RESEARCH ARTICLE

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Determination of Neonicotinoid Residues in Honey Samples Collected from The East-Southeast Anatolia Region of Türkiye

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Abstract

In this study, 30 honey specimens were collected from some provinces of East-Southeast Anatolia to investigate the residue of neonicotinoid type insecticide. After the collected honey specimens were extracted with the liquid-liquid extraction method, the analyzes were carried out in a High-Pressure Liquid Chromatography Mass Spectrometry (HPLC-MS/MS) device. According to the analysis results, Acetamiprid in honey samples was observed to be in the range of 0.93-4.98 µg/kg. These results were found to be below the Maximum Residue Limit (MRL) value of 5 µg/kg according to FAO/WHO and Turkish Food Codex (TGK). It was determined that the results obtained for other analytes were insignificant and were in accordance with the residue limits of neonicotinoids in honey.

Keywords: East-Southeast Anatolia, honey, LC-MS/MS, neonicotinoid, residue.

INTRODUCTION

Honey is a sweet, natural and very valuable food type designed to meet the energy needs of the human body at a high level and quickly. Due to its nutritional value and being a healthy food item, honey is a food of animal origin that is widely consumed by humans. Honey: It is a natural product that the nectars of plants, the secretions of the living parts of the plants or the secretions of the plant sucking insects living on the living parts of the plants are modified by the honeybees by combining them with their own specific substances, reducing the fluid content and storing them in honeycombs (Anonymous, 2012). Honey contains approximately 200 substances, water and mainly carbohydrates, as well as minerals, free amino acids proteins, enzymes, and various vitamins (A, B1, B2, B3, B5, B6, B8, B9, B12, C, E) (Rao et al., 2016). National and international standards have been developed to determine the quality of honey and it has become mandatory to comply with these standards by law. Compliance of honey according to these standards is controlled by analyzes made in food control laboratories (Anonymous, 2009a; Anonymous, 2009b). Honey, which is of great importance in terms of nutritional value, is open to cheating because the amount of quality honey is limited and expensive. It is clearly stated in the Turkish Food Codex (TGK) Honey Communique (2012/58) that no additional additives can be added to honey and that honey cannot contain any substances that threaten human health (Anonymous, 2012). Pesticide detection is necessary to monitor contamination in bee products and ensure consumer health (Morzycka, 2002).

Neonicotinoids are a new class of insecticides and their use in agricultural areas has been increasing in recent years. Although its toxic effects on mammals and birds are very low, its toxic effects on bees and insects are extremely high. Due to this difference in toxic effects, the use of neonicotinoids instead of organochlorine, organic phosphorus, carbamate and pyrethroid insecticides has become a rapidly growing insecticide class worldwide (Jeschke et al., 2011; Casida and Durkin, 2013). In the class of neonicotinoids; It includes imidacloprid (the most widely used neonicotinoid compound), acetamiprid, nithiazine, clothianidin, thiacloprid, thiamethoxam, and nitenpyram (Jeschke and Nauen, 2008). It has been reported that neonicotinoid type pesticides leave residues in soil and irrigation channels and cause environmental pollution (Krupke et al., 2012; Adak et al., 2012).

Neonicotinoids are insecticides that exert nicotine-like stimulant effects by binding to nicotinic acetylcholine receptors in the CNS. Nicotinic receptors are found in both the central and peripheral nervous system in mammals, while in insects they are found only in the central nervous system. Neonicotinoid type insecticides bind to central nervous system receptors more tightly and irreversibly in insects than in mammals. Therefore, in addition to having more toxic effects for insects than mammals and birds, some of its metabolites are also toxic (Matsuda et al., 2011).

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In this study, our aim is to investigate in terms of Neonicotinoid residues in honey samples taken from different localizations in Eastern and South-eastern Anatolian Regions.

MATERIALS AND METHODS

Chemicals and Standard Substances

Acetamiprid (99.9%), Clothianidin (99.9%), Clothianidin-d3 (Internal Standard) (99.9%), Dinotefuran (98.8%), Imidacloprid (99.9%), Nitenpyram (98.8%), Thiacloprid (99.9%), Thiamethoxam (99.6%). Standard materials were obtained from Sigma-Aldrich with a certificate. The purity of each standard substance is over 97.8%.

Other chemicals

Acetonitrile (analytical class, Fluka), dichloromethane (analytical class, Merck), acetic acid (analytical class, 100%, Merck).

Solutions

Mobile phase A: Acetonitrile, HPLC grade.
Mobile phase B: Acidified water (0.2% Acetic acid): 2 mL of acetic acid is taken into a 1 L flask and made up to the 1 L line with distilled water.
Stock Standard solution (S0): 10 mg of each standard substance separately, was weighed into 10 mL flasks and the top was filled with methanol up to the 10 mL line. All stock standard solutions used in the study were stored at -18^{0}C.

Equipments

a) Mutireaks (Multi mixer) (Heidolph Instrument, Germany)
b) Ultrasonic bath
c) Centrifuge
d) UHPLC system. Eksigent, expert Ultra LC 100.
e) Analytical column; Agilent Poroshell 120 SB: C18 2.7 µm 100x3.0 mm.
f) AB Sciex 3200 QTRAP, Tandem Quadrapol Mass Spectrometer (MS/MS).
g) Software: Analyst 1.6.1

Collection of Samples

Honey samples were collected for the detection of neonicotinoid residues from different locations of some provinces (Şanlıurfa, Van, Bitlis, Bingöl, Adıyaman, Diyarbakır, Mardin) in the Eastern and Southeastern Anatolia Regions. A total of 30 honey samples, including cotton honey, flower honey and thorn honey, were collected from beekeepers in different locations between July and September 2020. Collected honey samples were stored at +4^{0}C until analysis. No pollen analysis was performed to determine the honey type while taking honey samples. The provinces and districts where honey samples were collected from the Eastern and Southeastern Anatolia regions are shown in Table 1 and Figure 1 on the map.

Table 1. Provinces and districts where honey samples were taken.

No	Where the Sample Was Taken		Type of Honey	Number of Samples
	Province	District		
1.	Adıyaman	Çelikhan	Thorn	1
2.	Adıyaman	Gerger	Plateau	1
3.	Adıyaman	Center	Thorn	1
4.	Adıyaman	Center	Cotton	1
5.	Bingöl	Karlıova	Thorn	1
6.	Bitlis	Center	Thorn	1
7.	Diyarbakır	Çermik	Cotton	2
8.	Diyarbakır	Çermik	Plateau	1
9.	Mardin	Center	Cotton	5
10.	Şanlıurfa	Akçakale	Cotton	2
11.	Şanlıurfa	Bozova	Cotton	3
12.	Şanlıurfa	Harran	Cotton	2
13.	Şanlıurfa	Hilvan	Cotton	1
14.	Şanlıurfa	Siverek	Thorn	1
15.	Şanlıurfa	Siverek	Cotton	1
16.	Şanlıurfa	Siverek	Clover	2
17.	Şanlıurfa	Suruç	Cotton	1
18.	Şanlıurfa	Viranşehir	Thorn	2
19.	Van	Gürpınar	Thorn	1
				Total: 30

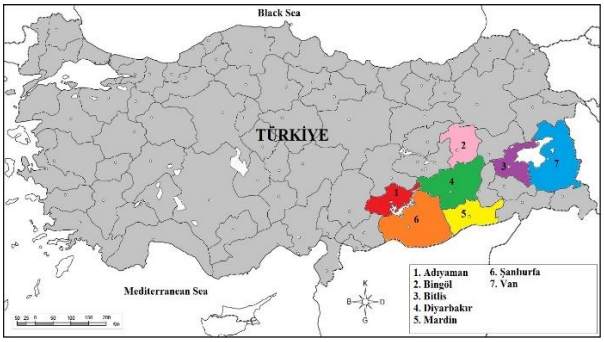

Figure 1. Display of the points where honey samples were taken on the map.

Extraction of Samples

For the extraction of honey samples, the extraction method used by Jovanov et al. was modified and used. 2 g honey sample was taken into 15 mL PPE tubes and 100 µL of internal standard, 0.5 mL of acetonitrile and 2.0 mL of dichloromethane were added. It was stirred at vortex for one minute. It was kept in an ultrasonic bath for 10 minutes and then mixed in vortex for 1 minute again. Then, the supernatant was taken by centrifugation at 2500 rpm for 5 minutes. It was evaporated at 40°C under nitrogen flow. 0.2 mL of mobile phase was dissolved with A/B (50/50) mixture and mixed with vortex for 2 minutes. 0.22 µ was passed through injector filters and transferred to insert vials (Jovanov et al., 2013).

UHPLC Device Requirements

UHPLC works directly connected to the mass spectrophotometry detector. Agilent Poroshell 120 SB: C18 2.7 µm 100 x 3.0 mm column was used for analytical separation. The flow rate is 0.3 mL/min. The mobile phase gradient flow program is shown in Table 2.

Table 2: Mobile phase gradient flow program.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
00:00	80	20	0.30
01:00	80	20	0.30
01:10	50	50	0.30
03:30	50	50	0.30
03:40	80	20	0.30
06:00	80	20	0.30

MS/MS Requirements

AB Sciex 3200 QTRAP (AB Sciex, Foster City, USA) was used as a mass spectrophotometry detector. Ionization was carried out in positive and negative ion mode and with the Electrospray Ionization Module (ESI). Scan type is set to MRM (Multiple Reaction Monitoring). Capillary voltage was set to 5500 V, nebuliser gas (7 psi), curtain gas (30 psi), heater gas (50 psi) and collision gas (50 psi) using nitrogen gas. The temperature of the TurboIon Spray Module was fixed at 400^0C. Analyte dependent parameters; Working standard solution containing 0.1 mg/kg of each standard substance was used for DP (declustering potential), CXP (cell exit potential) and CE (collision energy). MS/MS detector parameters of each analyte are shown in Table-3.

Table 3: MS/MS detector parameters.

Analytes	Q1 (Da)	Q3 (Da)	DP (volts)	EP (volts)	CEP (volts)	CE (volts)	CXP (volts)
Acetamiprid	223.07	126.20*	41	9	12	27	4
		99.20	41	9	12	47	4
Clothianidin	250.07	132.00*	41	7.5	14	19	4
		169.10	41	7.5	14	15	4
Clothianidin-d3 (IS)	253.01	132.00	41	8	14	23	4
Dinotefuran	203.08	129.1*	36	8	18	15	4
		114.1	36	8	18	17	4
Imidacloprid	256.10	290.10*	36	9	14	19	4
		175.10	36	9	14	21	4
Nitenpyram	271.12	225.20*	36	5.5	16	15	4
		56.00	36	5.5	16	49	4
Thiacloprid	253.06	126.20*	46	12	14	29	4
		99.10	46	12	14	53	4
Thiamethoxam	292.00	211.1*	31	11,5	16	15	4
		132.1	31	11,5	16	25	4

***** Confirmation ion

DP: Declustering potential, **CE:** Collision energy, **CEP:** Cell exit potential, **CXP:** Collision Cell Exit Potential, **EP:** Entrance Potential

RESULTS

After the honey harvest between September and October 2020, 30 strained honey samples were collected from the central and central districts of some provinces in the Eastern and South-eastern Anatolian regions. The samples were analyzed in the High-Pressure-Liquid-Chromatography-Mass-Spectrometry (HPLC-MS/MS) instrument in Tekirdağ Namık Kemal University Scientific and Technological Research Application and Research Center. Analyzes were performed in the mass spectrometry detector, in positive ion mode, in MRM (Multiple Reaction Monitoring) mode, using the ElektroSpray Ionisation (ESI) module.

According to the analysis results, it was observed that Acetamiprid was in the range of 0.93-4.98 µg/kg. It was determined that these results were below the Maximum Residue Limit (MRL) value of 5 µg/kg according to FAO/WHO and TGK. The results obtained for other analytes were found to be insignificant. Analysis results of neonicotinoids in honey are presented in Table 4.

Table 4. Analysis results of honey samples.

Analytes	SAMPLES																												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Acetamiprid	**0,9**	**1,1**	**3,8**	**2,1**	**2,5**	**4,3**	**3,2**	**11,**	**15,**	**1,0**	**1,0**	**3,5**	**1,2**	**1,1**	**2,7**	**3,1**	-	**1,6**	-	-	**4,9**	**0,9**	-	-	-	-	-	-	**4,0**	**2,0**
Clothianidin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dinotefuran	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Imidacloprid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitenpyram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thiacloprid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thiamethoxam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-) Not available

DISCUSSION AND CONCLUSION

According to the analysis results of the honey specimens made in the study, acetamiprid was found to be in the range of 0.93-4.98 µg/kg.

Çil et al., (2020) conducted a study to determine the level of pollution in the environment due to the presence of neonicotinoids in honey products produced in Türkiye. For this purpose, 44 honey samples were examined taken from beekeepers in different regions of Türkiye after the honey harvest. The samples were examined for the presence of acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam using Liquid Chromatography Quadrupole Time of Flight Mass Spectrometer (LC-MS Q-TOF). It was reported that neonicotinoid group insecticides were not found in any of the 44 honey samples analyzed (Çil et al., 2020).

Özdemir and Muz, (2023) collected 33 honey samples for neonicotinoid residue analysis from Tekirdağ province and some district centers. According to his analysis, he reported that there were neonicotinoid pesticide residues exceeding the maximum residue limits in 33 honey specimens (Özdemir and Muz 2023).

Mitchell et al., (2017) investigated the presence of neonicotinoids in honey samples collected from different countries. A total of 198 honey samples were collected, 37 from Africa, 41 from Asia, 22 from North America, 53 from Europe, 28 from South Central America and the Caribbean, and 17 from Oceania and the Pacific Islands. 5 major neonicotinoid (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) residues were analyzed by UHPLC-MS/MS method. They determined that at least one of the five compounds was present in 75% of all honey specimens. They reported that these neonicotinoid type compounds are acetamiprid, thiacloprid, imidacloprid, clothianidin and thiamethoxam. However, they observed that all the detected neonicotinoid compounds were below the maximum residual amount allowed for human consumption (Mitchell et al., 2017).

Sanchez-Hernandez et al., (2016) investigated thiamethoxam, clothianidin and imidacloprid neonicotinoids and their metabolites in honey specimens by LC/QTOF-MS method. While collecting honey samples, they took this into account when evaluating the results of the samples they selected especially from the sunflower and corn fields. Researchers who reported that 3 neonicotinoid species were not found in honey samples, reported that thiamethoxam metabolites TM5 and TM13 were found in all honey samples collected from beehives near sunflower fields (Sánchez-Hernández et al., 2016).

In a study investigating the presence of neonicotinoids in Irish honey, 30 honey samples were collected from beekeepers. As a result of the analysis, the presence of clothianidin, imidacloprid and thiacloprid was determined in honey samples. It has been reported that the neonicotinoid concentrations detected are below the maximum residue level allowed for human consumption and do not pose a threat to human health. However, it has been reported that the presence of these neonicotinoids in honey may have negative effects for honeybees, and their presence in nectars may have negative effects on other insect species (Kavanagh et al., 2021).

A total of 693 honey samples collected from across China were analyzed to examine neonicotinoid concentrations. As a result of the analysis, it was reported that 40.8 % of the honey samples contained at least one of the five neonicotinoids tested. It has been reported that neonicotinoid concentrations in honey samples have significant adverse effects on honeybee health, and the detected neonicotinoid levels are likely safe for human consumption (Wang et al., 2020).

Migrant beekeepers, who came from Adana to Harran and Akçakale (Harran Plain) districts of Şanlıurfa, placed their bees in the region to collect nectar from cotton. Beekeepers reported that there were intense bee deaths between 2-10 August 2018 and bee losses ranged between 30% and 70%. In the field investigations, it was determined that there was intensive pesticide use in this period.

Parallel to similar studies, it was observed that all the neonicotinoid compounds determined in the honey specimens analyzed in this study were below the maximum residue amount allowed for human consumption, but it was seen that these studies should be continued in detail and more comprehensively.

In this study, it was reported that the neonicotinoids detected in some honey samples were below the MRL value. These results show that there is a small use of neonicotinoids, especially in cotton farming areas. This result may have been caused by the fact that farmers still use neonicotinoids despite the prohibition or restrictions

on the use of neonicotinoid pesticides. It would be appropriate to inform the farmers engaged in agricultural activities about public health and prevent bee deaths, about biological control methods, and to provide training on the use of pesticides.

Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship contributions

Concept: H.D., N.Ö. and F.S.K. Design: H.D., N.Ö. and F.S.K. Data Collection or Processing: H.D., N.Ö. and F.S.K. Analysis or Interpretation: H.D., N.Ö. Literature Search: H.D., N.Ö. and F.S.K. Writing: H.D. and F.S.K.

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