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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 incidents

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ABSTRACT

A method for the determination of 200 pesticides and pesticide metabolites in honeybee samples has been developed and validated. Almost 98% of compounds included in this method are approved to use within European Union, as active substances of plant protection products or veterinary medicinal products used by beekeepers to control mites Varroa destructor in hives. Many significant metabolites, like metabolites of imidacloprid, thiacloprid, fipronil, methiocarb and amitraz, are also possible to detect. The sample preparation was based on the buffered QuEChERS method. Samples of bees were extracted with acetonitrile containing 1% acetic acid and then subjected to clean-up by dispersive solid phase extraction (dSPE) using a new Z-Sep+ sorbent and PSA. The majority of pesticides, including neonicotionoids and their metabolites, were analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) but some of pesticides, especially pyrethroid insecticides, were analyzed by gas chromatography tandem mass spectrometry (GC–MS/MS). The procedure was validated according to the Guidance document SANCO/12571/2013 at four concentration levels: 1, 5, 10 and 100 ng/g bees and verified in the international proficiency test. The analysis of bee samples spiked at the limit of quantification (LOQ) showed about 98% mean recovery value (trueness) and 97% of analytes showed recovery in the required range of 70–120% and RSDr (precision) below 20%. Linearity and matrix effects were also established. The LOQs of pesticides were in the range of 1–100 ng/g. The developed method allows determination of insecticides at concentrations of 10 ng/g or less, except abamectin and tebufenozide. LOQ values are lower than the median lethal doses LD_{50} for bees. The method was used to investigate more than 70 honeybee poisoning incidents. Data about detected pesticides and their metabolites are included.

1. Introduction

Pesticides are widely used as plant protection products (PPPs) in agriculture. In Poland, there are more than 1500 PPPs authorized to use, which contains at least one of 200 different pesticides, very diverse in terms of chemical structure and toxic effects on bees. Those pesticides belong to many different categories such as insecticides, fungicides, herbicides, growth regulators, acaricides etc., but in terms of chemical properties they belong to much more different classes, e.g., insecticides included in this study belong to 16 different chemical classes.

Within last years due to global decline in honeybee population the bee health is a matter of public concern. Since 2003 in North America and Europe the phenomenon named Colony Collapse Disorder (CCD) occurs. The European data from EPILOBEE project showed that yearly colony mortality rates reported between 2012 and 2014 reached up to 36% [1]. The data from the United States showed that annual colony losses reported by beekeepers reached up to 45% [2]. The same data showed that commercial beekeepers in the United States reports greater loose of bee colonies in the summer than in the winter. Summer loses are usually connected with poisoning incidents. The mechanism of CCD remains unknown, but there is an agreement between scientists that there are several

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factors that could interact possible causes of colony losses. The scientists should expand the knowledge and understanding the role of pesticides, as one of the main factors that affect bee health, by development of new very sensitive and reliable methods detecting as much as possible pesticides, that even at very low levels at environmental doses and by interaction could weaken bees defence systems allowing parasites or viruses to kill the colony.

Till now a limited number of methods for the determination of pesticide residues in honeybees have been published. There are only few papers describing methods that can analyse more than 100 pesticides in this difficult matrix. and indicating method validation data. The proper selection of pesticides, by looking for those compounds which are currently approved or used as PPPs or as veterinary medicinal products (VMPs) by beekeepers, is of the same importance as the quantity of analysed pesticides. This could help to establish the most likely pesticide related risk for honeybee health. The effectiveness of the method used for the investigation of honeybee poisoning incidents is limited by the number of currently approved and used substances and not by the number of compounds banned to use many years ago.

By one of existing multiresidue methods honeybee samples can be analysed for 153 pesticides with gas chromatography system with dual selective detectors for electron capture and nitrogen-phosphorous (GC-NPD/ECD) and confirmatory analysis with different polarity column [3]. Roughly 150 pesticides can be analysed by method employing GC–MS/MS [4]. The drawbacks of this method are that gas chromatography is a technique that is unable to detect a lot of modern pesticides actually permitted to use in agriculture. Many of presently used pesticides could be analysed only with the methods involving liquid chromatography. Application of only LC–MS/MS determination of pesticides in honeybee are focused mainly on neonicotinoids [5–7] but there are also procedures that could provide valid data about the occurrence of 115 pesticides in honeybee colonies [8].

Methods based on both gas and liquid chromatography have the potential to analyse the broadest spectrum of pesticides but it is a common issue that a share of actually approved pesticides is insufficient. There was described only one validated method involving both GC-ToF and LC–MS/MS determination of 80 environmental contaminants in honeybees [9]. This method was adopted to monitor presence of contaminants in France apiaries [10]. Simultaneous analysis both with GC–MS and LC–MS/MS, but without any validation data, was used to study the occurrence of 200 pesticides in different beehive matrices from North American apiaries [11]. The applicability of that method to European Union is limited because only about 50% of studied compounds is approved to use [12]. Very recently there was published second occurrence study that uses both GC–MS/MS and LC–MS/MS method to establish the exposure of native bees to pesticides [13], but similarly only about 60% from 136 examined pesticides is approved to use within EU [12].

Besides of detection system that is characterised by obvious abilities and limitations the second most important method related step is sample preparation. The QuEChERS method with dispersive solid phase extraction (dSPE) clean-up is a sample preparation technique that enable the multiresidue pesticide analysis of complex matrices. The QuEChERS is one of the most popular sample preparation approach in the area of pesticide residue analysis in food. There has been published methods of honeybee samples analysis with QuEChERS [4,9,11,14]. The matrix solid-phase dispersion (MSPD) technique is also popular approach for honeybee extract clean-up [3,15,16].

The analysis of pesticides in honeybees is challenging due to complexity of insect body and presence of all kind of natural compounds, like beeswax, chitin and proteins that in chromatographic analysis are difficult for clean-up impurities. Whatever dSPE or MSPD based method is used, a lot depends on the capabilities of sorbents used in clean-up technique. Sorbents commonly used to clean-up bee extracts are primary secondary amine (PSA), octadecylsilane (C18), Florisil and graphitized carbon black (GCB). GCB is useful for removal of pigments, but retains planar pesticides. The new promising sorbents, which have been applied to the determination of pesticide residues in avocado for a purification of high oil extracts, are zirconium dioxide coated silica sorbents Z-Sep and Z-Sep+ [17]. Due to presence of Lewis acid sites, Bronsted acid-base sites, and octadecylsilane group on the surface of these new sorbents they could be a good adsorbent of fatty acids and proteins.

The aim of this study was to develop and validate an analytical method for determination as much as possible pesticides currently approved to use within European Union and their metabolites in honeybee samples taking into account two sources of exposure: pesticides used in agriculture as PPPs, and pesticides intentionally introduced into hives by beekeepers as acaricides in order to control *Varroa destructor* mite. The development of this method is important because results of study with such a broad and most actual spectrum of pesticides analysed in honeybees, shown in Table 1, will help to assess the risk connected with the current used pesticides and their role in the bees decline.

2. Materials and methods

2.1. Selection of compounds

The selection of PPPs active substances to be included in this method was done after the verification of Polish database of plant protection products [18] and EU pesticide database [12]. Only carbendazim, nitenpyram and novaluron are not approved to use in EU as PPPs. Carbendazim and novaluron were included in this method because according to Regulation (EC) No 1107/2009 [19] these active substances are still temporary on the market. Nitenpyram is one of the neonicotinoid insecticide.

The number of VMPs for Varroa control authorised to use by European countries, besides ethereal oils and organic acids, is limited to a few pesticides like amitraz, coumaphos, tau-fluvalinate and flumethrin [20]. For the reason that resistance development of Varroa species against currently used VMPs is observed and there is a lack of new substances, other pesticides registered to use within non-EU countries (cymiazole) or formerly used (bromopropylate) were included in the method to check whether beekeepers uses them.

Among 200 pesticides and pesticide metabolites included in this method only five compounds, from both PPPs and VMPs groups, are not currently approved to use within EU.

2.2. Reagents

High purity pesticide analytical standards and internal standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Sigma–Aldrich (Seelze, Germany) and Toronto Research Chemicals (Toronto, Canada). Stock standard solutions (250–1500 μg/mL) were prepared in acetonitrile, acetone, methanol or dimethylformamide and stored in the dark at a temperature below −18°C. Individual standard solutions for optimization and mixed standard solutions for calibration and validation experiments were prepared by appropriate dilutions of stock standard solutions.

Ultra Resi-Analyzed purity acetonitrile used for the preparation of standards, LC–MS grade acetonitrile used as eluent in liquid chromatography, acetone and methanol were obtained from J.T. Baker brand of Avantor Performance Materials (Deventer, The Netherlands). Dimethylformamide, formic acid, ammonium formate, PSA, anhydrous magnesium sulphate, C18 sorbent—Discovery DSC-18, GCB sorbent—Supelclean ENVI-Carb

Table 1
Scope of analysed pesticides with their category of use (AC—acaricide, AL—algicide, VMP—veterinary medicinal product, FU—fungicide, HB—herbicide, IN—insecticide, MO—molluscicide, NE—nematicide, PG—plant growth regulator, RE—repellent, ST—soil treatment) and their metabolites (MET), acid-base properties [32,33] and LOQ values for the developed method.

Compound	Category	pK_a	Property	LOQ(ng/g)
1-Naphthylacetamide (1-NAD)	PG	0.41		1
2,4-D	HB, PG	2.73	Acidic	10
2,4-Dimethylanilin (DMA)	MET			100
N-2,4-Dimethylphenylformamide (DMF)	MET			5
N-2,4-Dimethylphenyl-*N*-methylformamidine (DMPF)	MET			10
6-Chloro-4-hydroxy-3-phenyl-pyridazine	MET			1
Abamectin	AC, IN			100
Acetamiprid	IN	0.7		1
Alpha-cypermethrin	IN	5	Acidic	5
Amidosulfuron	HB	3.58	Acidic	1
Azoxystrobin	FU	–	No diss.	1
Bentazone	HB	3.3	Acidic	1
6-Hydroxy bentazon	MET	3.3	Acidic	10
		11.7	Acidic	
Beta-Cyfluthrin	IN	–	No diss.	5
Bifenazate	AC	12.94	Basic	10
Bifenox	HB	–	No diss.	5
Bifenthrin	IN	–	No diss.	5
Bixafen	FU	–	No diss.	1
Boscalid	FU	–	No diss.	1
Bromopropylate	VMP	–		1
Bromoxynil	HB	3.86	Acidic	10
Bupirimate	FU	5	Basic	1
Carbendazim	FU	4.2	Basic	1
Carbetamide	HB	11.3	Acidic	1
Carboxine	FU	0.5	Acidic	1
Carfentrazone-ethyl	HB	–	No diss.	1
Chlorantraniliprole	IN	10.88	Basic	1
Chloridazon	HB	3.38	Acidic	1
Chlorothalonil	FU	–	No diss.	10
Chlorotoluron	HB	–	No diss.	1
Chlorpropham	PG, HB	–	No diss.	1
Chlorpyrifos	IN, AC	–	No diss.	1
Chlorpyrifos-methyl	IN, AC	–		1
Chlorsulfuron	HB	3.4	Acidic	1
Clethodim	HB	4.47	Acidic	5
Clofentezine	AC	–		5
Clomazone	HB	–	No diss.	1
Clothianidin	IN	11.1	Basic	5
Coumaphos	VMP			1
Cyazofamid	FU	–	No diss.	5
Cycloxydim	HB	4.17	Acidic	10
Cyflufenamid	FU	12.08	Acidic	1
Cymiazol	VMP	5.2	Basic	5
Cymoxanil	FU	9.3	Acidic	5
Cyproconazole	FU	–	No diss.	5
Cyprodinil	FU	4.44	Basic	10
Deltamethrin	IN	–	No diss.	5
Desmedipham	HB	–	No diss.	1
Dichlorprop-P (2,4-DP)	HB	3.67	Acidic	10
Difenoconazole	FU	1.07	Acidic	1
Diflubenzuron	IN	–		1
Diflufenican	HB	–		5
Dimethachlor	HB	–	No diss.	1
Dimethoate	IN, AC	–	No diss.	1
Dimethomorph	FU	−1.3	Acidic	1
Dimoxystrobin	FU	–	No diss.	1
Dithianon	FU	–	No diss.	100
Epoxiconazole	FU	–	No diss.	1
Esfenvalerate	IN	–	No diss.	5
Ethofumesate	HB	–	No diss.	10
Ethoprophos	NE, IN	–	No diss.	1
Etofenprox	IN	–	No diss.	1
Etoxazole	IN	–		5
Famoxadone	FU	–	No diss.	5
Fenazaquin	AC	2.44	Acidic	1
Fenbuconazole	FU	–	No diss.	5
Fenhexamid	FU	7.3	Acidic	10
Fenoxaprop-*P*-ethyl	HB	3.2	Acidic	5
Fenpropidin	FU	10.1	Acidic	5
Fenpropimorph	FU	6.98	Acidic	5
Fenpyroximate	AC	–	No diss.	5
Fipronil	IN	–	No diss.	1

Table 1 (*Continued*)

Compound	Category	pK_a	Property	LOQ(ng/g)
Fipronil-carboxamide	MET			1
Fipronil-desulfinyl	MET			5
Fipronil-sulfide	MET			1
Fipronil-sulfone	MET			1
Flazasulfuron	HB	4.37	Acidic	1
Flonicamid	IN	11.6	Basic	10
Florasulam	HB	4.54	Acidic	1
Fluazifop-P-butyl	HB	–		5
Fluazinam	FU	7.34	Acidic	5
Fludioxonil	FU			1
Flufenacet	HB	–	No diss.	1
Fluquinconazole	FU	0.9	Acidic	1
		−4.4		
Flurochloridon	HB	–	No diss.	5
Fluroxypyr	HB	2.94	Acidic	100
Fluroxypyr-1-methylheptyl ester	MET			10
Flusilazole	FU	2.5	Basic	1
Flutriafol	FU	2.3	Acidic	10
Foramsulfuron	HB	4.6	Acidic	1
Gibberellin A4	PG			100
Hexythiazox	AC, IN	–	No diss.	5
Hymexazol	FU	5.92	Acidic	100
Imazalil	FU	6.49	Basic	5
Imidacloprid	IN	–	No diss.	1
Imidacloprid-olefin	MET			1
Imidacloprid-urea	MET			1
6-Chloronicotinic acid	MET			100
Indole-3-butyric acid (IBA)	PG			10
Indoxacarb	IN	–	No diss.	5
Iodosulfuron-methyl-sodium	HB	3.22	Acidic	1
Ipconazole	FU	−5.43		5
Iprodione	FU, NE	–	No diss.	10
Isoproturon	HB	–	No diss.	5
Isopyrazam	FU	–	No diss.	1
Isoxaflutole	HB	–	No diss.	1
Kresoxim-methyl	FU	–	No diss.	5
Lambda-cyhalothrin	IN	–	No diss.	1
Lenacil	HB	10.7		1
Linuron	HB	–	No diss.	1
Mandipropamid	FU	–	No diss.	1
Malathion	IN, AC	–	No diss.	5
MCPA	HB	3.73	Acidic	5
MCPB	HB	4.5	Acidic	100
Mecoprop-P (MCPP-P)	HB	3.68	Acidic	10
Mepanipyrim	FU	2.7	Acidic	10
Mesosulfuron-methyl	HB	4.35	Acidic	1
Mesotrione	HB	3.12	Acidic	5
Metaflumizone	IN	–	No diss.	10
Metalaxyl-M/Metalaxyl	FU	–	No diss.	1
Metamitron	HB	–	No diss.	5
Metazachlor	HB	–	No diss.	1
Metconazole	FU, PG	11.38		5
		1.08		
Methiocarb	IN, MO, RE	–	No diss.	1
Methiocarb sulfone	MET			1
Methiocarb sulfoxide	MET			1
Methoxyfenozide	IN	–	No diss.	5
Methyl isothiocyanate (MITC)	MET	12.3	Basic	100
Metrafenone	FU	–	No diss.	5
Metribuzin	HB	1	Basic	5
Metsulfuron-methyl	HB	3.8	Acidic	1
Myclobutanil	FU	2.3	Acidic	100
Napropamide	HB	–	No diss.	1
Nicosulfuron	HB	4.78	Acidic	1
Nitenpyram	IN	3.1		5
Novaluron	IN	–		10
Oxyfluorfen	HB	–	No diss.	10
Pencycuron	FU	–	No diss.	1
Pendimethalin	HB	2.8	Acidic	1
Pethoxamid	HB	–	No diss.	1
Phenmedipham	HB	–	No diss.	5
Phosmet	IN	–	No diss.	5
Picoxystrobin	FU	–	No diss.	1
Pirimicarb	IN	4.44	Basic	1
Pirimicarb-desmethyl	MET			1
Pirimiphos-methyl	IN	4.3	Acidic	1
Prochloraz	FU	3.8	Basic	5
Propamocarb	FU	9.6	Basic	5

Table 1 (*Continued*)

Compound	Category	pK_a	Property	LOQ(ng/g)
Propaquizafop	HB	−2.3	Acidic	1
Propiconazole	FU	1.09	Basic	5
Propoxycarbazone-sodium	HB	2.1	Acidic	5
Propyzamide	HB	–	No diss.	1
Proquinazid	FU	–	No diss.	5
Prosulfocarb	HB	–	No diss.	5
Prothioconazole	FU	6.9	Acidic	100
Prothioconazole-desthio	MET			1
Pyraclostrobin	FU, PG	–	No diss.	1
Pyridate	HB	–	No diss.	5
Pyrimethanil	FU	3.52		5
Pyriproxyfen	IN	6.87		5
Quinoclamine	AL, HB	–	No diss.	5
Quinoxyfen	FU	–	No diss.	10
Quizalofop-P-ethyl	HB	–	No diss.	5
Quizalofop-P-tefuryl	HB	−1.25	Acidic	10
Rimsulfuron	HB	4	Acidic	1
S-Metolachlor	HB	–	No diss.	1
Silthiofam	FU	–	No diss.	1
Spinosyn A	IN	8.1		0.8
Spinosyn D	IN	7.87		1.2
Spirodiclofen	AC, IN	–		5
Spirotetramat	IN	10.7		1
Spirotetramat-enol	MET			1
Spirotetramat-enol-glucoside	MET			1
Spirotetramat-keto-hydroxy	MET			10
Spiroxamine	FU	6.9	Basic	1
Sulcotrione	HB	3.13	Acidic	10
Sulfosulfuron	HB	3.51	Acidic	1
tau-Fluwalinate	IN, VMP	–	No diss.	5
Tebuconazole	FU	5		1
Tebufenozide	IN	–	No diss.	100
Tebufenpyrad	AC	–	No diss.	5
Teflubenzuron	IN	9.2	Acidic	10
Tefluthrin	IN	9		1
Tembotrione	HB	3.18	Acidic	10
Tepraloxydim	HB	4.58	Acidic	10
Terbuthylazine	HB	1.9		5
Tetraconazole	FU	0.65	Acidic	5
Thiacloprid	IN	–	No diss.	1
Thiacloprid-amide	MET			1
Thiamethoxam	IN	–	No diss.	5
Thifensulfuron-methyl	HB	4	Acidic	1
Thiophanate-methyl	FU	7.28	Acidic	1
Tralkoxidym	HB	4.3	Acidic	5
Triadimenol	FU	–	No diss.	10
Tribenuron-methyl	HB	4.7	Acidic	100
Trifloxystrobin	FU	–	No diss.	1
Triflusulfuron-methyl	HB	4.4	Acidic	5
Trinexapac-ethyl	PG	4.57	Acidic	100
Triticonazole	FU	–	No diss.	5
zeta-Cypermethrin	IN	–	No diss.	5

No diss.: no dissociation.

120/400, zirconium (IV) oxide—ZrO_2, Z-Sep and Z-Sep+ were supplied by Sigma-Aldrich (Bellefonte, PA, USA). Anhydrous sodium acetate and glacial acetic acid were supplied by POCH brand, Avantor Performance Materials (Gliwice, Poland). Water was purified through a Mili-Q Plus system from Merck Millipore (Billerica, MA, USA).

2.3. Sample preparation procedure

The QuEChERS procedure after modification was applied. Sample of honeybees was frozen in liquid nitrogen and thoroughly homogenised for analysis. Furthermore, to know the average weight of one honeybee, 20 insects were weighted. A 5 g of homogenised sample was weighed in a 50 mL centrifuge tube and 50 μL of a mixture of internal standards (0.5 μg/mL—imidacloprid-D_4; 1 μg/mL—acetamiprid-D_3, carbendazim-D_3, chlotianidine-D_3, tiamethoxam-D_4 and chlorpyrifos-D_{10}; 50 μg/mL—deltamethrin-D_5) was added. Then 2 g portion of glass beads and 10 mL of water were added and sample was shaken in a MiniG vertical mechanical disrupter (SPEX SamplePrep, Metuchen, NJ, USA) for 3 min. After that 10 mL of 1% acetic acid mixture in acetonitrile were added and once more shaken in a mechanical disrupter for 3 min. Afterwards, 1 g of sodium acetate and 4 g of magnesium sulphate were added and the sample was shaken for 3 min. The mixture was then centrifuged at 3500 rpm, −12°C for 20 min. Of the 10 mL of the supernatant only 7 were collected and purified by means of dSPE with different mixture of sorbents shown in Table 2. DSPE sorbents for the optimisation were chosen based on a review of the literature and self-made modifications. The mixture of 350 mg of PSA, 350 mg of Z-Sep+ and 1050 mg of MgSO4 (G) was finally chosen for the validation.

The supernatant was shaken with a mixture of sorbents in a mechanical disrupter for 10 min and again centrifuged. Further, 100 μL of extract was added to a Mini-UniPrep amber glass chambers (Whatman, GE Healthcare Life Sciences, UK) containing 400 μL of LC water phase and the sample was mixed and filtered in a

Table 2
Portions of sorbents corresponding to 1 mL of supernatant used at the optimisation step of dSPE clean-up.

A [34]	B [35]	C [17]	C′	D [17]	E	F [4]	G
150 mg $MgSO_4$ 25 mg PSA	150 mg $MgSO_4$ 50 mg PSA50 mg C18	150 mg $MgSO_4$ 35 mg Z-Sep+	150 mg $MgSO_4$ 50 mg Z-Sep+	150 mg $MgSO_4$ 35 mg Z-Sep	150 mg $MgSO_4$ 35 mg ZrO_2	100 mg $MgSO_4$ 40 mg PSA 90 mg C18 10 mg GCB	150 mg $MgSO_4$ 50 mg Z-Sep+ 50 mg PSA

chamber through a 0.2 μm pore size PTFE filter media. In a such prepared sample, 1 mL of extract represents 0.1 g of honeybees (mean weight of 1 honeybee), was injected into the LC–MS/MS system. Subsequently for GC–MS/MS analysis, 4 mL of supernatant were solvent-exchanged to 0.5 mL of *n*-hexane: 1 mL of such hexanic solution represents 4 g of honeybees.

2.4. Analysis

2.4.1. LC–MS/MS analysis

Agilent 1260HPLC system (Waldronn, Germany), equipped with several types of columns of Agilent Zorbax (Eclipse Plus C18 2.1 × 150 mm 3.5 μm, Extend-C18 3.0 × 150 mm 3.5 μm, SB-Aq 2.1 × 150 mm 3.5 μm) and Phenomenex (Kinetex 1.7 micron C18 150 × 2.1 mm and Luna 3 μm Phenyl-Hexyl 150 × 2.0 mm) were tested at the optimization step. Phenomenex Luna Phenyl-Hexyl thermostated at 50 °C, with the gradient mobile phase consisted of acetonitrile and water with 5 mM ammonium formate (acidified with formic acid to pH 6.0) was finally chosen for the validation. The flow rate was 0.4 mL/min through entire analysis with gradient programme set as follows: 5% of acetonitrile hold for 1 min, increased to 95% in 26 min and hold to 32.5 min, decreased to 5% in 33 min and hold to 43 min for re-equilibration. The total run time of analysis was 43 min. The injection volume was 10 μL. AB Sciex QTRAP® 6500 LC–MS/MS system (Framingham, MA, USA) in a scheduled MRM advanced mode with Turbo Spray Ion Drive in both positive and negative ionisation was used for the mass spectrometric analysis. The source settings were as follows: temperature—550 °C, curtain gas (N_2)—20 psi, collision gas (N_2)—medium, ion source gas 1 (nebuliser gas, N_2) —50 psi, ion source gas 2–40 psi and capillary voltage 5000 V in positive and −4500 V in negative ionisation mode. LC–MS/MS system was controlled by Analyst software version 1.6.2. Quantitative and qualitative analysis was done with MultiQuant software version 3.0. The procedural matrix matched standards were used for calibration.

2.4.2. GC–MS/MS analysis

Agilent Technologies (Palo Alto, CA, USA) gas chromatograph, model 7890A+, equipped with 7693B series autosampler, split/splitless injector and 7000B tandem mass spectrometry detector were used. Chromatographic separation was achieved on a HP-5MS UI capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness, Agilent, USA). The following oven temperature program was used: initial temperature of 80 °C hold for 1 min, increased to 200 °C at 40 °C/min, increased to 210 °C at 2.3 °C/min and hold 5 min, increased to 266 °C at 3 °C/min and hold for 5 min, and then increased to 320 °C at 10 °C/min and hold for 10 min (post run). The total run time of chromatographic analysis was 52.4 min. The injector and transfer line were operated at 285 °C and 280 °C, respectively. The carrier gas was helium with constant flow rate of 0.9 mL/min. The injection volume was 1 μL in the pulsed splitless mode. The MS/MS system with electron ionisation (EI) source in positive ionisation was used for mass spectrometric analysis. The MS/MS settings were as follows: source energy—70 eV, source temperature—280 °C, temperature MS1 and MS2 quadrupoles—150 °C, collision gas (N_2)—flow 1.5 mL/min, quench gas (He)—flow 2.25 mL/min. GC–MS/MS system was controlled by Mass Hunter software version B.07.01. Quantitative and qualitative analysis was done also with Mass Hunter software. The procedural matrix matched standards were used for calibration.

2.5. Validation

The procedure was validated according to the Guidance document SANCO/12571/2013 [21]. The honeybee samples free from pesticides were used as blank, to spike aliquots for validation studies and to prepare procedural matrix matched standards for calibration. Honeybees were stored in a freezer (below −18 °C) until analysis. To assess the accuracy of the developed method, both trueness and precision were evaluated. The linearity was checked using procedural matrix matched standards by analysing each level in duplicate at six concentrations between 1 and 1000 ng/g. In order to evaluate matrix effects the calibration standards in solvent were also analysed. The limit of quantification (LOQ) was evaluated as the minimum concentration of analyte that can be quantified with acceptable trueness and precision by spiking sample at this concentration level.

2.6. Real samples application

Developed and validated method has been used for detection and identification of honeybee poisoning incidents, which are examined in the framework of "Monitoring the state of health and colony losses in the national apiaries" program since 2014. This program besides epidemiological studies consisted of passive monitoring of pesticides in honeybees. During the years 2014 and 2015 described method was used to analyse about 70 samples of honeybees poisoned with pesticides. In every single case there were established commission that involves in particular veterinary inspector, plant health and seed inspector and a member of beekeepers association. The commission, besides inspection of PPPs suitable use in surrounding area, was responsible for overall inspection of the colony health, immediate freezing of the collected samples and for sending them to the laboratory.

3. Results and discussion

3.1. Sample preparation optimization

Sample preparation is one of the most important and difficult part of each analysis. To analyse a wide range of pesticides the multiresidue QuEChERS method was applied for honeybee samples. Among pesticides permitted to use as plant protection products there are a lot of compounds with acid or base properties which require special conditions of analysis or even single residue methods. Acidic or basic compounds can be in neutral or ionized form, depending on the pH of the extract and their pK_a value (Table 1). At the QuEChERS extraction step ionized compound have a tendency to stay in polar water phase not in acetonitrile. Buffering of the extract was proposed by the authors of the original QuEChERS method to ensure good enough extraction recovery of pH dependent compounds. To improve stabilisation of acidic pesticides and protection of base-sensitive pesticides acetate buffering

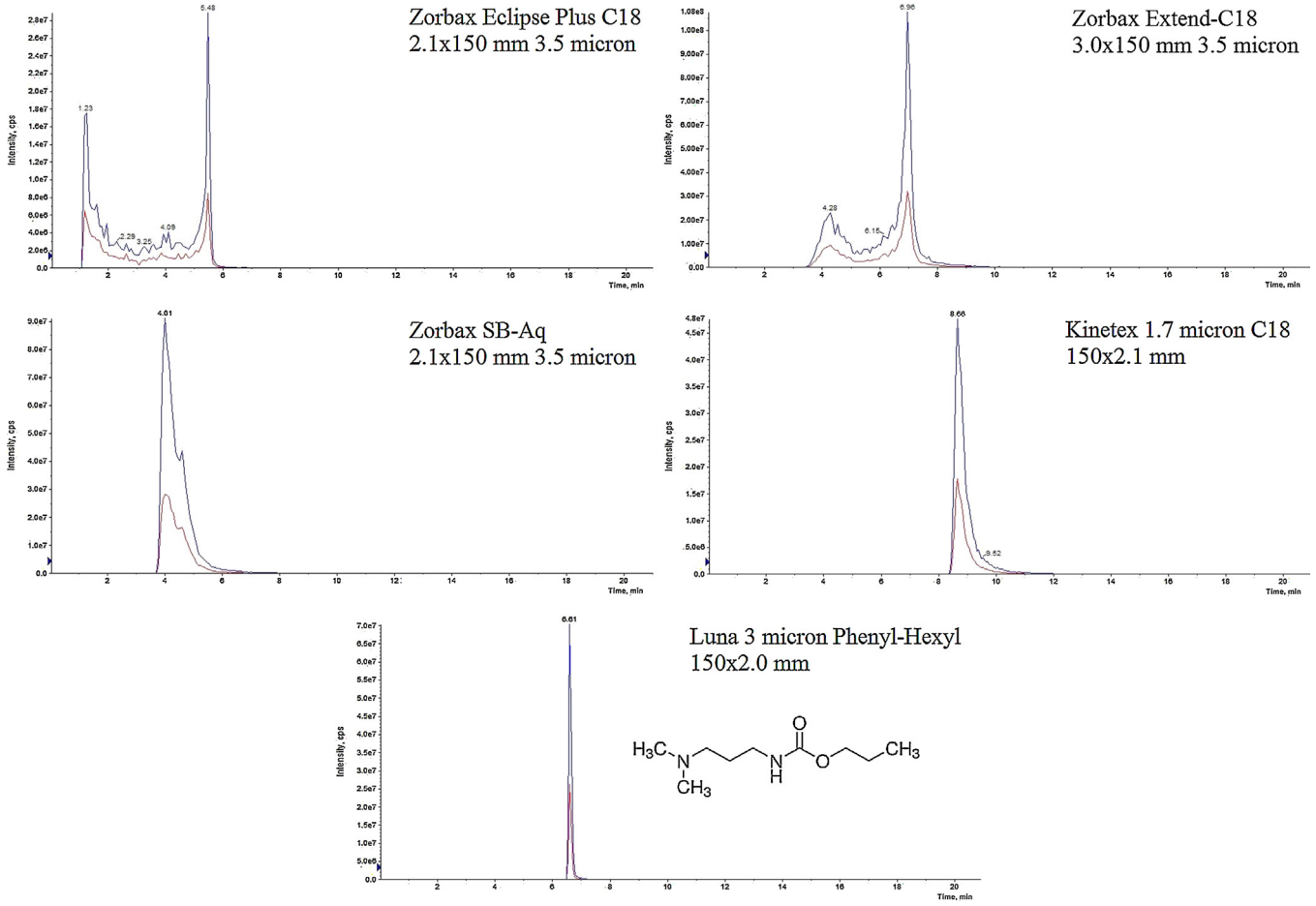

Fig. 1. LC–MS/MS chromatograms of propamocarb, basic pesticide, tested in the same conditions but at different chromatographic columns.

were introduced by Lehotay et al. [22] whilst Anastassiades used citrate salts for buffering [23].

Citrate buffered QuEChERS sample preparation protocol for acidic pesticides finishes without any clean-up just after the extraction step, because each sorbents mixture used for further dSPE clean-up contains PSA [23]. The clean-up with PSA and citrate buffering should be avoided in order not to lose acidic triazole fungicides during multiresidue analysis. Because of high amounts of proteins in insect body and their lower pK_a values than fatty acids the effectiveness of anions clean-up derived by PSA could be achieved even in the lower pH, such as in the acetate buffered QuEChERS method. This acetate buffering protocol were successfully used by Mullin in multiresidue analysis of pesticides in honeybees [11]. Introduced by Lehotay acetate buffering guaranteed the same pH value during extraction and clean-up of the samples [22]. In this constant low pH acidic pesticides are stabilised in their non-ionised form and are not retained during the PSA clean-up. QuEChERS method with acetate buffering extraction of honeybee samples was chosen for further dSPE optimisation step.

It was stated that Z-Sep and Z-Sep+ sorbents are the alternative and substitution for PSA sorbents but they could be especially useful in the adsorption of proteins and lipids [17]. It is the first study when both zirconium dioxide based sorbents (Z-Sep and Z-Sep+) were evaluated in a clean-up of honeybee extracts from matrix components, especially from beeswax and proteins. Recently only Z-Sep sorbent was proved to be effective in the analysis of limited number of 11 pesticides in honeybees in GC–MS/MS method [24]. Till now Z-Sep+ was not tested for clean-up of honeybee extracts. The excellent clean-up effectiveness of ZrO_2-coated sorbent among other traditionally used sorbents were shown for lipid removal from egg extract, but the losses of acidic pesticides and triazoles were observed [25]. The lack of control of pH dependent clean-up mechanisms could be the reason of very low recoveries of above mentioned pesticides. Clean-up step could be also matrix dependent whilst it was stated that the chemical composition of the eluent play an important role in retention mechanism of zirconium oxide chromatographic surfaces and they could build up secondary interactions [26].

The effectiveness of tested sorbents was evaluated by weighting matrix co-extractives after evaporation of 5 mL of acetate buffered extract to dryness. The comparison were done against buffered extract with no clean-up step, as indicated for acidic pesticides analysis in the citrate buffering QuEChERS protocol. The results of matrix removal efficiency are shown in Fig. 3. The outcomes showed that Z-Sep+ is a material that simplifies QuEChERS protocols used till now in honeybee analysis because it could be used instead of even three kind of sorbents. Only 35 mg of Z-Sep+ proved to be as much effective as 100 mg mixture of PSA and C18, and even more than 140 mg mixture of PSA, C18 and GCB used for honeybee extract clean-up respectively by Mullin et al. [11] and Walorczyk and Gnusowski [4]. Adsorption on the Lewis acid Zr(IV) sites is not the main clean-up mechanism of honeybee extract components because ZrO2 did not show almost any clean-up effect and Z-Sep, that contains on the surface only ZrO2, showed small potency in removing of honeybee matrix co-extractives. There is much difference between Z-Sep and Z-Sep+ sorbents. The same amount of

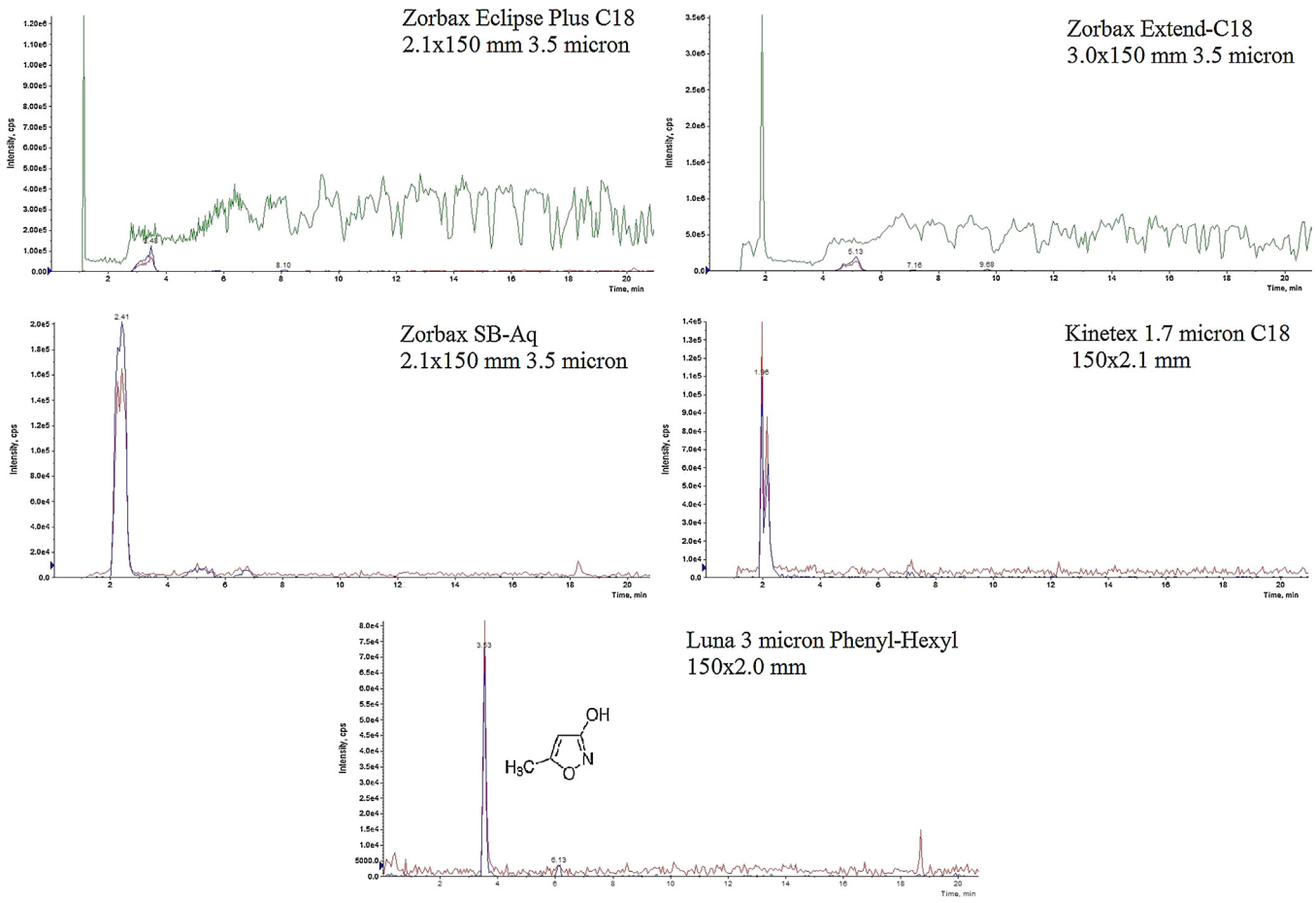

Fig. 2. LC–MS/MS chromatograms of hymexazol, aromatic acidic pesticide, tested in the same conditions but at different chromatographic columns.

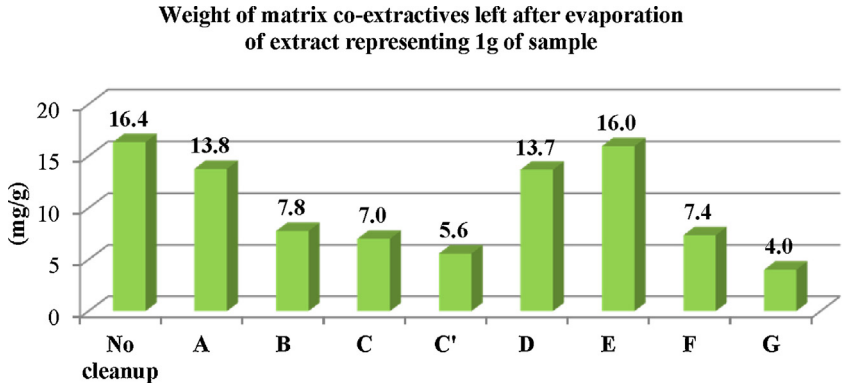

Fig. 3. DSPE clean-up efficiency achieved by tested sorbents in comparison to option with no clean-up (analysis of raw extract according to citrate buffering protocol for acidic compounds). Abbreviations A–G refers to Table 2.

Z-Sep+ has shown almost twofold decrease of matrix components in final extract in comparison to Z-Sep in our experiments.

In order to evaluate the clean-up mechanism differences between PSA and Z-Sep+ additional dSPE mixture G was prepared. This mixture contains Z-Sep+ and PSA in the same proportion as C18 and PSA in mixture B. The comparison of the G and C' clean-up efficiency results showed also that the clean-up mechanisms of PSA and Z-Sep+ differ from one another. Distinct mechanisms were additionally confirmed when these sorbents were used to clean-up the citrate buffered extract. The results are shown in Fig. 4 and clearly indicate that PSA clean-up mechanism is based mainly on pH dependent ionic interactions whilst Z-Sep+ is based on pH independent Lewis acid and hydrophobic interactions. Z-Sep+ efficiency almost did not change whether citrate or acetate buffered extract were cleaned-up. In our opinion for the first time this different and complementary mechanisms of PSA and Z-Sep+ were combined and applied for dSPE clean-up. PSA and Z-Sep+ used together showed excellent clean-up productivity of honeybee extract by about 99.6% of matrix removal efficiency. When PSA and Z-Sep+ were simultaneously used the amount of matrix co-extractives finally left is almost twofold less in comparison with the dSPE sorbent mixtures used for honeybee samples clean-up in the literature [4,11].

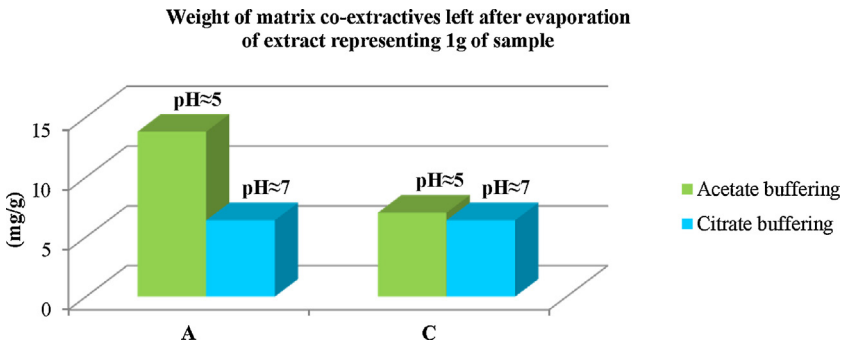

Fig. 4. Differences in dSPE clean-up efficiency achieved by PSA (A) and Z-Sep+ (C) sorbents depending the buffering protocol and pH value of extract during clean-up.

To check whether some pesticides are retained by any tested mixture of dSPE sorbents spiked honeybee samples were analysed. Z-Sep+ and PSA mixture of sorbents in acetate buffered conditions didnot cause losses of pesticides with acid groups or triazoles, e.g., cyproconazole, flutriafol, prothioconazole or triadimenol. Combination of Z-Sep+ with PSA build a correlation that even helps to recover more acidic pesticides. If the mixture of Z-Sep+ and PSA sorbents were used the recoveries of pesticides were much better in comparison to the same amount of Z-Sep+ used individually or when PSA + C18 mixture were used. The areas of MRM transitions were even twofold higher for many pesticides, even triazole fungicides like strong acid difenoconale ($pK_a = 1.07$) or neutral fenbuconazole (no dissociation), when to the same amount of Z-Sep+ sorbent the addition of PSA were used. Acid labile pesticides, like sulfonylureas has showed twofold better recoveries when combination of Z-Sep+ and PSA sorbents were used for clean-up instead of Z-Sep+ alone.

Finally, in contrary to previous findings about the negative impact of Z-Sep+ [25], dSPE clean-up with the newly developed mixture of Z-Sep+ and PSA together with acetate buffering gave excellent removing of matrix co-extractives without affecting acidic pesticides or triazoles and was chosen for the validation experiments and routine analysis.

3.2. LC optimization

Due to a large spectrum of analysed compounds, great differences between their physico-chemical properties and their acid-base properties, it was very difficult to obtain a good shape of chromatographic peak and reliable liquid chromatography analysis. The complexity of compounds requires selecting such chromatography column and conditions which enable analysis of a wide range of compounds in a honeybee matrix. The standard phase in pesticides analysis is octadecyl silane ligands (C18) bound to silica surface. This type of columns were tested during the optimisation step by analysing standard in solvent with concentration 0.8 μg/mL, prepared in the same way as the final extract for LC–MS/MS analysis. With the same conditions there was tested also Luna Phenyl-Hexyl, where phenyl groups are bound to silica surface using a 6-carbon chain for balanced aromatic and hydrophobic selectivity. The necessity for the application of LC column with selectivity other than provided by C8 or C18 ligands, particularly regarding polar or aromatic compounds, was previously observed during optimisation of the method for the determination of contaminants in honeybees [9]. Columns with phenyl-hexyl ligands were successfully used for the insecticide metabolites determination in beebread [27] and baby food [28] but not in multi-residue analysis of pesticides in honeybees, honey or even food [29,30]. Intensity of MRM transitions at tested Luna Phenyl-Hexyl column was higher than at Zorbax: Eclipse Plus C18, Extend-C18 and SB-Aq except Kinetex C18. Signals representing selected MRM's were more intensive at Kinetex C18 but for some analytes, like basic pesticide prapamocarb (Fig. 1) or pesticides with aromatic structure like hymexazol (Fig. 2), Luna Phenyl-Hexyl column gave more intensive and symmetric signals. Phenyl-hexyl phase enable analysis of a broader spectrum of compounds than C18 phase which offers only hydrophobic selectivity.

3.3. Optimisation of the MS/MS parameters

Each compound was separately optimised to obtain maximum sensitivity in MS/MS analysis. In LC–MS/MS analysis the following parameters were optimised: precursor ion, declustering potential (DP), product ions, collision energy (CE) and collision cell exit potential (CXP). To achieve highest sensitivity the ion source parameters such as source temperature, capillary voltage and both GS1, GS2 gas flows were also optimised. After setting the analyte retention times in LC–MS/MS analysis one another parameter, the time window, how long acquiring of each transition by the instrument would last, was optimised. Generally time windows were set at 60s (±30s from the retention time) but for some basic or acidic compounds, which retention time is sensitive for pH value, these windows were set at 120 s. The values of the LC–MS/MS optimized parameters for each MRM transition are shown in Supplementary materials in Table S1.

In GC–MS/MS analysis precursor ion, product ions, collision energy (CE) and dwell time for each analyte were optimised. The two most intensive precursor ion-product ion transitions were chosen for further procedure optimisation and validation. To achieve more sensitive analysis the total analysis time was divided through eight time segments, each acquiring different MRM transitions. The values of the GC–MS/MS optimized parameters for each MRM transition are shown in Supplementary materials in Table S2.

3.4. Method validation

To confirm that the developed method is suitable for its intended use the initial full validation according to SANCO guidance was carried out. Each evaluated parameter was verified against criteria indicated in the guidance document. All validation results obtained for pesticide determinations in spiked honeybee samples are listed in Table 3.

Calibration curves were generated by preparation of the procedural matrix matched standards. Each calibration standard in matrix were prepared twice at 6 concentration levels between 1 and 1000 ng/g. Almost all studied pesticides showed good linearity up to the level of 1000 ng/g, but some of GC–MS/MS analysed compounds like bifenox, bifenthrin, and chlorothalonil showed proper linearity up to the level of 500 ng/g. The obtained coefficients of determination (R^2) were higher than 0.99 and 0.98 respectively for 86% and 98% of analysed compounds.

Table 3
Validation results (linearity, trueness and precision) of pesticide determinations by LC-MS/MS and GC-MS/MS (bolded compound name) in honeybees.

Compound	Linearity range (ng/g)	R^2	Recovery,% (RSDr,%)			
			1 ng/g	5 ng/g	10 ng/g	100 ng/g
1-Naphthylacetamide (1-NAD)	1–1000	0.996	102 (8)	100 (5)	99 (8)	101 (6)
2,4-D	10–1000	0.998	–	–	102 (10)	112 (6)
2,4-Dimethylanilin (DMA)	100–1000	0.998	–	–	–	95 (11)
N-2,4-Dimethylphenylformamide (DMF)	5–1000	0.997	–	101 (5)	107 (5)	102 (6)
N-2,4-Dimethylphenyl-*N'*-methylformamidine (DMPF)	10–1000	0.997	–	–	96 (11)	100 (10)
6-Chloro-4-hydroxy-3-phenyl-pyridazine	1–1000	0.996	106 (11)	101 (4)	98 (3)	98 (8)
Abamectin	100–1000	0.994	–	–	–	111 (13)
Acetamiprid	1–1000	0.999	97 (8)	103 (6)	100 (3)	95 (6)
alpha-Cypermethrin	5–1000	0.987	–	109 (6)	88 (14)	110 (3)
Amidosulfuron	1–1000	0.999	108 (9)	104 (5)	110 (10)	94 (7)
Azoxystrobin	1–1000	0.992	103 (9)	105 (5)	94 (4)	84 (6)
Bentazone	1–1000	0.999	102 (13)	100 (3)	105 (5)	102 (8)
6-Hydroxy bentazon	10–1000	0.957	–	–	104 (4)	106 (9)
Beta-cyfluthrin	5–1000	0.980	–	112 (5)	90 (14)	108 (4)
Bifenazate	10–1000	0.982	–	–	98 (15)	88 (16)
Bifenox	5–500	0.995	–	90 (19)	97 (5)	92 (9)
Bifenthrin	5–500	0.999	–	96 (2)	102 (7)	101 (6)
Bixafen	1–1000	0.996	108 (12)	94 (7)	111 (5)	103 (7)
Boscalid	1–1000	0.991	85 (9)	94 (7)	93 (11)	99 (6)
Bromopropylate	1–1000	0.999	97 (4)	103 (6)	95 (14)	101 (7)
Bromoxynil	10–1000	0.998	–	–	110 (3)	98 (7)
Bupirimate	1–1000	0.996	90 (12)	82 (4)	98 (5)	103 (5)
Carbendazim	1–1000	0.999	104 (11)	102 (7)	96 (6)	90 (10)
Carbetamide	1–1000	0.999	103 (7)	99 (3)	105 (3)	106 (5)
Carboxine	1–1000	0.999	110 (9)	100 (7)	102 (4)	100 (9)
Carfentrazone-ethyl	1–1000	0.997	88 (12)	92 (9)	84 (8)	82 (12)
Chlorantraniliprole	1–1000	0.996	92 (16)	97 (4)	99 (6)	96 (7)
Chloridazon	1–1000	0.993	97 (17)	106 (6)	105 (5)	105 (6)
Chlorothalonil	10–500	0.994	–	–	85 (14)	96 (15)
Chlorotoluron	1–1000	0.999	81 (11)	95 (9)	98 (6)	100 (7)
Chlorpropham	1–1000	0.998	94 (8)	105 (2)	97 (10)	102 (2)
Chlorpyrifos	1–1000	0.999	96 (6)	98 (4)	98 (3)	97 (4)
Chlorpyrifos-methyl	1–1000	0.999	88 (6)	95 (3)	94 (3)	98 (3)
Chlorsulfuron	1–1000	0.999	96 (19)	99 (11)	106 (5)	108 (12)
Clethodim	5–1000	0.997	–	91 (5)	99 (5)	94 (6)
Clofentezine	5–1000	0.986	–	103 (6)	75 (12)	84 (8)
Clomazone	1–1000	0.999	114 (3)	98 (3)	99 (5)	104 (6)
Clothianidin	5–1000	0.999	–	66 (14)	94 (10)	103 (9)
Coumaphos	1–1000	0.992	100 (16)	88 (7)	104 (10)	111 (4)
Cyazofamid	5–1000	0.999	–	96 (6)	125 (6)	119 (6)
Cycloxydim	10–1000	0.995	–	–	99 (7)	81 (7)
Cyflufenamid	1–1000	0.981	70 (14)	85 (17)	123 (7)	110 (9)
Cymiazol	5–500	0.993	–	72 (11)	97 (10)	100 (9)
Cymoxanil	5–1000	0.999	–	101 (6)	102 (5)	98 (8)
Cyproconazole	5–1000	0.998	–	111 (6)	98 (2)	104 (5)
Cyprodinil	10–1000	0.997	–	–	97 (9)	98 (6)
Deltamethrin	5–1000	0.997	–	107 (6)	83 (8)	93 (6)
Desmedipham	1–1000	0.999	100 (16)	94 (5)	91 (7)	89 (13)
Dichlorprop-P (2,4-DP)	10–1000	0.998	–	–	95 (15)	95 (9)
Difenoconazole	1–1000	0.995	70 (18)	84 (5)	78 (11)	98 (8)
Diflubenzuron	1–1000	0.997	109 (15)	94 (7)	90 (4)	82 (10)
Diflufenican	5–1000	0.972	–	82 (11)	79 (9)	86 (13)
Dimethachlor	1–1000	0.999	104 (9)	98 (2)	99 (5)	94 (8)
Dimethoate	1–1000	0.999	108 (8)	102 (3)	99 (3)	101 (7)
Dimethomorph	1–1000	0.999	104 (15)	96 (5)	82 (5)	77 (7)
Dimoxystrobin	1–1000	0.999	114 (18)	101 (5)	103 (11)	95 (8)
Dithianon	100–1000	0.969	–	–	–	76 (9)
Epoxiconazole	5–1000	0.998	–	91 (7)	92 (6)	102 (4)
Esfenvalerate	5–1000	0.999	–	98 (5)	96 (10)	96 (5)
Ethofumesate	10–1000	0.999	–	–	83 (13)	91 (8)
Ethoprophos	1–1000	0.988	98 (9)	100 (6)	100 (5)	101 (11)
Etofenprox	1–500	0.999	93 (13)	94 (2)	93 (5)	100 (5)
Etoxazole	5–1000	0.999	–	86 (5)	88 (10)	69 (12)
Famoxadone	5–500	0.994	–	97 (9)	103 (11)	108 (5)
Fenazaquin	1–1000	0.997	119 (18)	94 (3)	105 (10)	96 (7)
Fenbuconazole	5–1000	0.993	–	84 (2)	101 (2)	104 (9)
Fenhexamid	10–1000	0.999	–	–	107 (7)	99 (6)
Fenoxaprop-*P*-ethyl	5–1000	0.985	–	95 (11)	101 (9)	83 (10)
Fenpropidin	5–1000	0.996	–	98 (8)	85 (6)	78 (8)
Fenpropimorph	5–1000	0.995	–	104 (2)	90 (6)	84 (13)
Fenpyroximate	5–1000	0.997	–	84 (9)	81 (16)	65 (14)
Fipronil	1–1000	0.995	104 (11)	95 (5)	100 (4)	96 (9)
Fipronil-carboxamide	1–1000	0.994	112 (15)	106 (10)	99 (9)	100 (7)
Fipronil-desulfinyl	5–1000	0.998	–	92 (7)	97 (8)	87 (7)
Fipronil-sulfide	1–1000	0.996	102 (14)	89 (4)	99 (8)	94 (10)

Table 3 (*Continued*)

Compound	Linearity range (ng/g)	R^2	Recovery,% (RSDr,%)			
			1 ng/g	5 ng/g	10 ng/g	100 ng/g
Fipronil-sulfone	1–1000	0.997	108 (12)	95 (4)	95 (8)	92 (12)
Flazasulfuron	1–1000	0.996	120 (10)	109 (3)	104 (5)	98 (6)
Flonicamid	10–1000	0.999	–	–	94 (11)	97 (8)
Florasulam	1–1000	0.999	86 (13)	110 (6)	106 (5)	87 (5)
Fluazifop-*P*-butyl	5–1000	0.990	86 (18)	95 (7)	82 (9)	70 (12)
Fluazinam	5–1000	0.983	–	70 (7)	88 (7)	77 (10)
Fludioxonil	1–1000	0.998	86 (18)	98 (9)	101 (6)	100 (9)
Flufenacet	1–1000	0.987	114 (19)	101 (10)	95 (12)	95 (10)
Fluquinconazole	1–1000	0.998	94 (16)	82 (11)	102 (5)	106 (5)
Flurochloridon	5–1000	0.996	–	105 (10)	103 (7)	108 (6)
Fluroxypyr	100–1000	0.997	–	–	–	80 (8)
Fluroxypyr-1-methylheptyl ester	10–1000	0.990	–	–	93 (9)	77 (16)
Flusilazole	1–1000	0.996	108 (12)	90 (5)	100 (7)	103 (6)
Flutriafol	10–1000	0.998	–	–	104 (9)	102 (7)
Foramsulfuron	1–1000	0.999	106 (5)	98 (6)	108 (6)	104 (8)
Gibberellin A4	100–1000	0.999	–	–	–	109 (7)
Hexythiazox	5–1000	0.998	–	92 (11)	73 (15)	60 (14)
Hymexazol	100–1000	0.999	–	–	–	103 (8)
Imazalil	5–1000	0.995	–	98 (6)	89 (9)	97 (12)
Imidacloprid	1–1000	0.988	100 (12)	105 (4)	105 (14)	94 (8)
Imidacloprid-olefin	1–500	0.993	110 (13)	107 (6)	99 (10)	90 (7)
Imidacloprid-urea	1–500	0.983	118 (14)	95 (12)	101 (13)	84 (7)
6-Chloronicotinic acid	100–1000	0.997	–	–	–	79 (10)
Indole-3-butyric acid (IBA)	10–1000	0.999	–	–	112 (10)	99 (7)
Indoxacarb	5–1000	0.986	–	107 (6)	91 (10)	82 (5)
Iodosulfuron-methyl-sodium	1–500	0.992	90 (18)	106 (8)	106 (10)	97 (12)
Ipconazole	5–1000	0.993	–	86 (7)	103 (8)	103 (8)
Iprodione	10–1000	0.992	–	–	100 (12)	90 (10)
Isoproturon	5–1000	0.999	–	96 (6)	96 (7)	99 (7)
Isopyrazam	1–1000	0.992	110 (11)	93 (2)	97 (9)	77 (10)
Isoxaflutole	1–1000	0.995	100 (10)	92 (13)	103 (9)	115 (9)
Kresoxim-methyl	5–1000	0.995	–	94 (6)	93 (11)	107 (6)
Lambda-Cyhalothrin	1–1000	0.992	106 (17)	108 (6)	96 (13)	112 (5)
Lenacil	1–1000	0.990	112 (4)	103 (4)	104 (7)	104 (6)
Linuron	1–1000	0.999	110 (14)	94 (9)	94 (10)	92 (9)
Mandipropamid	1–1000	0.998	106 (11)	102 (5)	91 (8)	98 (9)
Malathion	5–1000	0.999	–	94 (4)	95 (4)	99 (3)
MCPA	5–1000	0.997	–	108 (9)	97 (7)	103 (11)
MCPB	100–1000	0.999	–	–	–	106 (5)
Mecoprop-P (MCPP-P)	10–1000	0.999	–	–	91 (8)	90 (6)
Mepanipyrim	10–1000	0.988	–	–	96 (7)	111 (3)
Mesosulfuron-methyl	1–1000	0.999	122 (7)	103 (4)	106 (5)	100 (7)
Mesotrione	5–1000	0.996	–	96 (7)	110 (6)	89 (10)
Metaflumizone	10–1000	0.992	–	–	46 (16)	48 (6)
Metalaxyl-M/Metalaxyl	1–1000	0.999	98 (8)	99 (5)	102 (5)	93 (6)
Metamitron	5–1000	0.995	–	115 (13)	100 (8)	120 (8)
Metazachlor	1–1000	0.997	102 (8)	98 (3)	97 (10)	87 (12)
Metconazole	5–1000	0.995	–	102 (9)	103 (6)	101 (6)
Methiocarb	1–1000	0.997	90 (8)	98 (5)	101 (5)	96 (8)
Methiocarb sulfone	1–1000	0.998	108 (12)	100 (2)	97 (11)	99 (7)
Methiocarb sulfoxide	1–1000	0.992	98 (11)	101 (10)	102 (4)	97 (7)
Methoxyfenozide	5–1000	0.989	–	99 (9)	101 (9)	91 (7)
Methyl isothiocyanate (MITC)	100–1000	0.999	–	–	–	95 (9)
Metrafenone	5–1000	0.991	–	107 (7)	107 (10)	101 (16)
Metribuzin	5–1000	0.999	–	97 (8)	96 (5)	100 (7)
Metsulfuron-methyl	1–1000	0.999	110 (6)	106 (5)	110 (9)	102 (8)
Myclobutanil	100–1000	0.999	–	–	–	102 (6)
Napropamide	1–1000	0.999	98 (13)	89 (6)	98 (5)	100 (10)
Nicosulfuron	1–1000	0.999	104 (11)	106 (6)	105 (3)	98 (7)
Nitenpyram	5–1000	0.995	–	112 (5)	108 (10)	94 (9)
Novaluron	10–1000	0.989	–	–	86 (14)	89 (17)
Oxyfluorfen	10–1000	0.994	–	–	106 (9)	78 (12)
Pencycuron	1–1000	0.990	96 (16)	85 (7)	84 (13)	100 (8)
Pendimethalin	1–500	0.983	88 (19)	85 (7)	75 (10)	101 (4)
Pethoxamid	1–1000	0.999	100 (7)	98 (5)	99 (5)	94 (6)
Phenmedipham	5–1000	0.999	–	105 (7)	102 (4)	102 (9)
Phosmet	5–1000	0.997	–	93 (10)	109 (6)	112 (5)
Picoxystrobin	1–1000	0.992	114 (11)	95 (9)	86 (10)	104 (11)
Pirimicarb	1–1000	0.999	98 (11)	95 (6)	94 (6)	95 (6)
Pirimicarb-desmethyl	1–1000	0.999	114 (8)	102 (6)	99 (6)	94 (9)
Pirimiphos-methyl	1–1000	0.998	112 (8)	90 (8)	85 (5)	77 (8)
Prochloraz	5–1000	0.987	–	94 (7)	94 (11)	94 (19)
Propamocarb	5–1000	0.987	–	108 (9)	90 (4)	86 (8)
Propaquizafop	1–1000	0.980	116 (17)	98 (13)	82 (11)	72 (10)
Propiconazole	5–1000	0.996	–	111 (8)	110 (6)	103 (7)
Propoxycarbazone-sodium	5–1000	0.991	–	105 (2)	105 (11)	88 (9)

Table 3 (*Continued*)

Compound	Linearity range (ng/g)	R^2	Recovery,% (RSDr,%)			
			1 ng/g	5 ng/g	10 ng/g	100 ng/g
Propyzamide	1–1000	0.999	104 (16)	96 (7)	101 (9)	97 (9)
Proquinazid	5–1000	0.984	–	90 (3)	85 (13)	71 (12)
Prosulfocarb	5–1000	0.986	–	94 (5)	111 (13)	105 (11)
Prothioconazole	100–1000	0.984	–	–	–	110 (9)
Prothioconazole-desthio	1–1000	0.990	104 (19)	100 (10)	89 (8)	105 (7)
Pyraclostrobin	1–1000	0.993	99 (6)	86 (5)	80 (6)	78 (12)
Pyridate	5–1000	0.991	–	51 (16)	72 (5)	56 (8)
Pyrimethanil	5–1000	0.998	–	94 (7)	85 (5)	71 (7)
Pyriproxyfen	5–500	0.977	–	71 (9)	83 (6)	73 (8)
Quinoclamine	5–1000	0.999	–	102 (12)	108 (10)	93 (4)
Quinoxyfen	10–1000	0.999	–	–	112 (8)	82 (12)
Quizalofop-*P*-ethyl	5–1000	0.936	–	109 (8)	105 (12)	91 (8)
Quizalofop-*P*-tefuryl	10–500	0.994	–	–	90 (8)	94 (9)
Rimsulfuron	1–1000	0.996	80 (15)	94 (5)	99 (8)	98 (8)
S-Metolachlor	1–1000	0.994	98 (11)	104 (6)	104 (3)	105 (5)
Silthiofam	1–1000	0.996	102 (16)	81 (9)	99 (9)	96 (7)
Spinosyn A	0.75–750	0.975	110 (10)	95 (12)	102 (11)	90 (15)
Spinosyn D	1.25–250	0.989		123 (9)	78 (13)	76 (15)
Spirodiclofen	5–500	0.996	–	83 (9)	93 (6)	86 (8)
Spirotetramat	1–1000	0.995	68 (19)	101 (5)	102 (4)	101 (10)
Spirotetramat-enol	1–1000	0.991	110 (11)	107 (5)	96 (7)	98 (6)
Spirotetramat-enol-glucoside	1–1000	0.984	103 (14)	82 (8)	109 (5)	107 (13)
Spirotetramat-keto-hydroxy	10–1000	0.999	–	–	110 (4)	107 (4)
Spiroxamine	1–1000	0.990	116 (13)	100 (7)	101 (7)	92 (11)
Sulcotrione	10–1000	0.979	–	–	87 (12)	90 (7)
Sulfosulfuron	1–1000	0.996	110 (9)	98 (5)	104 (6)	102 (6)
tau-Fluwalinate	5–500	0.995	–	88 (8)	99 (5)	102 (6)
Tebuconazole	1–1000	0.992	100 (10)	98 (4)	99 (7)	94 (12)
Tebufenozide	100–1000	0.983	–	–	–	70 (13)
Tebufenpyrad	5–1000	0.991	–	82 (9)	94 (8)	84 (5)
Teflubenzuron	10–1000	0.988	–	–	88 (13)	80 (8)
Tefluthrin	1–500	0.999	92 (12)	98 (2)	102 (6)	103 (5)
Tembotrione	10–1000	0.997	–	–	–	97 (6)
Tepraloxydim	10–1000	0.997	–	–	97 (18)	82 (8)
Terbuthylazine	5–1000	0.992	–	88 (10)	106 (11)	111 (7)
Tetraconazole	5–1000	0.998	–	86 (12)	108 (5)	108 (4)
Thiacloprid	1–1000	0.998	94 (14)	105 (2)	102 (2)	99 (7)
Thiacloprid-amide	1–1000	0.997	92 (12)	99 (4)	113 (4)	100 (6)
Thiamethoxam	5–500	0.995	–	98 (8)	107 (6)	102 (6)
Thifensulfuron-methyl	1–1000	0.999	104 (16)	105 (5)	110 (7)	99 (9)
Thiophanate-methyl	1–1000	0.988	96 (12)	91 (11)	84 (3)	103 (9)
Tralkoxidym	5–1000	0.999	–	88 (12)	101 (6)	84 (13)
Triadimenol	10–1000	0.999	–	–	81 (11)	88 (8)
Tribenuron-methyl	100–1000	0.995	–	–	–	78 (11)
Trifloxystrobin	1–1000	0.977	100 (12)	95 (8)	92 (8)	96 (12)
Triflusulfuron-methyl	5–1000	0.998	–	104 (13)	76 (10)	86 (12)
Trinexapac-ethyl	100–1000	0.998	–	–	–	98 (7)
Triticonazole	5–1000	0.998	–	97 (4)	90 (6)	102 (7)
zeta-Cypermethrin	5–1000	0.985	–	84 (6)	86 (14)	111 (2)

Matrix effects were calculated by comparison between slopes of calibration curve in matrix and in solvent for each analyte. Standards in solvents either for LC–MS/MS or GC–MS/MS analysis were prepared in the same way as final extracts in sample preparation procedure. Among 200 tested pesticides 67 pesticides showed negligible matrix effects with ME(%) in the range from −20% to 20%. For LC–MS/MS analysed pesticides the suppression effect was rather observed, whilst for GC–MS/MS analysed pesticides both suppression and enhancement effects were observed. Therefore procedural matrix matched calibration was used to overcome this phenomenon and compensate for matrix effects.

For evaluation of trueness (recovery) and precision (repeatability) blank honeybee samples were spiked at four concentration levels of 1, 5, 10 and 100 ng/g bees. Spiked samples at each level were analysed in five replicates. The trueness determined by average recovery were calculated by comparison the determined concentrations of spiked samples to their target level. The precision was determined by calculating the relative standard deviation (RSDr). The recovery values obtained for majority of pesticides were within the satisfactory range 70–120% with RSDr value below 20%. Only metaflumizone and pyridate showed constantly lower recoveries but sufficient precision allowed to include these pesticides within the scope of the method. The RSDr values in all cases were below 20%.

The LOQ levels were in the range of 1–100 ng/g but, for 93% of pesticides were less or equal to 10 ng/g which makes this method very sensitive and suitable for its intended use in active and passive monitoring study. The analysis of bee samples spiked at the LOQ level showed that 97% of analytes showed recovery in the required range of 70–120% and RSDr (precision) below 20%.

Method was satisfactory verified in the international proficiency test organised by EURL for Residues of Pesticides in Food of Animal Origin and Commodities with high Fat Content (CVUA Freiburg, Germany) by analysing honey sample in the EUPT AO-10. The developed method for honeybee was also applied for analysis of honey. The laboratory analysed honey for 107 pesticides and for 18 detected compounds achieved the following qualification: category A and AZ^2 (the average of the squared *z*-Scores) 0.15.

Table 4
Pesticides detected in 73 samples of poisoned honeybees.

Compound	Number of detections	Min (ng/g)	Mean(ng/g)	Median(ng/g)	90th percentile(ng/g)	95th percentile(ng/g)	Max(ng/g)	LD_{50} [32](ng/g)[a]
Chlorpyrifos	38	1.5	272	26.0	1000	1210	3290	590
Dimethoate	30	1.4	399	298	757	1170	1596	1200
Clothianidin	22	5.3	17.1	12.0	26.0	47.9	76.2	440
Tebuconazole	18	1.6	93.1	25.9	44.6	228	1245	>2,000,000
N-2.4-Dimethylphenylformamide (DMF)	17	5.9	40.4	12.4	118	131	147	–
Cyprodinil	13	10.2	182	77.0	362	538	782	>7,840,000
Thiophanate-methyl	12	3.6	28.0	14.8	44.8	96.7	160	>1,000,000
N-2.4-Dimethylphenyl-*N*′-methylformamidine (DMPF)	11	17.1	216	210	504	625	746	–
Chlorothalonil	11	10.2	14.324	6034	35.030	45.415	55.800	>630,000
zeta-Cypermethrin	9	9.0	120	22.0	304	317	329	20
Carbendazim	8	1.7	10.6	11.6	15.9	19.2	22.5	>500,000
Azoxystrobin	7	2.3	78.1	90.0	141	143	146	>2,000,000
Difenoconazole	7	2.1	26.1	10.4	64.6	68.9	73.1	>1,000,000
Pyrimethanil	7	5.4	50.8	32.0	99.4	110	120	>1,000,000
MCPA	6	5.0	11.6	7.8	21.0	25.7	30.3	>2,000,000
Trifloxystrobin	6	1.9	32.2	22.3	68.2	72.6	77.0	>2,000,000
Fludioxonil	5	4.3	8.8	5.9	15.2	17.6	20.0	>1,000,000
tau-Fluwalinate	5	9.2	273	25.0	740	910	1080	120,000
Acetamiprid	4	2.0	4.2	3.9	6.3	6.7	7.0	80,900
Boscalid	4	1.7	3.8	2.7	6.8	7.5	8.2	>2,000,000
Etofenprox	3	3.0	17.7	6.1	36.4	40.2	44.0	>1300
Fipronil	3	34.2	158	170	251	261	271	59
Fipronil-sulfone	3	232	433	478	568	579	590	–
Imidacloprid	3	2.2	10.4	2.6	21.7	24.1	26.5	810
Thiacloprid	3	3.3	73.1	42.1	148	161	174	388,200
2.4-D	2	21.9	25.4	25.4	28.1	28.5	28.8	>1,000,000
Fipronil-carboxamide	2	1.8	2.0	2.0	2.2	2.2	2.2	–
Fipronil-sulfide	2	4.6	5.6	5.6	6.4	6.5	6.6	–
lambda-Cyhalothrin	2	11.0	12.5	12.5	13.7	13.9	14.0	380
Prochloraz	2	5.4	25.0	25.0	40.6	42.5	44.5	1,413,000
Propiconazole	2	9.3	38.7	38.7	62.2	65.2	68.1	>1,000,000
Prosulfocarb	2	5.7	10.7	10.7	14.6	15.1	15.6	>800,000
Thiamethoxam	2	44.0	160	160	252	264	275	240
Abamectin	1	–	–	–	–	–	588	
alpha-Cypermethrin	1	–	–	–	–	–	11.0	330
Beta-Cyfluthrin	1	–	–	–	–	–	9.7	10
Bifenazate	1	–	–	–	–	–	21.1	85,000
Clomazone	1	–	–	–	–	–	1.6	–
Cyflufenamid	1	–	–	–	–	–	1.8	>1,000,000
Deltamethrin	1	–	–	–	–	–	12.5	15
Desmedipham	1	–	–	–	–	–	6.9	>250,000
Dithianon	1	–	–	–	–	–	952	>1,000,000
Fipronil-desulfinyl	1	–	–	–	–	–	5.5	–
Flurochloridon	1	–	–	–	–	–	342	1,000,000
Flusilazole	1	–	–	–	–	–	4.4	1,650,000
Imidacloprid-urea	1	–	–	–	–	–	4.0	–
Metalaxyl-M/Metalaxyl	1	–	–	–	–	–	3	2,000,000
Metconazole	1	–	–	–	–	–	10.2	>1,000,000
Myclobutanil	1	–	–	–	–	–	109	–
Pendimethalin	1	–	–	–	–	–	12.1	1,000,000
Phenmedipham	1	–	–	–	–	–	6.7	>500,000
Propaquizafop	1	–	–	–	–	–	1.6	>2,000,000
Quinoclamine	1	–	–	–	–	–	8.3	>7500
S-Metolachlor	1	–	–	–	–	–	12.3	>2,000,000
Spinosyn A	1	–	–	–	–	–	1.8	36
Terbuthylazine	1	–	–	–	–	–	9.4	>320,000
Tetraconazole	1	–	–	–	–	–	7.3	630,000

[a] LD_{50}: conversion of units to ng/g done for mean honeybee weight equal to 0.1 g.

3.5. Real samples application

A total number of 74 poisoned honeybee samples were analysed during the years 2014–2015. The samples were send from different regions of Poland and represent apiaries with different number of hives. Apiaries that suffered from PPPs poisonings counted more than 2000 colonies in total.

In 73 samples there were determined at least one pesticide or metabolite. Only one sample was free from pesticides. The names of determined compounds together with number of positive samples, statistical evaluation of gathered results and contact acute 48 h LD50 value are included in Table 4. Four pesticides as average was determined simultaneously in honeybee sample. Up to thirteen pesticides were determined at the same sample. In total 57 pesticides and metabolites were determined in poisoned honeybee samples. The list of compounds detected in concentrations above LOQ includes 21 insecticides and their metabolites, 20 fungicides, 12 herbicides, 2 acaricides and 2 VMP metabolites. Top three of the insecticides detected in most poisoning incidents were organophosphates: chlorpyrifos (38 samples), dimethoate (30 samples) and neonicotinoid clothianidin (22 samples). The most commonly found fungicides were systemic ones as tebuconazole (18 samples), cyprodynil (13 samples) and thiophanate-methyl (12 samples). Tebuconazole is by itself low toxic to honeybees (LD50 above 200 μg/bee) but it belongs to an ergosterol biosynthesis inhibitor (EBI) fungicides which are known from a synergistic effects with different insecticides. Triflumizole for example in laboratory bioassays increased 244-fold honey bee toxicity of acetamiprid and 1141-fold toxicity of thiacloprid [31]. Non-systemic chlorothalonil (11 samples) was determined at very high concentrations. From the herbicide group only MCPA, 2,4-D and prosulfocarb were determined more than only once. Acaricides (abamectin and bifenazate) were determined in single cases. In many cases DMF and DMPF, metabolites of amitraz—VMP, were detected. MRM chromatograms of all detected pesticides are shown in Supplementary materials in Figs. S1–S57.

4. Conclusions

This newly developed multiresidue method allows simultaneous analysis of the broadest spectrum of pesticides currently approved in European Union to use as plant protection products and veterinary medicinal products in honeybee samples. Beyond the determination of active substances it is also possible to detect many significant metabolites (e.g., metabolites of imidacloprid, thiacloprid, fipronil, methiocarb and amitraz).

Only 76 pesticides included in this study do not dissociate which shows that pesticides with acid-base properties are in the majority of currently used pesticides. Thus there is necessity of developing multiresidue methods that includes as much as possible pH dependent compounds. The application of new Z-Sep+ sorbent together with PSA and acetate buffering conditions enable analysis of a broad spectrum of acidic pesticides the same as basic. For the first time different clean-up mechanisms of PSA and Z-Sep+ were combined and applied. These newly developed dSPE sorbent mixture provided more effective clean-up than sorbents till now used in honeybee samples analysis. Validation data together with satisfactory results reached at proficiency test of EUPT AO-10 for carbendazim and tebuconazole clearly showed that developed method, despite the use of Z-Sep+, enable very effective clean-up without losing basic pesticides or triazole fungicides. This proficiency test confirms also applicability of this new developed method in food analysis.

This new QuEChERS based method allows very sensitive and reliable simultaneous analysis of 200 pesticides and pesticide metabolites in honeybees, which makes this method a great important tool for establishing which actually used pesticides could have a negative impact on bee health. Fifty seven pesticides determined in poisoned honeybees clearly confirms the right selection of compounds and applicability of this method. It is the broadest spectrum of pesticides and their metabolites till now detected in honeybees. It is also a tool for establishing the cumulative risk for bee health caused by a mixture of pesticides that could be present in honeybee body, by interactions between themselves and between pesticides and other epidemiological factors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.01.045.

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