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Residue and Efficiacy Testing in Flash-Acting Amitraz Fumigation

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ABSTRACT

Due to global climate change, more attention needs to be paid to the control of some parasites. One of these, Varroa destructor, a honey bee parasite, is a pest that requires regular monitoring and effective treatment. At this point, healthy bees are vital for pollination and sustainable biodiversity. Honeybee colonies face threats like diseases and Varroa mite infestations. Beekeepers use acaricides such as amitraz for control. In flash-amitraz treatment trials on 14 mite- infected colonies, application resulted 95.1% antiparasitic effectiveness, with honey residues below MRL. Honey sampled from the colonies was tested for residues at the National Veterinary Reference Laboratory in the Etlik, Ankara. The reference laboratory conducted an analysis of amitraz residues utilizing the techniques of gas chromatography-mass spectrometry. Through this process, the laboratory was able to examine and identify these residues with the use of advanced GC-MS technology. Mite infestation level, antiparasitic efficiency and side effects of treatment were tested. The therapeutic efficacy of amitraz was evaluated using the Henderson-Tilton formula. This formula was employed as the method to effectively assess how well amitraz performs in treatment. The formulation and application of flash- amitraz are crucial for safety and effectiveness. Flash fumigation offers effective mite control while maintaining residue levels near the MRL, making it a reliable method for managing Varroa destructor in honey production. Amitraz-based acaricides are licensed veterinary preparations available in various commercial forms and remain widely preferred worldwide. Antiparasitic drug resistance poses a significant threat to bee health and colony productivity. Residue-free foods are crucial for global food safety and consumer health, particularly within the "One Health One Medicine" framework.

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INTRODUCTION

Honeybee breeding is an option against global hunger enabling rural people to sustain themselves participating in local production. Bees are also crucial pollinators and key driver of socioeconomic stability and environmentally sustainability. Recent research reveals alarming decline rates in honeybee colonies, raising serious concerns about the long-term implications for biodiversity conservation (Patel et al., 2021). The World Organisation for Animal Health (WOAH), formerly known as the OIE, has established control measures for the trade and movement of bees to prevent the introduction of new bee diseases into the territories of importing countries. These measures are detailed in the WOAH (OIE) Terrestrial Animal Health Code. Varroosis, a disease caused by the Varroa mite, is one of the six classified bee diseases included in this code. By adhering to the guidelines set forth by the WOAH, countries can effectively mitigate the risk of spreading bee diseases, such as Varroosis, through international trade and movement of bees, ultimately safeguarding the health of their bee populations and the sustainability of their apiculture industries (Fanelli & Tizzani, 2020). The epizootic ectoparasite mite Varroa destructor causes losses in Western honeybee (Apis mellifera) colonies. Mite-born viral infections reveal clinical symptoms called parasitic mite syndrome. Beekeepers must need an efficient Varroosis treatment against viral epidemics, colony collapses and irregular swarms (More et al., 2017).

Beekeepers need effective and residue-free miticides that should be non-toxic for bee colonies. If government authorities deem it necessary, they may impose additional restrictions or regulate the sale and use of registered pesticides within the country due to concerns regarding resistance, efficacy, toxicity and residue issues (Bubnic et al, 2021; EPA, 2020).

The amitraz, coumaphos, flumethrine, tau-fluvalinate, and other active substances have been registered as varroocides. Therapeutics including licenced chemical synthetics may result antiparasitic drug resistance risk, diminished efficacy and residue issues if not strictly applied as directed by the manufacturer's instructions (Almecija et al., 2022).

Amitraz, in the formamidine class, is the only acaricide chemical that blocks octopamine receptors in the mite CNS. Amitraz decomposes into volatiles during flash-fumigation. The three primary metabolites of amitraz in honey degrade within maximum ten days [N-(2,4-dimethyl phenyl) N'-methyl formamidine (DPMF), N-2,4-dimethyl phenyl formamide (DMF), and 2,4-dimethylaniline (2,4-DMA)] (Gupta, 2018).

Various fumigation approaches have own unique ways of exterminating ectoparasites whether ranging from gassy treatments to solid and liquid applications (Stejskal et al., 2021). The risk of residue accumulation in fumigation depends on the application process, chemical formulation, matrices and other conditions (Almecija et al., 2022). Unlike water-insoluble amitraz, the high solubility of many other synthetic varrocides in honey has resulted in cumulative residue problems.

As per EU regulations (EC) No 396/2005 and 2017/623, the accepted amitraz residue level in honey and bee products is 200 μg/kg (Efsa, 2016). At this point, the lower limit (the sum of all metabolites containing amitraz and 2,4-dimethylaniline group) as the Amitraz MRL standard by the Reference Laboratories of the Ministry of Agriculture and Food in Turkey has been accepted as 200 μgr/kg in honey (Http link: Official Gazzette Türkiye, 2017).

Certain treatments can also disrupt honeybees' physiological behavioural processes on colony level (O'Neal et al., 2017). Licenced Amitraz flash treatment (gas fumigation) which consists of seven smokes (Rulamit-VA), minimizes the risk of residue due to the active ingredient with negligible water solubility due to instant (only two minutes) fumigant exposure and has not been reported honeybee toxicity. So, we complemented test honey amitraz residue, acaricide efficiency and check the flash-fumigation if the bee life-threatening, to aiding beekeepers in choosing effective veterinary medicines.

MATERIALS and METHODS

The research was performed in a stationary apiary located in Sarkoy, Tekirdag, Türkiye after honey harvest where on 40° 47′ 50″ N 27° 21′ 56″ E, in August 2018. The amitraz flash fumigation trials were conducted in four-teen naturally mite-infested colonies (four colonies as control trials and ten for served as treatment).

Detection of Infestation Level

Colony infestation levels were evaluated before and after treatment using the powdered sugar test to count mites. The test was performed one hour before and after four-day flash-fumigation treatment process. First, the number of bees in each sample was accurately counted. Then, the infestation level (IL) was calculated using the formula: $IL = (VN / BN) \times 100$, where VN represents the number of Varroa destructor mites and BN represents the total number of bees in the sample. This standardized procedure ensures consistent and accurate assessment of infestation levels across all samples. The acaricide's effectiveness was also evaluated using this method. This standardized protocol guarantees a consistent and accurate evaluation of infestation levels across all specimens, allowing for the assessment of the acaricide's efficacy (Bava et al., 2022, Pietropaoli et al. 2021).

Flash-fumigation Treatment

The amitraz-based flash fumigation treatment uses impregnated cardboard plates measuring 20 x 10 cm, each containing 265 mg of the active ingredient, to treat up to 10 bee colonies. The treatment is highly effective, as the cardboard burns without a flame upon ignition, rapidly releasing amitraz.

Colonies were fumigation treated four times with an interval of three days to test antiparasitic effectiveness. The "Varroa destructor" samples fall-off to the bottom board were counted after an hour of each amitraz fumigation treatment. Since there was no ant issue in the apiary the sticky paper was not needed.

The application of Rulamit-VA is comparable to the use of a traditional beekeeper's smoker. Notably, the treatment only targets phoretic mites on adult bees, as the fumes cannot penetrate wax capping. When used in a well-ventilated colony, as directed in the product manual, the flash fumigation process utilizing amitraz-impregnated cartons leaves no residual odours, residues, or hazardous substances.

Flash-fumigation was performed using a bee smoker with a "fume blow tip." (Figure). The hive's flight hole was fully open during the amitraz fumigation. Colonies began wing ventilating inside the hive after the first fumigation. Seven flash fumes were administered consecutively through the flight hole of 12 hives, completing the each of process only in a minute. The bees were not negatively affected by the seven flash fumes. The treatment was repeated four times, with three-day intervals. After fumigation, Varroa mites that fell off were collected from the sticky bottom board after an hour. Treatment efficacy is determined by a predefined formula. Four colonies are used as the control group (Bava et al., 2022).

Honey Sampling and Residue Analysis

Sealed and unsealed honeycombs were sampled before and after the end treatment for residue analysis. Each flash fumigation was trialled in four tours, with an interval of three days (seven fanning of the application smoke). All honey samples were analysed for amitraz and its metabolites [N-(2,4-dimethyl phenyl) N'-methyl formamidine (DPMF), N-2,4-dimethyl phenyl formamide (DMF), and 2,4-dimethylaniline (2,4-DMA)] for the MRL. Honey samples were analyzed using GC-MS at the TÜRKAK - accredited ‘’National Reference Laboratory’’ The Veterinary Control Center Research Institue, Etlik, Ankara.

Figure. Bee hive smoker

RESULTS

This study investigated the metabolite markers of amitraz residues in honey when honeybee colonies were treated with Rulamit-VA, examining within antiparasitic efficacy. During treatment, no colony-level issues or symptomatic observations regarding honeybee health were reported. The Henderson-Tilton (Henderson and Tilton, 1955) formula calculated the miticide efficacy of the GMP-certified licensed amitraz-containing fumigant Rulamit-VA to be 95.1%.

Due to amitraz's well-documented instability in honey, specialized analytical methods were employed to determine total residues of amitraz, including its metabolites. The residue levels of amitraz and its metabolites in sealed and unsealed comb honey samples were found to be well below the reference Maximum Residue Limit (MRL). Amitraz marker residues did not exceed one-tenth of the MRL level in honey samples.

DISCUSSION

While the latest research on amitraz in the current world literature reveals the reliability of amitraz, it has been determined that there is a gap in this regard in Turkey. The commercial product named Rulamit-VA (265 mg amitraz included) which is the subject of this research, is a locally produced veterinary medical preparation subject to prescription, licensed by the Ministry of Agriculture and Food of the Republic of Turkey. Contrary to Aydin and Girisgin's previous publications (Aydin and Girisgin, 2010) in which they preferred imported Amitraz products with foreign commercial formulations now it is preferred to investigate a completely domestic amitraz product.

Global honey production reached 1,830,768 tonnes in 2022, with Türkiye contributing 118,292 tons. The honey is deemed unfit for human consumption and commerce if residues exceed the Maximum

Residue Limit (MRL). Most synthetic acaricides exhibit lipophilic properties, leading them to accumulate predominantly in beeswax. When it comes to beeswax, amitraz has a unique characteristic - it breaks down completely within 24 hours, making it the "only licensed" unstable acaricide in this matrix. On the other hand, all synthetic lipophilic acaricides are relatively stable in beeswax and their concentration increases with each additional treatment and wax recycling (Kast et al., 2021, Medici et al., 2015).

Unfortunately, the persistent presence of acaricides in the wax contributes to developing acaricide-resistant Varroa mites. On the contrary of wax, acaricides to leave residues in honey, they must possess water-soluble characteristics. Consequently, the water-insoluble acaricide origin residue risk is comparatively very low, often falling too below the established MRLs in honey (Gupta, 2018).

The parasitic mite known as Varroa destructor poses the gravest threat to honeybee populations among all the factors. A severe infestation by Varroa mites can lead to significant colony mortality within a few weeks in honeybee colonies that receive insufficient treatment. Amitraz employs a mite-specific mode of action, avoiding the induction of detoxification gene expression in honeybees (Boncristiani et al., 2012). Amitraz stands out as the only acaricide sensitive to acidic environments. Since honey is acidic, amitraz breaks down when mixed with honey. Moreover, if used in accordance with the package insert, it decomposes entirely within ten days, posing no risk threats to the brood or the end consumers (Korta et al., 2001).

A US-based study published in 2022 examined the residual levels of amitraz and its metabolites in honey and beeswax after administering a dose of Apivar (amitraz strip) five times higher than normal. Amitraz itself was not detected in any bee products after 42 days. However, trace amounts of its metabolites DMPF and DMA were found in samples taken 28 days after treatment. These findings show that even at high doses, amitraz metabolite residues in bee products remain within acceptable limits (Chaimanee et al., 2021).

Beekeepers often purchase amitraz-containing strips in bulk at a low cost, leaving them in the hive for extended periods, hoping for improved results. However, these strips have a shelf life of 24 months from the manufacturing date, and once opened, their potency decreases significantly within 1-2 weeks due to amitraz oxidation, leading to diminished effectiveness. When metabolized, amitraz binds to neuron receptors, modifying the behaviours of mites. This alteration prevents mites from interacting with bees, consequently halting the reproduction of Varroa destructor. Notably, amitraz is metabolized at a rate 7,000 times higher in V. destructor than in honeybees. Multiple amitraz-based treatments are commercially available, they vary in their galenic formulations and efficacy (Almecija et al., 2024) . Amitraz has been found to have low acute toxicity to honeybee larvae, with an LC50 of 461.4 mg/L in a study (Dai et al., 2017). Another study showed that even with a four-day exposure, Amitraz had low toxicity and did not impact their survival rate. Products approved for honeybee use are safe if the packaging instructions are followed (Dai et al., 2018).

Unlike long-acting registered pyrethroids, flash fume of amitraz fumigation can't linger as long in the hive environment, resulting in lower residue levels and less bee exposure. As exposure diminishes, the development of resistance progresses at a more gradual pace. Water-insoluble amitraz has distinct metabolomics from pyrethroids, and commercial formulations like fast-acting flash fumes cause tardy resistance among Varroa populations. These characteristics minimize the risk of toxic effects on bees and reduce the potential risk to human consumers of hive products. Three registered active ingredients, (Coumaphos, flumethrin and tau-fluvalinate) have been found to remain stable in honey for approximately nine months, while the risk is considerably lower for flash-acting amitraz (Bischoff and

Moiseff, 2023; Bubnič et al. 2021).

Amitraz, a varroa mite pesticide used in Spain since 1999, has maintained almost 100% effectiveness despite long-term use and has not shown any significant resistance development. In this context, updated new regulations in the USA allow Amitraz at a concentration of 3.33% to alleviate varroa mite infestations, minimize hive product residues and prioritize consumer welfare.

In a study conducted with Apivar strip formulation in Spain in 2015, the effectiveness of long acting amitraz strip was found to be 64.3% (Leza et al. 2015).

Researchers tried the same strips in Türkiye, declared that they found its effectiveness to be 99.43% (Aydin and Girişgin, 2010). Similarly, Adjlane and Haddad, who tried the same strips in 2017, found the effectiveness to be 39.23% (Adjlane and Haddad, 2017).

It is not known exactly how much the resistance genes expressed against amitraz in Varroa destructor are affected by commercial formulations of amitraz and similar reasons such as application errors, seasonal and management differences (Hernández-Rodríguez et al., 2022; Marsky et al., 2024).

The use of smouldering Amitraz tablets like Apiwarol in Poland (for brief durations (20-30 minutes) carries a minimal risk of residual traces. However, an analysis of honey samples collected from areas exposed to the smoke directly, including the brood chambers and supers, revealed only negligible residue accumulation, indicating a low potential for contamination (Pohorecka et al., 2018). A study conducted in Türkiye reported the effectiveness of amitraz fumigation as 30% (Girisgin et al, 2019).

Fumigation is a technique that involves using a range of different chemicals to eradicate and effectively rid an environment of pests and insects. The long-acting/slow-released amitraz strips (42-56 days) are acts in different. Solid fumigation of licenced Apiwarol tablet acts during short-term smouldering (20 minutes) treatment (Pohorecka et al., 2018; Semkiw et al., 2013) however the non-flammable carton-impregnated amitraz flash-fumigation acts via snap gaseous matrix (only a minute).

CONCLUSION

In conclusion, Ensuring the health of honeybees is crucial for combating global hunger, enhancing agricultural pollination, and promoting rural development. Only products that prioritize food safety should be employed to support bee populations.

Ethical Approval

The authors state that; Insects, being invertebrate animals, are not subject to the ethical considerations that typically require approval from ethics committees for research involving vertebrates.

Author Contributions

Research Design (CRediT 1) Author 1 (%50) – Author 2 (%50)
Data Collection (CRediT 2) Author 1 (%50) – Author 2 (%50)
Research - Data analysis - Validation (CRediT 3-4-6-11) Author 1 (%50) – Author 2 (%50)
Writing the Article (CRediT 12-13) Author 1 (%90) – Author 2 (%10)
Revision and Improvement of the Text (CRediT 14) Author 1 (%90) – Author 2 (%10)

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Conflict of Interest

The authors state that there is no conflict of interest between the authors.

Sustainable Development Goals (SDG)

3 Good Health and Well-Being

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