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Chromatographic determination of monoterpenes and other acaricides in honeybees: Prevalence and possible synergies

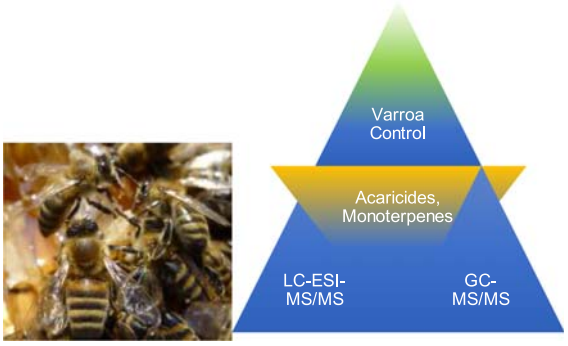
Konstantinos M. Kasiotis *, Zampia D. Tzouganaki, Kyriaki Machera *

Benaki Phytopathological Institute, Department of Pesticides Control and Phytopharmacy, Laboratory of Pesticides' Toxicology, 8 St. Delta Street, Kifissia, 14561, Athens, Greece

HIGHLIGHTS

- Novel GC-MS/MS method for thymol and carvacrol quantitation in honeybees
- Expanded LC-ESI-MS/MS method for acaricides and breakdown products quantitation
- Acaricides and breakdown products in honeybees after death incidents in Greece
- Preliminary risk assessment and possible synergistic effects discussion
- Acaricides residues were far below toxicity endpoint values.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the first targeted GC–MS/MS method for the detection and quantification of monoterpenic phenols, thymol and carvacrol in honeybees, employing a simplified sample preparation protocol, using ethyl acetate as the extraction solvent, is reported. The method was then applied to honeybees' samples after reported death incidents to evaluate the levels of the afore mentioned compounds in the course of 2015 early 2017. In parallel, other regularly used acaricides, namely amitaz, tau-fluvalinate, and coumaphos were also monitored using an LC-ESI-MS/MS multiresidue method based on modified QuEChERS technique. Breakdown products of amitraz; DMF and DMPF and coumaphos oxon were also investigated. The predominant acaricides detected were coumaphos, thymol, metabolites DMF and DMPF, and in less extent tau-fluvalinate, with concentrations for compounds varying from the low ng/g scale up to approximately 60,000 ng/g $_{\text{bee body weight}}$. The highest concentrations were observed for coumaphos and thymol. Preliminary risk assessment using hazard quotient (HQ) as the criterion, showed negligible risk from acaricides as individual components of bees. However, potential synergistic effects between acaricides or acaricides and other pollutants should not be disregarded.

1. Introduction

Honeybee (*Apis mellifera L.*) health issues and subsequent colony losses have multifactorial etiology, such as the ectoparasitic mite of *Varroa destructor*, the microsporidian (fungus) of *Nosema apis* and *Nosema ceranae*, viruses [such as the Dicistroviridae (acute bee paralysis virus (*ABPV*)], insecticides (such as the neonicotinoids) and habitat loss. However, the presence of *Varroa* has proved to be the dominating reason for a colony loss (Martin et al., 2012; Thrasyvoulou, 2005; Villalobos, 2016). *Varroa* impacts colonies through the consumption of hemolymph, the vectoring of honeybee viruses and its immunosuppression activity. In this direction, the mite is mostly controlled using acaricides of synthetic origin such as coumaphos (an organophosphate

* Corresponding authors.
E-mail addresses: K.Kasiotis@bpi.gr (K.M. Kasiotis), K.Machera@bpi.gr (K. Machera).

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compound), tau-fluvalinate (synthetic pyrethroid) and amitraz (a formamidine compound) accompanied by proper beekeeping practices. Although acaricides do not constitute the major culprit behind honeybee acute poisoning and death incidents, there are reports on serious effects on honeybees' fitness (Palmer et al., 2013), and impact on physiological functions and immune system, that render them more vulnerable to pathogens (Boncristiani et al., 2012). In this context, it is essential to enhance acaricides' efficiency to manage infestation at levels lower than those that may impact colony survival, and in parallel incorporate them in monitoring schemes to continuously monitor their concentration in bee bodies and inside the beehive. Honeybees' susceptibility to pesticides is attributed to their deficit in genes that encode detoxification enzymes. More specifically, it is reported that the honeybee genome has much fewer protein coding genes than other organisms such as *Drosophilla melanogaster* (Claudianos et al., 2006). In this context, the use of alternative chemicals which bees will be able to detoxify but would exhibit the desired activity was and is the challenge of researchers involved in apiculture science. Nonetheless, organic acids (e.g. oxalic acid) are also used in the same context (Milani, 2001; Rademacher and Imdorf, 2004), representing a more "subtle" chemical treatment compared to the use of traditional synthetic acaricides.

While, synthetic organic chemicals represent the forefront in apiculture applications, insecticidal and antifungal components that are produced by plants are a promising alternative as "natural treatments" (Boulogne et al., 2012). Essential oils of several plants and their components have been used as adequate alternatives to synthetic acaricides and are used by beekeepers to control *Varroa* mites in honeybee colonies (Damiani et al., 2009; Imdorf et al., 1999). Amongst them, thyme ((*Thymus vulgaris L.* (Labiatae)) and origanum essential oils are frequently used in this domain (Sammataro et al., 2009), with components such as thymol (Fig. 1) and carvacrol (Fig. 1) being prevalent. Thymol a phenol, originated from *Thymus vulgaris* or *Thymus zygis*, has been used as a botanical pesticide that tends to exhibit lower toxicity compared to conventional chemical pesticides. The significance and growing interest for these substances, also augmented the interest on their proper delivery to the bee colony. The latter has been a challenge due to their low water solubility and widespread diffusion, hence carriers were tested for the efficient delivery to bee hemolymph [indicatively see (LeBlanc et al., 2008)].

A number of studies showed accumulation of thymol in pollen and honey (Adamczyk et al., 2005), whereas other studies demonstrated, apart from its efficacy against *Varroa* mite, its diffusion to honey (Bogdanov et al., 1998; Tananaki et al., 2014). Although thymol is used for years, as an acaricide, its environmental as well as veterinary effects are not well established (Liu et al., 2017). Notwithstanding, thymol is absorbed by bees and bee products and is suspected of intoxication on colonies and especially on larvae depicting an 48 h–LD_{50} of 440,000 ng/g $_{\text{larvae body weight (bw)}}$, (Charpentier et al., 2014) or in other studies an LC_{50} 2,103,000 ng/g $_{\text{bee bw}}$, and 1,507,000 ng/g $_{\text{larvae bw}}$. Though not well established, it seems that thymol absorption in bees have adverse effects such as phototactic behavior e.t.c. (Gashout and Guzman-Novoa, 2009). Other organic compounds, such as antibiotics, have also attracted much attention since they have certain applications in apiculture. However, even for this promising class of compounds, it was found that they disturb the gut microbiota and increase mortality in honeybees (Raymann et al., 2017).

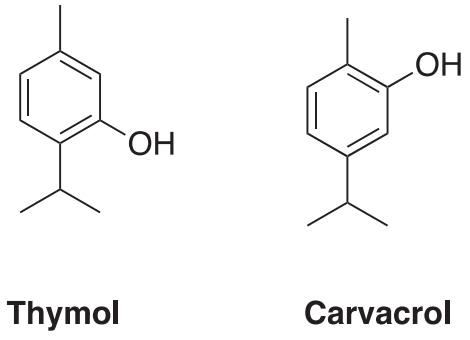

Fig. 1. Chemical structures of thymol and carvacrol.

Thymol and carvacrol are both achiral monoterpenes due to the aromatic ring that they possess. Their biosynthesis commences from geranyl pyrophospahate or neryl pyrophosphate that are transformed to γ–terpinene via the action of soluble enzyme γ-terpinene cyclase. Then, a subsequent aromatization to *p*-cymene and final hydroxylation produces the two compounds (Yamazaki and Usui, 1962). Chemical analysis of thymol and carvacrol is typically performed using gas chromatography tandem mass spectrometry (GC-MS/MS) (Armorini et al., 2016; Boubaker et al., 2016; Fiori et al., 2013), but also HPLC-UV, FD is used (Angelo et al., 2016; Vinas et al., 2006) regularly with other analytes as well.

In this study, thymol concentration levels in bees after reported death incidents were examined in order to contribute to the domain of thymol's toxicity to honeybees after its application as a *Varroa* mite control agent. The latter was partly driven by limited reports of beekeepers who complained of suspected mortality due to use of thymol based formulations. In parallel, carvacrol was monitored since it is an analogous compound to thymol that is used to control *Varroa*, mainly as a constituent of essential oils. Thus, a straightforward method for the determination of thymol and its related counterpart, carvacrol, with GC–MS/MS in/on bee samples was developed, validated, and applied to honeybee samples. Concomitantly, same samples were analyzed for coumaphos and its oxon metabolite, amitraz (including three of its metabolites), and *tau*-fluvalinate using an expanded LC-ESI-MS/MS multiresidue method built upon previous work of our group (Kasiotis et al., 2014). Although the sample preparation steps for the extraction of several contaminants from bees are reported (by our group as well), an experimental design (using central composite design, CCD) was used to identify the most important factors affecting the LC-ESI-MS/MS sample preparation (Li et al., 2017). CCD is a valuable tool that provides statistical models which help interpret the interactions between the parameters that were optimized within a certain process. The latter is used by a plethora of scientists to enhance their experimental approaches by improving certain parameters that affect performance [indicatively see (Nasirizadeh et al., 2012; Rizzetti et al., 2016)]. Results of rest of monitoring (for the complete list of pesticides) was not the objective of this work, however analytical figures of merit for *tau*-fluvalinate, coumaphos, its metabolite, coumaphos oxon, and amitraz (and its breakdown products) are presented. In the presented work, for risk assessment purposes, apart from the straightforward comparison of concentrations found on/in bees, with the acute oral and contact median lethal dose (LD_{50}) of each active substance, the HQ was also exploited. HQ is designated as the ratio between the environmental exposure with toxicity (Johnson and Gnanadhas, 2016). The latter has been described in the EFSA's Guidance Document on the risk assessment of plant protection products on bees (EFSA-Guidance, 2013). HQ is also reported in several works as a tool to estimate risk and evaluate pesticide residues detected in apiculture commodities (such as pollen) collected by honeybees (Stoner and Eitzer, 2013; Villa et al., 2000). Recently, HQ was used in preliminary exposure assessment as a consequence of detected pesticide residues in live and poisoned honeybees (Kiljanek et al., 2017).

Thus, to the best of our knowledge, we present the first targeted GC-MS/MS method for the detection and quantification of thymol and carvacrol in bees, employing a simplified extraction protocol. Concomitantly, other acaricides' concentrations are determined and reported in honeybee samples from Greece (2015 early 2017), utilizing an optimized and expanded LC-ESI-MS/MS method of our group. Overall, multiple acaricides prevalence is reported, corroborating their accumulation in bee tissues and in parallel highlighting possible synergistic effects. Such effects, as an outcome of exposure to multiple