Iranian Journal of Chemistry and Chemical Engineering (IJCCE)

Determination of bromopropylate (Folbex-VA) residue in honey produced in Iran (Damavand region)

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ABSTRACT

Varroa mite (Varroa destructor Anderson & Trueman) is considered one of the most important pests of honey bee colonies throughout the world. This mite feeds on honey bee hemolymph, transmits some viruses, and inflicts heavy economic losses on the beekeeping industry worldwide. Beekeepers use various miticides, including Folbex-VA fumigant strips, in bee colonies to control Varroa mite populations. This lipophilic compound dissolves in wax first. It is then released into honey. Pesticides used to control pests and diseases may remain in raw and processed products that are used by humans and livestock. These pesticide residues can cause health hazards because of their chronic or acute toxicity in humans. Consequently, this research measured Folbex-VA residues in six honey production regions in Damavand County (Ayeneh Varzan, Aru, Havir, Mosha, Tar Lake, and Damavand) in two months (the final spring and summer months). The GC-MS was used in the isolation and identification processes of Folbex-VA. A factorial experiment using a completely randomized design was performed to analyze the data, and a comparison of the means was carried out using Duncan's multiple range tests at the significance level of 1%. The results indicated that in the last month of spring, pesticide residues were higher than the maximum residue level (MRL) set by the European Union (EU) in the samples taken from Ayeneh Varzan, Havir and Masha but at the MRL in the samples taken from Aru, Tar Lake and Damavand. However, in the last month of summer, pesticide residues in the samples taken from all six regions were below the MRL adopted by the EU.

KEYWORDS: *Varroa destructor, honey, Folbex-VA, residue, Damavand.*

INTRODUCTION

Honey is one of the most important natural products that has been used for a long time due to its nutritional and therapeutic value. Carbohydrates, enzymes, proteins, amino acids, vitamins, lipids, and minerals constitute important compounds in various types of honey [1]. Honey has substantial antimicrobial properties and plant sources, environmental conditions, processing and storage conditions, and also its freshness, play an influential role in these properties. The hydrogen peroxide produced by glucose oxidase in honey is the most important factor giving it its antimicrobial properties. In addition, its pH and contents of methylglyoxal, proteins, peptides, flavonoids and other polyphenolic compounds play a part in these properties. Enzymes are the most important proteins found in honey the most well-known of which include glucose oxidase, amylase, invertase and catalase. Glucose oxidase catalyzes the conversion of glucose to gluconic acid (involved in the stability of honey) and hydrogen peroxide (responsible for the antimicrobial properties of honey). Very unstable hydrogen peroxide reacts with metal ions. The free radicals produced in this reaction destroy microorganisms. Diastase or amylase and invertase from the thoracic salivary glands of worker honey bees enter into nectar and then into honey in the process of nectar conversion into honey [2, 3]. Iran ranks first in honey consumption but seventh in its average yearly production in the world. The average annual honey consumption per capita is 235 g in the world whereas in Iran it is about 600 g. The largest annual amount of honey exported from Iran is 2000 tons, but in some years its export reaches 5000 tons. Quality, marketing and packaging are the main problems and barriers to honey export in this country. At present, Iran has five million and 100 bee colonies, 5.35% of the total in the world, that produce 45000 tons of honey per year (3.2% of the world's production). The mean annual honey production per colony is 10 kg in Iran, but the global average is 20-60 kg [4]. One of the biggest challenges in recent decades has been to ensure food security and safety for the increasing world population. Crises such as the appearance of new pests and diseases, resistance of pests and weeds to chemical pesticides, serious environmental harm and soil degradation and erosion, and threats to human health through the prevalence of skin diseases and various types of cancer and chronic diseases have happened due to excessive use of chemicals in the production of agricultural products. The use of pesticides causes one of the crises associated with the safety of agricultural products. Pesticides used to control pests and diseases of fruits and vegetables before and following crop harvest may remain in raw and processed products used by humans and livestock. Pesticide residues can contaminate agricultural products such as fruits, vegetables, cereals and aquatic life thereby creating potential health hazards via chronic or acute toxicity [5]. Insects are the most important pollinators in nature and pollinate about 70% of the angiosperms. Bees are the major pollinators among insects. They are classified into general and specific categories based on their food sources, foraging behavior and range of foods. In addition, some bee species differ from others in that their morphology has changed and they have adapted to living in colonies and to being managed and transported to different locations. These characteristics indicate the economic and ecological importance of bees. These valuable animal pollinators play an undeniable role in pollination. Consequently, the economic importance of honey bees in producing honey is negligible compared to their economic importance in pollinating and increasing the yield of crops. For example, the estimated value of honey bees in increasing the yield of crops in Iran is 90 times higher compared to the value of the honey they produce accounting for about 4% of the GDP in this country in 2001. Given the income that honey bees generate in agricultural production, they can be economically considered the fourth most profitable and valuable animal in several European countries. In addition, honey bees are profitable as they have a direct role in the production of products including honey, pollens, propolis, and royal jelly [6]. *Varroa destructor* Anderson & Trueman is the most dangerous pest of honey bees (*Apis mellifera* L.) and the major factor threatening the beekeeping industry worldwide. The reproductive stages of *V. destructor* take place in the pupal cells of honey bees inflicting irreparable damage to honey bees. *Varroa* mites feed on the hemolymph of honey bee pupae and adults and thus reduce their body water and protein and carbohydrate contents, which leads to their death. In addition to its direct damage, this ectoparasite causes deformation of the wings of drones and female worker bees and reduces their life span in the hive. Chemicals have been used in recent years to control *Varroa* mites, but their uncontrolled and incorrect use has caused problems including pesticide residues in honey and other honey bee products, environmental pollution and, most importantly, the appearance of resistant mites in apiaries [7]. Folbex-VA, a selective miticide of the benzilate group, is used as fumigant strips (2-3 strips/colony). Each strip contains 370 mg bromopropylate (w/w). The strip is lit and the resulting smoke contains bromopropylate that controls the mites causing acarine disease (*Acarapis woodi*) and *Varroa* mites (*Varroa jacobsoni*), but honey bee colonies tolerate this treatment well. It is recommended to use 2-3 fumigant strips three times at 7-10 intervals. However, the strips are used four times at 2-3-day intervals in spring. Each time, half a strip, one strip or three strips are used for weak hives, hives with 7-9 frames, and stronger hives, respectively. The fumigant strip must be attached securely to an empty frame with wire and lit using an alcohol lamp [8]. It is noteworthy that Folbex-VA was the first product registered in Switzerland for controlling *Varroa* mites in apiaries, but it has not been used in that country since 1992. Consequently, bromopropylate residues have been decreasing steadily since 1992 in Switzerland. Bogdanov et al. (2008) predicted that it would take almost 20 years for this miticide to disappear from honey bee wax [1]. Thirteen years later (in 2019) bromopropylate residue levels decreased to <0.01 mg/kg and could still be detected in honey bee wax [9]. Bogdanov et al. (1998) studied Folbex-VA (bromopropylate), Perizin (coumaphos), Apistan (fluvalinate) and Bayvarol (flumethrin) residues in honey and other bee products. The chromatography results revealed that the honeycombs containing larvae had miticide residues (1.8-48 mg/kg). In addition, the miticide residue levels in honey bee wax were 5-10 times less compared to the honeycombs on average [10]. Few studies have been conducted in Iran on pesticide residues in honey and other bee products. Talebi Jahromi et al. (2000) measured coumaphos residues in apiaries in Tehran Province and reported that coumaphos residues could be detected in all honey samples [11]. Akhlaghi and Nehzati Paghaleh (2016) monitored Apistan, Fabcosin, Bayvarol and also thyme essential oil residues in honey, honey bee wax, and propolis. They stated that the miticide residues in honey, honey bee and propolis were not statistically significant. They also found that, numerically, the highest miticide residue level was detected in propolis and the lowest in honey bee wax. Apistan residue levels in all products were significantly higher than the other miticides followed by those of Bayvarol and Amitraz [12]. It is worth mentioning that thyme essential oil had the lowest residue level. Hasheminia et al. (2018) measured Diazinon residues in honey produced in Damavand. The samples were taken from six locations (Ayeneh Varzan, Aru, Havir, Mosha, Tar Lake, and Damavand) in the final spring and summer months. The results demonstrated that Diazinon residues could be detected in all six locations. In some locations, the residues exceeded the MRL for this pesticide. In addition, pesticide residue levels were higher in the final month of spring than in the final month of summer in all the samples. The highest pesticide residue level was detected in Ayeneh Varzan in the last month of spring (0.03919 mg/kg) and the lowest (0.01714 mg/kg) in Havir in the final month of summer [13]. Fluvalinate residues were measured in honey produced in Damavand in another study. The highest residue level was detected in Ayeneh Varzan in the final month of spring (0.077 mg/kg) and the lowest (0.021 mg/kg) in Mosha in the last month of summer [14]. The analysis conducted by Brugnerotto et al. (2023) employed gas chromatography-mass spectrometry (GC-MS) to determine the occurrence and levels of seven pesticide residues within *Mimosa scabrella* honeydew honey (MHH) originating from the southern region of Brazil. Honey samples collected from the Paraná state exhibited no detectable pesticide residues. Conversely, among the fifteen samples analyzed from the Santa Catarina state, atrazine was identified in six samples at concentrations below 9 µg/kg. The measured values were lower than the currently established maximum residue limits (MRLs) for honey, set at 0.05 mg /kg. Moreover, τ-fluvalinate was detectable in just one sample; however, its content remained below the limit of quantification. Hence, the *Mimosa scabrella* honeydew honey (MHH) samples analyzed in this study exhibited either no presence or minimal levels of the seven pesticides investigated, and in all samples, the concentrations were below the prescribed MRLs [15]. In a study by Marti et al. (2022), the residue levels of pesticides in Swiss commercial beeswax were determined. The examination of individual samples exhibited considerable variability, with a wide range of residue concentrations. On the other hand, pooled samples allowed for calculating average annual residue values representing Swiss production. In the investigation, 13 pesticides were accurately quantified out of a total of 17 pesticides that were found. These included 13 acaricides and/or insecticides, two fungicides, a synergist, and a repellent. The mean concentrations for the beekeeping-related contaminants were determined as follows: coumaphos at 401 µg/kg, tau-fluvalinate at 236 µg/kg, bromopropylate at 106 µg/kg, and N-(2,4-Dimethylphenyl)-formamide (DMF), a breakdown product of amitraz, at 3 µg/kg [16]. Fuente-Ballesteros et al. (2023) have introduced and validated an analytical approach to quantify seven acaricides (namely atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate) in kinds of honey originating from diverse botanical sources (multifloral, heather, and rosemary) using gas chromatography-mass spectrometry. This study proposed an efficient and straightforward sample treatment methodology involving a solvent extraction process utilizing a 50:50 (v/v) mixture of ethyl acetate and cyclohexane. Ultimately, after an extensive examination of several honey samples for seven acaricides, only residues of τ-fluvalinate were found, with concentrations ranging from below the limit of quantification to 23 µg /kg [17].

As mentioned above, pesticide residues in food products are a serious challenge to human health and a threat to their export. Consequently, measures must be taken to reduce pesticide residues by correctly applying pesticides. In this regard, this research studied Folbex-VA residues in honey produced in Damavand in the last months of spring and summer.

EXPERIMENTAL SECTION

Damavand altitudes are located in Tehran Province, and honey is among its most important products. Many native people in this region are professional beekeepers. According to the statistics published by Damavand Jihad Agriculture Management, there are about 45,000 honey bee colonies in this county that produce about 300 tons of honey annually. The rangelands around Tar Lake, Eastern and Western Varin, Havir Lake, and the rangelands in Javard, Kahnak and Aru villages are the locations where bee hives are transported for the first six months of the year.

Sampling

Sixty honey samples were purchased, 10 from the main stores in each of the six regions (Ayeneh Varzan, Aru, Havir, Mosha, Tar Lake and Damavand) that sold the honey produced there. The samples in each region were bought once in the last month of spring and again in the last month of summer.

Sample preparation

Ten grams of each sample was weighed using a digital balance (Perten Co, Sweden, ±0.01 g), and poured in an Erlenmeyer flask with a screw cap to which 10 mL distilled water was added. The flasks were placed on a shaker for 30 min so that the honey dissolved completely in water to prepare uniform and homogeneous solutions. The solutions were then filtered using Whatman No.1 filter paper. Dichloromethane (10 ml) was poured into each solution which was put on the shaker for 15 min to be thoroughly mixed. The solution in each flask was poured into a test tube. Two layers were formed in each test tube with time with a yellow liquid on top (the mixture of water and honey) and dichloromethane on the bottom. The yellow liquid in each test tube was removed using an electric pipette and poured into another container. To each separated sample was added n-Hexane (Merck, Germany) (10 ml) and the solution was put on the shaker for 10 min. In each container, the n-Hexane formed a layer on top and the yellow liquid a layer on the bottom. The n-Hexane was removed and poured into the container which included dichloromethane from the previous stage. Finally, the prepared solution was placed in a rotary evaporator, the obtained solution was dried in an Erlenmeyer flask and 5 ml ethyl acetate (Merck, Germany) was added to the dried sample [18].

GC-MS instrument

The GC-MS instrument was Agilent 7890A/5975C GC-MS with an HP-5MS column 30 m long, film thickness 250 mµ and inner diameter 25 mm. The temperature program varied from 100 to 280 ℃ at the rate of 5 ℃ /min. The temperature program started at 100 ℃ and was raised to 280 ℃ at the rate of 5 ℃/min, and 99.99% purity helium gas was used as the carrier gas. The temperature program for the column set lasted a total runtime of 42 min. The pesticides were separately identified and their quantities were determined based on the various e/m ratios. For this purpose, a small amount of the evaporated sample was introduced into the ion source in the ion trap detector and ionization and ion decomposition took place with the help of the electrodes. Rigorous validation was conducted, covering multiple aspects, including selectivity, limits of detection (0.016 µg/kg), quantification (0.032 µg/kg), and mean recovery was 97%.

Statistical analysis

A factorial experiment using a completely randomized design was performed to analyze the data and Duncan's multiple range tests were used for comparison of the means at a 1% probability level in SPSS 17.

RESULTS AND DISCUSSION

The ANOVA results and the results obtained from the comparison of the means of the data related to the effects exhibited by the various treatments regarding Folbex-VA residues in the tested honey samples are presented in Tables 1-4. The individual effects of the various treatments including region, sampling time and their interactions on residue levels were significant (*$p \leq 0.01$*). It is noteworthy that, the maximum residue limit (MRL) for Folbex-VA adopted by the Codex Alimentarius Commission is 0.010 mg/kg [19].

Table 1: Analysis of variance of data obtained from the effect of different treatments on the residue of Folbex-VA in honey samples

Source of variable	df	SS	MS	F
Location	5	308.76	61.75	23.48^{**}
Time	1	30.11	30.11	11.45^{**}
Location × Time	5	125.188	25.04	9.52^{*}
Error	11	28.93	2.63	-

**Significant difference at the level of 1% ; CV= 4.39%

Table 2: Comparison of the average data obtained from the effect of geographical area on the residue of Folbex-VA in honey samples (means± SE) (mg/kg)

Pesticide	Havir	AyenehVarzan	Mosha	Damavand	Aru	Tar lake
Folbex	0.0169±0.0006[a]	0.0147±0.0005[b]	0.0136±0.0004[c]	0.0089±0.0007[d]	0.0056±0.0005[e]	0.0055±0.0004[e]

Means with at least one letter in common not significant at 1% according to Duncan's multiple range test

Table 3: Comparison of the average data obtained from the effect of sampling time on the residue of Folbex-VA in honey samples (means± SE) (mg/kg)

Pesticide	June	September
Folbex	0.0204±0.00038[a]	0.0013±0.00017[b]

Means with at least one letter in common not significant at 1% according to Duncan's multiple range test

Table 4: Comparison of the mean of data obtained from the interaction (geographical area × sampling time) on the residual Folbex-VA in honey samples (means± SE) (mg/kg)

Location	June	September
Tar lake	0.0110±0.00021[e]	Not detected
AyenehVarzan	0.0279±0.00018[b]	0.0015±0.0002[g]
Mosha	0.0257±0.00048[c]	0.0015±0.0002[g]
Aru	0.0113±0.00025[e]	Not detected
Damavand	0.0169±0.00014[d]	0.0009±0.0002[h]
Havir	0.0296±0.00031[a]	0.0042±0.0002[f]

Means with at least one letter in common not significant at 1% according to Duncan's multiple range test

The investigation involved comparing average data concerning the influence of different geographical regions on the levels of Folbex toxin (Folbex-VA) in honey samples, and the summarized results are presented in Table 2. Remarkably, the Havir area exhibited the highest poison residue concentration at 0.0169 mg/kg, while the Tar Lake area displayed the lowest concentration, measuring 0.0055 mg/kg. The data analysis revealed a statistically significant difference in the level of residual poison among all regions except Aro and Tar Lake ($p \leq 0.01$). In contrast, Table 3 reveals average data comparison to assess sampling time's influence on the remaining Fulbex toxin amount in honey samples. The results indicate that the highest poison concentration (0.0204 mg/kg) occurred in June, whereas the lowest concentration (0.0013 mg/kg) was observed in September, and this difference in measured values was found to be statistically significant ($p \leq 0.01$). Table 4 compares the average data, elucidating the combined influence of geographical region and sampling time on the concentration of Folbex toxin in honey samples. The data highlights that the highest poison concentration (0.0296 mg/kg) was measured in June in the Havir region, while the lowest concentration (0.0009 mg/kg) was found in September in the Damavand region. Furthermore, it was observed that during September, the amount of residual poison in honey samples from the Aro and Tar Lakes regions was below the detectable limit. The results showed that Folbex-VA residue for all the honey samples taken from all the studied regions in the last month of summer was lower than the MRL, but the residue levels in the honey samples taken from Havir, Ayeneh Varzan and Mosha in the last month of spring were higher than the MRL and in Lake Tar, Aru and Damavand regions were below or equal to the MRL. The higher residue levels in the last month of spring compared to the last month of summer can be attributed to the large number of Folbex-VA fumigant strips used in early spring. According to the recommendation of the manufacturers, these fumigant strips should be used 3-4 times in spring at 10-day intervals. Eissa et al. (2014) also studied bromopropylate (Folbex) residues in honey samples taken from different regions in Egypt and reported that the residues in the regions where this miticide was used were higher than the MRL in the European Union [18]. Bogdanov (2006) reported in his research that bromopropylate residue in honey was lower than the MRL in Switzerland (the MRL in Switzerland is 0.1 mg/kg), but its residues in honey bee products such as honeycomb and propolis exceeded the MRLs [20]. Of course, from 1991 to 2002 the residue levels declined gradually every year because the use of Folbex VA was banned in 1993. Wallner (1999) believes that since bromopropylate is a lipophilic and fat-soluble compound, it dissolves first in honeycombs and propolis and is then gradually released into honey [21]. Research has shown that Folbex VA residues were still detectable in honey produced in Germany eight years after its use was banned, although the residues exhibited a declining trend. Kast *et al*. (2021) also showed that although bromopropylate residues in honey decreased to 0.1 mg/kg (the MRL in Switzerland) they were still detectable in honey bee wax even 13 years after its use was banned [9]. The results of the research by Bogdanov et al. (1998) suggested that the amounts of Folbex-VA in honeycombs and propolis were much higher than the MRL [10]. It is noteworthy that the miticide residue did not decrease significantly even after the honeycombs and propolis were boiled for three hours or the samples were placed in an oven at 140°C for two hours.

CONCLUSIONS

Varroa mites are one of the most important pests of honey. They are controlled using chemical miticides. The use of chemical miticides influences honey quality. Folbex-VA fumigant strips are commonly used in Iran to control these mites although its use has been banned in many European countries. It is noteworthy that its residues remain for a long time in honey and other honey bee products. The findings of this research showed that its measured residues in most sampled areas exceeded the MRL adopted in the European Union. It seems that considering the undesired effects of this miticide, it must be replaced by miticides with lower persistence in honey and other honey bee products.

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