (SPE) followed by clean-up using gel permeation chromatography (GPC) is the most generally utilized for honeybee sample preparation. However, this procedure of preparing samples allows only for the determination of selected analytes from the group of pesticides. New ways of sample preparation for analysis are necessary to determine the widest possible spectrum of pesticides [49].

The QuEChERS method, first introduced by Anastassiades et al. [50] in 2003 with the aim of overcoming the practical limitations of multi-residue extraction methods existing pesticides. The method has been described as a quick, easy, cheap, effective, robust, and safe, which was proposed for the analysis of pesticide residues in fruits and vegetables [16,50]. The QuEChERS extraction method was developed in order to obtain a procedure that could be performed in any laboratory, using simple steps which that consisted of extraction via acetonitrile extraction, dehydration, and salting-out with anhydrous magnesium sulfate and sodium chloride. Thereafter, dehydration and clean-up of the extract are done using dispersive SPE with anhydrous magnesium sulfate and primary secondary amine (PSA), which is a weak anion exchange adsorbent [16,50,61]. Reportedly dispersive SPE using PSA reduces the sample interferences and the matrix effect as extremely effective for the removal of organic acids, polar dye components, and saccharides [62]. According to Anastassiades et al. [50] and Lehotay and Mastovská [61], some of the advantages of the QuEChERS method are high recoveries (greater than 85%) for pesticides, in a wide range of polarity and volatility, low consumption of solvents, rapid analysis (10 to 20 samples in 30 to 40 minutes), production of little

waste, use of little material, robustness and low cost of reagents used. Therefore, QuEChERS is a technology that contributes much to speed up and simplification of sample pre-treatment procedures. In addition, the amounts of organic solvents used are extremely small: about 10 mL of acetonitrile per sample. For those reasons, it can be concluded that technology has met all of the requirements described by Wan and Wong (1996). In this study, we describe the evaluation and adaptation of the QuEChERS approach in combination with high-performance liquid chromatography–diode array detector (HPLC–DAD) and LC-MS/MS used to determine pesticide residues in bee samples. The refined methodology was already used to determine other types of pesticide residues in different matrices (honey samples).

1.6 Green metrics

Besides that, QuEChERS is a suitable methodology for being incorporated into greener methods. Although the Green Chemistry approach is not new, the use of green chemistry metrics, especially for assessing analytical procedures, is still a big challenge [63,64]. Green metrics tools mainly include: (a) the National Environmental Methods Index (NEMI) pictograms [65] (the oldest tool, qualitative nature) [63]; (b) Eco scale table score [65], which encompasses information about the kind and amount of chemicals used, the energy used in the process, occupational hazards, and the amount of waste generated; and (c) the HPLC Environmental Assay Tool (HPLC-EAT) [66,67], which measures any impacts on the analysts safety/health and on the environment.

However, as none of those tools can solely take into consideration all the twelve principles of Green Chemistry, it is advisable to merge them into a single parameter, to determine the environmental performance of analytical procedures. Multi-criteria decision analysis (MCDA) is a powerful methodology for doing so. One example of MCDA is the Preference Ranking Organization Method for Enrichment of Evaluation (PROMETHEE), which ranks the available choices by giving different weights to the used parameters [68].

1.7 Determination of neonicotinoid insecticides based on chromatographic methods

Chromatography is a powerful technique for the determination of insecticides. In the general chromatographic determination, neonicotinoid insecticides are classified into two group's i-e HPLC and GC. However, because neonicotinoid insecticides are

generally degraded by heat, additional processes such as derivatization are necessary for GC determination, making sample pre-treatment procedures more complicated than HPLC determination.

As presented above, it is presumed that the utilization of HPLC rather than GC is more advantageous for the determination of neonicotinoid insecticides from the viewpoints of speedup and simplification of sample pre-treatment procedures. It also is readily apparent that most cases reported to date used HPLC for determination. In determination by HPLC, both HPLC-UV and HPLC-DAD contributed greatly to analyses of neonicotinoid insecticides until the 2000s, when MS and MS/MS began to become popularly used. Since the report by Ishii et al. (1994) [59], HPLC-UV and HPLC-DAD have been applied to the determination of neonicotinoid insecticides in various matrices. In addition to UV and DAD, methods were developed in which IMD and TMX were separated using column chromatography and then converted into electrochemically active compounds by ultraviolet irradiation to be detected by an electrochemical detector (ECD) or converted into fluorescent substances to be detected using a fluorescence detector (FLD). ECD and FLD are generally more sensitive than UV or DAD but the analyte needs derivatization. They are applied to residue analysis in the bodies of bees, where determination at low concentrations must be done. In fact, HPLC-UV and DAD have less measurement sensitivity and selectivity than either LC-MS or MS/MS, which are the most widely, used methods today. Utilization of MS enabled not only the detection of trace pesticide residues in various matrices with high accuracy but also the elucidation of their respective chemical structures. LC-MS and LC-MS/MS are suitable for highly sensitive determination of only slightly volatile and heat-unstable pesticides. The matrix effect is a phenomenon that is also observed in LC-MS and LC-MS/MS determination; it was shown earlier that when the target pesticide is eluted together with matrix components in the sample, ion suppression or ion enhancement occurs during the ionization process, engendering error in the determination result [69–72].

1.8 Acute Toxicity analysis of insecticides in bees

The aim of this risk assessment is to identify the main chemicals that may pose a threat to the life of bees in their natural environment, which is currently contaminated with a large array of insecticides and other chemicals. By highlighting the compounds with higher risk to bees, we hope that apiculturists, beekeepers, and policymakers

involved in agricultural production will be able to screen the products most harmful to bees and find the appropriate remedies to avoid further damage. LD stands for "Lethal Dose". LD50 is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD50 is one way to measure the short-term poisoning potential (acute toxicity) of a material [73–75].

In the past, bee poisoning by pesticides was associated only with lethal exposure (acute intoxication), which results in an accumulation of dead bees near the hives. However, pesticides affect bees in sublethal levels, not killing them immediately, but harming their behaviors or their ability to fight infections. Customarily, a dose lower than the LD_{50} (1/2,1/5 and 1/10 of LD_{50}) for a certain substance, is considered as sublethal, thus not involving mortality events. Sublethal effects may include a large number of physiological disturbances that are usually considered for all non target insects, whereas other sublethal endpoints have been specifically developed with regard to honey bees. With respect to neural effects, that are the most widely investigated sublethal impairments, a distinction can be done between intellectual disabilities, behavioural effects and physiological function related effects [76]. The impact of pesticide on intellectual disabilities has been mainly evaluated, testing the effects on olfactory and visual learning performances. Some pyrethroids, as deltamethrin, have been shown to have an effect on olfactory learning [24], but the most important effects have been related to neonicotinoids both *via* direct contact and *via* ingestion exposure [77–79]. IMD is the first active ingredient proven to affect honey bee in open field [80]. Lately, the RFID (Radio-Frequency Identification) technology has allowed to perform this kind of experience with a larger number of individuals and to ensure a reliable recording of data. Thus, Imidacloprid and thiamethoxam [30,81] have been demonstrated to be detrimental for spatial orientation capacities in forager bees. Some of the most relevant effects on physiological function are then represented by thermoregulation and muscle activity modifications [76]. Furthermore, sublethal effects might also be mitigated at a colony level. Behavioral effects of bees can be observed in laboratory conditions and are likely to be evaluated at a colony level, where a more pronounced adaptability is present.

1.9 Biochemical Biomarkers

A large number of studies have used biochemical biomarker's functional tools to assess the effect of various compounds on living organisms [82]. Biomarkers can be

defined as observable changes or measurable at the molecular, biochemical, cellular, physiological, or behavioral indications of present or past exposure of an organism to xenobiotics [83]. In the terrestrial environment, the bee is a particularly relevant model for the development of biomarkers in order to assess contamination because their intense foraging activity puts them in contact with a large number of contaminants around the hive [83,84]. Few studies have been done on the presence of biomarkers in bees, and most of them concern the enzyme acetylcholinesterase (AChE). However, an effective assessment of the ecotoxicological impacts of xenobiotics on bees requires an approach that combines several biomarkers as it will allow a more accurate diagnosis of the effects caused by exposure to stressors through a combination of different biological responses [83]. Examples of biomarkers used in toxicity and effects assessments of stressors on organisms include the activity of enzymes from biotransformation, antioxidant defense, and oxidative stress parameters [85]. The biotransformation process of xenobiotics is divided into three phases: phase I involves oxidation, reduction, or hydrolysis of contaminants; phase II consists of in conjugation reactions and in phase III, excretion occurs [85]. The process of biotransformation generates reactive oxygen species (ROS), which are free radicals that react with bimolecules causing their disruption, often with a consequent loss of function. Examples of ROS include the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and highly reactive hydroxyl radical ($OH^{\bullet}$) [86]. To minimize oxidative damage to cellular components, organisms developed antioxidant defenses. Important antioxidant enzymes are the superoxide dismutase enzymes (SOD, converts $O_2^{-\bullet}$ to H_2O_2), catalase (CAT, reduces H_2O_2 for water), and glutathione peroxidase (GPx, detoxifies H_2O_2 or hydroperoxides produced, for example, by lipid peroxidation). Glutathione S-transferase (GST) catalyzes the conjugation of glutathione (GSH) with various substances electrophilic and prevents oxidative damage by combining peroxide degradation products lipids to GSH [86]. Organisms can get used to increased ROS production by regulating positively the antioxidant defenses, such as enzyme activities antioxidants. Failure to defend antioxidants in detoxifying production Excessive ROS can lead to significant oxidative damage, such as damage to the DNA, protein and lipid degradation, enzyme inactivation, and peroxidation lipid [86]. Lipid peroxidation is one of the main mechanisms by which oxy radicals can cause tissue damage, impairing cell function, and causing changes in the physical-chemical properties of cell membranes, which in

turn disrupt vital functions [86]. Lipid peroxides break down and produce a variety of substances, with malondialdehyde (MDA) being the most important of them. Levels of MDA reflect the combined effects of exposure to oxidative stress and the ability or lack of them to resist oxidative damage through various repair mechanisms. The malondialdehyde biomarker is a common measure of oxidative stress in insects and vertebrate systems [87]. A group of biomarkers can be a valuable tool for finding physiological disturbances induced by stressors and to study the modes of action of stressors. The biomarkers involved in the main biological systems represent evidence of bee health and can also be used symptomatology and chemical analysis to establish a diagnosis of pesticide poisoning [83].

Table 1. Proposed biomarkers: description and relevance.

Biomarkers	**Description/Relevance**
SOD	Superoxide desmutase. Defense enzyme against oxidative stress. Altered by exposure to xenobiotics.
GPx-GR-GSH	Glutathione peroxidase- Glutathione reductase-reduced glutathione. Defense enzymes against oxidative stress. Altered by exposure to xenobiotics.
GST	Glutathione-S-transferase. Enzyme belonging to the Phase II metabolic system (CYP450) whose activity is increased / reduced in the presence of xenobiotics.
AchE	Acetylcholinesterase. Inhibition of this vital neurotransmitter for muscle function commonly affected by xenobiotics such as neonicotinoid insecticides.
LPO	Lipoperoxidation. It occurs in the cell membrane when the production of reactive oxygen species exceeds the antioxidant capacity. Membrane permeability and attack facilitating DNA attack.
CAT	Catalase. Defense enzyme against oxidative stress. Altered by exposure to xenobiotics.

1.10 Neurotransmitters

Neurotransmitters (dopamine, serotonin and octopamine) are chemical messengers between nerve cells and target cells which carry, boosts, and balance the signals throughout the body. These neurotransmitter presents in body in the forms of glands, muscles, or other neurons. It works on receptor site, and triggers changes in the receiving cell. So we can say that receptors and neurotransmitter acts like a lock and key system. Just as it takes the right key to open a specific lock, a neurotransmitter (the key)

will only bind to a specific receptor (the lock). Billion of neurotransmitter molecules work constantly to keep our brain functioning and manages everything properly working in body. They can also affect a variety of psychological functions such as fear, mood, pleasure, and joy.

Dopamine: it is chemical messenger play an important role in the coordination of body movements. It is also known as the feel good neurotransmitter and involved in reward, motivation and addition.

Serotonin: it is a hormone plays an important role in regulating and modulating mood, sleep, anxiety, sexuality, and appetite.

Octopamine: Octopamine is a neurotransmitter and found only in invertebrates such as insects, commonly released during high neural activities such as flight and fight response. It can effects flight muscles; heart muscles; endocrine glands; sensory receptors (taste, vision, and olfaction responses etc); reproductive organ (oviducts etc) and central nervous system such as activity related to learning, memory, motivation, and rhythmic motor behaviors. Octopamine has no physiological role in vertebrates but would selectively target insects, making it the focus of much pesticide research.

My research project has been divided into three chapters which will be discussed in the later part of my thesis;

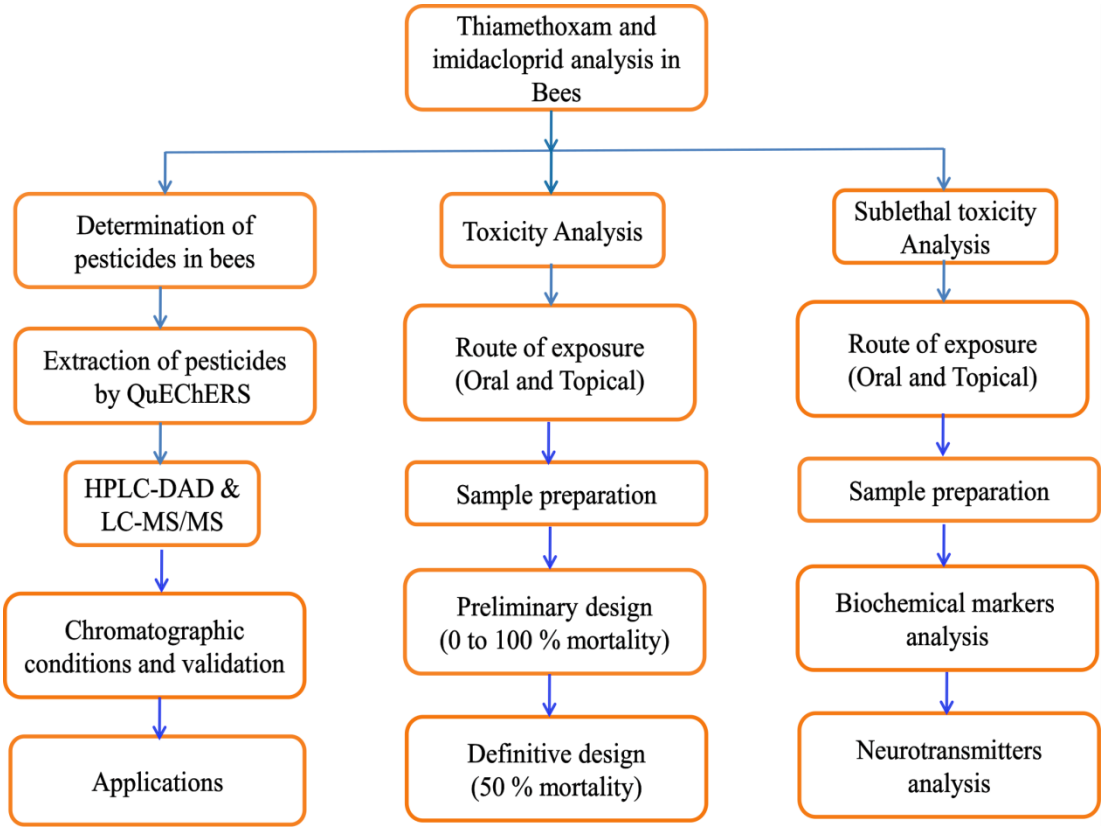
Chapter # 1. Modified-QuEChERS method for extracting thiamethoxam and imidacloprid from stingless bees: development, application, and green metrics (Manuscript has been already submitted).

Chapter # 2. Acute susceptibility of the stingless bee *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) to thiamethoxam and imidacloprid insecticides after oral and topical exposure (Manuscript almost ready for submission).

Chapter # 3. The effects of thiamethoxam and imidacloprid insecticides on stingless bee Melipona scutellaris: evaluation of biochemical-physiological changes in the assessment of sublethal toxicity (Manuscript under process).

Figure 6 shows the flow chart representing the overall scenario of the work done in this project which has been divided into three parts; determination of TMX and IMD, lethal concentration, physical and chemical effects on the bee health and behavior at low concentration of these insecticide.

Figure 6. Flow chart representing the determination, toxicity and sublethal toxicity effects of TMX and IMD on *M. scutellaris*.



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2. OBJECTIVES

2.1 General objective

The general objective of this research was to analyze the residues of insecticides thiamethoxam (TMX) and imidaclopired (IMD) of the tissue samples from native stingless bees from *Melipona scutellaris* Latreille, 1811 and to assess the effects of these compounds by using biochemical biomarkers.

2.2 Specific objectives

1. Develop and optimize QuEChERS extraction method to pre-concentrate of insecticides TMX and IMD, under study of bee's tissue samples.
2. Develop, validate and apply reliable analytical method to detect very low levels of concentration of insecticides TMX and IMD in the tissue samples of bees exposed in laboratory.
3. To assess the lethal concentration and lethal dose of these insecticides towards *Melipona scutellaris* Latreille, 1811.
4. To assess the degree of oxidative stress caused by the compounds studied through enzymatic antioxidant systems like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and in systems non-enzymatic antioxidants such as oxidized glutathione and reduced glutathione and levels of lipid peroxidation in the bee tissue samples.

3. Chapter # 1. Modified-QuEChERS method for extracting thiamethoxam and imidacloprid from stingless bees: development, application, and green metrics

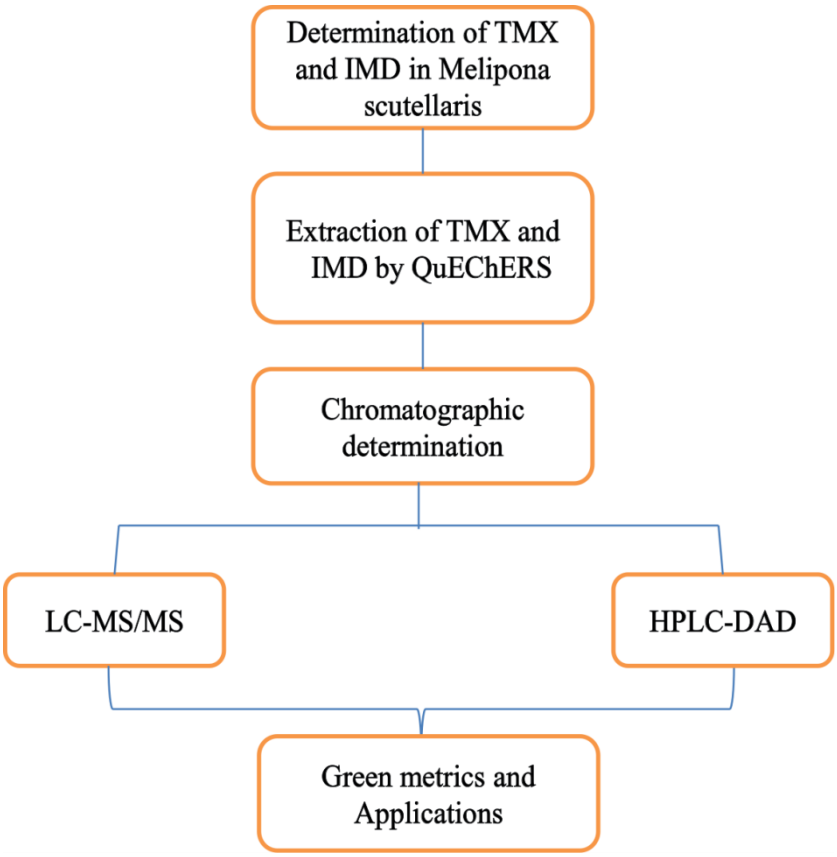
3.1 SUMMARY

In this work, a method for the determination of the neonicotinoid pesticides thiamethoxam (TMX) and imidacloprid (IMD) residues in the stingless bee *Melipona scutellaris* Latreille, (1811) was optimized, through factorial design, tested green metrics, and then applied to exposed bees. It combines the extraction with a modified QuEChERS method and the determination done by first HPLC-DAD and then by LC-MS/MS. Different parameters as the mass of sample, dispersive sorbents, and elution solvents were assessed. The applied method has a good sensitivity, specificity, and linearity for HPLC-DAD (from 10 to 100 μg L^{-1}) and LC-MS/MS (from 0.5 to 500 μg L^{-1}) with $R^2 > 0.99$. The limit of quantification (LOQ) for the LC-MS/MS system was 1.19 μg L^{-1} and 1.13 μg L^{-1} and for HPLC-DAD 306 μg L^{-1} and 315 μg L^{-1} for TMX and IMD, respectively. The method LOQ was 2.5 ng g bee^{-1} for both pesticides. Accuracy was between 94-100% with satisfactory intraday and interday precisions (RSD < 10%). The qualified method was applied to orally and topically exposed bee samples and the obtained results indicated that it is suitable for the determination and quantification of neonicotinoid pesticide residues in this species. Moreover, green analytical metrics were calculated (NEMI, Eco scale, HPLC-EAT, waste generated, and amount of sample) and compared to methods described in the literature involving neonicotinoids in honeybees. As a result, the present work displayed the highest Eco score and HPLC-EAT score, and the second smaller amount of sample and of waste generated. In this sense, besides the application, the multi-criteria decision analysis tool employed suggests that this work is a good option as a green analytical method.

3.2 EXPERIMENTAL PROCEDURE

The experimental procedure has been divided into four parts as shown by the flow chart in Figure 7. Insecticides were extracted from bee body via QuEChERS extraction method; determination and quantification were done by two chromatographic methods (HPLC-DAD and LC-MS/MS); reliability of method (green analytical metrics) and application of this method.

Figure 7. Flow chart representing the extraction, chromatographic determination, green metrics and application of TMX and IMD in stingless bee *M. scutellaris*.



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3.2.1 MATERIALS AND METHODS

3.2.1.1 Materials and Reagents

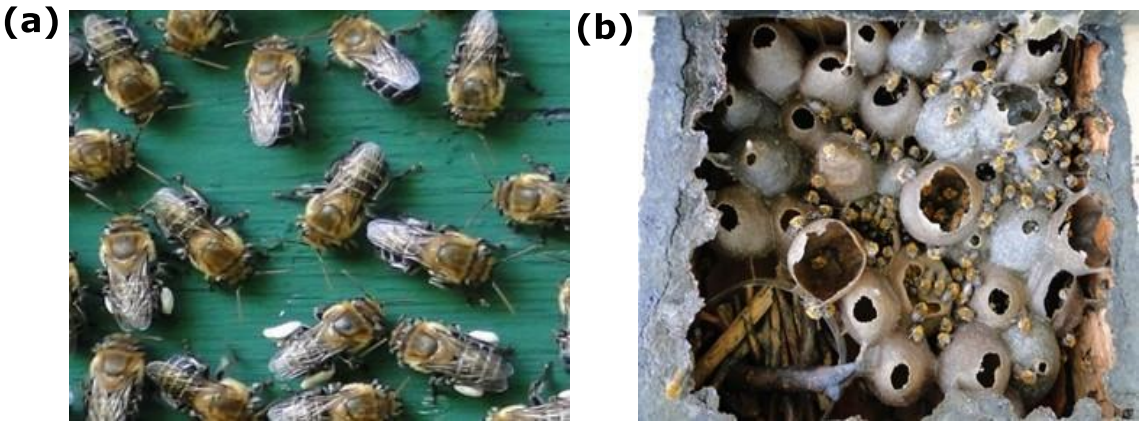
Ultra-pure water (18 MΩ cm at 25°C) was obtained from a Milli-Q Direct-0.3 purifier (Millipore). High-purity analytical standards (TMX and IMD) (> 98%) were purchased from Sigma-Aldrich (Germany). Stock solutions were prepared in methanol (HPLC grade, Mallinckrodt Backer, USA). Other chemicals used in the development of the method were: acetonitrile (ACN, Tedia Company, USA), primary and secondary amine (PSA – Agilent Technologies, USA), octadecylsilane (C18 – Agilent Technologies, UK), anhydrous magnesium sulfate (Mallinckrodt Backer, Japan), and sodium acetate (Sigma-Aldrich). All solvents used in this study were HPLC-grade. All reagents were used as received.

Stock solutions of the working standards (TMX and IMD) were prepared by dissolving 10 mg of each standard in 10 mL of methanol, so that the final concentrations were 1 mg mL^{-1}. From the stock solution, a work solution of 1 μg mL^{-1} was prepared and then dilutions from 0.0005 to 0.5 μg mL^{-1} were prepared with a 20:80 (in volume) mixture of methanol and ultra-pure water, respectively. All solutions were kept at 4ºC.

3.2.1.2 Bees collection

Melipona scutellaris bees were taken from beehives located at the experimental "melliponary" located at the Center for Water Resources and Environmental Studies (CHREA) from the University of São Paulo, São Carlos, Brazil (22°10'00.74" S 47°54'07.86"W). For the method optimization, bees were transported to the laboratory and stored frozen (– 80°C) until analysis. For oral and topical exposure bioassays, bees were collected at the first hours of the day and transported alive in plastic cages to an temperature-control chamber (28 ± 1°C). The experiments were performed in the dark. During the acclimation period (48 h), bees were fed with sucrose: water solution (50:50 in volume) *ad libitum*. The feeders were removed 2 h before the beginning of the experiments. Figure 8 shows the photo of *Melipona scutellaris* bee and their honey and pollen pots.

Figure 8. (a) General aspect of *M. scutellaris* bees. (b) Honey and pollen pots inside the hive.



Source: Adopted from BRIGANTE (2017)

3.2.1.3 Sample preparation — QuEChERS method

The QuEChERS method used was a modification of the original one described by Anastassiades et al. [50] which was developed for the determination of pesticides in fruits. The stingless bees (*M. scutellaris*) were weighed (± 0.2 g) into a polypropylene centrifuge tube (50 mL) and crushed with a glass rod. Acetonitrile (5 mL) and Milli-Q water (10 mL) were added to the tube. The tube content was thoroughly mixed and sonicated for 15 min. Afterwards, 6.0 g $MgSO_4$ and 1.5g NaOAc were added to the tube, which was then centrifuged (Eppendorf Centrifuge 5702, Germany) at 4400 rpm for 5 min. One milliliter of the supernatant was transferred to a 2-mL dSPE polypropylene tube containing 150 mg $MgSO_4$, 25 mg PSA, and 25 mg C18. The tube was then shaken by hand, vortexed for 1 min, and centrifuged (Eppendorf Centrifuge 5702, Germany) at 5000 rpm for 5 min. Finally, 0.75 mL of the second supernatant was filtered through a PTFE (polytetrafluoroethylene) membrane filter (13 mm diameter, 0.22 µm pore size) and transferred to a glass autosampler vial, dried with nitrogen gas, and re-suspended with 0.2 mL of a 80:20 (in volume) mixture of water and methanol for LC-MS/MS analysis.

3.2.1.4 2^3 Factorial design

Two 2^3 full factorial designs were performed (duplicates of all points), leading to two sets of 16 experiments each, carried out in random order: one for TMX and another

for IMD. The objective of the designs was twofold: i) to estimate the experimental error and ii) to determine whether the studied factors were statistically significant (within tested levels) at the 95% confidence level. The experimental error was calculated by Equation 1, in which s is the standard error (experimental error), n_i is the number of replicates, m is the number of different experiments, $\nu_i = n_i - 1$ is the number of degrees of freedom, and s_i^2 is the variance of each replicate [88].

$$s = \sqrt{\frac{\nu_1 s_1^2 + \nu_2 s_2^2 + \cdots + \nu_m s_m^2}{\nu_1 + \nu_2 + \cdots + \nu_m}} \quad (1)$$

The statistical significance of the factors can be determined with the aid of a Pareto chart. In this type of chart, the standardized effects estimate of each factor (and their interactions) is represented by horizontal bars, from the largest effect to the smallest one. Bars that cross the reference red line (whose position depends on the confidence level chosen during the calculations) are statistically significant. Also, the magnitude of the effects is shown next to each bar.

Three factors were assessed at two levels, coded as –1 and +1: i) sample amount (0.2 and 0.5 g of bees), ii) solvents total volume (15 and 20 mL), and iii) solvents ratio (1:2 and 2:1 of ACN: H_2O in volume). The *Statistica* software (version 6.0) was used for performing the necessary calculations, assuming confidence level of 95%.

3.2.1.5 Chemical analysis

The determination of neonicotinoids was performed by high performance liquid chromatography (HPLC), an Agilent 1200 series liquid chromatography equipped with automatic injector, quaternary pump, degasser system, diode array detector (DAD) and ChemStation ver. Software was used. B.03.01 for data acquisition and processing. The diode array detector (DAD) are based on the absorption of ultraviolet light or visible by the sample components when subjected to electromagnetic radiation, so photometers and spectrophotometers as also used as detectors for HPLC. While HPLC parameters are listed in Table 2.

Table 2. HPLC conditions for chromatographic determination of TMX and IMD.

	HPLC-DAD
Column	ZORBAX SB-C 18 column 4.6x250nm, 5micron
Wavelength	254 nm
Temperature	25 ºC
Flow rate	1.0 ml min^{-1}
Detector	DAD
Injection volume	20 µl min^{-1}
Isocratic Mobile phase	Water with 1 % Phosphoric acid , Acetonitrile (60:40)

Some advantages presented by high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) are by CASS et al, 2001. However LC-MS/MS are suitable for highly sensitive assessment of slightly volatile and heat-unstable insecticide such as neonicotinoid, therefore we used high performance liquid chromatography (HPLC- Agilent 1260 Series) coupled with a mass spectrometer (ABSciex-QTRAP 5500 triple linear quadrupole) with a Turbo VTM electrospray (ESI) as ionization source, operating in a positive mode at 550 V. A Zorbax SB-C18 chromatographic column (4.6 × 250mm, 5µm) was used at 25^{o}C. The injection volume was 20 µL. The mobile phase was 60:40 mixture (in volume) of H_2O with 0.1% HOAc (solvent A) and ACN (solvent B) was pumped at 0.8 mL min^{-1}, in isocratic mode (run time 8 min). For TMX and IMD, MRM transitions were 292 → 132; 211 (t_R 4.12 min) and 256 → 175; 209 (t_R 5.2 min), respectively. Other LC-MS/MS parameters are listed in Table 3.

Table 3. LC-MS/MS parameters for the analysis of TMX and IMD.

Insecticides	**Q1 Mass (m/z)**	**Q2 Mass (m/z)**	**Dwell Time (ms)**	**DP (V)**	**CE (V)**	**C × P (V)**
TMX	256	175	100	51	31	10
	256	209	100	51	19	10
IMD	292	211	100	26	33	12
	292	132	100	26	19	21

Q1= precursor ion, Q2= product ion, DP = desolation potential, CE = collision energy, C x P = collision cell exit potential.

3.2.1.6 Method qualification

To ensure reliable results, the performance of the used method was assessed according to the SANTE 11813/2017 guideline ("EC. European Commission. 2017. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. SANTE/11813/2017. Available at: <https://www.eurl-pesticides.eu/docs/public/tmplt_article",) for pesticides, including the figures of merit: specificity, selectivity, linearity, accuracy, precision (repeatability and reproducibility), stability, limit of detection (LOD), and limit of quantification (LOQ).

The specificity of the method was checked using reagent and matrix blanks. To evaluate linearity, five standards were injected, first in the solvent mixture (methanol: H_2O 20:80 in volume), and second in the bee matrix. Recovery (%) was calculated by comparing the analytes peaks areas after the extraction of a spiked matrix with the peak area of the added standard. Accuracy was calculated by comparing the average of recoveries from the experimental concentration with the nominal one, which was assessed at two concentration levels: 0.03 (low) and 0.3 (high) µg mL^{-1}, both in triplicate. Precision was expressed in terms of the relative standard deviation (RSD) and was evaluated in the same way as accuracy, allowing to obtain both intra-day and inter-day precisions. Stability was also checked by testing bees extracts over time with samples kept refrigerated until testing [63,64].

Sensitivity was expressed in terms of the limits of detection (LOD) and quantification (LOQ), considering the calibration curve parameters. The method LOD (LOD_m) was estimated considering the number of replicates and the standard deviation (SD) of the blanks, the lowest fortification level, and the sensibility (slope). The method LOQ (LOQ_m) was set as the lower spiked concentration in which recovery was higher than 80% and RSD lower than 20%, considering the mass used and the final volume of the extracts.

3.2.1.7 Green metrics calculations

For assessing the degree of greenness of the herein proposed analytical method, five other papers using the QuEChERS method as a step for quantifying neonicotinoid pesticides in bees were selected [39,71,90–92]. Six metrics were considered: (a) NEMI

pictograms, (b) Eco Scale Score, (c) HPLC-EAT, (d) Waste generated (in grams), (e) the amount of sample required in the method, and (f) the number of analytes quantified by the method. Those six metrics were used as inputs for calculating the PROMETHEE ranking.

NEMI circular pictograms are divided into four sections, which are related to i) whether the method uses any reagent that is or has been on any hazardous waste list from the United States Environmental Protection Agency (EPA) (1^{st} quadrant), ii) uses persistent, bio-accumulative, and toxic (PBT) chemicals, also according to the EPA (2^{nd} quadrant), iii) generates corrosive media (pH above 12 or below 2) (3^{rd} quadrant), or iv) generates more than 50 g of wastes (4^{th} quadrant). To the methods that used one, two, or three or more hazardous substances, it was given the weights 1, 2, or 3, respectively (1^{st} quadrant). The Eco Scale Score is a tool based on penalties given to the use of hazardous chemicals, the level of energy consumption, occupational risks, and the amount of waste generated [93]. The penalty for each hazardous chemical is obtained by the product between the penalty points (PP) given to the amount of that chemical, its signal word ("warning" and "danger" are 1 and 2, respectively), and the number of pictograms, according to the Global Harmonized System (GHS). The final score is given by subtracting the sum of penalties from one hundred. Analytical methods with scores equal to or greater than 75, between 74 and 50, and less than 50, are green, moderately green, and not green, respectively [93]. HPLC-EAT is the sum of the safety, health, and environmental impact factors weighted by the mass of each solvent used [94]. The HPLC-EAT freeware from Lund University was used for making the necessary calculations [66]. Calculations were made using the Visual PROMETHEE software, giving the same weight to the six metrics (preference function: Level).

3.2.1.8 Oral and topical experiments

M. scutellaris individuals were orally and topically exposed to both TMX and IMD following OECD Guideline 213 [95] and OECD Guideline 214 [96], respectively. In the oral tests, oral ingestion was performed by feeding the bees with water and sugar solution (50% w/v) containing small doses of the pesticides: 0.1 ng μL^{-1} (Oral A) and 0.25 ng μL^{-1} (Oral B) while the control group has received only sugar solution during the experiment. In the acute topic tests, topical exposure was performed by the direct application of 1 µL of an aqueous solution containing different concentrations of test

compounds in a Triton X-100 (1.0%) solution: 1 ng μL^{-1} (Topic A) and 2.5 ng μL^{-1} (Topic B). The solutions were applied with a micro applicator to the dorsal side of the thorax of each bee. Prior to that application, bees were anesthetized with carbon dioxide for 10 seconds. Experiments were carried in dark an temperature-controlled chamber (28 ± 1°C). For all tests performed, three replicates of 10 bees per tested dose of insecticides and the control sample were used. After being exposed for 24 h, the dead bees were collected and stored frozen at – 80°C until further analysis, as described above.

3.3 RESULTS AND DISCUSSION

3.3.1 2^3 Factorial Design

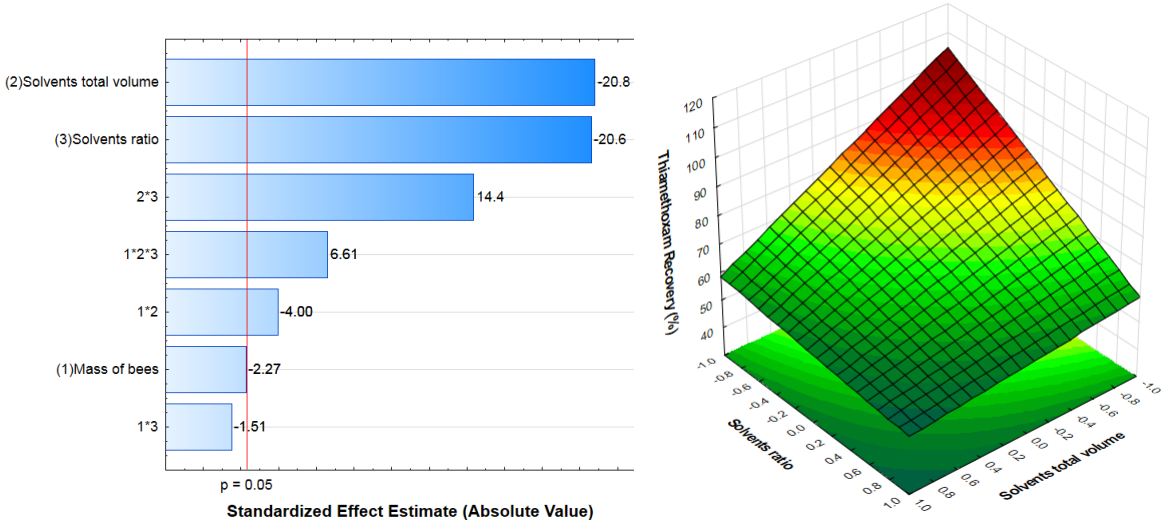
The experimental design matrix and the respective results are presented in Table 4. By using Equation 1, the estimated experimental error was approximately 1.0% for both pesticides.

Table 4. Experimental design matrix and respective results.

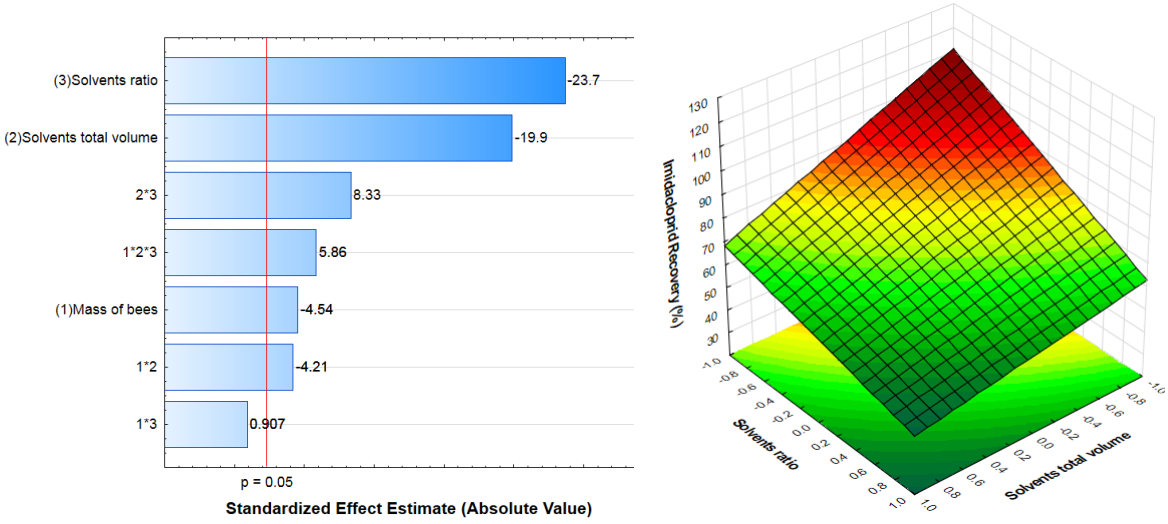
Run #	Mass of Bees (g)	Solvents total volume (mL)	Solvents ratio (ACN: H_2O)	Average Recovery (%)	
				IMD	TMX
1	0.2	15	2:1	108	97
2	0.2	15	1:2	65	70
3	0.2	20	2:1	65	79
4	0.5	20	2:1	50	54
5	0.2	15	2:1	100	107
6	0.5	15	1:2	54	57
7	0.2	15	1:2	61	63
8	0.5	15	2:1	108	112
9	0.5	15	1:2	55	60
10	0.5	20	2:1	52	59
11	0.2	20	1:2	52	45
12	0.5	15	2:1	115	117
13	0.5	20	1:2	47	41
14	0.5	20	1:2	52	46
15	0.2	20	2:1	67	81
16	0.2	20	1:2	50	48

Within tested levels, the “mass of bees” factor was not statistically significant for TMX and barely significant for IMD as shown by the results in Figure 9. That means that either 0.2 or 0.5 g of bees gave rise to approximately the same recovery.

Figure 9. Pareto chart and response surface for (a) TMX and (b) IMD (R^2 = 0.993).



(a)



(b)

For both TMX and IMD, the most important factors were “solvents ratio" and “solvents total volume”. Their effects were negative, meaning that recovery increased when the extraction was performed with a 1:2 mixture of ACN:H_2O, and when the total volume of solvents was 15 rather than 20 mL. Thus, for the stingless bee *M. scutellaris*, the extraction using ACN:H_2O was successfully performed. The final extraction

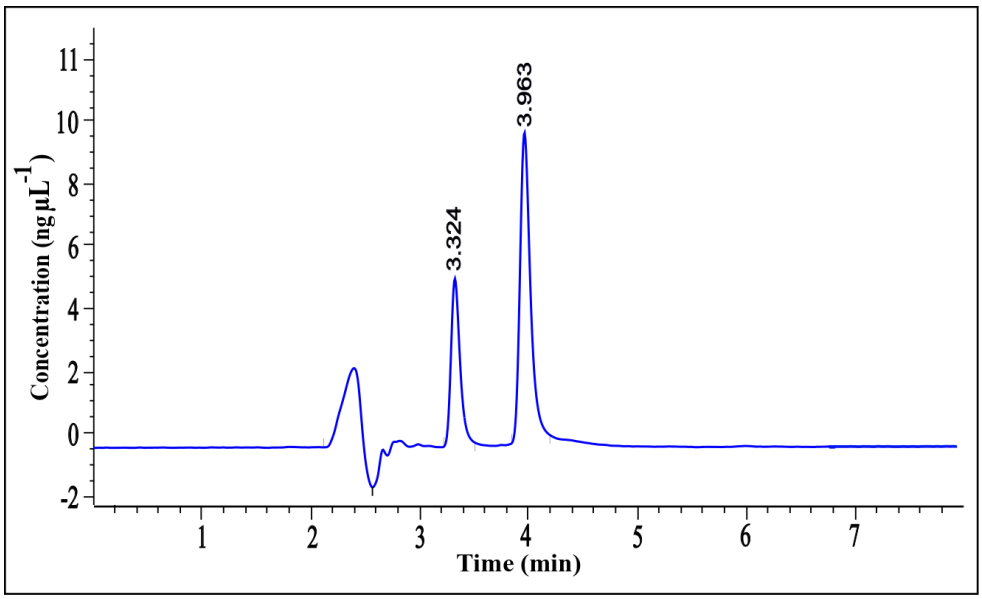
methodology was defined as follows: 0.2 g of bees, 5 mL ACN, and 10 mL H_2O, as the most acceptable recoveries (see section 3.2.1) with the least mass of bees required were achieved with under these conditions. NaOAc has been reported as the most efficient substance for performing the salting out step during the extraction of neonicotinoids in bees and associated matrices (pollen and honey) [39,91]. Using *M. scutellaris* as the matrix, Prado et al. [97] tested the QuEChERS method for the determination of abamectin and difenoconazole and obtained the best results using NaOAc together with $MgSO_4$, as observed in the present study for the neonicotinoids tested (6.0 g $MgSO_4$ and 1.5 g NaOAc).

3.3.2 Analytical performance of the optimized chromatographic method

3.3.2.1 Method development for HPLC-DAD

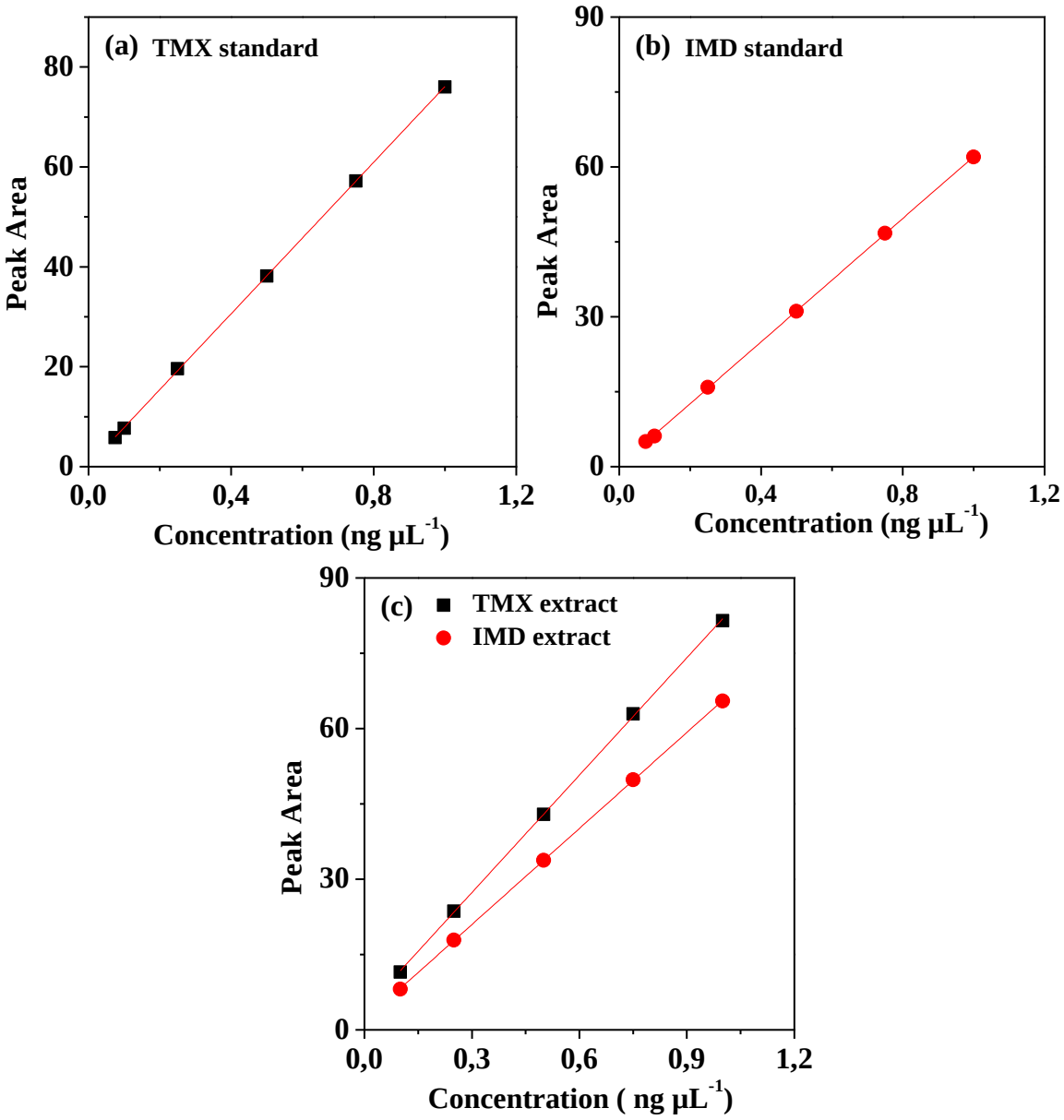
Various parameters were applied for the optimization of HPLC conditions such as selection of mobile phase, flow rate, injection volume, and column temperature as presented in the Table 3 above, which gives excellent identification of individual peaks, resulting in good and well-resolved separation of the analytes, as shown by the results in Figure 10. It was necessary to use a delay of 2 to 3 minutes to avoid the interference of substances that are present in the synthetic medium. The peaks of thiamethoxam and imidacloprid were obtained at a retention time of 3.324 and 3.963 minutes; respectively [36,37,98].

Figure 10. Standard chromatogram representing the retention time of TMX and IMD pesticides.obtained by using HPLC-DAD.



Good linearity range and satisfactory values of correlation coefficients (r) were obtained for TMX and IMD pesticides standard as well as extract as shown by the results in Figure 11 (a), (b) and (c) which indicates that the regression line perfectly fits the experimental data, showing a linear response over concentration range tested. The Limit of Detection (LOD) and Limit of Quantification (LOQ) for TMX was 1.01 ng μL^{-1} and 3.06 ng μL^{-1} and for IMD was 1.04 ng μL^{-1} and 3.15 ng μL^{-1} respectively. The correlation coefficients were 0.993 and 0.998 for both pesticides as shown in Table 5.

Figure 11. Calibration curves of (a) TMX (black line) and (b) IMD (Red line) standards. (c) Calibration curves of TMX extract (black line) and IMD extract (red line) obtained by HPLC-DAD.



The LOD and LOQ of the method reached values considerably lower than the maximum residue limits (MRL) established by the European Union and Brazilian Legislation. In addition, the LOD values were lower or similar to those reported in the literature, which depends on the instrumentation and analytical conditions used.

Table 5. Method parameters for TMX and IMD calibration curves.

Insecticides	**Linear range (ng µL $^{-1}$)**	**R^2**	**Linear equation**	**LOD (ng µL $^{-1}$)**	**LOQ (ng µL $^{-1}$)**
TMX	0.1-1	0.99	83.26x+0.1	1.01	3.05
IMD	0.1-1	0.99	66.18x+0.1	1.04	3.15

Recovery and repeatability studies were variable in three concentration levels, 0.1 ng μL^{-1}, and 0.75 ng $\mu L^{-1}$and 1 ng μL^{-1}. According to European Commission SANCO NCCLS guideline 12495/2011, recovery intervals between 70.0 and 120.0% are considered acceptable for analysis of residues, with a repeatability of up to 99%. All spiked samples presented satisfactory recovery rates for the target pesticides, ranging from 85% to 110% as shown in Table 6. Approximately 93.0% achieved recoveries were above 100%. These recoveries indicate good accuracy of the method. In relation to the repeatability, the relative standard deviations (RSDs) ranged from 2.2% to 16.6%. More than 60.0% of pesticides had RSDs below 10.0%, which verifies the good precision of the method [36,37,98].

The results showed broad agreement with data from other researchers who also developed and validated different versions of QuEChERS method, for the determination of pesticides TMX and IMD in different fruits and vegetables. The QuEChERS provides several benefits compared to conventional extraction methods, such as simplicity of the steps, ease of execution with greater sensitivity, accuracy and precision, which allow its affordable application/usage in pesticides analysis laboratories for monitoring ever smaller levels of residues in food [36,37,98].

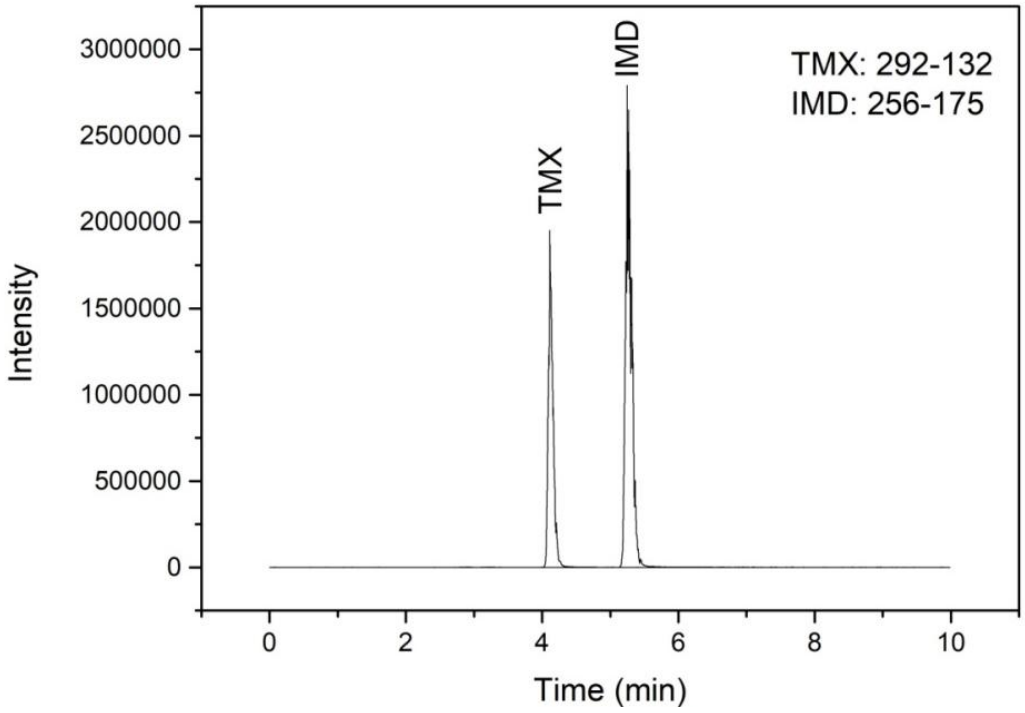
Table 6. Inter and intraday precision and accuracy of TMX and IMD.

Insecticides	Levels	Precision intraday (RSD %)	Precision interday (RSD %)	Accuracy
TMX	Low	5.12	4.96	97%
	Mid	1.06	0.97	100%
	High	0.49	0.53	100%
IMD	Low	3.17	3.73	94%
	Mid	1.53	1.49	99%
	High	0.46	0.51	99%

3.3.2.2 Method development for LC-MS/MS

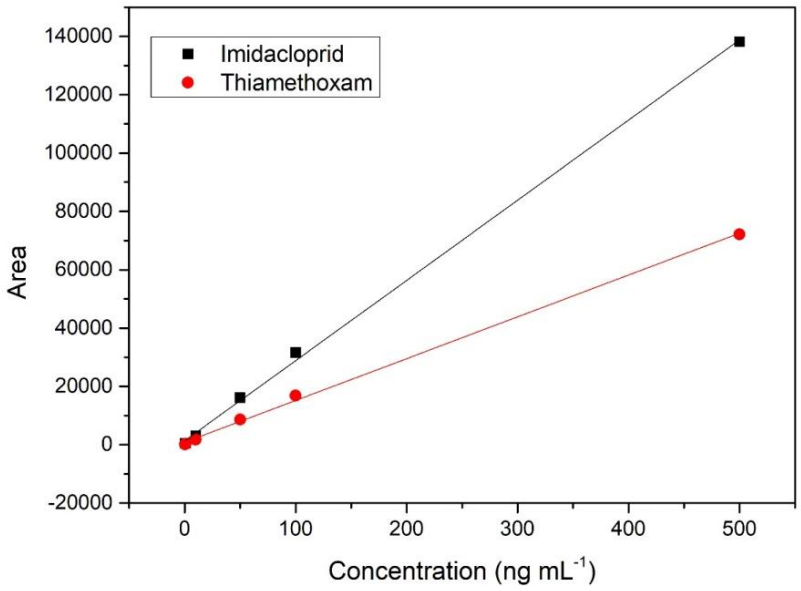
Figure 12 shows a typical chromatogram representing the retention time of TMX and IMD pesticides.

Figure 12. Chromatogram of the neonicotinoids TMX and IMD obtained by LC-MS/MS.



For the system and the matrix, good linearity range (0.0005 ng μL^{-1} to 0.5 ng μL^{-1}) and satisfactory values of determination coefficients ($R^2 > 0.99$) were obtained for TMX and IMD prepared in the matrix as shown by the calibration curves in Figure 13.

Figure 13. Calibration curves prepared in the matrix (bee tissue) for TMX and IMD by LC-MS/MS.



Reagent blanks did not show the presence of the investigated pesticides, while in matrix blanks TMX and IMD were detected in residual amounts (< LOD). Table 7 and Table 8 summarize the correspondent figures of merit.

Table 7. Method parameters for TMX and IMD calibration curves in solvent and matrix.

Analytes	Linear range (ng µL^{-1})	R^2	Linear Equation	ME (%)	LC-MS/MS	Method	
					LOQ (ng µL^{-1})	LOD_m (ng g^{-1})	LOQ_m (ng g^{-1})
TMX	0.0005 – 0.5	0.99	$y = 143.4x + 832.9$	– 87	0.0012	0.62	2.5
IMD	0.0005 – 0.5	0.99	$y = 274.9x + 1332.1$	– 90	0.0011	0.35	2.5

ME = matrix effect

Table 8. Accuracy and precision for TMX and IMD analysis in *M. scutellaris* by LC-MS/MS.

Insecticides	Levels	Precision (RSD %)		Accuracy (%)
		Intraday	Interday	
TMX	Low	5.12	4.96	97
	High	0.49	0.53	100
IMD	Low	3.17	3.73	94
	High	0.46	0.51	99

The QuEChERS method provides several benefits compared to conventional ones, such as simplicity of the steps, ease of execution with greater sensitivity, accuracy, and precision. Those benefits make it a good choice for determining small concentrations of pesticides in biological matrices. The calculated LOQ_m (2.5 ng g^{-1} for

both compounds, Table 7) were considerably lower than the maximum residue limits (MRL) for crops established by the European Commission (EC 1107/2005): 10 and 50 ng g^{-1} for TMX and IMD, respectively [99]. The method qualification results were in good agreement with the data from other researchers who also developed and tested different versions of QuEChERS methods for determining the TMX and IMD in bees as shown in Table 9. The use of only 0.2 g in the extraction procedure is important, as the stingless bee *M scutellaris* has a small body mass compared with *A. mellifera*. With a LOQ_m of 2.5 ng $g^{-1}$for both compounds, the herein described method is comparable to others in the literature, although higher recoveries (%) were obtained, demonstrating its efficiency.

Table 9. Methods described in the literature for the determination of neonicotinoids in bees.

	Extraction (Modified QuEChERS)	Mass (g)	System	LOQ (ng g^{-1})	Recovery (%)	Reference
Bumblebee (*Bombusterrestris*)	500 µL ACN + 250 mg $MgSO_4$/NaOAc (4:1)	0.098	UPLC-MS/MS	0.9 (TMX) 2.2 (IMD)	89-90 (TMX) 88-92 (IMD)	David et al. [91]
Honey Bee (*Apismellifera*)	12 mL H_2O + 15 mL triethylamine 2% in ACN + 6 g $MgSO_4$ +1.5 g NaOAc	15	LC-MS/MS	0.66 (TMX) 0.66 (IMD)	69-89 (TMX) 95-100 (IMD)	Kamel [39]
Honey Bee (*Apismellifera*)	10 mL ACN + 4 g $MgSO_4$ + 1 g NaCl + 1 g citrate trisodium	5	LC-MS/MS	n.i.	> 70% TMX and IMD)	Codling et al. [92]
Honey Bee (*Apismellifera*)	10 mL H_2O + 10 mL ACN + 3 mL*n*-hexane + 4 g $MgSO_4$ + 1 g NaCl	1	LC-MS/MS	3.4 (TMX)	70-74 (TMX)	Barganska et al. [71]
Honey Bee (*Apismellifera*)	3 mL H_2O + 3 mL heptane +10 mL ACN (with 2% TEA) + $MgSO_4$ + citrate	5	UPLC-MS/MS	0.18 (TMX) 0.54 (IMD)	102 (TMX) 108 (IMD)	Daniele et al. [90]
Stinglessbee (*M. scutellaris*)	10 mL H_2O + 5 mL ACN + 4 g $MgSO_4$ + 1.5 g NaOAc	0.2	LC-MS	2.5 (TMX) 2.5 (IMD)	97-100 (TMX) 94-99 (IMD)	This work

n.i. = not informed

Recovery and repeatability studies were performed at two concentration levels: 0.03 ng μL^{-1} and 0.3 ng μL^{-1}. According to the European Commission (SANCO NCCLS guideline 11813/2017), recoveries between 70 and 120% are acceptable for several matrices. All spiked samples presented acceptable recoveries for the target pesticides, ranging from 94 to 100%. These recoveries thus indicate good accuracy of

the method. Considering repeatability, the RSD ranged from 0.46 to 5.12%, demonstrating good inter and intraday precisions as shown in Table 8. Matrix effects of – 87 and – 90% for TMX and IMD, respectively, were observed. As those percentages are greater than – 50% (absolute values), matrix effects were strong [100,101]. Although the analytical method is selective and sensitive, pronounced matrix effects were observed. Ion suppression, resulting from matrix effects, is quite common in LC-ESI-MS analyses, as matrix components can compete with the target analyte for the available charges in spray ionization [101,102].

3.3.3 Green metrics

They are tools used as qualitative/quantitative figures to make clear how sustainable a process or a procedure is [63,64] Particularly in Analytical Chemistry, green metrics are very difficult to be implemented, because analytical procedures generate diffuse pollution [64]. To determine what is the real damage analytical procedures pose to the environment and to human health, a myriad of metrics is available in the literature. Each one of them has its pros and cons.

NEMI:

The National Environmental Methods Index (NEMI) was the first green metrics designed by US agencies and private companies [103]. Initially, it was a qualitative tool, but La Guardia et al. [104] turned it into a semiquantitative technique creating a color scheme in accordance with the level of environmental damage caused by the chemicals used. The NEMI is a visual tool, consisting of a circle divided into four quadrants:

(a) The first quadrant is related to the generation of hazardous wastes, listed by the United States Environmental Protection Agency (EPA). That information was codified in this work to make the comparison with the other papers possible. That codification depends on the number of hazardous chemicals used in each procedure (Table 10).

Table 10. NEMI Codification.

Number of Chemicals	Color in the pictogram	Codification Number
1	green	1
2	yellow	2
3 or more	Red	3

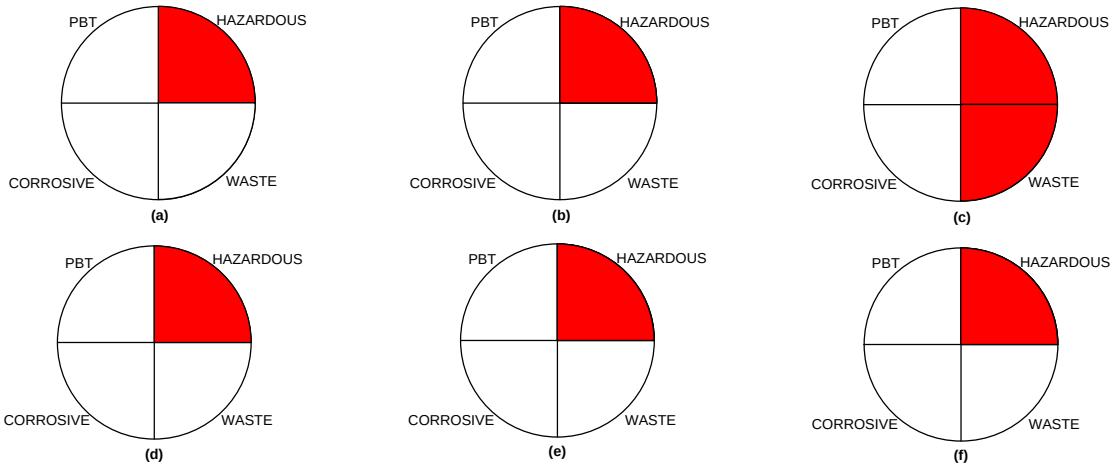
(b) The second quadrant is related to the use of chemicals present on the EPA list of Persistent, Bioaccumulative, and Toxic (PBT) Chemicals.

(c) The third quadrant refers to the corrosive nature of the analytical procedure. A method is said to be corrosive if its pH is below 2 or above 12.

(d) The fourth quadrant refers to whether an analytical procedure generates more than 50 g of waste.

Neither this work nor the papers used for comparison purposes fulfilled criteria (b) and (c); only one paper fulfilled criterion (d).

Figure 14. NEMI pictograms for: (a) this work; (b) David et al. [91]; (c) Kamel [39]; (d) Bargańska, Ślebioda, and Namieśnik [71]; (e) Codling et al. [92] and (f) Daniele et al. [90].



This tool offers a visual analysis of the features the methods under assessment have. However, that analysis is somewhat incomplete because the amount of chemicals used in the procedures is not taken into account. This may lead to misinterpretations regarding how green an analytical procedure really is. The NEMI pictograms for the assessed papers and this work are presented in Figure 14.

EcoScale:

Eco Scale is a quantitative green metrics tool based on a penalty system. It is more complete than NEMI because it takes into consideration the amount of each chemical used, the amount of energy used, and the risks to the analyst [93]. To calculate the penalty points of the chemicals used, it is necessary to know their Global Harmonized System (GHS) classification [105] or each chemical, the partial penalties given to the amount of chemicals used, to the signal word (both in Table 11), and the number of GHS pictograms in its Material Safety Data Sheet (MSDS) are multiplied. The calculated Eco Score for this work is shown in Table 12. The Eco Scale Final Score is one hundred minus the sum of all partial penalty points. The Final Score of green methods is equal to or greater than 75. Partially green methods have Final Scores between 74 and 50. Methods with Final Scores lower than 50 are not green [93].

Table 11. Eco Scale Table Score.

Classes	Factors	Criteria	Penalty Points (PP)
Chemicals	Amount (mL or g)	< 10 (1)* 10 – 100 (2)* > 100 (3)*	Number of GHS pictograms × amount used score × signal word score
	Hazard (GHS)	None (0)* Warning (1)* Danger (2)*	
Instruments	Energy (kWh/sample)	Titration, UPLC, UV-vis, FTIR (≤ 0.1) AAS, LC-UV, GC, ICP-MS (≤ 1.5) NMR, LC-MS, GC-MS, DRX (> 1.5)	0 1 2
	Waste (mL or g)	None < 1 1 − 10 > 10 Recycling Degradation Passivation No treatment	0 1 3 5 0 1 2 3
Operator	Occupational Hazard	The analytical process is hermetic. Emission of vapors or gases into the air.	0 3
Eco Score	**100 − ∑ PP**		

* (score)

Table 12. Calculated Eco Score for this work.

Classes	Factors	Penalty Points (PP)								Eco Score
		0	1	2	3	4	5	6	∑ PP	(100 –∑ PP)
Chemicals	Acetonitrile					×			22	**78**
	Methanol							×		
	Anhydrous magnesium sulfate	×								
	Sodium acetate		×							
	Primary-secondary amine (PSA)	×								
	Silica-C18	×								
	Acetic acid					×				
Instruments	LC-MS/MS			×						
	Waste						×			
Operator	Occupational hazard	×								

This tool offers a better understanding of why a system is green or not. Differently from NEMI, it is a quantitative metric. However, the Eco Scale does not take into consideration the amount of sample needed for performing the analytical procedure.

HPLC-EAT:

It is a tool that allows for determining how sustainable a chromatographic method is. The HPLC-EAT is the sum of the S (safety), E (environmental), and H (health) factors, weighted by the mass of each solvent in the mobile phase (Equation 1), in which m_i is the mass of solvent i and $S_i, E_i,$ and $H_i$are the respective factors. Those factors were calculated using the models developed by Koller et al. [94].

$$\text{HPLC-EAT Score} = \sum_{i=1}^{N} m_i(S_i + E_i + H_i) \qquad (2)$$

The calculations were performed with the aid of the HPLC_EAT freeware, from Lund University (Sweden) [66]. The freeware has its own table of calculated S, E, and H factors, and it allows the user to add more data for solvents, not present in its database. The program is fed with the following mobile phase features: types of solvents, gradient or isocratic mode, composition, and flow rate. The output is a table (Table 13) of partial HPLC-EAT scores for each component of the mobile phase and the final score, which is the sum of the partial scores. The table is also displayed as a histogram.

Table 13. HPLC-EAT Score Table for this work.

Solvent	Mass (g)	Factors			HPLC-EAT Score
		S	E	H	
Water	14.8	0	0	0	0
Acetonitrile	6.44	2.72	1.06	0.77	29.326
Total	21.2	–	–	–	29.326

The six calculated figures of merit are shown in Table 14. Regarding the NEMI figures, all the selected works, including the present one, scored 3 in the 1st quadrant, because three or more substances that generate hazardous wastes were used. Only Kamel [39] scored in the 4th quadrant (one point). The NEMI score for the present work was 3 because ACN, CH_3OH, and HOAc were used.

Table 14. Green metrics parameters calculated for the selected papers.

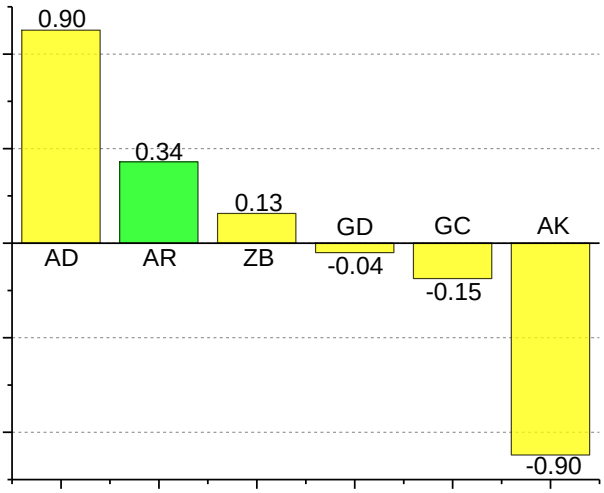
Paper	NEMI	Eco Scale	HPLC-EAT	Waste mass (g)	Amount of Sample (g)	Number of analytes
AR	**3**	**78**	**28.1**	**29.7**	**0.2**	**2**
AD	3	74	11.7	6.14	0.1	20
AK	4	63	105.9	65.3	15	13
GC	3	78	42.0	40.0	5.0	13
GD	3	53	68.5	35.3	5.0	58
ZB	3	71	64.0	33.0	1.0	19

Note: AR – this work; AD – David et al. [91]; AK – Kamel [39]; GC – Codling et al. [92]; GD – Daniele et l. [90] ; ZB –Bargańska, Ślebioda, and Namieśnik [71].

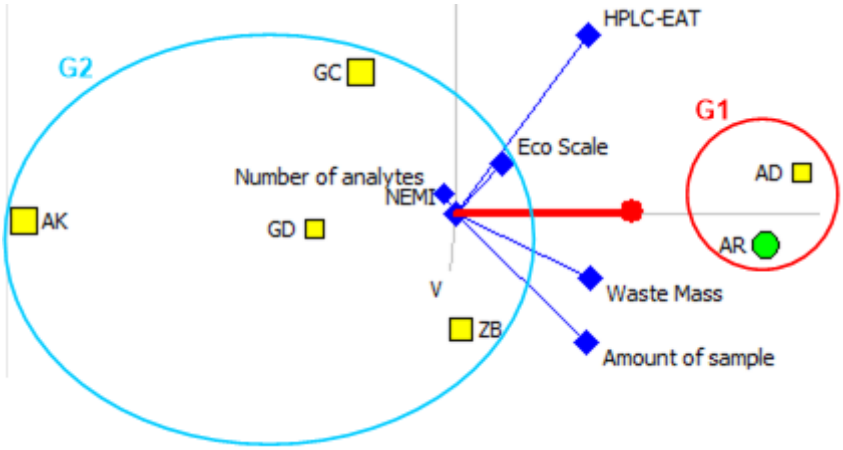
According to Galuzka et al., [93] methods with Eco Scores greater than 75 are excellent for green analyses, while methods between 50 and 75 are just acceptable for that purpose. Therefore, among the five works being compared, four are acceptable and one is excellent, like the present work.

The PROMETHEE analysis showed (Figure 15) the existence of two groups of methods. Group 1 comprises this work and the one of David et al., [91] Group 2 the other four [39,71,90,92]. The plots generated by the PROMETHEE analysis are comparable to the biplots used in Principal Component Analyses (PCA). Similar works are grouped together, and the decision axis points out to the direction of the greenest methods.

Figure 15. PROMETHEE analysis: (a) ranking and respective MCDA weights; (b) biplot with decision axis (red). Group 1: AR – this work and AD – David et al. [91] Group 2: AK –Kamel [39]; GC– Codling et al. [92]; GD – Daniele et al. [90]; and ZB – Bargańska, Ślebioda, and Namieśnik [71].



(a)



The figures of merit that caused the methods to be split into two groups were HPLC-EAT, waste mass, and amount of sample. Inside Group 1, which is aligned with the decision axis, although the present work has the highest Eco Score, David et al. [91] used less sample, generated less wastes, and quantified more analytes, making it the best method among the selected ones.

3.3.4 Method application

The application of the developed method in bees orally and topically exposed to the investigated neonicotinoids is described in Table 15. Upon the highest oral test concentration (0.25 μg mL^{-1}), TMX was detected (0.005 μg bee^{-1}) and the observed consumption during 48 h was 0.009 μg bee^{-1}. Regarding the topic exposure, TMX was observed in both applied concentrations (1 and 2.5 μg mL^{-1}) with the concentrations 0.010 and 0.019 μg bee^{-1}, respectively. No IMD was detected (< LOD_m) in the bees via oral exposure, while in topic exposure concentrations were 0.07 and 0.09 μg bee^{-1} for the low (1 μg mL^{-1}) and high (2.5μg mL^{-1}) levels, respectively.

According to those results, TMX and IMD can be taken up by the bees, mainly when topic exposure is considered. This fact can be related to the metabolic pathway of insects that can depurate those compounds over the 48 h exposure. Regarding the topic way, due to the cuticle barrier [106], the uptake and further depuration of those pesticides can be slow. This discussion can be better performed in further studies in which bees (*M. scutellaris*) be exposed to a wide range of concentrations, followed by the application of this method for the quantification in tissues. For instance, the method can be considered as feasible for this matrix and these target compounds.

The observed concentration of TMX after oral exposure is comparable to the lethal doses/concentrations (LD_{50}/LC_{50}) for *A. mellifera* reported in the literature: 0.0034 μg bee^{-1} [107]; 0.0112 μg bee^{-1} [106] during 48 h of exposure. The same finding can be observed for the topic exposure, in which the LD_{50} reported in previous works for *A. mellifera* were 0.029 μg bee^{-1} for 24 h exposure [108]; or even 0.051 μg bee^{-1} for 48 h [83]. For IMD, the toxicity data (LD_{50}) towards *M. scutellaris* observed by Costa et al. [109] was 0.0013 μg bee^{-1} for topic exposure (48 h) and the LC_{50} was 0.81 μg mL^{-1}. For another stingless bee species (*Scaptotrigona postica*), the endpoints (LD_{50} and LC_{50}) for oral and topic exposure were 0.024 μg bee^{-1} and 14.3 μg mL^{-1} respectively [110]. For *A. mellifera*, the LD_{50} for 48 h of oral exposure can involve a concentration of 0.103 μg bee^{-1} [107], while for topic exposure the already reported concentration was 0.0179 μg bee^{-1} [108]. Those toxicity data for different bees also demonstrate the relevance of determining the endpoints for TMX and IMD for *M.*

scutellaris in association with the quantification in tissues, for elucidating the toxicokinetic action of those pesticides in this species.

Table 15. Exposure concentration, consumption and observed concentrations (obtained by LC-MS/MS) of TMX and IMD for 48h exposed bees (*M. scutellaris*).

	Exposure concentration (ng μL^{-1})		Consumption (ng bee^{-1})		Observed concentration			
					(ng bee^{-1})		(ng g_{bee}^{-1} ± RSD)[a]	
	TMX	IMD	TMX	IMD	TMX	IMD	TMX	IMD
Oral A	0.10	0.10	4.0	1.0	–	–	< LOD_m	< LOD_m
Oral B	0.25	0.25	9.0	7.0	5.0	–	48.0 ± 0.42	< LOD_m
Topic A	1.00	1.00	[B]	[B]	10.0	70.0	89.0 ± 0.21	570 ± 0.03
Topic B	2.5	2.5	[B]	[B]	19.0	90.0	162 ± 0.21	760 ± 0.02

Note: < LOD_m = 0.62 ng g^{-1} (TMX); 0.35 ng g^{-1} (IMD); < LOQ_m = 1.25 ng g^{-1}; [a] n = 3; [b] not applied

In environmental studies, the occurrence of TMX and IMD in bee bodies has been reported for *Apis mellifera*: 4.1 ng g^{-1} for TMX [71]; from 0.2 to 6.2 and from 2.5 to 41.1 ng g^{-1} for IMD and TMX, respectively [91]. Also for *Bombus terrestris*: < 0.30 to < 0.90 ng g^{-1} for TMX and < 0.72 ng g^{-1} for IMD [92], indicating the uptake of these compounds are different for different bee species. In Brazil, although *M. scutellaris* naturally occurs in the Northeast region, this species is well adapted to the Southeast region as well, where these neonicotinoids are broadly used in many crops. In those crops, the pollination is essential or can increase the production of cultivars as coffee, citric fruits, papaya, and strawberry. The MRL can range from 0.01 to 4 mg kg^{-1} for IMD and from 0.01 to 1 mg kg^{-1} for TMX in the Brazilian normative [111,112]. Considering the wide distribution of neonicotinoids in plant tissues and that their uptake by bees was already reported [113], the uptake capacity observed via topical exposure can lead to an urgent need for environmental risk studies to estimate the impact of these contaminants in the bees metabolism and life. However, the impact of spray drift applications can be also taken into account, once the detection in tissues via topic exposure was observed.

4. Chapter # 2. Acute susceptibility of the stingless bee *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) to thiamethoxam and imidacloprid insecticides after oral and topical exposure

4.1 SUMMARY

The stingless bee species *Melipona scutellaris* has high potential as a pollinator, significant honey production among the meliponids and is particularly essential for some agricultural plants of the genus *Solanaceae* (eggplant, tomatoes, and peppers) which require the bee to have the capacity to perform pollination by vibration. The neonicotinoid insecticides thiamethoxam (TMX) and imidacloprid (IMD) are widely used in crops in Brazil, and may present a risk of exposure during the foraging of bees, either through the interception of the spray plume, or through the collection and consumption of contaminated pollen. Thus, this study aimed at determining the toxicity effects of TMX and IMD for the native bee *Melipona scutellaris*. For the assessment of acute, topical and oral toxicity, insecticides were tested individually, in the form of active ingredients and as a commercial formulation, so that the mean lethal dose (LD_{50}) and the mean lethal concentration (LC_{50}) over time were determined for 24 and 48 hours of exposure. The values for the topical LD_{50} of the IMD obtained for *M. scutellaris* were 6.0 and 3.0 ng a.i./bee , and for TMX were 2.2 and 0.9 ng a.i./bee, for 24 h and 48 h, respectively. While the dietary oral LC_{50} for IMD obtained were 1.0 ng a.i./µL and 0.5 ng a.i./µL, while for TMX were 0.3 ng a.i./µL and 0.06 ng a.i./µL for 24 h and 48 h, respectively. The exposure bioassays for *M. scutellaris* were observed for all tested concentrations in the following order: 24 h < 48 h, according to the increase of exposure dose. For 24 h topical exposure, the LD_{50} for commercial product Actara (TMX) was 1.0 ng a.s./bee and, the estimated oral LD_{50} was 4.4 ng a.i./bee, which indicates that topically Actara (TMX) product is more toxic to stingless bee. While for commercial product Nortox (IMD), topical LD_{50} was 3.1(± 2.4) ng a.i./bee, and the estimated oral LD_{50} was 20.0 ng a.i./bee, which indicates that topically Nortox is more toxic than oral exposure. Subsequently to the acute tests, sublethal doses of these substances were used for the analysis of biochemical markers, which will be discussed later in Chapter 3.

4.2 MATERIALS AND METHODS

4.2.1 Collection of bees

The bees used in this research came from the experimental meliponary belonging to the University, located in the rural area of the municipality of Itirapina, São Paulo-SP. Bees from 19 sister hives were used, and the condition for their use was that they had good health and strength of the colony. For all tests performed, 30 bees were used with three replicates of 10 bees per tested dose of insecticides, in addition to the control sample, and the rejoinders were from different colonies.

Figure 16. A photograph of the colonies of stingless bee *M. scutellaris* at CRHEA-Water Resources and Environmental Studies Center, at the São Carlos School of Engineering.

The collections of bees occurred, mainly, in the period of spring and summer (between September and May). The bees were collected at the exit of the hives, mainly during the peak hours of the workers' activity, which corresponds from 7 am to 9 am, directly in 250 mL plastic cages, provided with aeration and with emergency food to reduce stress, and kept in the dark until arriving at the laboratory. All the experiments were carried out in a temperature-controlled chamber (28 ± 1°C).

The bioassays were carried out at the Ecotoxicology and Aquatic Ecology Laboratory of the Nucleus of Ecotoxicology and Applied Ecology, at the Water Resources and Environmental Studies Center, at the São Carlos School of Engineering, University of São Paulo.

4.2.2 Insecticides and reagents

Analytical standards for pure chemicals, TMX (99.3% purity, from Sigma Aldrich) and IMD (100% purity, from Sigma Aldrich) were purchased from Germany. Commercial formulations used for these insecticides were ACTARA 250 WG, ia thiametoxam (75%), registered with the Ministry of Agriculture, Livestock and Supply - MAPA under nº 10098, produced by Syngenta, and IMIDACLOPRID NORTOX, 480 SC, ai imidacloprid (70%), registered under MAPA under nº 11012 , produced by NORTOX S/A.

Stock solutions of 1000 ng μL^{-1} in acetone (HPLC grade, Mallinckrodt Backer, USA) were prepared for the active ingredients. For commercial formulations, stock solutions and subsequent dilutions were prepared directly in sucrose solution (50%) for oral tests and in water-triton X-100 solution (0.1%) for topical tests. All solutions were kept in a refrigerator at 4 °C.

4.2.3 Insecticide susceptibility bioassays

The susceptibility of bees to insecticides was evaluated using two means of exposure: oral ingestion and topical or contact exposure. In both cases, preliminary tests for each of the insecticides were carried out in order to determine the bee's sensitivity range to the product, and for later definition of the concentrations applied in the definitive tests. For preliminary tests, doses were used: 0.01; 0.1; 1; 10 and 100 ng μL^{-1}, according to Medrzycki et al. [114], from the standard solution of 1000 ng μL^{-1}. Once the sensitivity range was defined, six to eight concentrations were established for the definitive tests in order to cover an interval between 100% and a mortality level not significantly different from that of the controls.

For the definitive oral exposure tests, the following doses of TMX active ingredient were applied: 0.0075; 0.01; 0.025; 0.05; 0.075; 0.1 and 0.25 ng μL^{-1}. For IMD active ingredient, the doses were: 0.05; 0.086; 0.2; 0.5; 1.25; 3 and 7.5 ng μL^{-1}.

For the definitive tests of topical or contact exposure, the doses of TMX active ingredient were: 1; 2.5; 3.5; 5; 7.5 and 10 ng μL^{-1}, and for IMD active ingredient were: 0.5; 0.75; 1.2; 5.5; 7.5 and 10 ng μL^{-1}. Oral doses for the commercial product Actara and Nortox were: 0.01; 0.025; 0.05; 0.075; 0.1 and 0.25 ng μL^{-1}, and 0.25; 0.5; 0.75; 1.0; and 2.5 ng μL^{-1}respectively. However topical doses for Actara and Nortox were: 0.5; 0.75; 1.0; 2.5; and 3.5 ng μL^{-1}, and 1.0; 2.5; 5.0; 7.5 and 10.0 ng μL^{-1} respectively.

4.2.4 Acute oral toxicity bioassays

The procedures for oral bioassays were based on the Organization's Protocol for Economic Cooperation and Development, number 213 [95], with modification, for *M.scutellaris* changes in the original protocol includes only the temperature (28°C in this work , instead of 25°C) , since this protocol was developed to be applied in toxicity studies with the bee *Apis mellifera*.

To obtain the desired concentrations using the active ingredients of the insecticides, initially the stock solutions were prepared in acetone and the subsequent dilutions were made directly in a 50% (w / v) sucrose solution. The bees were kept in a temperature-controlled chamber (28 ± 1°C) without food for the period of 2 hours before exposure to insecticides. These, in turn, were administered to each sample through 1.5 mL Eppendorf tubes, containing 1 mL of sucrose + insecticide solution, and for a period of 6 hours, being subsequently replaced by sucrose solution without Contamination. The food consumption during this 6 h period was calculated by subtracting the amount of food left in the Eppendrof tube, which allowed us to estimate the dose of insecticides to which the insects were exposed. A control group was carried out without oral exposure to pesticides. After, exposure mortality of bees were verified in 24 h and 48 h and dead bees were removed and stored frozen (−80 °C) until analysis.

Bees were considered dead when they remained completely immobile during a 10-second observation period after being poked lightly with a fine brush. At the end of the test, the bees were immediately lyophilized and stored at − 80 °C for chromatographic and protein content analysis (enzymes).

4.2.5 Acute topical toxicity bioassays

The procedures for determining acute topical toxicity were based on the Organization for Economic Cooperation and Development protocol, number 214 [96], with same modification, since it was also developed for the *A. mellifera* bee. The insecticides were initially diluted in a solution of 0.1% of Triton X-100 solution and, by means of successive dilutions; the desired doses were prepared for application in the test. The bees were anesthetized with CO_2 for 10 seconds, receiving a topical application of 1.0 µL of the solution containing the tested substance. A control group received only 1.0 µL of water: triton X-100 solution (0.1%). At the end of the test, the bees were immediately lyophilized and stored frozen at −80 °C for chromatographic analysis and protein content (enzymes).

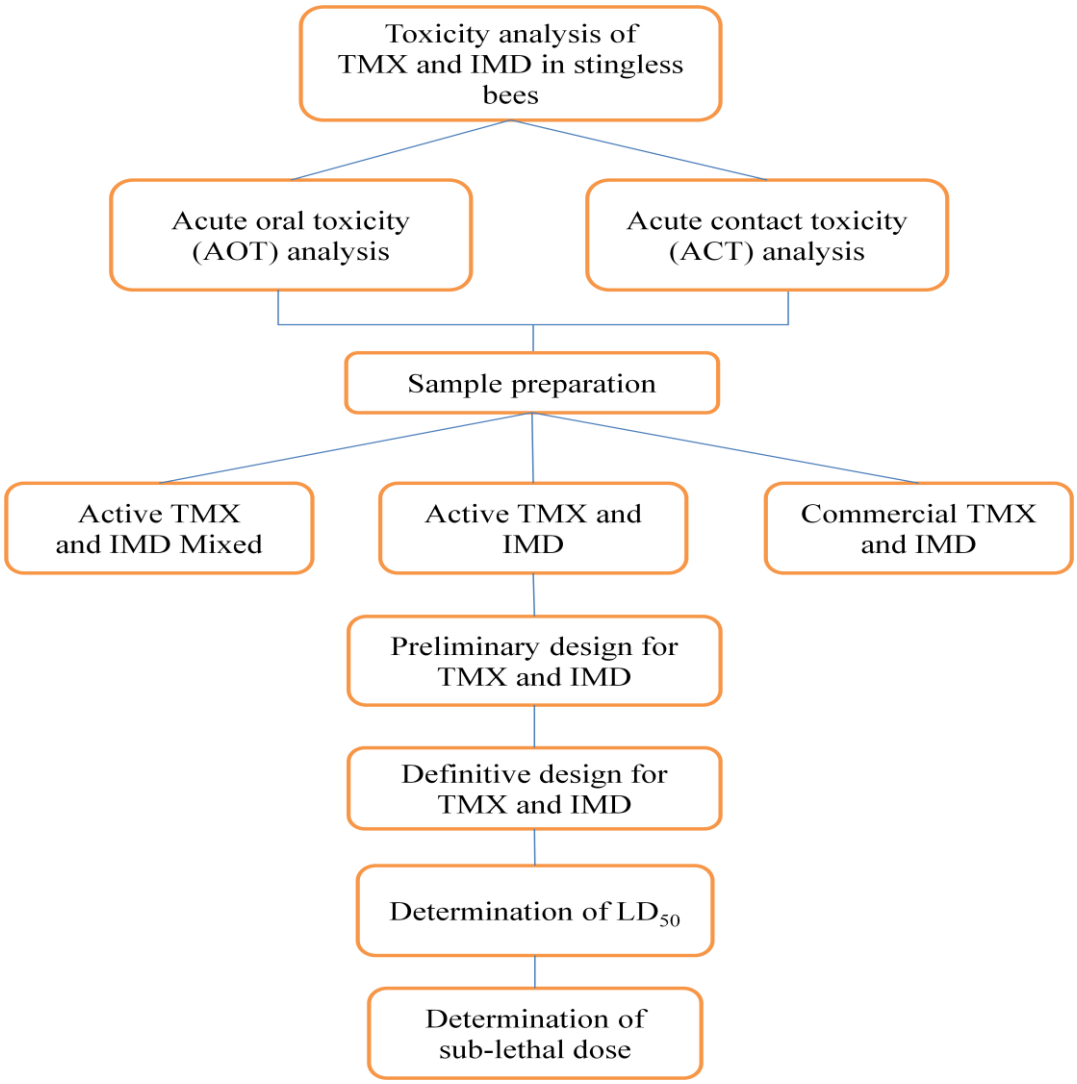
4.2.6 Statistical analysis

Mortality data obtained from the assays were subjected to the statistical program Sigma Plot 11.0 (from Systat Software, Inc., San Jose California USA, www.systatsoftware.com) to calculate the lethal concentration (LC_{50}) and lethal dose LD_{50} with 95% confidence limits. The dose curve or concentration versus mortality was estimated by using the Origin Pro 8.5 software.

4.2.7 Experimental procedure

The experimental procedure has been divided into five parts: route of exposure; sample preparation; preliminary design; definitive design and lethal concentration/dose determination as shown by the flow chart in Figure 17.

Figure 17. Representing the overall experimental procedure of toxicity analysis for TMX and IMD insecticides in stinglessbee (*M. scutellaris).*



Source: Self-made figure

4.3 RESULTS AND DISCUSSIONS

4.3.1 Preliminary concentration- mortality bioassays of TMX and IMD

The LC_{50} after 24 h and 48 h of exposure to TMX was 0.09 (± 0.02) ng of active ingredient / μL of diet offered and 0.04 (± 0.03) ng of active ingredient μL^{-1} of diet offered, respectively. For IMD the LC_{50} after 24 and 48 h exposure was 4.0 (± 0.02) ng of active ingredient μL^{-1} of diet offered and 0.8 (± 0.3) ng of active ingredient μL^{-1} of diet offered, respectively as shown in Table 16.

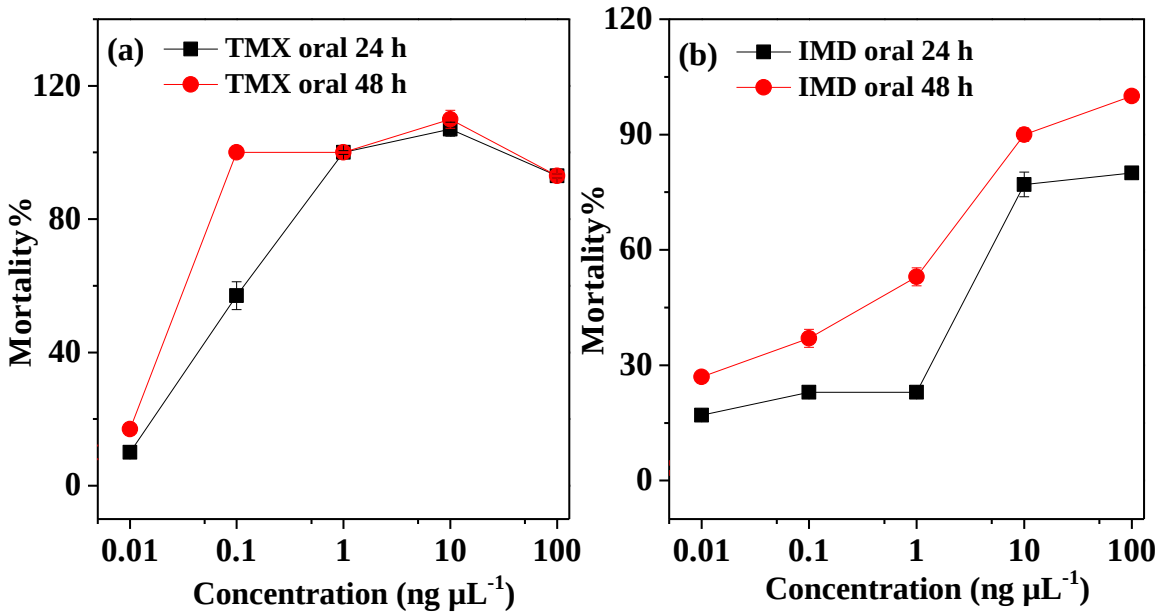
Table 16. LC_{50} value of TMX and IMD in preliminary design to oral exposure.

Pesticide	**Time[a]**	**LC_{50}[b](ng$\mu L^{-1}$of diet)**	**CI $_{95\%}$[c]**
TMX	24	0.09 (± 0.02)	0.07 – 0.11
	48	0.04 (± 0.03)	0.01 – 0.07
IMD	24	4.0 (± 0.02)	3.98 – 4.02
	48	0.8 (± 0.3)	0.5 – 1.1

[a] Time in hours after the administration of insecticides; [b] lethal concentration 50 %; [c] confidence interval at 95 %.

Figure 18 shows 50 % mortality of stingless bees observed between the concentrations; 0.01 to 0.1 ng μL^{-1} of TMX and 1.0 to10 ng μL^{-1} of IMD, where an increase in the concentration of IMD and TMX causes an increase in the mortality observed after 24h exposure. However, after 48 h exposure with high concentration of IMD and TMX, the mortality ratio is almost 100 % for stingless bees which indicates that these insecticides become more toxic with the passage of time. The TMX was orally more toxic to *M. scutellaris* when compared to IMD pesticide as indicated in Figure 18.

Figure 18. Mortality % of *M. scutellaris* versus different concentrations of (a) TMX and (b) IMD insecticides orally exposed to 24 h (black line) and 48 h (red line), n = 3.



The LD_{50} after 24 h, 48h and 72 h of topical exposure to TMX was 3.8 ng a.i/bee, 4.0 ng a.i/bee and 4.0 ng a.i/bee, respectively. For IMD the LD_{50} after 24, 48 and 72 h exposure was 6.0 ng a.i/bee, 0.8 ng a.i/bee and 0.3 ng a.i/bee, respectively as shown in Table 17. The results indicated that initially at 24 h TMX was topically more toxic to *M. scutellaris* when compared to IMD but with the passage of time like 48 h and 72 h, the IMD became more toxic topically.

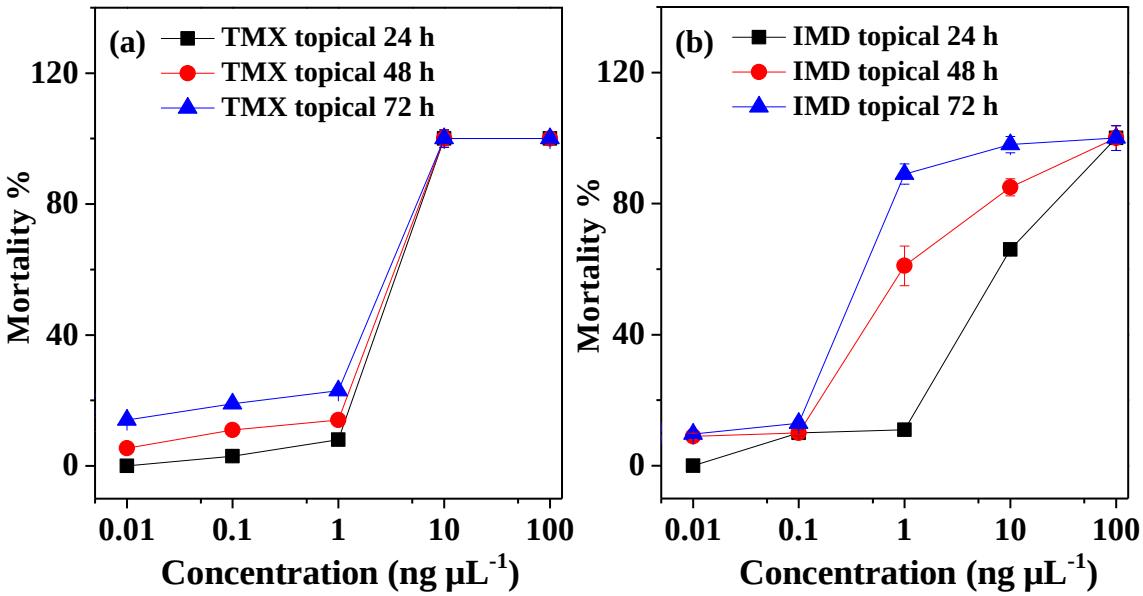
Table 17. LD_{50} value for TMX and IMD in preliminary design to topical exposure.

Insecticide	**Time** [a]	**LD_{50}(ng a.i/bee)**[b]	**CI $_{95\%}$**[c]
TMX	24	3.8 (± 0.02)	3.78 – 3.82
	48	4.0 (± 0.03)	3.97 – 4.03
	72	4.0 (± 0.03)	3.97 – 4.03
IMD	24	6.0 (± 0.02)	5.98– 6.02
	48	0.8 (± 0.3)	0.5 – 1.1
	72	0.3 (± 0.2)	0.1– 0.5

[a] Time in hours after the administration of insecticides; [b] lethal dose 50 %; [c] confidence interval at 95 %.

Figure 19 shows the concentration versus mortality bioassays of TMX and IMD of topical exposure for 72 h. The results indicated that both TMX and IMD were equally topically toxic to *M. scutellaris*. The 50% mortality of stingless bees was observed in the range 1.0 to10 ng μL^{-1} for 24 h. For 48 h exposure the 50% mortality of stingless bees observed in the range of 0.1 to 1.0 ng μL^{-1} for IMD while for TMX the range of concentration was 1.0 to 10 ng μL^{-1} which indicates that after 48 h and 72 h exposure the toxicity of IMD was increased compared to TMX.

Figure 19. Mortality % of *M. scutellaris* versus different concentrations of (a) TMX and (b) IMD insecticides topically exposed to 24 h (black line), 48 h (red line) and 72 h (blue line)**,** n = 3.



4.3.2 Definitive concentration-mortality bioassays of TMX and IMD (active ingredients)

4.3.2.1 Definitive concentration-mortality evolution of TMX oral and topical exposure

TMX and IMD insecticides susceptibility was assessed for *M. scutellaris*. The insecticide susceptibility varied significantly with the type of exposure (oral ingestion and topical). The oral LC_{50} obtained were of 0.3 (± 0.1) and 0.06 (± 0.04) ng a.i. /µL (Table 18 and Figure 20), for 24 and 48 h, respectively. The values for the topical LD_{50} of the TMX obtained for *M. scutellaris* were: 2.7(± 0.3) and 1.7 (± 0.9) ng a.i. /bee (Table 18 and Figure 21), for 24 h and 48 h, respectively.

Table 18. Summary of the acute toxicity tests of TMX against *M. scutellaris* exposures.

Exposure route	Time[a]	LD_{50}[b]	LC_{50}[c]	X^2 [d]	D.F[e]
Topical (ng a.i/bee)	24	2.7(± 0.3)	-	27.14	2
	48	1.7 (± 0.9)	-	14.71	2
Oral (ng a.i/µL diet)	24	-	0.3 (± 0.1)	2.40	2
	48	3.5 (± 0.3)	0.06 (± 0.04)	7.14	3

[a] Time in hours after the administration of IMD; [b] lethal dose 50 %; [c] lethal concentration 50 %; [d] Chisquare from model; [e] degrees of freedom.

Figure 20. Plot of concentration versus mortality of TMX active ingredient orally exposed for (a) 24 h (black line) and (b) 48 h (red line).

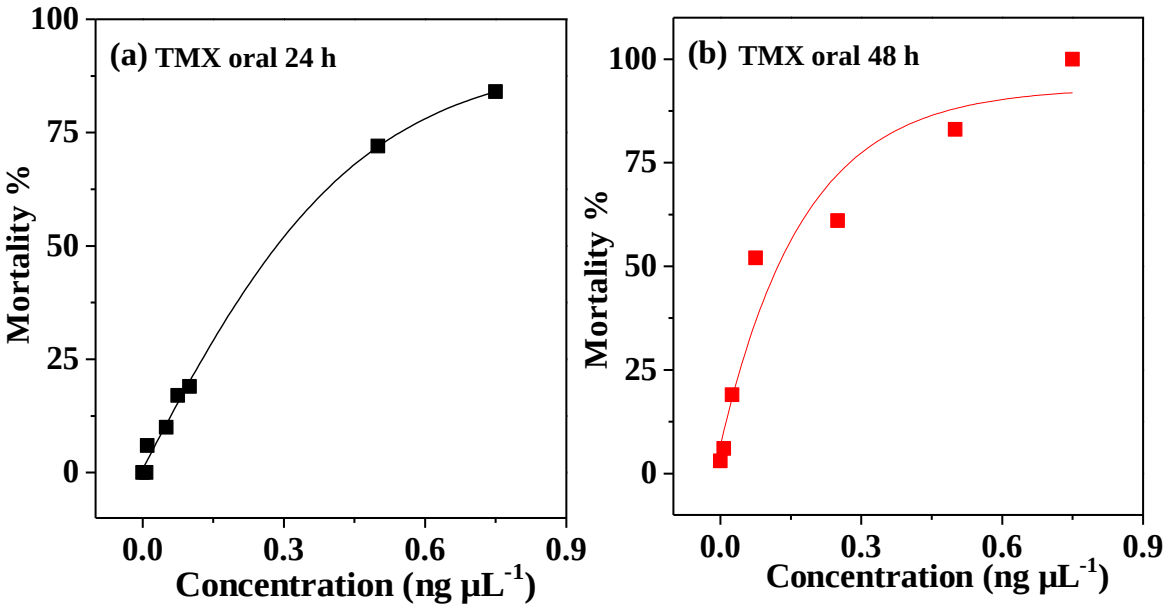
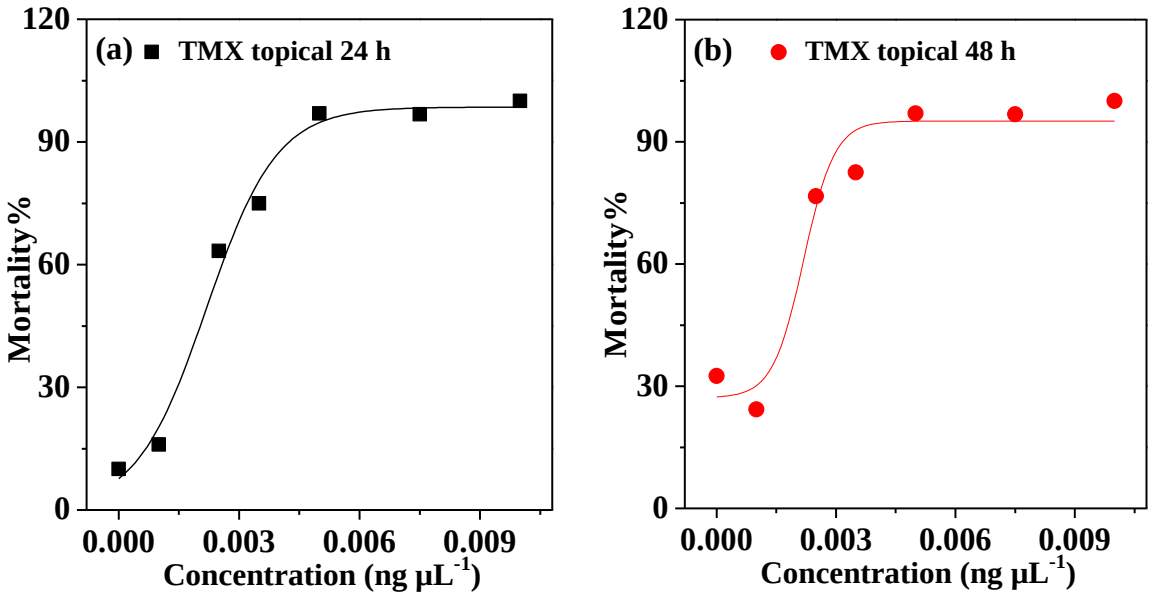


Figure 20 and 21 shows the direct relationship between increased mortality due to increased concentration of the insecticide for both topical and dietary oral exposure for 24 and 48 h. However 100% mortality was observed at 5.0 ng μL^{-1} concentrations for topical exposure at 24 h and 48 h, while for oral exposure the highest mortality was - 80% and 100% was observed at 7.5 ng μL^{-1} in 24 h and 48 h respectively. For the exposure time of 48 h, topical LD_{50} was 1.7 ng a.i./bee and oral LC_{50} was 0.06 ng a.i./µL of diet offered. In the level of comparison, the estimated oral LD_{50} was 3.5 ng a.i./bee, which indicates that TMX was 2 times more toxic to stingless bees topically.

Figure 21. Plot of Concentration versus mortality of TMX topical exposure for (a) 24 h and (b) 48 h.



The observed concentration of TMX after oral and topical exposure is comparable to the lethal doses/concentrations (LD_{50}/LC_{50}) for *A. mellifera* reported in the literature: observed LC_{50} were 0.0034 µg bee^{-1} [107]; 0.0112 µg bee^{-1}[106] during 48 h of exposure. The same finding can be observed for the topic exposure, in which the LD_{50} reported in previous works for *A. mellifera* were 0.029 µg bee^{-1} for 24 h exposure [108]; or even 0.051 µg bee^{-1} for 48 h [83].

4.3.2.2 Definitive concentration-mortality evolution of IMD oral and topical exposure

The values for the dietary oral LC_{50} of the IMD obtained for *M. scutellaris* were; 1.0 (±0.3) and 0.5 (±0.2) ng a.i./µL (Table 19 and Figure 22), for 24 h and 48 h, respectively. However, the values for the topical LD_{50} of the IMD obtained were: 6.0 (±2.0) and 3.0 (±1.0) ng a.i./bee (Table 19 and Figure 23), for 24 and 48 h, respectively. These results show a small variation between 24 h and 48 h when IMD was administered orally, but a greater than two-fold difference when administered topically.

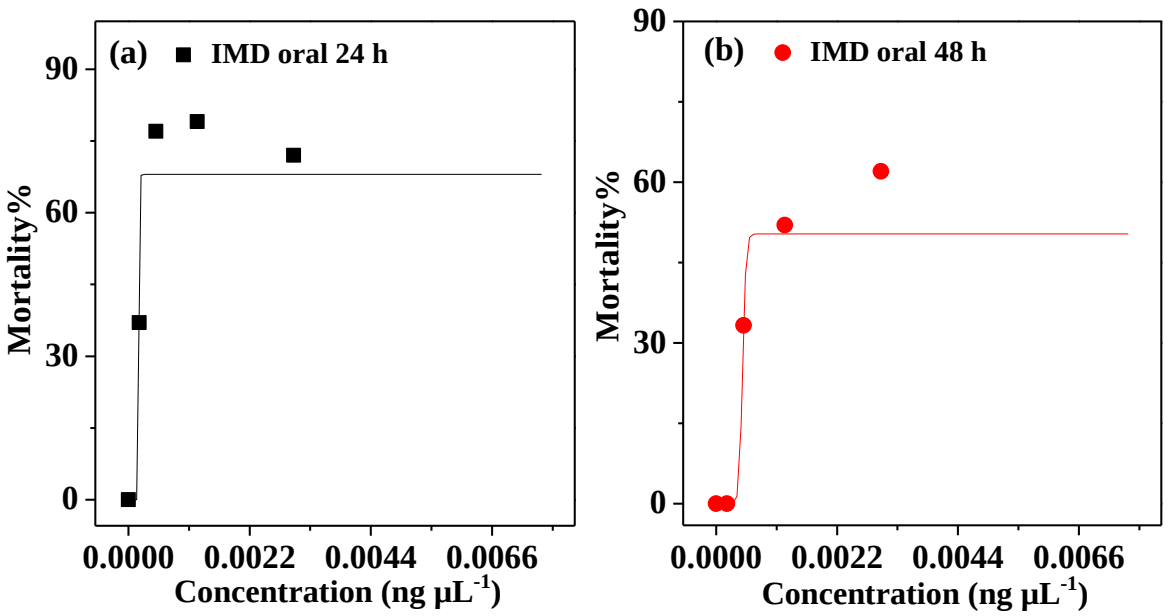
Table 19. Summary of the acute toxicity tests of IMD against *M. scutellaris* exposures.

Exposure route	Time[a]	LD_{50}[b]	LC_{50}[c]	X^2 [d]	D.F[e]
Topical (nga.i/bee)	24	6.0 (±2.0)	-	7.92	3
	48	3.0 (±1.0)	-	76.96	3
Oral (ng a.i/µL diet)	24	-	1.0 (±0.3)	54.62	2
	48	5.0 (±1.0)	0.5 (±0.2)	36.23	2

[a] Time in h after the administration of IMD; [b] lethal dose 50 %; [c] lethal concentration 50 %; [d] Chisquare from model; [e] degrees of freedom

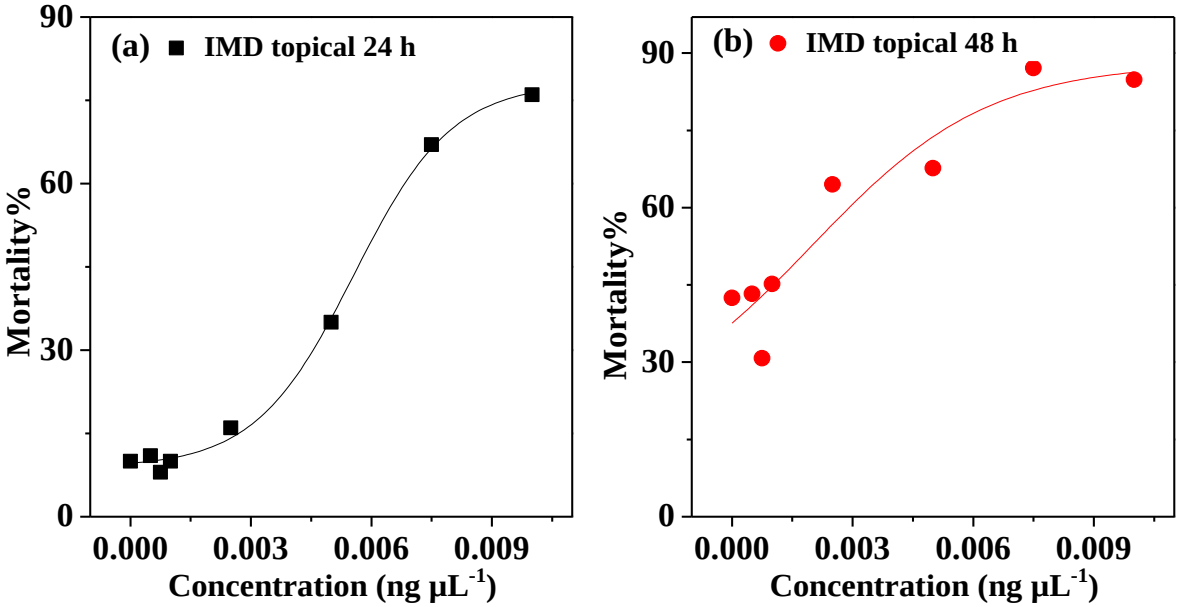
The evolution of bee mortality after topical exposure to IMD progressed only from the concentration of 2.5 ng μL^{-1}, resulting in values close to 20% and 40%, respectively for 24 and 48 h. From this value there were small increases with increasing concentration, reaching a maximum of approximately 60 and 80% mortality for 24 and 48 h respectively, at the highest concentration tested (10 ngμL^{-1}) (Figure 22c). After ingesting the IMD, it showed variation in the toxic response according to the tested concentrations. Mortalities reached values close to 50% and 80%, respectively, in 24 and 48 h, in the first tested concentrations (0.1 and 0.25 ng μL^{-1}), with little increase in these percentages with increasing concentration and not reaching 100% mortality (Figure 23c). For the 48 h exposure time, topical LD_{50} was 3 ng a.i./bee and the oral LC_{50} was 0.5 ng μL^{-1} (Table 19) of the offered diet. At the level of comparison of lethal doses, the estimated oral LD_{50} was 5 ng a.i./bee which indicates that IMD was 2 times more toxic to stingless bees topically. .

Figure 22. Plot of concentration versus mortality for IMD oral exposure for (a) 24 h (black line) and (b) 48 h (red line).



A comparison of toxicity data from our study with literature values for honey bees shows a greater sensitivity of the stingless bee to this neonicotinoid. Our 24 h and 48 h LD_{50} value of IMD for *M. scutellaris* was 3.0 and 6.0 ng a.i./bee. Considerably higher LD_{50} values of 17.9 ng a.i./bee (24 h) [108]; 24 ng a.i./bee (24 h and 48 h) [115]; 42 – 1041 ng a.i./bee (48 h) [116]; 49 – 1022 ng a.i./bee (48 h) [117]. Similarly, our 48 h (0.5 ng a.i./bee) dietary LC_{50} for *A. mellifera* are: 81 ng a.i./µL diet (48 h) [117] and 40.9 ng a.i./µL diet (48 h) [118]. These values show that the *M. scutellaris* stingless bees are more sensitive to IMD than *A. mellifera*. Others studies that compare the tolerance between stingless and africanized honey bee species showed that the former are usually more sensitive to insecticides [36,119,120].

Figure 23. Plot of concentration versus mortality of IMD topical exposure for (a) 24 h (black line) and (b) 48 h (red line).



Our results corroborate the work of Soares et al. [110], which determined the topical LD_{50} and oral LC_{50} of IMD for native bee S*captotrigona postica* Latreille, 1807 (Hymenoptera: Apidae). The obtained values were: topical LD_{50} of 25.20 (24 h) and 24.46 ng a.i./bee (48 h) and oral LC_{50} of 42.5 (24 h) and 14.3 ng a.i./ µL diet (48 h), indicating that this species is also more susceptible to these neonicotinoid pesticide than *A. mellifera*. Comparing values of LD_{50} and LC_{50} (48 h) of IMD for S. postica and *M. scutellaris*, it was noted that this bee is 19 times more sensitive when compared to the other bees. According to Johansen and Mayer classification [121], which consider insecticides with a LD50 < 2.000 ng/ bee as highly toxic to stingless bees, therefore we can say that IMD and TMX are considered highly toxic for *M. scutellaris* both orally and topically. The toxicity of neonicotinoid insecticides for stingless bees can be classified in two groups based on the presence of nitro or cyan grouping. The insecticides with nitro group are the most toxic ones, such as TMX and IMD, because the presence of this functional group grants to the pesticide great affinity with the nicotinic receptor of acethylcholine and, therefore, its high toxicity [122].

The route of intoxication is an important factor for risk assessment, as it may determine the time for an insecticide to reach its target site. It was observed that a dietary exposure (24 h or 48 h) to TMX and IMD were comparatively less toxic to *M. scutellaris* than a 48 h topical exposure. This may have been due to the fact that the high topical toxicity of these insecticides (TMX and IMD) to *M. scutellaris* might be caused by the fact that these molecules are orally not rapidly metabolized in olefin and 5-hydroxyIMD due to the resistance of antioxidant enzyme in bee midgut. Such metabolites are toxic with acute exposure, highly suggesting that 5-hydroxyIMD and/or olefin contribute for an increased action of IMD in stingless bees. This hypothesis was discussed by Suchail et al. [115] considering the mortality kinetics and neurotoxicity symptoms in *A. mellifera*.

4.3.3 Oral and topical exposure bioassays of binary mixture of TMX and IMD (analytical standard)

A binary mixture of TMX and IMD was investigated; tests were conducted considering Co (control) as treatment 1; NOEC as treatment 2; and LD_{50} or LC_{50} (mean lethal dose/concentration) as treatment 3. Herein, NOEC stand for "No Observed Effect Concentration" which is the test concentration immediately below the lowest tested concentration that produced statistically significant adverse effects and it can be determined statistically. All these treatments were observed for 24 h, 48 h and 72 h of exposure, validating the test according to with OECD protocol. In topical exposure, topical doses for treatment 2 had binary mixture of TMX and IMD; 1.0 and 0.75 ng μL^{-1} respectively, and for treatment 3 binary mixtures of TMX and IMD; 2.7 ng and 2.5 ng μL^{-1} respectively, as shown in Table 20. In oral exposure, oral doses for treatment 2 had binary mixture of TMX and IMD; 1.0 and 0.5 ng μL^{-1} respectively, and for treatment 3 had binary mixture of TMX and IMD; 4.0 and 2.5 ng μL^{-1} respectively, however the control group (treatment 1) has received just sucrose solution during the experiment as can be seen in Table 20.

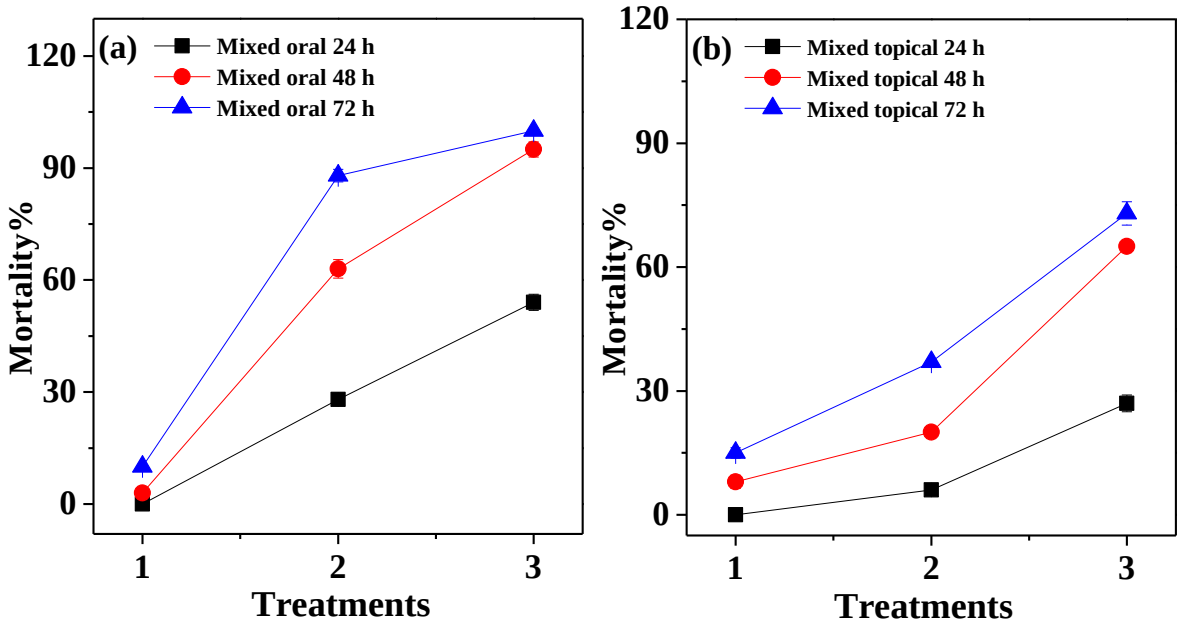
Table 20. Average mortality (%) of stingless bees during oral and topic exposure: NOEC; LC_{50}; LD_{50}; and control. no = 30.

Exposure route	Treatments	TMX (ng µL^{-1})	IMD (ng µL^{-1})	24h[a]	48h[b]	72h[c]
Topical	1	00	00	0	8.0	15
	2	1.0	0.75	6.0	20	37
	3	2.7	2.5	27	65	78
Oral	1	00	00	0	3.0	10
	2	1.0	0.5	28	66	88
	3	4.0	2.5	53	94	100

[a] Mortality % for 24 h; [b] Mortality % for 48 h; [c] Mortality % for 72 h

Mortality (%) during the oral and topic exposure can be observed in Table 20 and Figure 24a and 24b. It is possible to verify that control groups have no significant mortality during the 48 h exposure. For other groups 100% mortality was observed only for oral exposure, only for treatment 3 (LC_{50}) while for treatment 2 (NOEC) in oral exposure, the highest mortality (88%) was observed for the mixture of analytical standard (TMX and IMD). Oppositely, topic exposure has the highest mortality percentage for treatment 3 (LC_{50}) was 78%.

Figure 24. Mortality % of *M. scutellaris* versus different concentrations binary mixture of TMX and IMD insecticides (a) oral and (b) topical expose to 24 h (black line), 48 h (red line) and 72 h (blue line)**.**



The exposure bioassays were based on endpoint levels (72 h) in *M. scutellaris* was observed for all tested concentrations (Figure 24a and 24b) and follows the order: 24h<48h<72h, according to the increase of exposure dose. Mortality observed in investigated stingless bees was up to 5 times higher when bee was 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 insecticides and the effect of them in metabolism will depend first on the permeation through the cuticle [123].

4.3.4 Topical and Oral bioassays of TMX and IMD (Commercial products Nortox and Actara)

The values for the oral LC_{50} of the commercial product of TMX named as Actara and that of IMD named as Nortox obtained for *M. scutellaris* were; 0.07(±0.05) and 0.4 (± 0.2) ng a.i. /µL of diet offered (Table 21 and Figure 25a and 25b), for 24 h, respectively. Similarly, the values for the topical LD_{50} of Actara and Nortox obtained for *M. scutellaris* were: 1.0(± 0.7) and 3.1(± 2.4) ng a.i. /bee (Table 21 and Figure 26a and 26b), for 24, respectively.

Table 21. Summary of the acute toxicity tests of the Actara (TMX) and Nortox (IMD) against *M. scutellaris* exposures.

Insecticides	**Time[a]**	**Exposure route**	**LD_{50}[b] (nga.i/bee)**	**LC_{50}[c] (nga.i./µL)**	**X^2 [d]**	**D.F[e]**
Actara	**24**	**Topical**	1.0(± 0.7)	-	24.9	3
		Oral	4.4(± 3.1)	0.07(±0.05)	3.1	3
Nortox	**24**	**Topical**	3.1(± 2.4)	-	6.5	2
		Oral	20.0(±10.0)	0.4 (± 0.2)	43.4	2

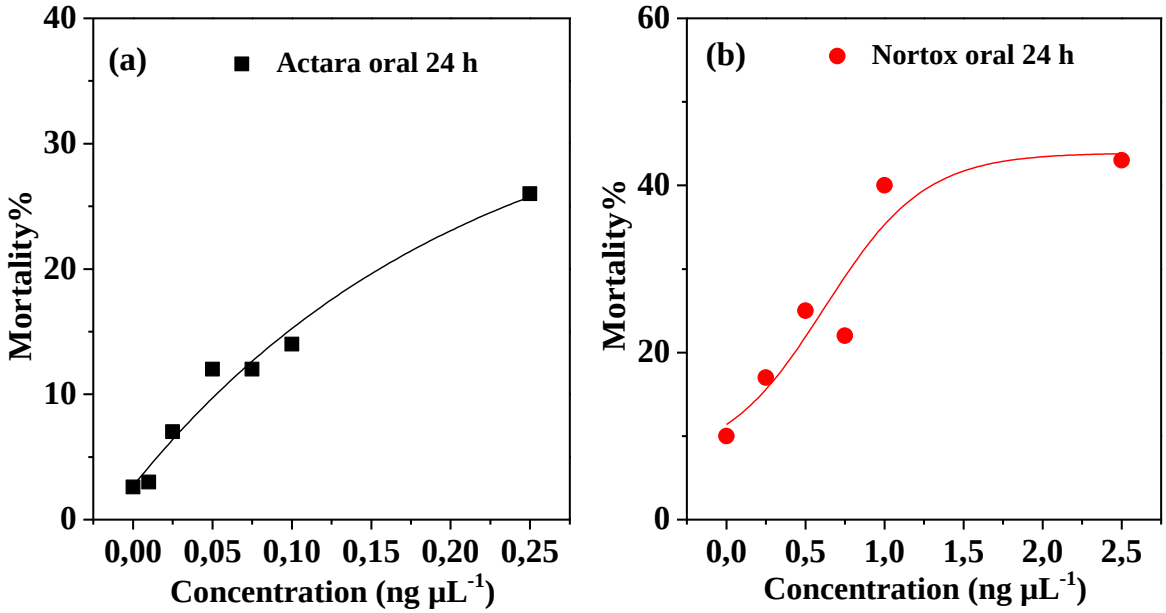
[a] Time in h after the administration of insecticides; [b] lethal dose 50 %; [c] lethal concentration 50 %; [d] Chi-square from model; [e] degrees of freedom

According to Johansen and Mayer classification [121], mean lethal dose with a LD_{50}<*2000* ng /bee as highly toxic to bees, therefore Actara and Nortox both are considered highly toxic for *M. scutellaris.* For the exposure time of 24 h, topical LD_{50}

for Actara was 1.0(± 0.7) ng a.s./bee and oral LC_{50} was 0.07(±0.05)ng a.s./µL of diet offered. In the level of comparison of lethal doses, the estimated oral LD_{50} was 4.4(± 3.1) ng a.i./bee, which indicates that Actara was 4 times more toxic to stingless bees topically when compared to oral exposure. While for Nortox, topical LD_{50} was 3.1(± 2.4) ng a.i./bee and oral LC_{50} was 0.4 (± 0.2) ng a.i./µL of diet offered, at the exposure time of 24 h. for the level of comparison estimated lethal doses, LD_{50} was 20.0(±10.0) ng a.i./bee, the result shows that topically Nortox was 7 times more to stingless bees (*M. scutellari).*

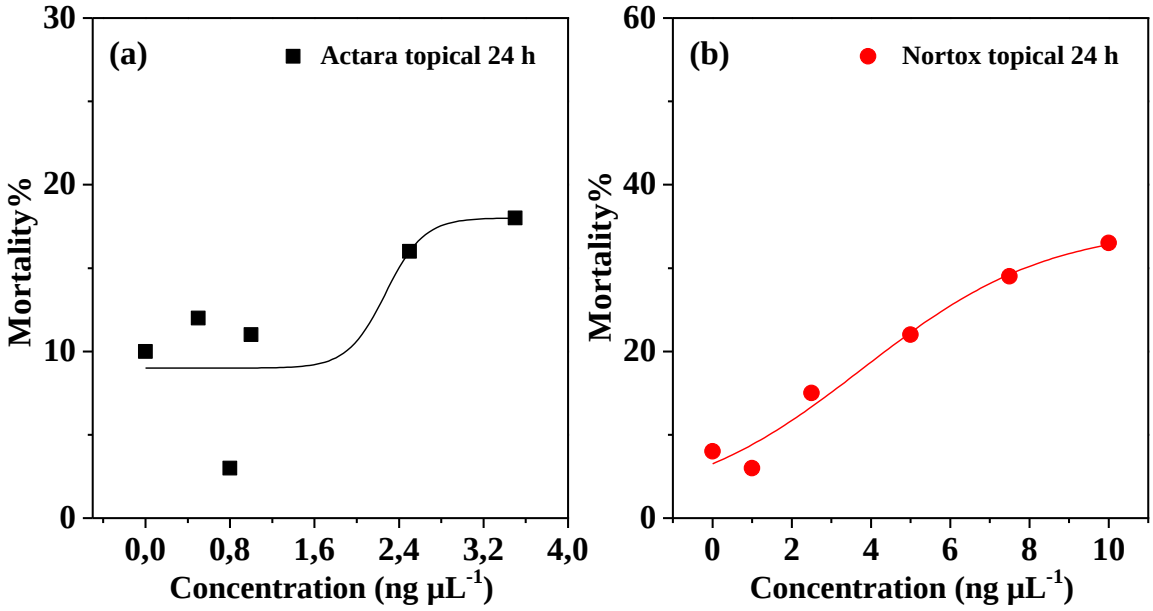
It was observed that a topical exposure to these insecticides was more toxic to *M. scutellaris* than 24h dietary exposure. This may have been due to the fact that these are neurotoxic insecticides with action by contact, and that it readily penetrated the integument of the insect when diluted in triton. When exposure was by ingestion, the insecticide present in the midgut likely encountered a variety of enzymes involved in the metabolisum of neonicotinoids (e.g. cytochrome P450, aldehyde oxidase and gluthation S-transferase) [124]. However, *M. scutellaris* both topically and orally more sensitive to the commercial product Actara when compared to the commercial product Nortox.

Figure 25. Mortality % of *M. scutellaris* versus concentration after the intoxication with (a) Actara (black line) and (b) Nortox (red line) by oral ingestion for 24 h.



Our results revealed that *M. scutellaris* is usually more susceptible to these insecticides via topical and oral than *A. mellifera* [108,115,117,118]. The toxicity of these insecticides was higher to wild bees than *A.mellifera*. This difference in the responses of various bee species to insecticides exposure was previously described by Desneux et al., [125]. Changes in insecticides susceptibility among bee species were also observed by several others [126], with the most of the results indicated that the honey bee *A. mellifera* was more tolerant to insecticides in comparison with species of stingless bees.

Figure 26. Mortality % of *M. scutellaris* versus concentration after the intoxication with (a) Actara (black line) and (b) Nortox (red line) by topical administration for 24 h..



The symptoms resulting from poisoning by Active and commercial products were similar for both the topical and oral treatments, such as decreased mobility, shivering of wings and body, paralysis, tremors, prostration and death, which are common symptoms of intoxication by neonicotinoid insecticides observed by Medrzycki et al. [127] and Faria [128] since the target organ of this substance is the nervous system. This is an indication that the stingless bees present in Brazil have a higher sensitivity to insecticides than species identified as surrogates in toxicological tests, showing the importance of properly assessing the impact of these molecules for species that are found in our forests and farmland to ensure their conservation.

In conclusion, our study showed that *M. scutellaris* is highly sensitive to the action of the commercial product of insecticide TMX (Actara) and that of IMD (Nortox) after topical and oral intoxication. Because of this and of the economic and ecological importance, native species of stingless bees should be more studied, especially in relation to pesticide impact.

5. Chapter # 3. The effects of thiamethoxam and imidacloprid insecticides on stingless bee Melipona scutellaris: evaluation of biochemical-physiological changes in the assessment of sublethal toxicity

5.1 SUMMARY

The meliponines, also known as stingless bees, exhibit biological characteristics ideal for pollination [131], making them efficient pollinators in some cultures [132]. Stingless bee *Melipona scutellris* Latreille, 1811 (HYMENOPTERA: APIDAE) is distributed in the North and Northeast regions of Brazil, with records from Rio Grande do Norte to Bahia. This species also seems to be well adapted to the climate and ecology of the State of São Paulo and although they show great potential as pollinators, research on the susceptibility of this bee to insecticides is limited. Among the threats to bees, disintegration and loss of habitat [133,134], migratory apiculture, high levels of parasites, and intensive use of pesticides are highlighted [134-137].

Thiamethoxam (TMX) and imidacloprid (IMD) belongs to neonicotinoid class and both are classified as systemic insecticides, because they have the ability to absorb in all plant tissues through the sap. [142,143] including pollen [92,144] and nectar [138,144]. Neonicotinoids are used to control agricultural crop pests [138] and they are based on nicotine [139], acting as agonists of the acetylcholine in the nicotinic receptor [140] not hydrolyzed by acetylcholinesterase, which causes hyperexcitation of the insect's nervous system [76]. Relatively low mammal toxicity and systemic activity contribute to it being one of the most widely used insecticides in the world [145]. However due to their extensive application and persistence in both soil and water, neonicotinoids become bioavailable to pollinators in sublethal concentrations for throughout the year [141].

Pesticides are known to cause oxidative stress in a broad range of animals, including insects. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the natural defense mechanism antioxidants in an organism and this inequity can result from an over abundance of stressful ROS producers [146]. Antioxidant enzymes such as SOD and CAT are essential in the defense against oxidative stress and both are allied with the toxicity of pesticides on insects. In addition to these two antioxidant enzymes, the GST enzymes, GPx and /GR have also been reported to occur in insects. Increasing levels of antioxidant enzymes would, therefore,

be an indication of the attempt of organisms in dealing with an oxidative stress environment [146]. Recently, articles evaluating the effects of the neonicotinoid on pollinators have pointed out sublethal effects on behavior, locomotion, and memory [144,147-149], metabolism and immunity [150,151], reproduction [152,153], and synergistic effects of additional pesticides with neonicotinoids [106,154]. The efficiency of these insecticides can be measured by their action under target insects and also non targets such as bees [79].

In this study, we focused on of the TMX and IMD active and commercial products, ACTARA 250 WG, i.a thiametoxam (75%) and IMIDACLOPRID NORTOX, 480 SC, i.a imidacloprid (70%), to measure its relatively long term sublethal impacts on stinglees bee M.scutilares workers, and examine bees' biochemical markers at sublethal concentrations LC_{50} (50% lethal concentration) for oral exposures (1/2 LC_{50}; 1/5 LC_{50}; and 1/10 LC_{50}) and sublethal doses LD_{50} (50% lethal concentration) for topical exposures (1/2 LD_{50}; 1/5 LD_{50}; and 1/10 LD_{50}).

5.2. MATERIALS AND METHODS

5.2.1 Sublethal toxicity analysis

5.2.1.1 Stingless bees

M. scutellaris bees were taken from bee hives situated at the experimental "melliponary" located at the Center for Water Resources and Environmental Studies (CHREA) from the University of São Paulo, São Carlos, Brazil. For oral and topical exposure bioassays, bees were collected at the first hours of the day and transported alive in plastic cages and kept in a temperature-controlled chamber (28 ± 1°C) for all the duration of the measurement. To encourage consumption of the solutions, bees were starved for 2 h before the start of the experiments. After 2 h of adaptation to rearing environment, dead bees and the sub-chronic treatment was administered. During the acclimation period (24 h), bees were fed with sucrose: water solution (50:50 in volume) ad libitum and each treatment consisted of five replicates with 10 bees in each replicate.

5.2.1.2 Sample preparation and route of exposure

Insecticides stock solutions of 1000 ng/µL were prepared in acetone and preserved at - 4°C. To obtain the 4 concentrations (LD_{50}, LD_{50}/2, LD_{50}/5and LD_{50}/10) as shown in Table 1, Work solutions were prepared by dilution of reserved solutions in sugar syrup (50%) for AOT(acute oral toxicity) and in Triton X-100 for ATT (acute topical toxicity). The LD_{50} and LC_{50} were obtained from prior work (chapter 2). The susceptibility of a stingless bee to each insecticide (TMX and IMD) was tested by means of two routes of exposure: oral and topical. Both treatments were conducted under the international guidelines for pesticide toxicity testing in honeybees [95,96]. In oral exposure; bees were allowed access to 1000 µL of treated food. 6 hours later, the feeder was replaced with one containing a sucrose-only solution. While in topical exposure the bees were anesthetized with CO_2 for 8 sec. Then, a micropipette was used to apply 1 µL of each dose at the thorax of each bee. Two control groups were used: a solvent control group, which received Triton X-100 alone, and an unexposed control group, to which no substances were applied. After exposure, bees were kept in BOD with access to food (sucrose solution) ad labitum.

Table 22. Insecticides, routes of exposure and concentrations used in sub-chronic treatments.

Insecticides	Exposure route	LD_{50} [b] (ng a.i/bee)	LC_{50} [d] (ng a.i/µL diet)	½	1/5	1/10
TMX	**Topical** [a]	2.0	-	1.0	0.4	0.2
	Topical [c]	1.0	-	0.5	-	-
	Oral [a]	-	0.4	0.2	0.08	0.04
	Oral [c]	-	0.1	0.05	0.02	0.01
IMD	**Topical** [a]	6.0	-	3	1.2	0.6
	Topical [c]	3.1	-	2.0	1.0	-
	Oral [a]	-	2.0	1.0	0.4	0.2
	Oral [c]	-	0.4	0.2	0.1	-

[a] Active TMX and IMD products ; [b] lethal dose 50 %; [c] Commercial TMX and IMD products; [d] lethal concentration 50 %

5.2.2 Enzymatic activity assays

5. 2.2.1 Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is an analytical method that enable protein separation based on their molecular mass. SDS is an anionic detergent, which facilitate the denaturation of the indigenous proteins by disturbing the non-covalent forces. A polyacrylamide gel electrophoresis was used to assess an expression of enzymes in the Colors of the bee tissue. A tissue sample from all over the organism was taken in Eppendorf and macerated using a glass stick, followed by 250 µL of purified water in the Milli-Q system with resistivity 18.2 MΩ cm^{-1} and centrifuged at 13000 rpm for 30 minutes at 25 ºC. The supernatant was collected and mixed with denaturing sample buffer (Tris-HCl 100×10^{-3} mol L^{-1} pH 6.8; 0.2% bromophenol blue; glycerol 30%, SDS 4%, and merc-mercaptoethanol 200×10^{-3} mol L^{-1}) with a proportion of 20 µL of sample: 10 µL of buffer (2: 1) and heated for 5 minutes in a thermostatic bath at 95 ºC. Thereafter, 15 µL of the sample and 8 µL of the

molecular mass marker Pierce Molecular weight marker for unstained proteins from Thermo Scientific were incapable to apply stacking gel made. The gel was subjected to an initial voltage of 90 V until it entered the gel resolution (SDS-PAGE 15%) and 120 V from then until the end of the race. After a run, gel was stained with Coomassie Brilliant Blue from BioRad and then bleached with a 75% acetic acid solution and 10% glycerol dissolved in 1000 mL of distilled water.

5.2.2.2 Evaluation of lipid peroxidation (MDA) levels

Free radicals induce lipid peroxidation, playing an important role in pathological processes. The injury mediated by free radicals can be measured by conjugated dienes, malondialdehyde (MDA), 4-hydroxynonenal, and others. However, MDA is one of the most known secondary products of lipid peroxidation, and it can be used as a marker of cell membrane injury.

MDA as an indicator of lipid peroxidation and has a key role in oxidative stress. Most assays determine malondialdehyde (MDA) by its reaction with thiobarbituric acid (TBA), which can be measured by chromatographic methodologies. The assessment of lipid peroxidation levels was performed using the product produced between malondialdehyde (MDA) and thiobarbituric acid (TBA) according to the works of Almeida et al. [155,156]. The tissue samples from the entire organism were weighed and homogenized (Ultra Stirrer homogenizer, model Ultra 380) in 0.1 Tris-HCl buffer solution mol L^{-1}, pH 8.0 in a 1: 3 (m / v) ratio. Then, 300 µL of 0.4% TBA solution was diluted in HCl 0.200 mol L^{-1}. The samples were incubated by 40 minutes at 90 ºC. Then, the bee extract after the reaction of MDA with TBA formed a pink solution. Subsequently, the extract was placed in an ice bath to cool to -10 ºC and then 1.0 ml of n-butyl alcohol was added to each of them. Soon after, they were centrifuged at 3500 rpm for 3 minutes and 700 µL of supernatant for further examination. The investigation of the samples was done using an HPLC-DAD-UV. The column chromatographic used was C18 (150 x 4.6 mm, 5 µm pore diameter). The volume sample injection was 20 µL, with a column temperature of 30 ºC and a flow rate mobile phase of 1 mL min^{-1}. The wavelength selected to get the chromatograms was 532 nm. The mobile phase used was composed of 60% A (buffer monobasic potassium phosphate 50.0 mmol L^{-1} pH 7.0) and 40% B (methanol) in mode isocratic. For the quantification of the formed MDA, the analytical curve was used, also injected into HPLC-DAD-UV, with known

concentrations of MDA derivative with TBA, in which the data were articulated in p mol mg^{-1} of tissue.

5.2.2.3 Evaluation of the enzymatic activity of GST

Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm. Glutathione S-transferase (GST) activity assays in insects are usually performed by spectrophotometric kinetic measurements of conjugated product formation with substrates such as reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). This requires a spectrophotometer that can measure absorbance in the UV range and micro centrifugation to remove the particulates from crude homogenates which absorb light at 340 nm.

The enzymatic activity of GST was measured by monitoring the conjugate of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalyzed by GST in the thorax plus bee abdomen sample, according to the method described by Keen et al. [157]. The tissue samples of the bees were weighed and homogenized in buffer Tris HCl 0.200 mmol L^{-1}, pH 7.5 containing 1.00 mol L^{-1} of protease inhibitor Phenylmethanesulfon Fluoride (PMSF) in a 1: 4 (m / v) ratio. Then the extract was centrifuged at 9,000 RCF (relative centrifugal force) for 30 minutes at 4 °C. The supernatant was transferred to the reaction medium containing 2.22 m mol L^{-1} of CDNB (dissolved in phosphate buffer 0.200 mol L^{-1}, pH 6.5) and 2.22 m mol L^{-1} of GSH (dissolved in phosphate buffer 0.200 mol L^{-1}, pH 6.5) and absorbance was monitored at 340 nm in a microplate reader.

5.2.2.4 Determination of the enzymatic activity of acetyl cholinesterase

The enzymatic activity of AChE was determined by monitoring the degradation of acetyl thiocholine by acetyl cholinesterase activity in samples of whole bee tissue, with the formation of thiocholine, according to the method described by Ellman et al. [158] modified for a microplate reader. The tissue samples from the whole organism were weighed and homogenized in buffer Tris HCl 0.100 mol L^{-1}, pH 8.0 in the proportion 1: 4 (m / v). Then they were centrifuged for 30 minutes at 10,000 rpm at 4 ° C. The supernatant was collected and added to a reaction medium containing DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) dissolved in potassium phosphate buffer 0.100 mol

L^{-1} pH 8.0 and 0.500 mmol L^{-1} of acetylcholine iodide and absorbance was monitored at 405 nm in microplate reader.

5.2.2.5 Total protein analysis

The estimate of the concentration of total proteins in the extracts produced in GST and AChE tests were performed using the Bradford method [159] using albumin bovine serum (BSA) as standard and read at 595 nm in a microplate reader.

5.2.2.6 Statistical analysis

The normality and homogeneity of the results were verified using Shapiro Wilk and Levene. The non-parametric data were transformed into a log and, in If they remain non-parametric, the non-parametric analysis was performed, Kruskal Wallis. Parametric data were analyzed by ANOVA-one way with Hoc Tukey's post for the comparison between the exposed groups. The software used for the analysis was *Statistica* (version 6.4).

5.2.3 Neurotransmitters

5.2.3.1 Standards and solutions

The procedures for the analysis of neurotransmitters in samples of bees exposed to pesticides were based on Benedetto et al. [160]. The analytical standards used were with a high degree of purity, octopamine (98.0%), dopamine (98.0%), and serotonin (98.0%) and purchased from Sigma Aldrich. At octopamine and dopamine stock solutions (1000 ng mL^{-1}) were prepared individually in milli Q water and the serotonin stock solution (200 ng mL^{-1}) was prepared in MeOH : H_2O (1:1) . All solutions were kept in the dark and in a refrigerator (- 4 ºC). As intermediate mix solutions (10 ng mL^{-1}) were prepared by diluting the individual stock solutions in perchloric acid 0.200 mol L^{-1} containing 3.00 mmol L^{-1} cysteine (homogenization buffer). Subsequently futher diluted to the following concentrations of neurotransmitters: 15; 30; 60; 120; 240; 480; 920 and 1200 ng μL^{-1} in homogenization buffer for the construction of the analytical curve. Each concentration level was prepared in triplicate.

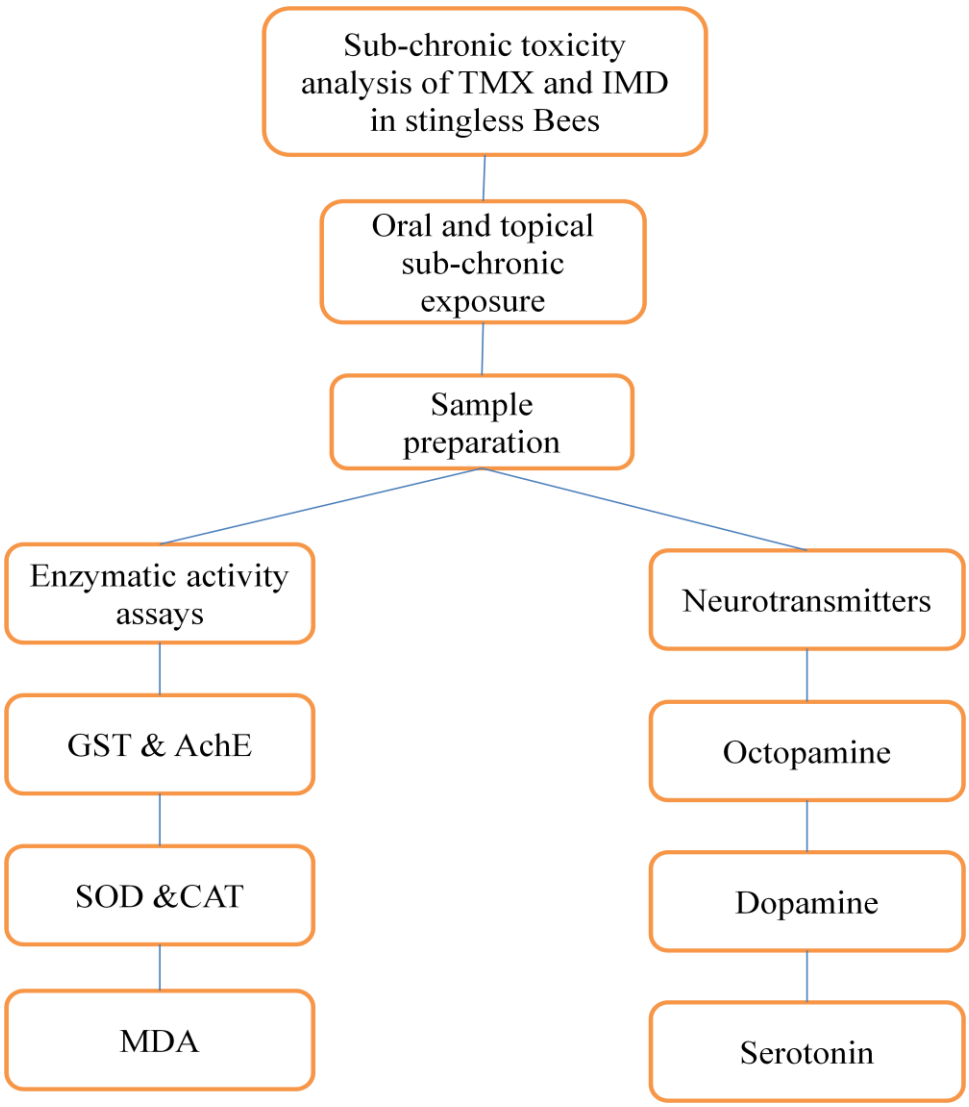
5.2.3.2 Sample preparation and analysis of neurotransmitters

The head samples of the bees were weighed separately and homogenized in homogenization buffer (100 µL of perchloric acid (0.200 mol L^{-1}) with cysteine (3.00 mmol L^{-1})). The homogenate was centrifuged (12,000 rpm, at 4 ºC for 5 minutes) and the supernatant was filtered using a 0.22 µm PVDF filter and analyzed in the system HPLC with fluorescence detection (FD). For the separation of neurotransmitters substitute a C18 chromatographic column (250mm x 4.6mm, 5µm) from Nano Separation Technologies and maintained at 35 ° C during analysis. The mobile phase used was composed of 95% A (perchloric acid 5 mmol L^{-1}) and 5% B (acetonitrile) in isocratic mode. The flow rate of the mobile phase was 1.0 mL min^{-1} and the volume sample injection was 20 µL. Neurotransmitters were monitored with a fluorescence detector set with a 279 nm wavelength of excitation and 320 nm emission. The concentration of neurotransmitters was calculated based on the analytical curve. The developed method was used to determine octopamine, dopamine, and serotonin in bee head samples from the bioassays of acute oral and topical toxicity. In addition to these samples, bees were collected from out of the hive and then kept in liquid nitrogen for the preservation of the biological components and analysis of neurotransmitters in hive environment.

5.2.4. EXPERIMENTAL PROCEDURES

The experimental procedure has been divided into three parts: (1) sub-lethal toxicity analysis, (2) Enzymatic activity assays**,** and (3) neurotransmitters as shown by the flow chart in Figure 27.

Figure 27. Flow chart representing the overall experimental procedure of Biochemical biomarkers functional tools to assess the effect of TMX and IMD insecticides on stingless bee *Melipona scutellaris.*



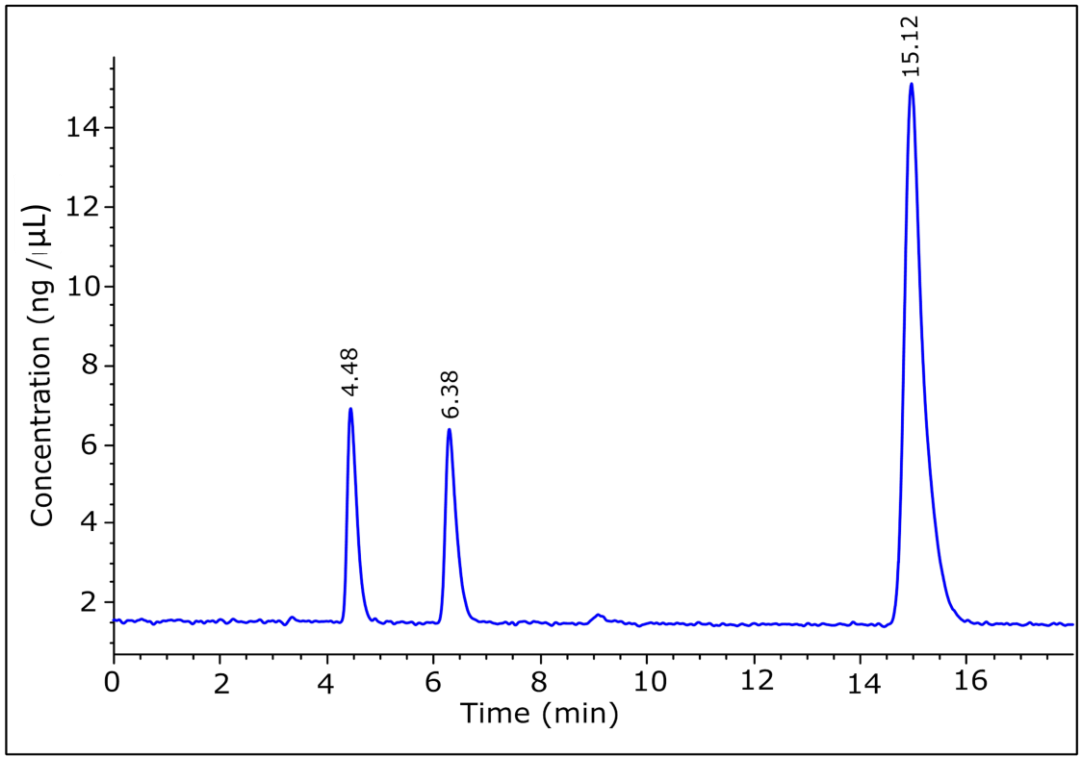
Source: Self-made figure.

5.3 RESULTS AND DISCUSSION

5.3.1 Neurotransmitter

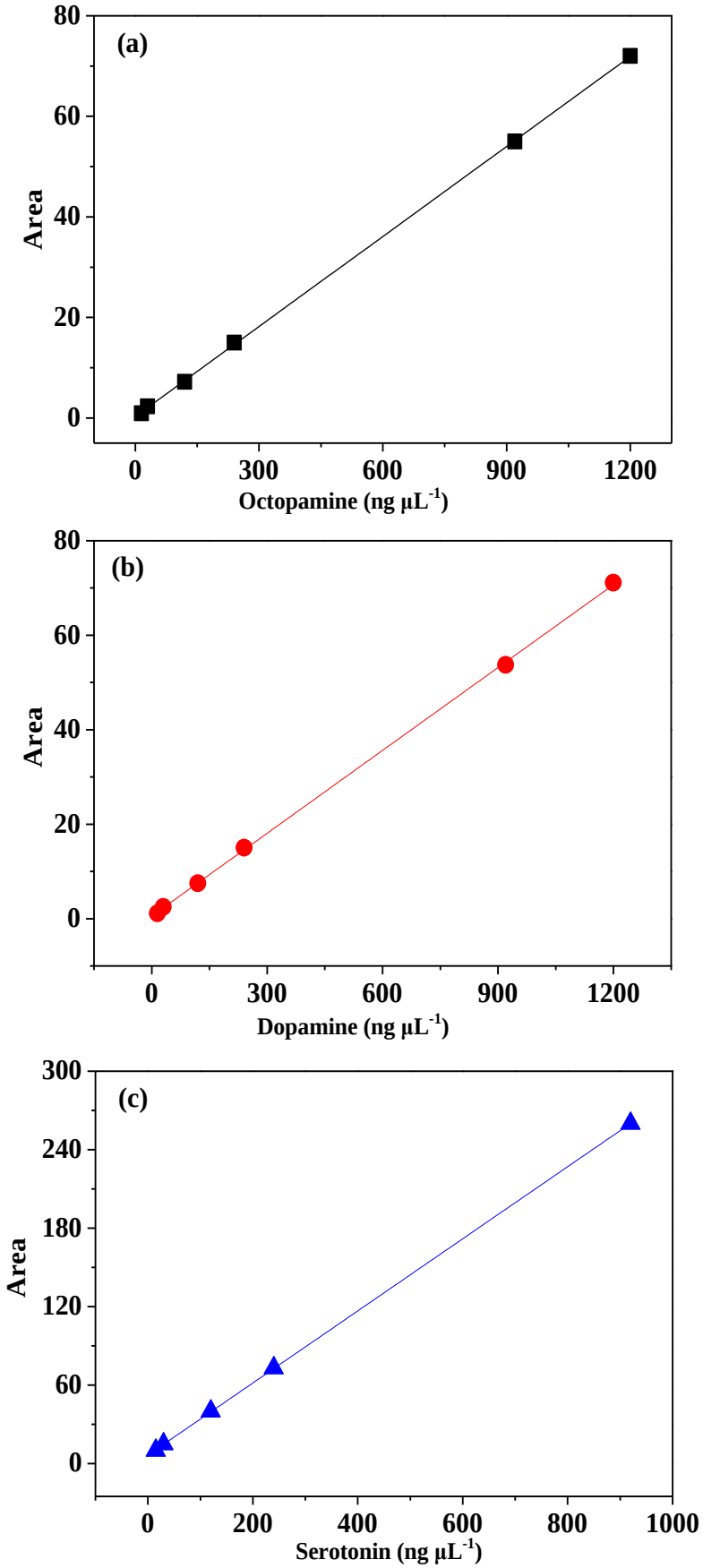
The neurotransmitters; octopamine, dopamine and serotonin concentrations were detected by HPLC-FD. Figure 28 shows the standard chromatogram for a solution of the octopamine, dopamine and serotonin standards at a concentration of 120 ng μL^{-1} in homogenization buffer of perchloric acid 0.200 mol L^{-1} containing cysteine 3.00 x 10^{-3} mol L^{-1}.

Figure 28. Chromatogram of a standard solution of 1–octopamine, 2–dopamine and 3–serotonin in concentration of 120 ng μL^{-1} using HPLC-FD, λ_{ex} = 279 nm, λ_{em} = 320 nm.



The retention time were 4.5 minutes, 6.4 minutes and 15.1 minutes for octopamine, dopamine and serotonin respectively, the total analysis time was 18 minutes. The analytical curves were made with six concentrations of neurotransmitters in perchloric acid 0.200 mol L^{-1} containing cysteine 3.00 x 10^{-3} mol L^{-1} in triplicate as shown in Figure 29. The concentration range of neurotransmitter was from 0.015 to 1.2 ng μL^{-1}.

Figure 29. Analytical curves obtained for (a) octopamine (black line), (b) dopamine (red line) and (c) serotonin (blue line).



The analytical curves in Figure 29 shows a linear increase in chromatographic peak area as a function of analyte concentration in the interval of concentration studied, presenting linear correlation coefficients (r^2) of 0.999. The Huber test was used to assess the linearity of the curves and also to determine the limit of quantification of the method. The data related to the linear interval, the regression equation of the line and the linear correlation coefficient are shown in Table 23. The LQ of the method was 0.015 ng μL^{-1} for the three neurotransmitters.

Table 23 - Analytical parameters obtained from the analysis of the calibration curve for neurotransmitters.

Analytes	**Linear range (ng μL^{-1})**	**Line equation**	**r^2**
Octopamine	0.015 – 1.2	y = - 0.289 + 0.059 * x	0.999
Dopamine	0.015 – 1.2	y = - 0.55 + 0.059 * x	0.999
Serotonin	0.015 – 0.920	y = - 6.5 + 0.27 * x	0.999

Live bees samples from acute oral and topical toxicity bioassays were used for the determination of neurotransmitters while in control group bees were directly collected from the hive and placed immediately in liquid nitrogen. The data described included exposure to commercial products (Actara and Nortox) and as well as individual high purity standards of TMX and IMD. The concentrations of neurotransmitters were determined using the straight equations (Table 23) obtained for the analytical curve of the three compounds. Figure 30 shows the concentrations of octopamine, dopamine and serotonin found in samples of the bee's heads.

Figure 30 – Average concentration of octopamine, dopamine and serotonin in extracts from the head of *M. scutellaris* for (a) IMD oral; (b) IMD topical; (c) Nortox oral; (d) Nortox topical; (e) TMX topical; (f) Actara topical; (g) Actara oral in the samples: control (Co); 1/10LD_{50} for topical and 1/10LC_{50} for oral (1); 1/5LD_{50} for topical and 1/5LC_{50} for oral (2); and 1/2 LD_{50} for topical and 1/2 LC_{50} for oral (3); live bees at 24h.

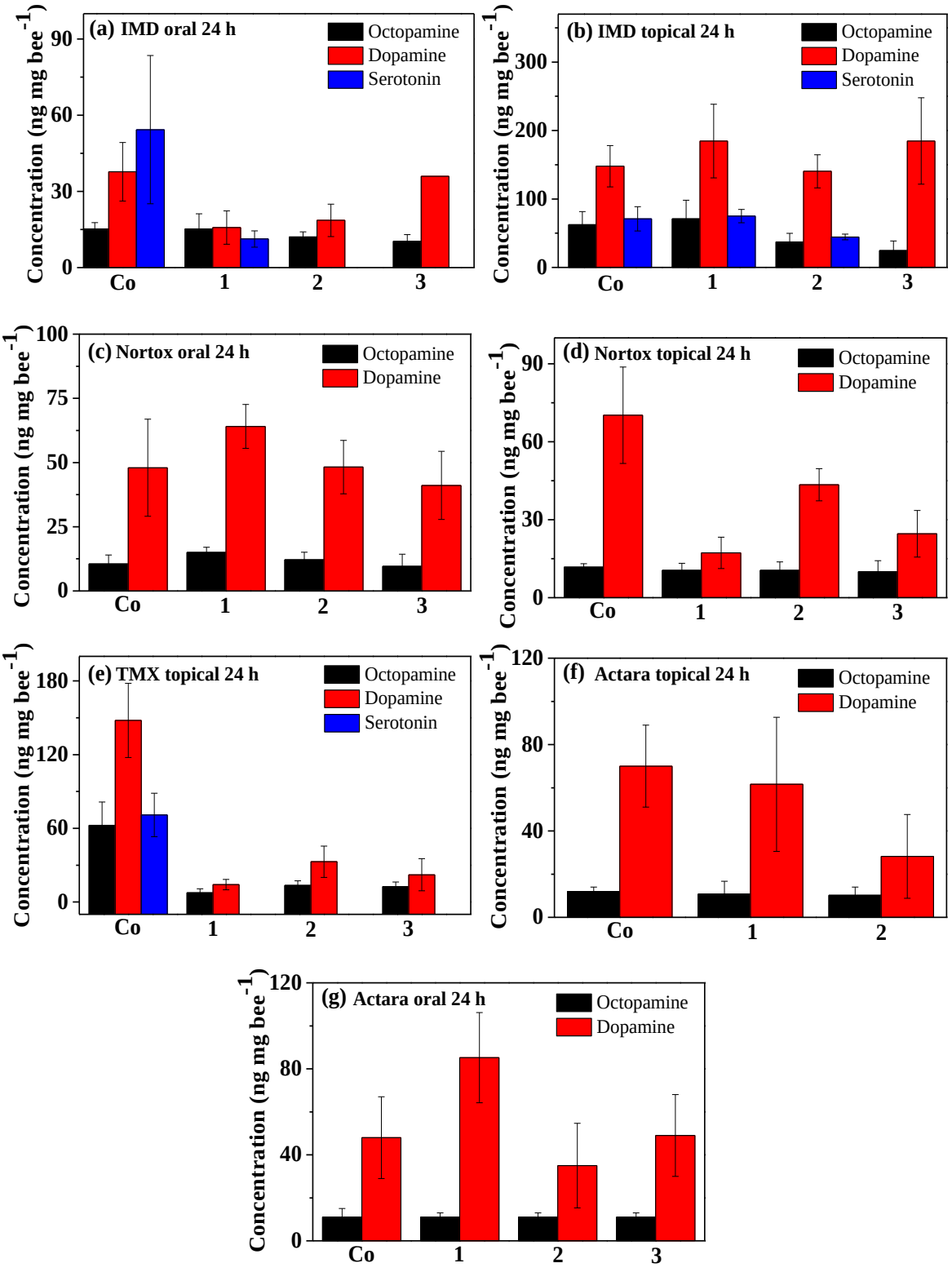


Figure 30 showed that dopamine and octopamine were detected in all the analyzed samples (Figure 30a-g). However, serotonin was detected only in topical and oral exposures of the active products IMD as shown in Figure 30a and 30b. Octopamine showed lower concentration in all the analyzed samples because the bees nervous system had 2 to 3 times higher concentration of dopamine [161]. Figure 30a showed the oral exposure of bees to IMD, where an increase in the concentration of IMD, serotonin concentration decreases (treatment 1) while dopamine concentration increases (treatment 3). Similarly, the same effect was observed when IMD was topically exposed to bees as can been by the results in Figure 30b (treatment 3). Figure 30e showed the topical exposure to the high purity standard of TMX, where lower concentration of dopamine and octopamine was observed (treatment 1, 2, 3) as compared to the control group, while serotonin was suppressed in all the analyzed samples (treatment 1, 2, 3).

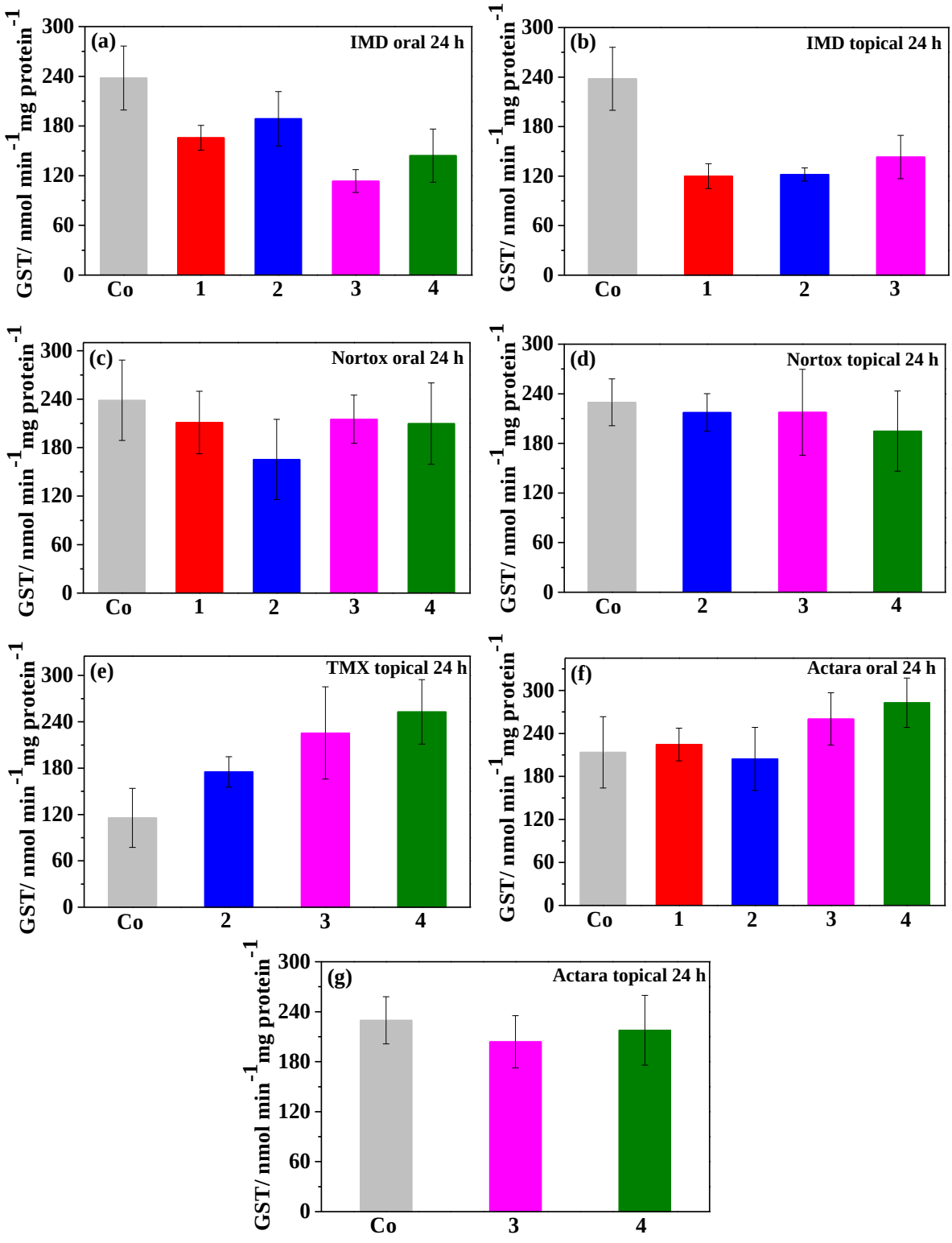
When the bees were exposed to the commercial products of IMD named as Nortox (Figure 30c and Figure 30d); topically, the dopamine concentration was lower than the control group (treatment 3) at higher concentration of Nortox, while at lower concentration of Nortox dopamine concentration ≥ control group (treatment 1 and 2). The same effect was observed for TMX commercial product named as Actara, when orally exposed to bees (Figure 30g). However, Figure 30f showed the periodic decrease in dopamine concentration while no effect of Actara was observed in octopamine concentration (treatment 1, 2, 3).

5.3.1 Determination of the enzymatic activity of GST

The results obtained for the measurement of the enzymatic activity of GST in tissues of *M. scutellaris* bees exposed orally and topically to commercial products, as well as the high purity standard of individual TMX and IMD are shown in Figure 31. Figure 31 shows the GST activity in bee tissue samples for oral IMD (Figure 31a); topical IMD (Figure 31b); Nortox oral (Figure 31c); Nortox topical (Figure 31d); topical TMX (Figure 31e); Actara oral (Figure 31f); Actara topical (Figure 31g); in the samples: Control (Co), 1/10 LD_{50} for topical and 1/10 LC_{50} for oral (1); 1/5 LD_{50} for topical and 1/5 LC_{50} for oral (2); 1/2 LD_{50} for topical and 1/2 LC_{50} for oral (3); and LD_{50} for topical and LC_{50} for oral (4); live bees at 24 h . For samples of oral exposure to the high purity standard of IMD (Figure 31a and Figure 31b), it was observed that the enzymatic activity of GST decreased to control for treatment 1 up to treatment 4 for live

bees exposed for 24 h. However, the GST activity is lower in topical exposure when compared oral exposure.

Figure 31 - GST activity in bee tissue samples for oral IMD (Figure 31a); topical IMD (Figure 31b); Nortox oral (Figure 31c); Nortox topical (Figure 31d); topical TMX (Figure 31e); Actara oral (Figure 31f); Actara topical (Figure 31g); in the samples: Control (Co), 1/10 LD_{50} for topical and 1/10 LC_{50} for oral (1); 1/5 LD_{50} for topical and 1/5 LC_{50} for oral (2); 1/2 LD_{50} for topical and 1/2 LC_{50} for oral (3); and LD_{50} for topical and LC_{50} for oral (4); live bees at 24 h.



When the bees were exposed orally to the commercial product of IMD (Nortox) (Figure 31c), a small difference in the GST activities was observed between the samples and the control group in the order Co>3>1>4>2. When the bees were topically exposed to Nortox (Figure 31d) there was no significant difference between the samples and the control group. When the bees were topically exposed to TMX, the GST activities were increased with the increase in TMX concentration compared to the control group in the order Co<2<3<4. For samples of oral exposure to the commercial product of TMX (Actara) (Figure 31f), the activity of the GST enzyme was increased for the samples of treatment 1, 3 and 4 while decreased for sample of treatment 2. Similarly, when the bees were topically exposed to Actara no significant difference between the samples and control group was observed.

6. CONCLUSIONS

The refined and optimized QuEChERS method enabled the evaluation of important parameters that affect the extraction efficiency of insecticides in bee's samples. The changes applied to the methodology resulted in increased detectability and satisfactory values of linearity, accuracy, and precision. Meanwhile, the developed method and the detection system have shown satisfactory LOD and LOQ values and good recoveries. The analysis proceeded in exposed bees has resulted in the quantification of TMX and IMD in oral and topical exposure in order to demonstrates the applicability of this method and the relevance of exposure studies. Besides this application, green metrics could rank the selected methods using an MCDA approach, even considering the difficulties of applying them to analytical chemistry. In this context, the need for incorporating environmental features to judge the performance of an analytical method became evident and the QuEChERS approach had demonstrated to be a feasible alternative for green sampling preparation.

Acute toxicity tests results showed that both TMX and IMD were highly toxic to stingless bees; moreover TMX was even more lethal to *M. scutellaris* when compared to IMD. The mixture as well as the commercial products of TMX and IMD such as Actara and Nortox has shown high oral and topical toxicity in stingless bee as compared to active ingredient. It was finally concluded that topically *M. scutellaris* is more sensitive towards these insecticides. To minimize the topical exposure of these insecticides, we can spray the insecticides at night time rather than day time due to the high foraging activities of bees. The use of bee species other than *A. mellifera* must be encouraged as a model in toxicological studies, as well as in programs to assess the effects of anthropogenic activities in the environment. This statement should be considered in view of the fact that single bee species are unable to represent the array of behavioral, morphological and physiological attributes of the natural community. Toxicological data reported that the development of new research on TMX and IMD cause sublethal effects, and contribute toward the policies with a goal of reducing the hazard of these insecticides to stingless bees. 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.

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