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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 Ayaş, 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*

Ballarda Amitraz (Varroaset) Kalıntısının Yüksek Performans Likit Kromatografi Yöntemi (HPLC) ile Belirlenmesi

Öz: Amitraz (Varroaset) bal arılarında (*Apis mellifera* L.) (Hymenoptera: Apidae) sorun olan *Varroa destructor* Anderson & Trueman (Acarina: Varroidae) 'e karşı kullanılan bir akarisittir. Ballardaki amitraz kalıntısı HPLC ile tespit edilmiştir. Bal örnekleri arı yetiştiricilerinden Ankara'nın ilçeleri olan Ayaş, Kızılcahamam ve Polatlı'dan hasat dönemi boyunca toplanmıştır. Toplanan 32 örnekten 6 (%15) adedinde amitraz kalıntısına rastlanılmıştır. Örneklerde tespit edilen kalıntı miktarları sırasıyla 5.35, 0.34, 0.23, 1.27, 0.92 ve 0.40 ppm. dir.Tespit edilen kalıntı limiti 0.0643 ppm ve elde edilme oranı %56,71 dir. Bu sonuç doğrultusunda Dünya Sağlık Örgütü (WHO)' nün izin vermiş olduğu limit 1 mg/kg iken bazı bal örneklerinde daha yüksek düzeyde amitraz kalıntısı olduğu görülmektedir.

Anahtar Kelimeler: Amitraz, kalıntı, HPLC, bal, *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 of the *Apis* species in Turkey (Kaftanoğlu et al. 1992). There are four important *Apis* species known in the world, namely *Apis cerena*, *Apis dorsata,* A*pis 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 (Firatli 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.

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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 (Kaftanoğlu 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).

There is considerable data from around the world related to pesticide residue problems in honey and other crops (Stoeppoler et al. 1986, Cavallora 1989, Berzas et al. 1991, Imdorf et al. 1995, Garcia et al. 1996).

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 fluvalinate 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%), Bromoprophylate (98%), Coumaphos (96%), Fluvanilite (98-100%), Flumethrin (99.8-99.9%), acid formic (96.8%), Tymol (96%) and other plant extracts (97%) (Abbed and Ducos 1993 Kolonkaya et al. 2001).

In this study, residues of amitraz in honey were investigated. The samples were taken from Ayaş, Kızılcahamam and Polatli 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

Materials

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

Common name: Amitraz (BSI, E-ISO, ANSI, ESA, BAN, JMAF) .

Chemical abstract name: (N, N'-[(methylimino) dimethylidyne] 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 (PH≤7) and slowly decomposes in prolonged storage under moist conditions (Taccheo et al. 1988).

Some stabilizators were added during formultaion of Varroset for preventing early decomposition of Amitraz (Tutkun and Boşgelmez 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.

HPLC: Thermo Finnigan Model, Surveyor

Pump: Thermo Finnigan surveyor, Analytical pump, 4 gradient

Detector: Termo Finnigan, Surveyor Model, Photo Diode Array Detector (PDA)

Column: Phenomenex Luna 5μ C18 (250 x 4.6 mm, ID)

Column oven: Thermo Finnigan Surveyor, Autosampler,

Column oven temperature: 30 ^{0}C

Injection: Thermo Finnigan Surveyor Autosampler20 μl/l

Injection volume: 20 μL (sample and standard)

Detection: 220-360 nm

Loop: 20 μL

Flow rate: 1 mL/minute

Mobil phase: H_2O + acetonitril (20/80; v/v) isocratic

Running time: 17 minutes

For Amitraz Analysis;

Rotary evaporator: Büchi R-200

Simple Shaker: Gel, 3017 Stirring hot plate

Scale: Scaltec SBC21

Chemicals: Acetone, acetonitryl, dichloromethane, sodium chlorate, silicagel, active carbon and toluene.

Methods

Laboratory experiments: Treated and non treated honey samples (without residues) were obtained from apiarists in Ayaş, Beypazari, and Nallihan towns in Ankara. Thirty-two samples were treated with amitraz at the apiarists' application dosage while 8 samples were left as controls. Amitraz (Varroset) was applied 1200 mg/per hive for tree times in seven days interval. Amitraz was applied at the beginning of May. Two weeks after applications, honey samples were taken to the laboratory for analysis through by HPLC. During analysis, samples were kept in dark condition (+4ºC).

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; 10,190; 10,190; 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).

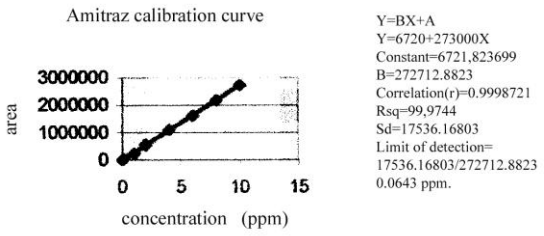

Figure 1. The curve of the amitraz standard for the experiment.

Table 1. Retention time and areas (their flow times and areas) for different concentrations of the amitraz standard by HPLC for honey samples

Concentration of the standards	Retention time (minutes)	Areas
10 ppm	10.187	2723635
8 ppm	10.190	2178762
6 pmm	10.190	1607974
4 ppm	10.268	1101109
2 ppm	10.268	554138
1 ppm	10.257	233391
0.5 ppm	10.247	10478
0.1 ppm	10.230	25535
0.05 ppm	10.282	11443
0.01 ppm	8.058	1250

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.

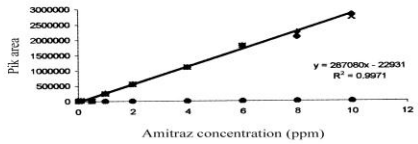

Figure 2. The calibration curve of the amitraz standard in honey (Constant=22931; ppm (X)=287080; Correlation (R)=0,9971; Rsp=99,71%).

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 = 1.14ppm.The recovery ratio was 1.14/2.01X100= 56,71 %, which is illustrated in Figure 3.

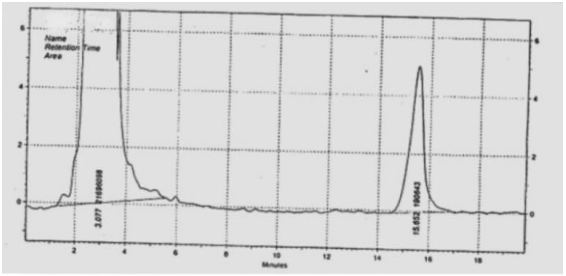

Figure 3. HPLC chromatogram of recovery ratio of amitraz in honey samples.

Controls: Eight samples were left as controls (without any amitraz application). They showed only mobile phase acetonitril (80%) and H_2O (20%) (Figure 4).

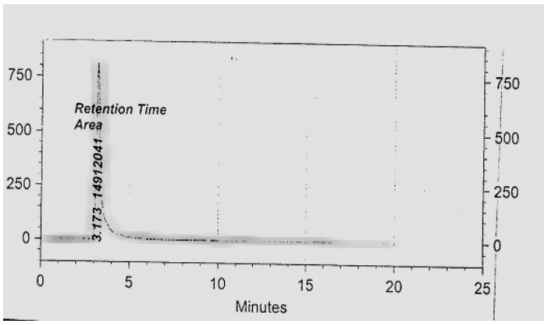

Figure 4. HPLC chromatogram of control samples (without amitraz application in honey samples).

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; 092 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).

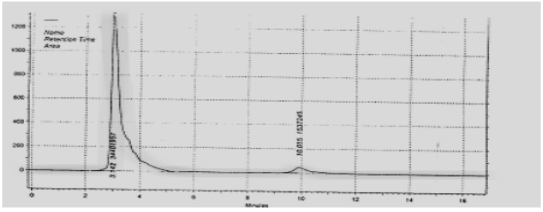

Figure 5. HPLC chromatograms of amitraz detected in honey (Sample 3)

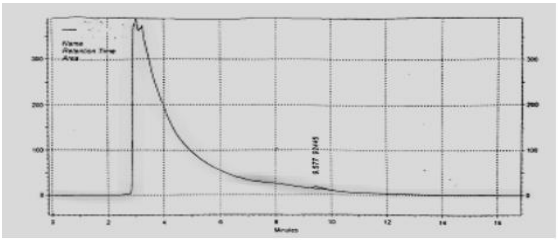

Figure 6. HPLC chromatograms of amitraz detected in honey (Sample 11)

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, whereas other researchers recovered amitraz from apples and grapefruits at 88.9-92.1 % and 87.2-90.9%, respectively (Tseng et al. 1999). In our study, this value was 56.6% in honey. The maximum tolerance limit of amitraz for mammals is 0,2 mg/kg (Baxendale and Keith 1993, Anonymous 2002). According to our results, the minimum detectable level of amitraz in honey was 0.0643 ppm, and this is lower than the maximum tolerance limit of amitraz for mammals. Amitraz use can result in residue 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-dimethylaniline; *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.

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