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Development of a Gas Chromatography - Tandem Mass Spectrometry Procedure for Determination of Pesticide Residues in Honey and Honeybee Samples

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Abstract

A new analytical procedure of determination of multiresidue of pesticides based on application of the modified QuEChERS approach at the step of sample preparation and technique GC-MS/MS has been developed and applied for studies the real honey and honeybee samples. Samples were collected from northern Poland (Pomerania). The proposed procedure enables for the determination of 34 and 30 pesticide residues respectively in honey and honeybee samples. The recovery of analytes was between 70 and 120% with relative standard deviation ≤ 20%. The limits of quantification were in the range of 2.3-27 ng/g and 2.2-29 ng/g respectively for honey and honeybee samples. The proposed procedure was proven to be a powerful, highly sensitive, and environmentally friendly analytical tool that requires minimal sample preparation.

Keywords: Environmental monitoring; Honey; Honeybees; GC-MS/MS; Pesticides; QuEChERS Method

Abbreviations

ACN: Acetonitrile; CCD: Colony Collapse Disorder; CME-UABE: Microextraction Ultrasound-Assisted Back-Extraction; CRMs: Certified Reference Materials; DDD: 1,1-dichloro-2,2-bis(4-chlorophenyl) ethane; DLLME: Dispersive Liquid–Liquid Microextraction; DSPE: Dispersive Solid Phase Extraction; GCB: Graphitized Carbon Black; GC-MS/MS: Gas Chromatography-Tandem Mass Spectrometry; GPC: Gel Permeation Chromatography; HMF: 5-hydroxymethylfurfural; IS: Internal Standard; LLE: Liquid-Liquid Extraction; LOD: Limit Of Detection; LOQ: Limit Of Quantification; LPME: Liquid-Phase Micro Extraction; MDL: Method Detection Limit; $MgSO_4$: Magnesium sulfate anhydrous; MQL: Method Quantification Limit; MRLs: Maximum Residue Levels; MRM: Multiple Reaction Monitoring; MSPD: Matrix Solid-Phase Dispersion; NaCl: sodium chloride; Na_2EDTA: disodium ethylenediaminetetraacetic; ND: Not Detected; NH_4OH: Ammonium hydroxide solution; PLE: Pressurized Solvent Extraction; PSA: Primary Secondary Amine; PTV: Programable Temperature Vaporizer; PVDF: Membrane of Polyvinylidene fluoride; QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe; RSD: Residual Standard Deviation; SD: Standard Deviation; SPE: Solid Phase Extraction; TPP: Triphenyl Posphate

Introduction

Pesticides are widely present in the environment, including water and soils, and foodstuffs, as a result of the application of phyto sanitary products in modern agriculture. In the last years, new pesticides, which show a more specific mode of action and have a higher polarity and lower persistence than old ones, have been developed [1]. The slow degradation of these compounds in the environment and extensive or inappropriate use by farmers can lead to the contamination of ecosystem in which the honeybees operate. Therefore, these insects can constitute reliable indicators of environmental quality because their intense foraging activity brings them into contact with a large number of pollutants within a radius that generally ranges from 1.5 to 3 km around the hive, depending on food abundance [2]. Moreover, the contaminants can be transported on bee bodies or with the forages to the hive, from where they can be transferred into honey [3].

Honey consists mainly of monosaccharides (ca. 70%), oligosaccharides (ca. 7%), water (ca. 18-20%) as well as other compounds from different chemical classes (essential elements, organic acids, proteins and amino acids, enzymes, flavonoids, anthocyanins, vitamins, sterols, phospholipids, essential oils and pigments), a total of approximately 300 compounds [4]. Honey is a food product with world-wide consumption especially among children and in terms of food safety concern it must be free of chemical contaminants particularly from pesticide residues [5]. The presence of such xenobiotics in honey can decrease their quality and devalue their properties [6-12]. In the recent years in many countries is observed increased mortality of bee colonies. Honeybee's death incidents are of great concern, because declines in bee populations in lesser extent might have detrimental impact on agriculture and environment. The increased mortality may affect for some crops, pollination and disturb the stability of the agricultural ecosystems [13]. The phenomenon of mass extinction of bees has been called Colony Collapse Disorder (CCD) [14]. CCD may be triggered not only by incompetent use of pesticides but also by mistakes made by the beekeepers in fighting pathogenic microorganisms and parasites. For example, *Varroa mite* [15] is treated with varroacides applied to plywood strips suspended between brood frames. Many synthetic varroacides (tau-fluvalinate, coumaphos, amitraz, and fenpyroximate) that are available today to beekeepers are lipophilic and may remain in hives for years following treatment [16-18]. Chronic exposure of honeybees to pesticides at concentrations that could approximate field-level exposure impairs natural foraging behavior and increases worker

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mortality leading to significant reductions in brood development and colony success [4]. Consequently, determination of pesticide residues in honey and honeybee samples is very important. Determination of pesticides in honey and honeybees at trace levels is a challenging task due to the complex sample matrix. Honeybees contain a large amount of beeswax, proteins and other interfering compounds which have an adverse effect on the results of analysis [19]. Therefore, clean-up stage prior to analysis is often necessary. Matrix solid-phase dispersion (MSPD) [15,16,17] or solid phase extraction (SPE) followed by clean-up using gel permeation chromatography (GPC) [4,19] and other modification this technique is the most frequently utilized for honeybee samples preparation stage. In turn, for honey samples in addition to the already mentioned extraction techniques during the preparation of the sample are also used liquid-liquid extraction (LLE), pressurized solvent extraction (PLE), dispersive liquid–liquid microextraction (DLLME), microextraction ultrasound-assisted back-extraction (CME-UABE) [1,5,20,21] or solid-phase microextraction (SPME) [22,23], liquid-phase microextraction (LPME) and their modification [24]. However, most of presented procedure of preparing samples for analysis allows only for the determination of selected analytes from the group of pesticides. Therefore, the new ways for preparation of the sample for analysis are necessary in order to determine the widest possible spectrum of pesticides. One of the most popular and useful techniques of extraction of analytes from the group of pesticides used in suitable analytical procedures is QuEChERS approach (stands for, Quick Easy Cheap Effective Rugged Safe) first introduced in 2003 [25]. This technique including a lot of it modifications ensures excellent extract clean-up and high analytes recovery [26] in application to different food matrices such as fruits and vegetables [27-32], fruit juices [33], raisings and wheat flour [34], cereals and fish tissue [35], rice paddies [36], soil [37], olives and olive oil [38], milk, eggs, avocado [39], honey [40-43], pollen [44] and honeybees [45,46]. In this paper the new procedure of determination of pesticide residues in honeybee and honey samples has been proposed and validated. The procedure is based on application of the QuEChERS approach in combination with gas chromatography–tandem mass spectrometry (GC–MS/MS) used to determined pesticide residues in honeybee and honey samples. The developed methodology allows determining 34 pesticides in honey and 30 pesticides in honeybee samples. The samples of dead honeybees and honey was collected from the most contaminated areas of Pomerania in Poland (Tczew, Gdansk, Kartuzy) from suspected pesticide poisoning [46,47]. The methodology was optimized and next it was validated according to the regulation "Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed" [48]. This methodology allows for the monitoring of pesticides belonging to various classes for use of bee organisms and their products as the indicators of environmental contamination.

Materials and Methods

Reagents and chemicals

The solution of triphenyl phosphate (TPP), analytical grade, used as an internal standard was purchased from Sigma Aldrich (Schnelldorf, Germany). The Certified Reference Materials (CRMs) standard solutions of bifenthrin, diazinon, dimethoathe, heptenophos, pyrazophos, tau-fluvalinate, vinclozolin, cypermethrin, fenitrothion, azinphos ethyl and malathion were purchased from LGC Standards (Łomianki, Poland), CRM solution of alachlor, aclonifen, ancymidol, chlorpyrifos-methyl, dazomet, dieldrin, endosulfan (alfa isomer), o,p`-DDD, parathion, parathion-methyl, pentachlorophenol, phenthoate, pirimiphos-methyl, prothioconazole, pyriproxyfen and triticonazole were obtained from Sigma Aldrich (Schnelldorf, Germany), CRM solution of fenthion, carbosulfan, quinalphos, metconazole, methidathion, prosulfocarb and triazophos were obtained from Ultra Scientific (North Kingston, RI, USA) and CRM solutions of dimoxystrobin and haloxyfop-R methyl were purchased from Dr Ehrenstorfer GmBH (Germany). The stock standard solutions were stored at -18°C. The calibration standards and working standards were prepared by dilution with acetonitryle on the day of analysis.

Acetonitryle (Chromosolv®, ≥ 99.9%) and n-hexane (Envisolv®, 95%) were obtained from Fluka (Sigma–Aldrich, Germany). Water was purified with a Milli-Q water system (Millipore Corporation, USA). The QuEChERS kits with salt packets containing 4 g of anhydrous $MgSO_4$ and 0.5 g of sodium chloride, as well as, two-milliliter centrifuge tubes with 150 mg anhydrous magnesium sulphate and 25 mg primary secondary amine (PSA) for dispersive solid phase extraction (dSPE) were purchased from Agilent Technologies (USA).

Sample collection

Fifteen honey and fifteen honeybee samples were collected from the apiaries located area in northern part of Poland (Pomerania). The samples were supplied to the Department of Analytical Chemistry by representatives of the Regional Beekeepers Association in Gdansk (Poland). Honey samples were packaged in glass vessels and were kept at -10°C until analysis. Honeybee samples were immediately freeze-dried and stored at -18°C until analysis. Figure 1 presents location of the samples collection area in northern part of Poland (Pomerania).

Sample preparation

The samples were prepared in accordance with modification of already developed and published procedure [46,47]. Honey samples were thoroughly homogenized and approximately 1 g of the homogenate was weighed into a polypropylene centrifuge tube (50 mL) containing 10 mL of ACN, 10 mL of water and 50 µL of internal standard solution (TPP at 100 mg/mL). The content was shaken manually and the QuEChERS salt kit was added. The mixture was immediately hand-shaken for 1 min and subsequently centrifuged at 4400 RPM for 3 min. Thereafter, 1 mL of the acetonitrile fraction was transferred into

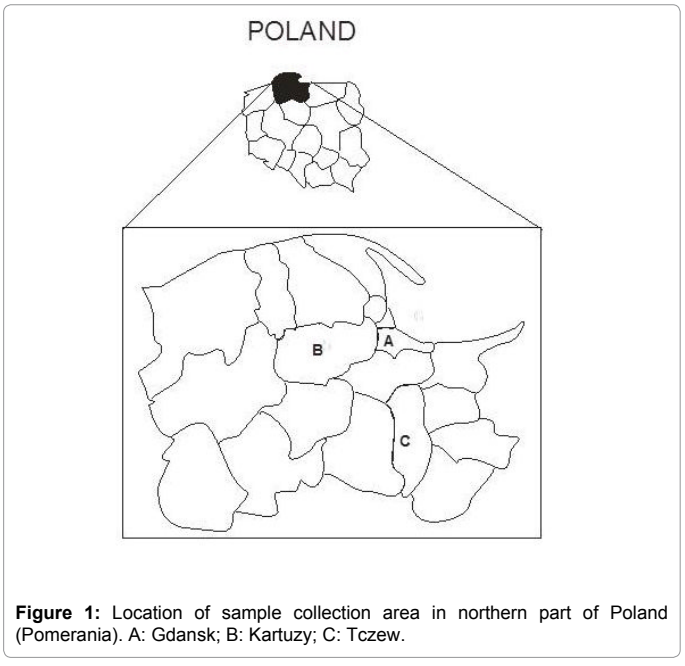

Figure 1: Location of sample collection area in northern part of Poland (Pomerania). A: Gdansk; B: Kartuzy; C: Tczew.

a 2 mL dSPE polypropylene tube. The tube was shaken for 1 min and centrifuged for 1 min at 5000 RPM. Finally, 0.5 mL of the supernatant was taken into a glass autosampler vial. The laboratory samples of freeze-dried honeybees were thoroughly homogenized. Approximately 1 g of sample was weighted into a polypropylene centrifuge tube (50 mL) and 10 mL of ACN, 10 mL of water, 3 mL of n-hexane, and 50 µL of internal standard solution (TPP at 100 µg/mL) were added. The tube content was hand-shaken. Subsequently, content of the salt kit QuEChERS was added. The mixture was immediately hand-shaken for 1 min and centrifuged at 4400 rpm for 3 min. Afterwards, 1 mL of the acetonitrile fraction (below the n-hexane fraction) was transferred to 2 mL dSPE polypropylene tube containing. The tube was shaken by hand, vortexed for 1 min and then centrifuged at 5000 rpm for 1 min. Finally, the 0.5 mL aliquot of the supernatant was transferred into a glass autosampler vial.

GC-MS/MS analysis

The prepared extracts were measured using a GC–MS/MS system from Agilent Technologies (USA). The system was equipped with a 7890A GC system, a 7000 MS/MS system, a programmed temperature vaporizer (PTV) injector, and a 7693A autosampler. The chromatographic separation was performed on a HP-5MS UI 0.25 µm, 0.25 mm × 30 m column (Agilent Technologies, USA) with precolumn (quartz capillary tube, 1 m). The column was set at a constant pressure. The mass spectrometer was operated in MRM mode. Helium gas (>99.999%) was used as carrier gas and quench gas, while nitrogen gas (>99.999%) was used as collision gas. The flow rate of collision and quench gas was 1.5 mL/min and 2.25 mL/min, respectively.

The column temperature was programmed as follows: the initial temperature was 60°C (for 1 min) and increased to 120°C at 40°C/min, ramped to 310°C at 5°C/min (for 0 min). The total run time was 40.5 min. The injection was programmed as follows: the initial temperature was 60°C (for 1.06 min) and ramped to 280°C at 600°C/min (for 0 min). The flow of gas during the evaporation was 100 mL/min. The flushing dispenser was 60 mL/min at 3.56 min and the dosing rate was 25 mL/min. The injection volume of extract sample was 10 µL. For MS/MS detection, the electron impact (EI) was used with 70 eV. For the multiple reaction monitoring (MRM) measurements, the collision energies and retention time are shown in Table 1. Example MRM chromatogram obtained during analysis of extracts from blank sample in acetonitrile spiked at 100 ng/mL (mix of pesticides) are illustrated in Figure 2.

Results and Discussion

Matrix effects

Literature information and results of our previous studies of honeybee and honey extracts [46,47] revealed large amounts of lipids and pigments especially in honeybees, which proved to be the most serious interferences during the analysis. Wax is a very complex mixture of lipophilic compounds, including esters of long-chain aliphatic alcohols with fatty acids or hydroxy-fatty acids, long-chain hydrocarbons, freelong-chain fatty acids, and carotenoids [19,49]. High molecular weight compounds that are lipid-like and long-chain fatty acids can reduce signal and/or cause column damage; therefore, additional cleanup is necessary to reduce the matrix constituents before injection [50]. In the current study, the analytical curves in acetonitrile and blank matrix extract were constructed to evaluate and compensate for the matrix effect (ME) and to assess the linearity according to the procedures for method validation suggested by SANCO [48]. So the matrix effects (ME) were evaluated by comparing the slope of the calibration curve in matrix with the slope of the calibration curve in solvent according to the following equation [51]

$$ME(\%) = \left(\left(\frac{\text{slope of calibration curve in matrix}}{\text{slope of calibration curve in solvent}} \right) - 1 \right) \times 100 \quad (1)$$

On the Figure 3 are shown the range of matrix effects for selected pesticides that can be determined in honeybees and honey using the dispersive SPE cleanup step in QuEChERS techniques. When the values found for the ME are between -20% and +20%, it is considered low; if they are between -50% and -20% or between +20% and +50%, it is considered medium; and if these values are below -50% or above +50% the ME is considered high [52]. The variation in matrix effects was dependent on the physical and chemical properties of the pesticides and the matrix. Moreover we can see that the influence of the matrix is considered low or medium.

Analytical protocol performance

The proposed procedure has been evaluated according to the Method Validation and Quality Control Procedures for Pesticides Residues Analysis in Food and Feed [48] in terms of repeatability, linearity and recovery. The limits of detection (LOD) and quantification (LOQ) were estimated based on the preliminary calibration curve in acetonitrile, in the concentration range of 2-100 ng/g. The LOD was calculated using the fallowing dependence LOD=3.3 × SD/b, where b is the slop of calibration curve and SD is a residual standard deviation of the calibration curve. The limit of quantification was calculated as LOQ=3 × LOD. The appropriate matrix-matched calibration was made at two levels of concentrations: 3LOD, 20 and 50 ng/g with addition of 100 ng/g TPP as the internal standard. Calibration curves were constructed by normalization of peak area of each analyte to IS at each calibration level were in accordance with the specifications of SANCO (lower than 20%). Information of the method detection and quantification limits for honey and honeybee samples are given in Table 2. The recovery of the analytes and repeatability studies were performed at three levels of fortification, 3LOD, 20 ng/g and 50 ng/g, by adding known quantities of pesticides and TPP to honey or honeybee samples, each in five replicates (n=5). The mean recovery ranged from 70-120% with relative standard deviation ≤ 20% as recommended by the SANCO Guideline (Figure 3). Table 3 compares selected parameters of presently used analytical methods based on the application of QuEChERS approach for the determination of pesticide residues in honey and honeybee samples including the parameters the proposed method (Figure 4A and 4B).

The main differences between the developed procedure and other published methods for determination of pesticides residues in honey and honeybee samples are on the stage of extraction and clean-up (Table 4). Based on the data presented in Tables 3 and 4 it can be concluded that the developed methodology is the new research concerning the possibilities for determination of compounds from group of pesticides in honey and honeybee samples in comparison with other already published methods. In addition, the analytical procedure characterized by an estimated low cost of analysis, short time of sample preparation and uses small amounts of the sample. The major advantages of this method are analysis of solvent extracts of honey and honeybee samples for determination of a wide range of pesticide residues in one analytical cycle.

Application to real samples

The multiresidue method for pesticide residue analysis described above was used to monitor content of 34 pesticides in honey and 30

No.	Compounds	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Colision energy [eV]
1	Heptenophos	124.0	89.0	11.740	10
		124.0	63.0		35
		108.9	78.9		5
2	Dazomet	161.9	89.0	14.790	5
		89.0	46.0		15
		129.1	86.0		15
3	Dimethoathe	86.9	46.0	14.790	15
		86.9	86.0		10
		92.9	63.0		5
4	Pentachlorophenol	265.9	167.0	15.661	25
		267.9	167.0		
		265.9	165.0		
5	Diazinon	137.1	84.0	16.302	10
		137.1	54.0		20
		199.1	93.0		15
6	Chlorpyrifos-methyl	124.9	47.0	18.065	15
		124.9	78.9		5
		285.9	92.9		20
7	Fenitrothion	125.1	47.0	18.065	15
		125.1	79.0		5
		277.0	260.1		5
8	Fenthion	278.0	109.0	18.067	15
		124.9	47.0		10
		124.9	79.0		5
9	Parathion-methyl	262.9	109.0	18.067	10
		125.0	47.0		10
		125.0	79.0		5
10	Vinclozolin	197.9	145.0	18.092	15
		187.0	124.0		20
		212.0	172.1		15
11	Alachlor	188.1	160.2	18.360	10
		188.1	132.1		15
		160.0	132.1		10
12	Prosulfocarb	91.0	65.0	18.716	15
		128.0	86.1		0
		251.0	128.2		5
13	Pirimiphos-methyl	290.0	125.0	19.214	20
		232.9	125.0		5
		232.9	151.0		5
14	Malathion	126.9	99.0	19.580	5
		157.8	125.0		15
		172.9	99.0		5
15	Parathion	138.9	109.0	19.972	5
		138.9	81.0		15
		290.9	109.0		10
16	Quinalphos	146.0	118.0	21.580	10
		146.0	91.0		30
		157.0	129.1		15

17	Phenthoate	121.0	77.0	21.586	25
		274.0	125.0		15
		274.0	121.0		10
18	Methidathion	144.9	85.0	22.075	5
		144.9	58.1		15
		85.0	58.0		5
19	Haloxyfop-R methyl	374.9	315.9	22.283	10
		315.9	90.9		20
		315.9	271.9		20
20	Endosulfan (alfa isomer)	194.9	159.0	22.352	5
		194.9	160.0		5
		194.9	125.0		20
21	Dieldrin	262.9	193.0	23.309	35
		277.0	241.0		5
		262.9	191.0		35
22	o,p'-DDD	235.0	165.2	23.663	20
		235.0	200.2		10
		237.0	165.2		20
23	Prothioconazole	186.0	53.0	23.864	20
		186.0	117.0		10
		186.0	89.0		10
24	Ancymidol	228.1	121.1	24.375	15
		121.1	78.1		25
		215.1	107.1		10
25	Aclonifen	212.1	182.2	24.944	10
		212.1	155.1		25
		264.1	194.2		15
26	Triazophos	161.2	134.2	25.652	5
		161.2	106.1		10
		161.2	91.0		15
27	TPP	326.0	325.0	26.987	5
		214.9	168.1		15
		232.9	215.1		10
28	Carbosulfan	118.0	76.0	28.019	5
		164.0	149.0		10
		164.0	103.1		25
29	Dimoxystrobin	116.0	89.0	28.115	15
		116.0	63.0		30
		205.0	116.0		10
30	Bifenthrin	181.2	165.2	28.217	25
		181.2	166.2		10
		166.2	165.2		20
31	Triticonazole	234.8	217.1	29.312	5
		234.8	182.1		10
		216.9	165.1		25

32	Pyriproxyfen	136.1	78.1	29.539	20
		136.1	96.0		15
		321.0	222.0		10
33	Pyrazophos	221.0	193.1	30.601	10
		221.0	149.0		15
		232.0	204.1		10
34	Azinphos ethyl	132.0	77.1	30.612	10
		160.0	77.1		20
		160.0	132.1		0
35	Cypermethrin	163.0	91.0	33.700	10
		163.0	127.0		5
		164.9	91.0		10
36	tau-fluvalinate	250.0	55.0	35.479	40
		250.0	200.0		
		181.0	152.0		

Table 1: The specific MRM transitions for all the analytes (retention time and ion transition for quantification).

		Honeybees		Honey	
MRLs for honey [ng/g] [53]	**Compounds**	**MDL [ng/g]**	**MQL [ng/g]**	**MDL [ng/g]**	**MQL [ng/g]**
-	Aclonifen	3.2	9.7	3.3	10
-	Alachlor	-		2.7	8.2
-	Ancymidol	3.2	9.5	3.3	9.9
-	Azinphos ethyl	4.0	12	4.0	12
-	Bifenthrin	2.7	8.2	2.8	8.5
-	Chlorpyrifos-methyl	4.0	12	4.3	13
50	Cypermethrin	2.7	8.2	2.9	8.8
-	Dazomet	4.0	12	4.0	12
-	Diazinon	-		2.8	8.3
10	Dieldrin	3.7	11	4.0	12
-	Dimethoathe	2.8	8.5	2.8	8.5
50	Dimoxystrobin	4.0	12	4.0	12
10	Endosulfan (alfa isomer)	2.6	7.9	2.8	8.3
10	Fenitrothion	2.7	8.2	3.0	9.0
10	Fenthion	3.3	10	-	
50	Haloxyfop-R methyl	4.3	13	4.3	13
-	Heptenophos	2.7	8.1	2.6	7.7
-	Carbosulfan	-		1.9	5.7
-	Quinalphos	5.0	15	5.0	15
20	Malathion	5.3	16	5.0	15
10	Metconazole	4.0	12	4.0	12

20	Methidathion	1.2	3.7	1.3	3.8
50	o,p'-DDD	3.7	11	3.7	11
-	Parathion	3.7	11	2.6	8.0
10	Parathion-methyl	4.7	14	-	
-	Pentachlorophenol	9.7	29	9.0	27
-	Phenthoate	7.7	23	-	
-	Pirimiphos methyl	4.7	14	4.7	14
50	Prosulfocarb	3.7	11	3.7	11
50	Prothioconazole	-		3.7	11
-	Pyrazophos	1.2	3.6	1.2	3.7
50	Pyriproxyfen	2.6	7.8	2.6	7.9
10	tau-fluvalinate	5.0	15	5.3	16
10	Triazophos	0.73	2.2	0.77	2.3
10	Triticonazole	-		4.3	13
-	Vinclozolin	5.0	15	5.0	15

Table 2: Procedure detection and quantification limits for analytes with values of Maximum Residue Limits (MRLs) for honey samples.

No.	Type of sample	Mass of sample [g]	Number of analytes	LOQ [ng/g]	Recovery [%]	References
1	A	1.5	12 organophosphorus and carbamates insecticides	24-1155	82-104	[54]
2		10	fipronil, imidacloprid, thiamethoxam, dimethoate, carbendazin, tebuconazole, amitraz, t-fluvalinate and 5-hydroxymethylfurfural (HMF)	5-100	70-120	[55]
3		2.5	26 multiclass pesticides	10-20	71-118	[56]
4		5.0	6 neonicotinoids pesticides	0.1-4.0	75-114	[57]
5	B	1	insecticides and 5-hydroxymethylfurfural (HMF)	only for analytical standards <2 ng/mL	58-120	[58]
6		2	150 pesticides	-	47-92	[45]
7	A	5	19 pesticides	0.1-10	69-119	[58]
	B	2				
8	A	1	34 multiclass pesticides	2.3-27	70.1-119.4	This work
	B		30 multiclass pesticides	2.2-29		
A: Honey samples B: Honeybee samples						

Table 3: Comparison of analytical performance of different procedures based on the application of QuEChERS approach for determination of pesticide residues in honey and honeybee samples.

pesticides in honeybee samples obtained from the Regional Beekeepers Association in Gdansk (Poland). The concentration levels of pesticide residues found in the samples are given in Table 5.

Honey samples

In most cases on the tested honey samples pesticide residues were below a level the MQL of investigated pesticides. Cypermethrin and metconazole were detected above the MRLs value (51.92 ± 0.10 ng/g and 62.02 ± 0.10 ng/g, respectively). Chlorpyrifos-methyl (insecticide from the group of acaricides) and carbosulfan were detected of honey samples in 93.3% and 66.7% respectively. Other pesticides were below the MQL. In honey samples where was determined pesticide residues were varieties of honey multiflorous or rapeseed. Therefore, it can be concluded that honey bees collected the substrates for the production of these honeys during spraying with the pesticides applied to protect the plants and crops (e.g., canola, apple, pear, etc.) by farmers. In addition,

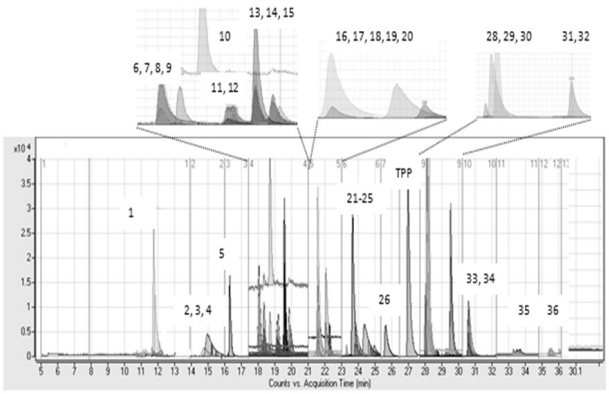

Figure 2: GC-MS/MS (MRM) chromatogram obtained during analysis of extracts from blank sample spiked at 100 ng/mL. Peaks identification are shown in Table 1 (number of peak reply ordinal number).

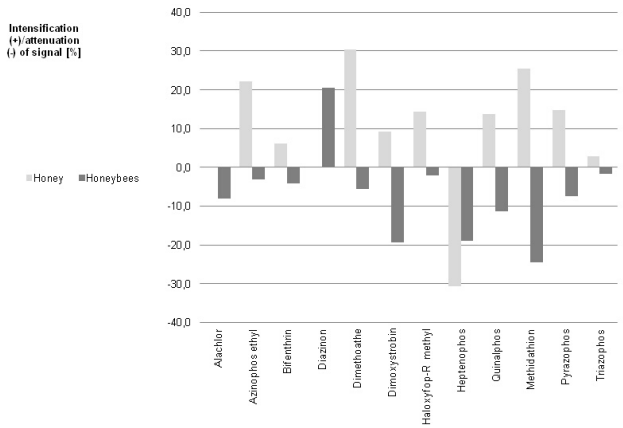

Figure 3: Changes in the value of the signal (strengthening or weakening) obtained for selected pesticides which can be determined in the honey and honeybee samples.

the results confirm the fact that the honey bees and their products can be used as indicators of environmental contamination.

Honeybee samples

The 8 pesticide residues were detected in all tested honeybee samples in the range: LOQ to 44.35 ± 0.24 ng/g (pyrazophos) 43.56 ± 1.21 ng/g (prosulfocarb). 11.58 ± 0.22 ng/g (heptenophos), 83.2 ± 1.9 ng/g (endosulfan), 14.85 ± 0.23 ng/g (dazomet) 26.28 ± 1.26 ng/g (cypermethrin).

It is worth noting that the honeybees do not have an established value of MRLs for pesticide residues which can be accumulate in their body. Ancymidol, bifenthrin, dimoksystrobina oraz pyriproxyfen were detected in 66.7% of honeybee samples. Bifenthrin is very highly toxic to bees (neurotoxic, typically causing paralysis in target pests) with a reported oral LD50 of 0.1 µg/bee and contact LD50 of 0.01462 µg/bee (about 1000 ng/g and 150 ng/g, respectively). Azinphos ethyl was detected in 46.7% of honeybee samples in the range from 12.69 ± 0.34 ng/g to 34.4 ± 2.6 ng/g. The toxicity of this pesticide for honeybees is high, because of the value reported oral LD50=0.958 mg/bee. Other pesticides were below the MQL.

Analysing the obtained results it can be concluded that the death of bees collected from the investigated areas occurred mainly in a result of poisoning by pesticides used near the apiaries.

Conclusions

The proposed new analytical procedure allows for determination of 34 multiclass pesticides in honey and 30 multiclass pesticides in honeybee samples in a single analytical run. The modified sample work-up procedure based on the QuEChERS methodology is effective, economical, and fast. The method was validated according to the regulation "Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed" [48] and then was applied to determine pesticide levels in real samples from the northern part of Poland (Pomerania). The obtained results confirm that the death of honeybees occurred mainly in a result of poisoning with the pesticide residues remaining near the apiaries and that the bees and honey can be used as environmental bioindicator. Honeybees produce honey from the nectar and/ r honeydew. The transformation of these substrates occurs in the bees body by means of suitable enzymes secreted from the salivary glands (e.g., invertase, amylase or glucose oxidase). Thus honey is a product already "processed" by the honeybees. Therefore, content of pesticide residues in honey is less than in the body of bees. However, to establish the cause of bee poisonings, we must have a sensitive analytical method that allows for the determination of a wide range of pesticides; the method presented in this paper fulfills this requirement.

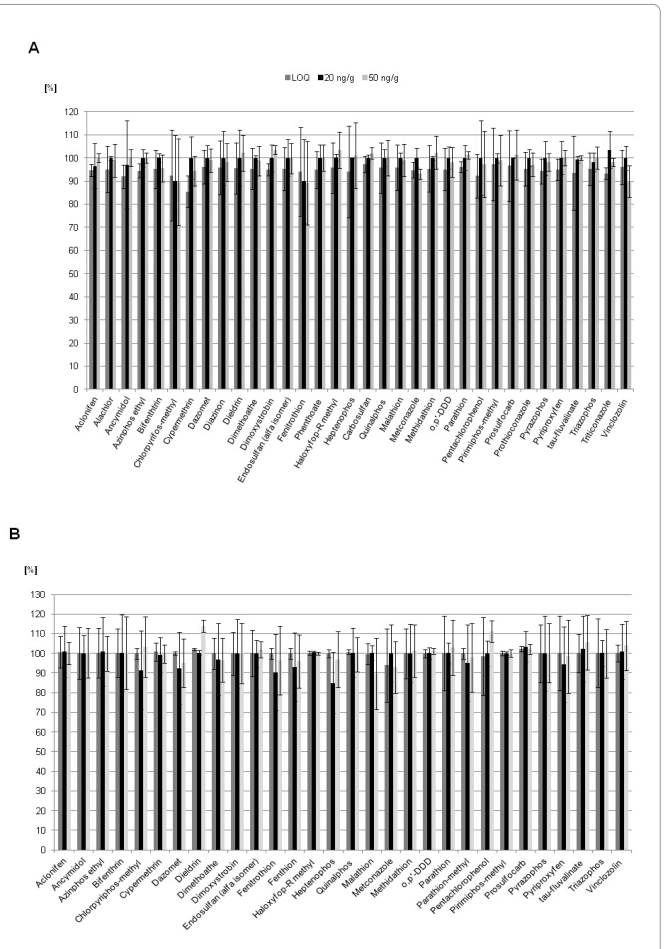

Figure 4: Pesticide recoveries (%) with error bars representing the standard deviation (n=5) obtained using QuEChERS extraction method. Samples were spiked at 3 LOD, 20 ng/g and 50 ng/g. A: Honey; B: Honeybee samples.

Type of sample	Preliminary sample preparation	Extraction QuEChERS	Clean-up dSPE	Preparation of purified extract for analysis	Time of sample preparation	References
A	- add 3 mL of hot water - dissolution	- add 3 mL of ACN - shake (30 s) - add 6 g of $MgSO_4$ and 1.5 g of NaCl and shake (1 min) - centrifugation (3000 rpm, 2 min)	- 1 mL of solvent extract - add of 150 mg of $MgSO_4$, 50 mg of PSA and shake (30 s) - centrifugation (3000 rpm, 2 min)	0.5 mL of final extracts for analysis	6 min	[54]
	- add 10 mL of water, 150 µL of NH_4OH and homogenization (1 min)	- add 10 mL of ACN - homogenization (1 min) - add 4 g of $MgSO_4$ and shake (1 min) - centrifugation (8000 rpm, 10 min)	-	10 µL of final extracts for analysis	13 min	[55]
	- heating the sample in water bath at 45°C (1 min) and homogenization	- add 5.0 mL of aqueous 0.1 mol/L Na_2EDTA and 5.0 mL of ACN - shake (3 min) - add 6 g of $MgSO_4$ and 1.5 g of NaCl and shake (1 min) - centrifugation (2200 rpm, 8 min)	- 2 mL of solvent extract - add of 120 mg of $MgSO_4$, 100 mg of PSA and shake (3 min) - centrifugation (1800 rpm, 8 min)	- filtration (PVDF, 0.2 µm) - 1 mL of final extracts for analysis with IS	24 min	[56]
	-	- add of 10 mL of water and 10 mL of ACN - shake (1 min) - add 4 g of $MgSO_4$,1 g of NaCl, 1 g of trisodium citrate and 0.5 g of disodium citrate and shake (1 min) - centrifugation (3000 rpm, 5 min)	- 6 mL of solvent extract - add of 900 mg of $MgSO_4$, 150 mg of PSA and shake (1 min) - centrifugation (3000 rpm, 5 min)	-	13 min	[57]
B	freeze drying	- add of 7.5 mL of water and 10 mL of ACN - shake (1 min) - add of 0.5 g of disodium citrate, 1 g of trisodium citrate, 4 g of $MgSO_4$,1 g of NaCl and shake (1 min) - centrifugation (600 rpm, 5 min)	- 1 mL of solvent extract - add of 750 mg of $MgSO_4$, 125 mg of PSA, 15 mg of GCB and shake (2 min) - centrifugation (600 rpm, 5 min)	- filtration (PVDF, 0.2 µm)	14 min	[58]
B	-	- add 15 mL of an ACN–water mixture (2:1, v/v) and homogenization (30 s) - add of 0.5 g of disodium citrate, 1 g of trisodium citrate, 4 g of $MgSO_4$,1 g of NaCl and shake (1 min) - centrifugation (4500 rpm, 2.5 min) -freezing out clean-up (−26°C for 2 h)	- 5 mL of solvent extract was decantation and filtration through a cotton wool - add 500 mg of $MgSO_4$, 450 mg of C18, 200 mg of PSA and 50 mg of GCB and shake (2 min) - centrifugation (4500 rpm, 2.5 min)	- 3 mL of extract was evaporated under a stream of nitrogen and the residue was re-dissolved in 1.5 mL toluene	~ 2.5 h	[45]
A	-	- add 10 mL of water and 10 mL of ACN - add of 0.5 g of disodium citrate, 1 g of trisodium citrate, 4 g of $MgSO_4$,1 g of NaCl and shake (1 min) - centrifugation (3000 rpm, 5 min)	- add 25 mg mL of $MgSO_4$, 150 mg/mL of PSA and shake (30 s) -centrifugation (3000 rpm, 5 min)	-	11.5 min	[58]
B	frozen		- add 25 mg mL of $MgSO_4$ and C18, 150 mg/mL of PSA and shake (30 s) - centrifugation (3000 rpm, 5 min)			

A	- add 10 mL of water - dissolution	- add 10 mL of ACN - add the salts kit (4 g $MgSO_4$ and 0.5 g NaCl) and shake (1 min) - centrifugation (4400 rpm, 3 min)	- 1 mL of solvent extract - add a purification kit (150 mg $MgSO_4$, and 25 mg PSA) and shake (1 min) - centrifugation (5000 rpm, 1 min)	0.5 mL of final extracts for analysis	6 min	This work
B	freeze drying	- add 10 mL of water and 10 mL of ACN and shake (30 s) - add of 3 mL of n-hexane and salts kit (4 g $MgSO_4$ and 0.5 g NaCl) and shake (30 s) - centrifugation (4400 rpm, 3 min)				
A – honey samples B – honeybee samples						

Table 4: The main differences between developed procedure and other published method based on the application of QuEChERS approach for determination of pesticide residues in honey and honeybee samples.

Pesticides	Matrix	No. of positive samples (%)	Min level [ng/g] (RSD)	Max level [ng/g] (RSD)	MRLs for honey [ng/g] [53]
Ancymidol	Honeybee	10 (66.7)	<MQL	54.52 (0.58)	-
	Honey	3 (20)	<MQL	12.662 (0.016)	-
Azinphos ethyl	Honeybee	7 (46.7)	12.69 (0.34)	34.4 (2.6)	-
Bifenthrin		10 (66.7)	ND	<MQL	
Chlorpyriphos-methyl		5 (33.3)	<MQL	43.11 (0.16)	
	Honey	14 (93.3)	<MQL	22.02 (0.21)	-
Cypermethrin	Honeybee	15 (100)	<MQL	26.2 (1.2)	-
	Honey	15 (100)	<MQL	51.92 (0.10)	50
Dazomet	Honeybee	15 (100)	<MQL	14.85 (0.23)	-
Dimoxystrobin		10 (66.7)	<MQL	13.5 (1.1)	
Endosulfan (alfa isomer)		15 (100)	<MQL	83.2 (1.9)	
Fenthion		9 (60)	<MQL	45.6 (2.5)	
Haloxyfop-R methyl		8 (53.3)	<MQL	73.3 (1.4)	
Heptenophos		15 (100)	<MQL	11.58 (0.22)	
	Honey	5 (33.3)	<MQL	10.0 (1.2)	-
Metconazole	Honey	1 (7)	ND	62.02 (0.10)	10

Parathion-methyl	Honeybee	6 (40)	<MQL	43.19 (0.18)	-
Prosulfocarb		15 (100)	<MQL	43.6 (1.2)	
	Honey	1 (7)	ND	13.38 (0.16)	50
Pyrazophos	Honeybee	15 (100)	<MQL	44.35 (0.24)	-
	Honey	7 (46.7)	<MQL	9.76 (0.15)	-

Table 5: Pesticide residues determined in honey and honeybee samples collected from apiaries in Pomerania region of Poland (concentration with RSD).

Furthermore, knowledge about the contamination and poisoning of bees by pesticides is very important and still needed.

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