Determination of Amitraz (Varroaset) Residue in Honey by High Performance Liquid Chromatography (HPLC)*

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Abstract: Amitraz (Varroaset) is an acaricide applied against Varroa destructor Anderson & Trueman (Acarina: Varroidae) infestations on honeybees (Apis mellifera L.) (Hymenoptera: Apidae). Amitraz residue in honey was determined by HPLC in Ankara, Turkey. Honey samples were collected from beekeepers during the harvesting period in Ayağ, Kızılcahamam and Polatlı towns in Ankara. Amitraz residue was found in various levels in six (15%) out of 32 honey samples. The residue amounts in the 6 samples were 5.35, 0.34, 0.23, 1.27, 0.92 and 0.40 ppm. The limit of detection was 0.0643 ppm and the recovery ratio was 56.71 %. According to the results, some honey samples showed higher residue levels of amitraz than the World Health Organisation (WHO) limit of 1 mg/kg.

Key Words: Amitraz, residue, HPLC, honey, Varroa destructor, Apis mellifera

Introduction

The honey bee Apis mellifera L. (Hymenoptera: Apidae), is classified in the Order Hymenoptera of the Class Insecta, and is well adapted to different ecological conditions in different regions of the world (Rutner 1988). A. mellifera is the only known Apis species known in the world, namely Apis cerena, Apis dorsata, Apis florea and Apis mellifera. The first three species are found in the Far East countries and India (Rutner 1988). A. mellifera is distributed through Europe, Africa and Asia.

The honeybee is beneficial to humans by producing honey, wax and propolis, besides pollination (Özbek 1990). Anatolia is in an important position in the world regarding honey bee strains and ecotypes, because of the wide variation of its climatic and ecological conditions and its very rich flora. There are several strains of honey bee distributed all over Turkey (Sirali 2002). There are 4.3 million bee colonies found in Turkey and mainly located in the Aegean, Black Sea and Mediterranean regions of country (Sirali 2002). Depends on the environmental condition a small hive contains about 20,000 bees which comprise the Queen, drones and workers. In recent times, migratory bee keeping has become widespread in Turkey (Kaftanoğlu et al. 1992).

Honey is among the important foods of Turkish people. Turkey is in fourth place in the world in honey production (Firatlı et al., 2000). Despite the high number of colonies and suitability of our country for beekeeping, migratory beekeeping is failing to control pests and diseases, which results in reduced production.
A. mellifera is susceptible to Varroa destructor Anderson and Trueman (Acarina: Varroidae), which was known Varroa jacobsoni Oudemans, the most important problem for beekeeping. The mite has caused a significant reduction in the number and quality of colonies in Turkey (Kafanoglu et al. 1992). V. jacobsoni was reported in Turkey in 1976 for the first time and spread through the country in a short time (Tutkun and Inci 1992). Many pesticides are used in controlling V. destructor and Amitraz is the one most preferred in Turkey and worldwide. (Cavallaro 1989, Kolankaya et al. 2001, Aydin et al. 2003). However, the overuse of this compound can cause contamination in honey. Amitraz is used by 53 percent of apiarists, while formic acid is the least used (4%) in Turkey (Aydin et al. 2003).


Fernandez et al. (1993), detected residue at 1-40 ppb/kg of some acaricides, including amitraz, by spectrophotometric and gas chromatographic methods in Spain. In Germany, 320 honey samples were extracted, of which 8.5% were included amitraz residue (Hammerling 1991). In Belgium, fulvalinate residues were checked in 215 honey samples and residue was detected in only one sample (Greef et al. 1994). Atienza et al. (1993) found that HPLC was the most effective method for the detection of fulvalinate residue in honeys.

Garcia et al. (1996), determined acaricide residues by high-performance liquid chromatography in Honey.

Various methods have been developed for the determination of acaricide residues in honey samples, mainly gas chromatography (GC), liquid chromatography (LC) and direct analysis by high-performance liquid chromatography (HPLC) has been used for amitraz and fulvalinate analysis of honey.

A HPLC multiresidue method has been developed for determination the residual effect of amitraz from honey. This method is very rapid and highly sensitive and permit the determination of acaricides residues at levels close to residues tolerances. This method, simple and economical alternative to GC for the separation and determination of acaricides All acaricides are identified by reversed-phase high-performance liquid chromatography (Martel and Zeganne 2002).

Floris et al. (2001), to evaluate the effectiveness and the persistence of amitraz against V. destructor no amitraz residue higher than 0.01 mg kg-1 was detected in honey.

Korta et al. (2001), carried out possible degradation rate of amitraz, bromopropylate, coumaphos, chlordimeform, cymiazole, flumethrin, and fluvalinate by HPLC. All acaricides except amitraz, are stable in this medium for at least 9 months. Degradation products are; 4-dimethylaniline 2,4-dimethylphenylformamide (DMF) and N-(2,4-dimethylphenyl)-N’ methylformamidine (DPMF) of amitraz.

In Turkey, honey and honey products have important marketing problems, especially the issue of chemical residues in export material (Firatli et al. 2000). There are minimal data available related to pesticide residue problems in honey in Turkey. There was no amitraz residue found honey samples in Ankara / Turkey, using the GC method (Kolonkaya et al. 2001).

One hundred and thirty four honey samples were investigated for pesticide residues and Malaoxone was found in 27 samples from the eastern part of Turkey (Bulakari and Tufan 1986).

The most effective insecticides for bee mites are Malathion (99%), Amitraz (98%), Bromopropylate (98%), Coumaphos (96%), Fluvalanulate (98-100%), Flumethrin (99.8-99.9%), acid formic (96.8%), Tymol (96%) and other plant extracts (97%) (Abbed and Ducas 1993 Kolonkaya et al. 2001).

In this study, residues of amitraz in honey were investigated. The samples were taken from Ayas, Kızılcahamam and Polatlı towns in Ankara. For the determination of amitraz. The HPLC multiresidue analysis method was performed. The goal of this study was for determining amitraz residue in honey. The detection limit of this method was expected to be lower than the tolerance levels announced by WHO.

Materials and Methods

This research was carried out in Ankara, Turkey to determine amitraz residue in honey by using HPLC.


Chemical abstract name: (N, N’-[(methylinino) dimethyldyne] di-2,4-xylidine) (IUPAC), “amitraz” Amitraz (Varroaset) is both an acaricide and insecticide which contains 97% pure material (Tomlin 2002-2003).

It is used to control animal ectoparasites, including ticks, mites and lice on cattle, dogs, goats, pigs and sheep. It has EC and WP formulations. Amitraz is unstable in acidic media (PHs7) and slowly decomposes in prolonged storage under moist conditions (Tacchoe et al. 1988).
Some stabilizers were added during formulation of Varroset for preventing early decomposition of Amitraz (Tutkun and Bögelmez 2003).

The maximum residue limit in honey is 1.0 mg/kg and in waxes 0.6 mg/kg (Cabras et al. 1993). The ADI (daily intake) is 0.003 mg/kg (Baxendale and Keith 1993). The maximum residue level of amitraz in honey is 0.2 mg/kg according to the Turkish Alimentary Codex (Anonymous 2002).

Instrumental Conditions: The HPLC system used for honey analysis is shown below.


Preparation of amitraz standard: 100mg amitraz (97 %), dissolved in acetonitril (ACN), and distilled water were used for adjusting 100ml in the HPLC as isocratic solvent system. All reagents were HPLC grade. This solution was used as a stock solution. The solution was diluted to obtain 10, 8, 6, 4, 2, 1, 0.5, 0.1, 0.05 and 0.01-ppm concentrations for the honey standards.

Amitraz extraction from honey samples: Amitraz was extracted from honey by using the method of Tseng et al. (1999) and Pass and Mogg (1991), with slight modification. The extracts were injected into the HPLC for determination of the detection limit (minimum limit).

Results and Discussion

Standards: Minimum determination limit, residue amount and recovery ratio of amitraz in honey samples were calculated by standard chromatogram (Figure 1).

Retention time ranged from 8.058 to 10.282 minutes, depending on the concentration of the amitraz standards. It shows linear relationships between standard concentrations, retention times and the areas under the standard curve. For a concentration of 10 ppm; the retention time was 10,187 minutes and the area was 272,3635 units. These values changed according to the concentrations: for 10;8;6;4;2;1; 0.5;0.1;0.05 and 0.01ppm. concentrations, retention times were 10.187; 10.19; 10.19; 10.268; 10.268; 10.257; 10.247;10.230; 10.282 and 8.58 minutes respectively. The values are 2723635, 2178762, 1607974, 1101109, 554138, 233391, 10478, 25535, 11443 and 1250 for each concentration of the amitraz standard.(Table 1)

The detection limit of amitraz was 0.0643 ppm.

Calibration: For calibration, ten different amitraz standard concentrations were prepared. Each standard was analysed three times (Figure 2).
Table 1. Retention time and areas (their flow times and areas) for different concentrations of the amitraz standard by HPLC for honey samples

<table>
<thead>
<tr>
<th>Concentration of the standards</th>
<th>Retention time (minutes)</th>
<th>Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ppm</td>
<td>10.187</td>
<td>2723635</td>
</tr>
<tr>
<td>8 ppm</td>
<td>10.190</td>
<td>2178762</td>
</tr>
<tr>
<td>6 ppm</td>
<td>10.190</td>
<td>1607974</td>
</tr>
<tr>
<td>4 ppm</td>
<td>10.268</td>
<td>1101109</td>
</tr>
<tr>
<td>2 ppm</td>
<td>10.268</td>
<td>554138</td>
</tr>
<tr>
<td>1 ppm</td>
<td>10.257</td>
<td>233391</td>
</tr>
<tr>
<td>0.5 ppm</td>
<td>10.247</td>
<td>10478</td>
</tr>
<tr>
<td>0.1 ppm</td>
<td>10.230</td>
<td>25535</td>
</tr>
<tr>
<td>0.05 ppm</td>
<td>10.282</td>
<td>11443</td>
</tr>
<tr>
<td>0.01 ppm</td>
<td>8.058</td>
<td>1250</td>
</tr>
</tbody>
</table>

From Figure 2, the equation for the line is, \( Y = 287080x - 22931 \), if the concentration \( X \) = 4; \( Y = 1101109 \), if \( X = 6 \), \( Y = 1607974 \) and if \( X = 8 \), \( Y = 2178762 \). From the calibration curve, the correlation coefficient was calculated to be 0.997. There is a linear relationship between concentration and area.

Recovery: For recovery calculations, 2.01 mg of amitraz was added to the honey (sample number 8, without spray) and analysed. The calibration process was repeated. At the end of the process, the equation \( Y = 177220x - 11287 \) was obtained. From the chromatogram of recoveries, the area under the curve was 190643 units and a retention time of 15.652 minutes.

From the formula, \( X \) (recovery) was = 1.14 ppm. The recovery ratio was 1.14/2.01X100 = 56.71%, which is illustrated in Figure 3.

Controls: Eight samples were left as controls (without any amitraz application). They showed only mobile phase acetonitril (80%) and \( \text{H}_2\text{O} \) (20%) (Figure 4).

Treatment:

Investigation of amitraz residue in honey samples: Determination of residue of amitraz in honey was performed in 32 samples. Amitraz residues were detected in 6 out of 32 samples (numbers 3, 4, 5, 6, 8 and 11), and their chromatograms are shown (Figures 5, 6). The residual amounts of amitraz, retention time and areas were calculated from the calibration curve. These samples had 5.35; 0.34; 0.23; 1.27; 0.92 and 0.40 ppm amitraz residue respectively.

For these samples, retention times were determined as 10.055, 10.120, 10.135, 10.238, 10.088 and 9.557 min. respectively. The values were 1537245, 74900, 44951, 344056, 241338 and 92445 for the contaminated samples (Figure 5, 6).
Conclusions

The purpose of this study was to apply an analytical method for determining amitraz residue in honey. In this study, the detection limit of amitraz was determined as 0.0643 ppm, which is lower than the tolerance level set by WHO at 1.0 mg/kg. This means that this method was sensitive and could be used as an official method to determine amitraz residue in honey.

According to the results, 5.35 mg of residue was determined in sample 3. This value is 26.75 times higher than the Turkish code limit (0.2 mg/kg) and 5 times higher than the American Chemistry Association limit (1 mg/kg). Other detected residue values were 0.34, 0.23, and 0.40 mg/kg. These are higher than the Turkish limit and lower than the American chemistry association limit. The residue value in sample 6 was 1.27 mg/kg, 6.35 times higher than the Turkish limit and 1.27 times higher than the American limit. For sample 8 (0.92 ppm.), the determined residue value was 4.5 times higher than the Turkish limit.

When we compared our recovery result with other researchers some differences were observed. The reason may be related to its detection in honey, although some degradation occurs during the storage period. As for amitraz, it is necessary that to give at least 21 day interval between last spraying and harvesting time for other crops. Degradation processes of amitraz have been studied and the main degradation products are 2,4-dimethylamidine, N-(2,4-dimethylphenyl)-N-methylformamidine and 2,4-dimethylphenylformamide (Korta et al., 2001). The residue limit for Varroaset is 1mg/kg in honey and 0.6 mg/kg in wax. This value is 0.05 mg/kg for Bromoprophylate, 0.05 mg/kg for Fulvanilate and 0.05 mg/kg for Malathion (Cabras et al., 1993). Maximum daily intake limit (ADI) is 0.003 mg/kg for amitraz, 0.0005 mg/kg for Perizin and 0.005 mg/kg for Malathion (according to the FAO/WHO; Baxendale and Keith 1993).

As shown in this study, amitraz can cause residue in some honey samples. Some of the honey samples contained amitraz at higher than 1 mg/kg, which is the maximum tolerance limit of WHO (Baxendale and Keith 1993). The residue problem is a reality concerning amitraz. For this reason, new research should be planned for honey and wax products for the determination of human health risks. HPLC can be used for further determination of residue levels. In this method the minimum detectable level of amitraz in honey was lower than the maximum tolerance level detection limit. Amitraz residue were found higher in six samples which compare to Turkish limit.

Acknowledgements

The University of Ankara Research Foundation financially supported this research. We are grateful to the ATABAY Company, for providing the data on amitraz. The authors wish to thank Prof., Dr. Nevzat Artik (Ankara University, Food Engineering Faculty) for his guidance and comments during the study, and to Didem Kahya and Nilüfer Vural (Ankara University, Science and Tehnology Center) for technical assistance during these experiments. Many thanks to Dr. Ertac TUTKUN for his valuable advice, suggestions and help in taking honey samples. We also thank the beekeepers, whose colonies we sampled, for their help. We are also grateful to Greg Sullivan for early reading the manuscript (University of Ondokuz Mayis, Samsun).

References


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