Natural Products and Biotechnology

Vol. 2 No. 1
pp. 1-13
(2022)

Comparison of Extraction and Clean-up Methods Modified in Different Ways for the Determination of Pesticide Residues in Bees

Orhan Dincay [1*], Hasan Sungur Civelek [2]

[1] Proanaliz Alaşehir Food Control Laboratory, Manisa, Turkey
[2] Department of Biology, Faculty of Science, Muğla Sıtkı Koçman University, Muğla, Turkey

Article History

Received : *Nov 03, 2021*
Revised : *Feb 06, 2022*
Accepted : *May 09, 2022*

Keywords

Bee,
Pesticide,
Residue,
Extraction,
Clean-up

Abstract

As a result of the unconscious use of pesticides, honeybees are adversely affected, thus causing colony losses all over the world. In this research, different extraction and clean-up methods were compared on bee samples based on the QuEChERS method which is the most widely used pesticide analysis method in food. This research includes application studies for the multi residue method (MRM), which allows analysis of more than one pesticide. During the extraction phase, 7 different methods were compared, while the method with the most suitable repeatability and recovery values among these methods was taken to the clean-up phase. In the clean-up phase, 5 different clean-up methods were tried on the method which yielded the best values in extraction. In terms of average percentage recovery values, the 1st extraction method ranks first with 72 active substances between 70-120%, and the 3rd clean-up method with 92 active substances. Thus, while the number of active substances with suitable recovery values in the selected extraction method was 72, it increased to 92 with the continuation of the clean-up method, achieving a success rate of 92%. It is recommended to use this method in pesticide residue analyzes in bees with its outstanding features such as completing the analyzes in a short time, working easily with the infrastructure that can be found in all control laboratories, low analysis costs, ease of use, and reliability.

Corresponding Author:
Orhan Dincay, Proanaliz Alaşehir Food Control Laboratory, Manisa, Turkey, orhan_dincay@hotmail.com
Cite this article as:
Dincay, O. & Civelek, H. S. (2022). Comparison of Extraction and Clean-up Methods Modified in Different Ways for the Determination of Pesticide Residues in Bees. *Natural Products and Biotechnology*, *2*(1), 1-13.

1. INTRODUCTION

In the present day, pesticides are indispensable products used to fight against diseases and pests in crop production. Millions of tons of pesticides are produced and released into the environment each year. For this reason, pesticides are one of the most common and dangerous chemicals for non-target human, animal, and environmental health. The main usage area of pesticides is agricultural control. Biocidal products which are used to control pests and vectors on humans and pets are also a source of exposure to non-target organisms (Yavuz and Aksoy, 2016). During the spray application of pesticides, some amount of them evaporated and dispersed into the air while the remaining amount reaches the plant and soil surfaces. The evaporated pesticides are carried to other regions by the winds and then they may return to the earth with rain, fog, or snowfall. In this way, pesticides reaching other non-target organisms may cause residue and toxicity (Akdogan *et al*., 2012). Usage of pesticides, industrial agricultural practices, honeybee pathogens and global climate changes are among the activities that threaten the life of honeybees. Especially since the beginning of the 1990s, the use of pesticides has increased rapidly due to population growth and the need for agricultural products. The increase in pesticide use is one of the factors that negatively affect the lives of honeybees.

ISSN: 2791-674X Research Article

As a result of the reckless use of pesticides, the negative effects of honey bees on their lives cause colony losses in the world. Pesticides used against agricultural pests not only affect honeybees flying over or pollinating the plants but also indirectly affect other individuals in the colony by carrying substances such as pollen and nectar containing pesticide residues to the hive (Polat *et al*., 2020). In a research, investigating the effects of neonicotinoid group pesticides on bees, it was determined that all the bees died within 24 hours after the pesticides with active ingredients Imidacloprid, Thiacloprid, and Thiamethoxam were used at the recommended dose for agricultural control; therefore it was determined that they were very harmful to bees. In this study, when acetamiprid was used at the recommended dose, 74% of the bees died within 24 hours. It has been determined that the survivors cannot use the necessary organs (Karahan *et al*., 2019). In another research, the effect of imidacloprid from the neonicotinoid group in winter loss on Anatolian honeybee (*Apis mellifera anatoliaca*) was investigated. In this research, 5 mL/100 L water and 6 different doses of this dose diluted 50% (2.5 mL/100 L water, 1.25 mL/100 L water, 0.625 mL/100 L water, 0.312 mL/100 L water, 0.156 mL/100 L water) was used. At the end of this research, 75.66% of the bees treated with different doses of imidacloprid died, while 49% of the control group bees died. In addition, it was observed that the bees treated with imidacloprid suffered more winter loss than the control group bees (Karahan *et al*., 2018). Alaux *et al*. (2010) conducted, exposed honeybees to imidacloprid, one of the neonicotinoid group pesticides and the nosema parasite. Co-exposure has been shown to severely weaken honeybees. Thus, it is thought that pesticides and parasites may have synergistic effects. Wu *et al*. (2012) found that bees growing on comb with high pesticide residue were infected with nosema parasite at a higher rate than those growing on comb with low residue. Kiljanek *et al*. (2016) carried out a method validation in which 200 pesticides can be screened with LC-MS/MS and GC-MS/MS with an extraction method they developed based on QuEChERS. They analysed more than 70 poisoned honeybee samples according to this method. The QuEChERS method is currently the most widely used sample preparation method for the analysis of pesticides by both research and control laboratories, especially in plant food samples, and it is reported that this method is used in 98% of scientific publications. In addition, articles on the use of complex matrices such as honey and pollen have also been published. In the coming years, it is predicted that it will be a predominantly preferred method by making small changes to improve the detection spectrum (Yavuz and Aksoy, 2016). In this research, different extraction and clean-up methods were compared on bee samples based on the QuEChERS method, which is the most widely used method in pesticide analysis in foods and application studies were carried out for the multi-residue method (MRM). Thus, it is aimed to be a pioneer in the validation studies for the MRM method, where hundreds of pesticides can be analysed together in bees.

2. MATERIAL and METHODS

2.1. Material

Since using as a blank sample in extraction and clean-up stages for the residues of some selected pesticides, it is very important that the bees to be used in the analyses do not contain any pesticide residues. For this reason, worker bees, which were destroyed at -18 °C, were supplied from BIOBEST, one of the important bumblebee production facilities in Antalya, to be used as blank samples in this research. The samples, which were transported to the laboratory in the cold chain, were kept in the deep freezer at -18 °C for the homogenization process. Afterward, the samples taken from the deep freezer were treated with dry ice at a temperature of -78.5 °C for another 3 hours and the samples were allowed to freeze thoroughly. Thus, a good homogenization (fragmentation) was achieved. After the blank samples were homogenized, the weighing phase started to be used in different extraction and clean-up methods. All certified pesticide standards supplied from Dr. Ehrenstorfer GmbH (Augsburg,

Germany) used in the analysis have a purity of over 90%. The chemicals used, such as water, methanol, ethyl acetate, n-hexane, formic acid, and ammonium formate, are HPLC grade and acquired from Merck KGaA (Darmstadt, Germany). The extraction and clean-up kits used belong to Agilent Technologies (California, USA). The device on which the analyses were made by Agilent Technologies (California, USA) 1260 Infinity LC, 6460 Triple Quadrupole LC/MS (Jetstream ESI) model.

2.2. Method

Methods have been modified in different ways based on the QuEChERS method, which is the most commonly used method for pesticide analysis in plant-based foods. While choosing the methods, the physiological characteristics of the bees' bodies (water and fat ratios) were taken into consideration. Therefore, solvents with different properties were tested due to the addition of water in different amounts in different weights and the body structure of the insects. During the extraction phase, 7 different methods were tried, and the method with the most suitable recovery values among these methods was taken to the clean-up phase. In the clean-up phase, 5 different clean-up methods were tried on the method with the best values in extraction.

2.2.1. Extraction Methods

Table 1. Extraction steps.

Extraction methods	1	2	3	4	5	6	7
Sample amount	10 g	5 g	10 g	5 g	10 g	5 g	15 g
Addition of water	2.5 mL	6 mL	2.5 mL	6 mL	2.5 mL	6 mL	3.75 mL
Addition of acetonitrile	10 mL	10 mL	7 mL	7 mL	7 mL	7 mL	15 mL (1% Acetic Acid)
Addition of ethyl acetate	-	-	3 mL	3 mL	-	-	-
Addition of N-hexane	-	-	-	-	3 mL	3 mL	-
Internal Standart (10 mg/kg Triphenyl Phosphate)	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	75 µL
Pesticide mixture (1 mg/kg)	500 µL	250 µL	500 µL	250 µL	500 µL	250 µL	750 µL

Homogenized samples were weighed into 50 mL centrifuge tubes according to the values specified in Table 1, after adding water, they were shaken for 1 minute and held for 5 minutes. Then, solvents of the relevant method, internal standard and pesticide mixtures were added and shaken for 2 more minutes. In the first 6 extraction methods, after the shaking step, 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate buffer salts were added to the tubes. It was shaken for 1 min and centrifuged at 3000 rpm for 5 min. In the seventh extraction method, after shaking, 6 g of anhydrous magnesium sulfate and 1.5 g of sodium acetate were added to the tubes, shaken for 1 min and centrifuged at 1500 rcf for 1 min. After centrifugation, 1 mL was taken from the supernatant of the tubes to determine the recovery values. It was filtered with a 0.45 µm polytetrafluoroethylene (PTFE) filter and transferred to vials. 10 µl of 5% formic acid (prepared in acetonitrile) solution was added to the vials for injections in the LC-MS/MS.

2.2.2 Clean-up Methods

After centrifugation at the extraction stage, 6 mL of supernatant was taken into 15 mL tubes.

1. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes.

2. Clean-up method: In order to purify the extract from organic acids, lipids and sugars, 900 mg magnesium sulfate and 150 mg primary secondary amine (PSA) were added to the tubes and shaken for 30 seconds.

3. Clean-up method: In order to purify the extract from organic acids, lipids and sugars, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent were added to the tubes and shaken for 30 seconds.

4. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes. Then, 900 mg magnesium sulfate and 150 mg PSA were added to purify the extract from organic acids, lipids, and sugars; then shaken for 30 seconds.

5. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes. Then, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent were added to purify the extract from organic acids, lipids, and sugars; then shaken for 30 seconds.

After shaking, the tubes were centrifuged at 3000 rpm for 5 minutes in all clean-up methods. Then, 1 mL was taken from the supernatant of the tubes to determine the recovery values. It was filtered with a 0.45 µm PTFE filter and transferred to vials. 10 µl of 5% formic acid (prepared in acetonitrile) solution was added to the vials for injections in the LC-MS/MS. The working conditions of LC-MS/MS are given in Table 2.

Table 2. Instrument parameters.

Parameters	
Column	Poroshell 120 SB-C18, 3.0x100mm, 2.7µm
Column temperature (°C)	35
Mobile Phase A	Water (5 mM ammonium formate, 0.1% formic acid)
Mobile Phase B	Methanol
Gas temperature (°C)	300
Gas flow (L/min)	10
Nebulizer (psi)	40
Sheath gas temperature (°C)	250
Sheath gas flow (L/min)	11
Binary pump flow (mL/min)	0.45
Binary pump timetable	
0.5 min	A: 70% B: 30%
2.5 min	A: 30% B: 70%
11.5 min	A: 5% B: 95%
13.9 min	A: 5% B: 95%
14 min	A: 70% B: 30%
16.5 min	A: 70% B: 30%

Calibration curves with six levels and two replications were prepared at 5, 10, 25, 50, 100 and 200 µg/kg concentrations for each active ingredient using the matrix-matched calibration method. Correlation coefficients of all pesticide active ingredients analyzed were calculated as $R^2 > 0.99$. Data calculations and results on the device were made using the built-in software named MassHunter.

3. RESULTS and DISCUSSION

Pesticide mixtures prepared for 100 selected pesticide active ingredients in LC-MS/MS device were spiked into blank samples. Recovery studies at 50 µg/kg level with five replications were performed for seven different extraction methods. Relative Standard Deviation (RSD) values of the measured values were calculated and it was checked whether these values met the ≤ 20% condition. The RSD values for all analyzed pesticides met the condition of ≤ 20% and the average percent recovery values of five repeated measurements are given in Table 3.

Table 3. Average recovery values were determined in extraction methods.

	Average Recovery in Extraction Methods (%)						
Pesticides	1	2	3	4	5	6	7
Acetamiprid	99	95	114	99	99	91	100
Ametoctradin	67	68	81	68	70	54	67
Amitraz	78	64	68	62	58	54	60
Azoxystrobin	117	107	127	101	103	99	105
Bifenthrin	74	63	70	56	51	55	54
Boscalid	72	79	96	85	81	83	72
Bupirimate	102	91	106	85	78	80	87
Buprofezin	90	82	96	79	68	68	84
Carbendazim	74	83	71	66	67	69	82
Chlorpyrifos	76	68	73	67	60	50	57
Chlorpyrifos Methyl	78	64	82	58	61	64	80
Clofentezine	84	66	46	45	57	67	49
Clothianidin	82	95	80	84	96	109	87
Cyantraniliprole	69	75	63	80	68	98	55
Cyazofamid	77	72	90	82	67	77	81
Cyflufenamid	91	111	112	100	101	113	104
Cyflumetofen	85	73	84	71	70	63	78
Cymoxanil	74	68	80	68	66	61	69
Cypermethrin	120	147	157	156	151	159	149
Cyprodinil	78	73	91	73	54	59	66
DEET	95	91	100	87	82	75	84
Deltamethrin	127	129	122	136	58	148	100
Diafenthiuron	132	131	139	125	110	101	135
Diazinon	98	75	90	66	75	72	83
Difenoconazole	122	171	164	177	184	176	189
Dimethoate	81	79	96	84	75	71	83
Dimethomorph	132	139	143	136	123	125	125
Dodine	74	93	90	63	90	64	77
E-Fenpyroximate	95	97	88	88	68	76	82

Emamectin Benzoate	72	67	76	69	64	65	65
Famoxadone	66	107	101	137	80	107	88
Fenamidone	83	82	102	110	94	96	82
Fenbutatin oxide	68	60	63	58	63	66	63
Fenitrothion	93	101	109	65	91	73	76
Fenpropathrin	131	141	143	147	121	135	129
Fenthion	100	113	115	101	101	90	95
Fenthion Oxon	90	85	98	75	79	68	76
Fenthion Oxon Sulfone	92	86	107	92	72	77	78
Fenthion Oxon Sulfoxide	91	83	48	50	77	76	87
Fenthion Sulfone	113	106	126	92	103	90	100
Fenthion Sulfoxide	100	97	107	91	90	89	86
Fipronil	140	149	158	158	155	174	167
Flonicamid	73	94	103	101	94	92	81
Flubendiamide	150	178	183	163	163	158	144
Fludioxonil	72	94	77	84	71	67	72
Flufenoxuron	110	120	145	182	133	122	113
Fluopicolide	79	84	87	84	72	79	76
Fluopyram	90	78	89	80	80	71	77
Flusilazole	62	72	86	102	73	63	63
Fluxapyroxad	95	88	103	87	91	87	84
Hexythiazox	68	70	74	88	66	65	67
Imazalil	78	71	87	74	68	68	67
Imidacloprid	78	134	72	70	122	129	101
Indoxacarb	96	135	136	124	136	130	144
Isopyrazam	35	22	32	20	28	22	28
Kresoxim Methyl	102	84	101	82	90	86	87
Lambda Cyhalothrin	127	145	130	133	144	121	123
Malaoxon	96	92	102	83	87	82	88
Malathion	100	82	98	72	80	75	91
Methoxyfenozide	81	79	92	74	75	72	77
Metrofenone	101	77	87	73	83	89	95
Novaluron	109	162	146	152	175	152	169
Omethoate	64	64	76	70	63	65	65
Penconazole	67	88	93	102	79	79	77
Permethrin I	68	42	42	45	57	41	46
Permethrin II	62	48	52	58	60	48	44
Phenthoate	112	98	112	98	92	75	96
Phosalone	127	128	124	103	113	100	123
Phosmet	127	127	127	118	106	113	108
Phosmet Oxon	81	82	88	78	78	76	78
Pirimicarb	79	80	95	79	72	74	76
Pirimicarb Desmethyl	78	73	48	49	70	67	72
Propargite	89	93	97	86	81	74	82
Propiconazole	124	167	161	157	151	157	154
Proquinazid	89	86	91	91	42	44	85

Pyraclostrobin	83	61	74	60	67	69	72
Pyridaben	118	133	149	124	110	103	123
Pyrimethanil	54	63	67	56	41	43	59
Pyriproxyfen	79	82	86	76	71	69	75
Spinetoram	78	64	75	66	65	60	69
Spinosyn A	80	65	73	57	72	65	68
Spinosyn D	75	52	63	53	53	50	55
Spirodiclofen	118	112	111	101	92	88	101
Spiromesifen	70	57	72	52	45	47	47
Spirotetramat	115	106	156	161	139	142	108
Spirotetramat Enol	96	116	118	166	145	153	108
Spirotetramat Enol Glucoside	54	79	49	41	73	64	76
Spirotetramat Ketohydroxy	156	157	142	162	146	152	150
Spirotetramat Monohydroxy	113	105	158	165	149	138	113
Sulfoxaflor	93	77	90	68	71	71	87
Tau Fluvalinate	159	102	101	91	95	111	155
Tebuconazole	96	146	155	171	129	126	119
Tebufenozide	82	76	91	74	80	65	82
Tebufenpyrad	91	105	115	125	103	102	99
Teflubenzuron	131	109	126	85	99	101	89
Thiabendazole	60	66	52	57	60	62	65
Thiacloprid	81	76	92	82	71	73	73
Thiamethoxam	108	113	112	106	100	101	108
Thiophanate Methyl	72	64	104	85	78	72	64
Trifloxystrobin	100	60	79	61	71	63	74

When the Table 3 is examined, the extraction methods have respectively 72, 60, 64, 51, 55, 46, and 61 active substances in the recovery range of 70-120%. In this case, the highest recovery was obtained with the 1st extraction method. For this reason, the clean-up stage was continued with the 1st extraction method. The selected extraction method was tested via five different clean-up methods with five repetitions of recovery studies at 50 µg/kg. RSD values of the measured values were calculated and it was checked whether these values met the ≤ 20% condition. The RSD values for all analyzed pesticides met the condition of ≤ 20% and the average percent recovery values of the five repeated measurements are given in Table 4.

Table 4. Average recovery values are determined in clean-up methods.

	Average Recovery in Clean-up Methods (%)				
Pesticides	1	2	3	4	5
Acetamiprid	119	96	98	123	112
Ametoctradin	92	84	82	82	85
Amitraz	60	50	82	58	80
Azoxystrobin	126	115	112	128	118
Bifenthrin	15	25	86	20	80
Boscalid	94	92	92	100	103
Bupirimate	112	94	95	104	103
Buprofezin	96	93	92	97	95
Carbendazim	94	67	78	91	84

Chlorpyrifos	73	68	71	75	76
Chlorpyrifos Methyl	67	66	88	84	96
Clofentezine	75	78	78	78	89
Clothianidin	105	81	92	114	115
Cyantraniliprole	59	56	68	74	68
Cyazofamid	91	51	80	76	82
Cyflufenamid	103	89	107	120	116
Cyflumetofen	93	93	90	84	92
Cymoxanil	78	64	114	94	83
Cypermethrin	160	191	110	174	161
Cyprodinil	84	72	81	83	86
DEET	106	93	91	105	102
Deltamethrin	180	163	108	200	144
Diafenthiuron	140	139	153	152	151
Diazinon	110	99	92	114	114
Difenoconazole	138	119	120	143	176
Dimethoate	92	79	75	91	87
Dimethomorph	150	128	116	152	156
Dodine	75	48	70	53	33
E-Fenpyroximate	95	101	95	99	96
Emamectin Benzoate	82	76	71	79	71
Famoxadone	60	64	73	92	78
Fenamidone	99	90	95	96	109
Fenbutatin oxide	64	63	72	58	54
Fenitrothion	111	60	88	100	124
Fenpropathrin	141	153	124	170	148
Fenthion	107	100	102	112	120
Fenthion Oxon	88	88	75	96	85
Fenthion Oxon Sulfone	93	61	82	86	94
Fenthion Oxon Sulfoxide	84	65	96	99	98
Fenthion Sulfone	97	83	100	123	82
Fenthion Sulfoxide	113	91	92	108	110
Fipronil	138	168	118	166	159
Flonicamid	88	65	76	92	101
Flubendiamide	150	99	137	138	128
Fludioxonil	72	91	72	91	99
Flufenoxuron	104	125	102	144	118
Fluopicolide	90	93	100	113	111
Fluopyram	97	86	94	99	98
Flusilazole	64	60	71	68	66
Fluxapyroxad	93	86	87	104	106
Hexythiazox	72	75	76	80	84
Imazalil	80	75	75	83	82
Imidacloprid	133	90	102	137	153
Indoxacarb	142	102	100	164	167
Isopyrazam	56	53	68	58	60
Kresoxim Methyl	119	102	87	111	112
Lambda Cyhalothrin	118	137	118	146	118

Malaoxon	107	91	91	110	107
Malathion	108	84	87	113	113
Methoxyfenozide	89	88	87	94	98
Metrofenone	107	100	107	121	118
Novaluron	148	147	94	132	78
Omethoate	74	65	72	71	70
Penconazole	77	70	76	79	81
Permethrin I	58	60	72	50	68
Permethrin II	62	66	76	58	62
Phenthoate	106	99	89	111	111
Phosalone	132	107	124	149	145
Phosmet	86	107	116	133	142
Phosmet Oxon	132	85	87	97	99
Pirimicarb	84	90	91	97	97
Pirimicarb Desmethyl	84	75	73	87	86
Propargite	96	97	98	106	104
Propiconazole	133	123	120	142	141
Proquinazid	89	90	91	91	86
Pyraclostrobin	92	89	84	87	96
Pyridaben	122	130	114	144	118
Pyrimethanil	56	60	64	55	63
Pyriproxyfen	90	89	91	95	96
Spinetoram	88	87	77	75	83
Spinosyn A	91	87	80	84	89
Spinosyn D	73	77	82	64	75
Spirodiclofen	123	113	103	118	105
Spiromesifen	46	58	80	55	74
Spirotetramat	143	96	116	127	132
Spirotetramat Enol	124	93	106	130	125
Spirotetramat Enol Glucoside	120	57	72	77	82
Spirotetramat Ketohydroxy	69	90	118	159	108
Spirotetramat Monohydroxy	162	123	104	148	160
Sulfoxaflor	92	67	96	103	93
Tau Fluvalinate	116	162	114	127	120
Tebuconazole	99	88	118	114	102
Tebufenozide	83	90	91	87	92
Tebufenpyrad	96	81	89	125	106
Teflubenzuron	120	162	154	93	111
Thiabendazole	74	65	78	76	70
Thiacloprid	88	77	79	89	91
Thiamethoxam	125	93	96	131	128
Thiophanate Methyl	86	99	107	112	118
Trifloxystrobin	111	104	93	97	109

When Table 4 is examined, the clean-up methods have respectively 66, 61, 92, 61, and 74 active substances in the recovery range of 70-120%. In order to get accurate results in multi-residue analyzes and to eliminate the matrix effect in chromatographic devices, matrix-matched calibrations should be used. For this reason, it has a great importance to pay attention to the fact

that the blank bee samples are not contaminated with any pesticides during the collection. In addition, it is one of the most important issues to keep the samples in dry ice for at least three hours to achieve a good homogenization. Otherwise, as the unfrozen bee bodies will remain soft, and effective homogenization process will not be possible. The main principle of the QuEChERS method is the extraction of the sample homogenized with the help of acetonitrile in foods of plant origin. However, in this method, it is necessary to add water at certain rates in samples with water content below 80% (EN 15662, 2018). According to this research, the water ratio of the body of bees is below 80% on average (Burdine and McCluney, 2019; Li *et al*., 2012). For this reason, homogenized samples were weighed and different solvent combinations were tried with different amounts of water additions. 100 active substances selected in LC-MS/MS were spiked to the blank samples at the level of 50 µg/kg. As a result of this process, the RSD values of the recovery studies with 5 replications met the condition of ≤20%. In terms of average percentage recovery values, the 1st extraction method ranks first with 72 active substances between 70-120%. Therefore, since this extraction method was found to be more successful than other methods, the clean-up was continued with this method. In addition, different clean-up methods in the QuEChERS method were tested by modifying the waiting times and adding chemicals. After the first extraction step, clean-up methods were tested for average percent recovery values. The third clean-up method is in the first place, with 92 active substances between 70-120% meeting the RSD ≤20% condition. While the number of active substances with suitable recovery values in the selected extraction method was 72, it increased to 92 with the continuation of the clean-up method. Thus, a success rate of 92% was achieved in general. The working principle of this method is formed by combining extraction and clean-up methods as follows: The samples are treated with dry ice at a temperature of -78.5°C for 3 hours to allow them to freeze thoroughly. From the homogenized samples, 10 g is weighed into 50 mL centrifuge tubes, 2.5 mL of cold water is added to them, shaken for 1 minute and waited for 5 minutes. Then, 10 mL of acetonitrile, 50 µl of ISTD (10 mg/kg Triphenyl Phosphate) are added and shaken for 2 minutes. After this stage, 4 g anhydrous magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate buffer salts are added to the tubes, shaken for 1 more minute and centrifuged at 3000 rpm for 5 minutes. Then, 6 mL of the supernatant part of the extract is taken into 15 mL tubes after centrifugation to purify the extract from organic acids, lipids, and sugars. Then, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent are added and shaken for 30 seconds. Then it is centrifuged again at 3000 rpm for 5 min. 1 mL is taken from the tube and transferred to vials by passing through a 0.45 µm PTFE filter. 10 µl of 5% formic acid (prepared in acetonitrile) solution is added to the vials to be given to the LC-MS/MS device.

When this research is compared with similar research, it is seen that the results are supported. For example, Bargańska *et al*. (2018) conducted method studies to determine a broad spectrum of pesticide residues in honey bee samples after modification of QuEChERS extraction with GC-MS/MS and LC-MS/MS. The results proved that the QuEChERS protocol together with LC and GC techniques meets the requirements of green analytical chemistry and thus can be used as a tool in environmental monitoring. The recovery was found to be 85.5-103.5% for honey bee samples. Vernich *et al*. (2016) made a comparison between QuEChERS and other pesticide extraction procedures for honey and honeybee matrices. The results proved that the QuEChERS protocol is the most effective method for the detection of selected pesticides in both matrices. When reviewing at the qualitative studies in Turkey, conducted between 2006-2010 in the Laboratory of Pharmacology-Toxicology Department of Pendik Veterinary Control and Research Institute, it was aimed to evaluate the results of pesticide analysis because of suspicious bee deaths. In the suspected poisoning case, pesticide analyzes were performed on materials such as bee, honeycomb, sunflower, grass, and tree leaves. Analyzes were made qualitatively using Gas Chromatography, Gas Chromatography Mass

Spectrometer, Liquid Chromatography, and Liquid Chromatography Mass Spectrometer devices. In the analyzes, 15 insecticides, 6 naphthalene, 3 herbicides, 1 fungicide, 1 antiseptic/disinfectant and 1 growth hormone were determined (Unal *et al*., 2010). In another research between 2015-2018, 188 different dead bee samples, which came to Cukurova region for wintering, were collected from beekeepers and brought to Adana Veterinary Control Institute on suspicion of pesticide poisoning were examined. Qualitative analysis was performed with a gas chromatography (GC) device. In the examination of dead bee samples, tau-fluvalinate residues were found in 2 samples from 2015, and cypermethrin residues were found in 1 sample from 2016 (Segmenoglu, 2020).

When these researches on bees in our country are examined, it is seen that the results have been determined qualitatively. However, it has great importance to carry out quantitative analyzes in order to evaluate the results according to the maximum residue limits in pesticide analysis. Checking the suitability of pesticide analyzes in foods is carried out according to maximum residue limits. If the results are below these limits, it is considered not to be hazardous to health. It is mentioned in the literature that a similar approach is applied in pesticide analyzes to be performed on bees (Kasiotis *et al*., 2014; Naggar *et al*., 2015; *Kiljanek et al*., 2016). In these studies, LD_{50} values in bees are examined to determine if the pesticides detected cause the death of bees. In addition, six bee samples were sent to a laboratory in Germany by the Ministry of Agriculture and Forestry of Turkey for neonicotinoid analysis because the conditions of the domestic laboratories were not suitable in 2018 (Oruc and Cayci, 2019). Up to date, there is no residue laboratory in Turkey that has accreditation by the Turkish Accreditation Agency in pesticide analysis of bees.

4. CONCLUSION

It can be accepted that pesticides detected above LD_{50} values may cause bee death. Otherwise, qualitative analysis cannot be a proof that pesticides that have low toxicity cause death in bees because of non-absolute numerical results. Therefore, it would not be a correct approach to use the phrase that pesticides with low LD_{50} values and low toxicity for bees caused the death of bees.

Pesticides with LD_{50} values above the colony health of bees will create a pesticide load on bees, so the use of pesticides with low LD_{50} values and high toxicity should be avoided in plant production areas. Farmers should spray in areas with hives, especially in the evening when the bees are not flying. In addition, beekeeping activities should be carried out carefully in regions where polyculture agriculture is abundant. It is of critical importance that farmers do not apply pesticides during the flowering periods of the plants and during the hours when the bees are active. If it is not possible to do all these, colonies should be removed from these regions. Despite all the precautions to be taken, in case of bee deaths in front of the hive, samples should be taken quickly with the suspicion of possible pesticide poisoning. It is recommended to validate using this modified QuEChERS method, which is presented to the residue laboratories that will analyze the samples. It is then recommended to determine pesticide exposure in bees and to take prompt measures early without causing serious losses. This presented method will not only guide method validation studies for pesticide analysis in bees in Turkey but also avoid the need to send bee samples abroad on the grounds that laboratory conditions are not suitable.

As a result of using proposed modified QuEChERS method, it will be possible to obtain both qualitative and quantitative results in multi residue analyzes in bees. Consequently, it is possible to obtain fast, accurate and reliable results with the validation studies to be carried out with the aforementioned modified QuEChERS method, which is a combination of the 1st extraction and 3rd clean-up methods, which allows quantitative analysis of pesticides in bees. It

is recommended that the modified QuEChERS method be used in pesticide residue analyzes in bees, with its remarkable advantages such as completing the analyzes in less than six hours, working easily with the infrastructure available in all control laboratories, low analysis costs, and being easy and reliable.

Acknowledgements

This study includes a part of Orhan Dincay's Ph.D. thesis named "Determination of Pesticide Residues by LC-MS/MS and GC-MS/MS Mass Spectrometry Using the Modified QuEChERS Extraction Method in Mass Mortalities Observed Honey Bees and Honey".

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Orhan Dincay: Investigation, Method development, Sample preparation, Method validation, LC–MS/MS analysis, Statistical analysis, Writing-review & editing. **Hasan Sungur Civelek:** Investigation, Writing-review & editing, Supervision.

Orcid

Orhan Dincay https://orcid.org/0000-0002-7915-1068
Hasan Sungur Civelek https://orcid.org/0000-0003-2316-9813

5. REFERENCES

Akdogan, A., Divrikli, U., & Elci, L. (2012). Importance of pesticides and their effects on ecosystem. *Academic Food Journal, 10(1),* 125-132.

Alaux, C., Brunet, J.C., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P., & Le Conte, Y. (2010). Interactions between Nosema microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology, 12(3)*, 774-782. https://doi.org/10.1111/j.1462-2920.2009.02123.x

Bargańska, Ż., Konieczka, P., & Namieśnik, J. (2018). Comparison of two methods for the determination of selected pesticides in honey and honeybee samples. *Molecules*, *23(10)*, 2582. https://doi.org/10.3390/molecules23102582

Burdine, J.D., & McCluney, K.E. (2019). Differential sensitivity of bees to urbanization driven changes in body temperature and water content. *Scientific Reports, 9:1643*, 1-10. https://doi.org/10.1038/s41598-018-38338-0

EN 15662. (2018). European Standard, Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method.

Karahan, A., Yıldırım, F., Karahan, M., & Karaca, İ. (2018). Does Imidacloprid Cause Winter Losses in Honey Bees? *Arıcılık Araştırma Dergisi, 10(2),* 54-60.

Karahan, A., Acar, İ., Kutlu, M.A., & Karaca, İ. (2019). Effect of Prohibited or Restricted Neonicotinoids on Bees in Turkey. 2nd International Agricultural Congress, Ankara, Turkey, 21-24 Nov 2019, p. 11-16.

Kasiotis, K.M., Anagnostopoulos, C., Anastasiadou, P., & Machera, K. (2014). Pesticide Residues in Honeybees, Honey and Bee Pollen By LC–MS/MS Screening: Reported Death

İncidents in Honeybees. *Science of the Total Environment, 485–486,* 633–642. https://doi.org/10.1016/j.scitotenv.2014.03.042

Kiljanek, T., Niewiadowska, A., Semeniuk, S., Gawel, M., Borzecka, M., & Posyniak, A. (2016). Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry-Honeybee poisoning incident. *J. Chromatogr. A, 1435*, 100-114. https://doi.org/10.1016/j.chroma.2016.01.045

Li, Y., Wang, S., & Qin, Y. (2012). Supercooling Points of *Apis mellifera ligustica* when Performing Different Age-Related Tasks. *Journal of Apicultural Science, 56 (1),* 107-114. https://doi.org/10.2478/v10289-012-0012-z

Naggar, Y.A., Codling, G., Vogt, A., Naiem, E., Mona, M., Seif, A., & Giesy, J.P. (2015). Organophosphorus Insecticides in Honey, Pollen and Bees (*Apis mellifera* L.) and Their Potential Hazard to Bee Colonies in Egypt. *Ecotoxicology and Environmental Safety, 114,* 1–8. https://doi.org/10.1016/j.ecoenv.2014.12.039

Oruc, H.H., & Cayci, M. (2019). Honey Bee Deaths with Suspected Poisonings in Turkey. I. International VI. Veterinary Pharmacology and Toxicology Congress, Kayseri, Turkey, 4-7 Sep 2019, p. 161-170.

Polat, B., Ozuicli, M., Cetin, H., & Aydin, L. (2020). The Effects of Pesticide Usage on Honey Bee Health and Products. *J Res Vet Med., 39(2),* 128-134. https://doi.org/10.30782/jrvm.634586

Segmenoglu, M.S. (2020). The Effect of Pyrethroid Group Pesticides on Honey Bee Deaths in Çukurova Region. *TURJAF, 8(3),* 594-597. https://doi.org/10.24925/turjaf.v8i3.594-597.3025

Unal, H.H., Oruc, H.H., Sezgin, A., & Kabil, E. (2010). Determined Pesticides After Honey Bee Deaths Between 2006 and 2010 in Turkey. *U. Bee J., 10(4),* 119-125.

Vernich, P.C., Calatayud, F., Simó, E., & Picó, Y. (2016). Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees. *MethodsX, 3,* 452-458. https://doi.org/10.1016/j.mex.2016.05.005

Wu, J.Y., Smart, M.D., Anelli, C.M., & Sheppard, W.S. (2012). Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to Nosema (Microsporidia) infection. *Journal of Invertebrate Pathology, 109,* 326-329. https://doi.org/10.1016/j.jip.2012.01.005

Yavuz, O., & Aksoy, A. (2016). The Methods Used in Pesticide Analysis. *Türkiye Klinikleri J Vet Sci Pharmacol Toxicol-Special Topics. 2(2),* 89-100.