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**MONITORING OF MULTI-CLASS PESTICIDES IN HONEY SAMPLES
FROM MACEDONIA BY ULTRA HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY – TANDEM QUADRUPOLE MASS
SPECTROMETRY**

ABSTRACT

The possible presence of pesticide residues in honey has impelled the need for setting up monitoring programs to determine the proper assessment of human exposure to pesticides. This paper describes an effective multi-class method using a modified QuEChERS sample preparation for detection and quantification of 18 pesticides with Ultra-high-performance liquid chromatography (UHPLC) – tandem quadrupole mass spectrometry. The method was validated according to the requirements laid down in DG SANCO 12571/2013 document. Levels of detection and quantification were lower than the established MRLs, the obtained precision was better than 20 %, and the recovery values were between 74.4 and 104.1 %. Fifty honey samples within the national monitoring program were collected from August to November, 2014 and tested for presence of carbaryl, carbofuran, fenvalerate, cypermethrin, deltamethrin, permethrin, bifenthrin, amitraz, coumaphos, bromopropylate, dichlorvos, diazinone, malathion, parathion, dimethoate, omethoate, methomyl and thiametoxam. Trace levels of methomyl, diazinone, bromopropylate and fenvalerate were detected somewhat above the reporting level for these pesticides (10 µg/kg). This contamination indicates on existing of possible moderate cross-contamination during pollen and nectar collection by bees, and residues of substances used in beekeeping. Although traces of pesticides were found in 16 of the samples tested (32 %), the levels found did not pose increased toxicological risk for the population.

Keywords: pesticides, honey, multi class method, liquid chromatography, tandem quadrupole, QuEChERS.

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INTRODUCTION

Honey is a valuable commodity that can be used as a final product or food ingredient. It is a highly-energetic natural carbohydrate product containing significant number of compounds with high benefit for the humans (Eissa et al. 2014). The quality of honey is mainly determined by its sensorial, physicochemical and microbiological characteristics. The honey quality criteria are well defined by the EC Directive 2001/110 (EC, 2001). Agricultural contamination of food with pesticides is a challenging problem that could not be neglected. In recent years, analysis of pesticide residues in food becomes a substantial requirement for consumers, producers and food safety authorities (Eissa et al. 2014). Pesticide residues might cause genetic mutations and cellular degradation, thus presenting a potential risk for the human health, mainly due to their cumulative properties (Blasco et al. 2003).

The analysis of pesticides residues in honey is significant analytical challenge, due to the complex and variable honey composition dependable on the type of plant where bees collect nectar (Tomasini et al. 2012). It is considered that there are two forms of contamination of honey: during pollen and nectar collection of bees, indirect contamination and through treatment of hives, in which pesticides can migrate in honey, i.e. direct contamination (Orso et al. 2014). The evidence for the presence of pesticide residues in honey (Blasco et al. 2003, Orso et al. 2014) has impelled the need for setting up monitoring programs for obtaining a proper assessment of human exposure to pesticides through honey consumption (Wallner, 1999). Different national regulations have established maximum permitted concentrations of pesticides (MRLs) in honey, but the lack of harmonized regulative cause problems in international trade (Blasco et al. 2003).

Many publications have reported various extraction methods for determination of pesticide residues in honey, employing solid phase-extraction (SPE), matrix solid-phase dispersion (MSPD), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) (Amendola et al. 2011). The pesticide determination is usually performed by GC or LC coupled with various detectors. Only few of these methods allow multi-class detection of pesticide residues, as well as their quantification at very low concentration levels. In the recent years MSPD sample preparation has been identified as powerful tool for multi-residual analysis of pesticides in honey samples due to its compatibility both with GC and LC chromatographic systems and mass spectrometric detection systems (Tomasini et al. 2012; Orso et al. 2014, Rodríguez-López et al. 2014; Fidente et al. 2005; Tanner and Czerwenka, 2011).

The lack of information about pesticide residues in honey samples from Macedonia implies the necessity to determinate the pollution of this bee product.

Accordingly, the aim of this study was to identify the current honey contamination with 18 pesticides applying validated multi-class ultra high-performance liquid chromatography (UHPLC) – tandem mass spectrometry method. On basis of pesticides found in a representative number of 50 honey samples, we could conclude whether the honey samples were exposed to direct or indirect contamination, as well as their safety from the toxicological point of view.

MATERIAL AND METHODS

Chemicals and materials

As an analytical standards carbaryl, carbofuran, fenvalerate, cypermethrin, deltamethrin, permethrin, bifenthrin, amitraz, coumaphos, bromopropylate, dichlorvos, diazinone, malathion, parathion, dimethoate, omethoate, methomyl and thiametoxam were purchased from Fluka (Pestanal quality). Acetonitrile was with HPLC grade, while methanol and water were with LC/MS grade, all supplied from Carlo Erba. Formic acid (Suprapur grade) and ammonium formate were products of Sigma-Aldrich. The sorbents used for MSPD were magnesium sulfate anhydrous (Merck), sodium chloride (Carlo Erba), primary-secondary amine - PSA and C-18 (Supelco).

Sample collection

A total of 50 multifloral honey samples were collected at individual beekeepers from different geographical locations in Macedonia in the period August-November, 2014. Upon collection, all honey samples were placed into clean glass jars, labeled and placed on +4 °C, transferred to the laboratory and kept at 4 °C until analysis. The sample size needed for obtaining the required sample homogeneity was at least 500 g.

Sample preparation: modified QuEChERS procedure

The extraction method used was based on the original QuEChERS method developed by Anastassiades et al. (2003). Briefly, for the extraction, 5.0 g of honey was weighed in a 50 mL polypropylene centrifuge tube and 10 mL of water was added. The mixture was vortexed for 1 min. Afterwards 10 mL acetonitrile was added and the sample was homogenized. After adding 4 g magnesium sulfate and 1 g sodium chloride, the sample was vortexed again and centrifuged at 8000 rpm for 10 min. Six mL of the upper layer was placed into 15 mL polypropylene tube containing 900 mg magnesium sulfate, 150 mg PSA and 150 mg C-18 sorbent. After 1 min homogenization the tube was centrifuged at 3000 rpm for 5 min. Four mL of the solution were evaporated to dryness, re-dissolved in 2 mL mobile phase, filtered through 0.45 µm filter and placed into autosampler vial and further analyzed with UHPLC-TQD system.

Instrument and experimental conditions

UHPLC with mass spectrometric detection was performed on Acquity – TQD Waters system (Waters, Milford, MA, USA), utilizing Ascentis® Express of 2.7 µm particle size, 50 mm length and 2.1 mm. i.d. (Supelco, Bellefonte, PA, USA). Source temperature was 150 °C, desolvation temperature 450 °C. Detection by MS/MS was performed using electrospray ionization (ESI) operating in positive mode. For each compound the optimum collision energies with aim of getting two multiple reaction monitoring (MRM) transitions with the best signal intensity were selected.

The mobile phase components for the UHPLC system were methanol (A) and water (B), both modified with 0.1 % formic acid and 5 mM ammonium formate, applying suitable gradient program. The flow rate was 0.6 mL/min and the total run time 23.10 min.

Method validation

The optimized UHPLC-MS/MS method employing modified QuEChERS sample preparation was validated regarding the requirements laid down in SANCO Guidance document on analytical quality control and validation procedures for pesticide residue analysis in food and feed (SANCO, 2013). Accordingly, the method validation includes the following parameters: limits of detection and quantification (LODs and LOQs), linearity, precision and recovery. The range of determination was 5-250 ng/mL, the precision and accuracy was estimated at reporting level 10 µg/kg and five times reporting level i.e. 50 µg/kg.

RESULTS AND DISCUSSION

Optimization of the LC-ESI-MS-MS parameters

The chromatograms were recorded in MRM mode with ESI interface in positive ionization mode. The MRM analysis introduces high method specificity based on the detection of both parent ion and one of its known fragments (Sampaio *et al.* 2012). For each compound, the in-house optimized collision energies were selected with the aim of getting two characteristic MRM transitions with the best intensity (Table 1).

The main advantage of the method was the application of MS/MS since it provides high confidence level for identification of the target pesticides. The monitoring of the second fragmentation products allows more efficient discrimination of the interfering matrix, than the products of the primary fragmentation (Sampaio *et al.* 2012). The first MRM transitions exhibited higher signals and accordingly higher sensitivity, thus, they were used for quantification of the compounds examined. The second MRM transitions are so-called target ions and are mandatory for confirmatory purposes.

Table 1. MRM conditions for the simultaneous determination of pesticides with electrospray ionization in positive mode (dwell time 0.025 s)

Pesticide	Parent ion/(m/z)	Product ion/(m/z)	CV ^a /(V)	CE ^b /(eV)	RT ^c /(min)
Carbaryl	202.0	202>127.1	22	15	5.87
		202>145.1	22	10	
Carbofuran	222.1	222.1>123.1	80	20	5.47
		222.1>165.1	80	5	
Fenvalerate	420.4	420.4>145.1	12	32	5.72
		420.4>219.2	12	10	
Cypermethrin	165.1	165.1>55.0	46	18	5.38
		165.1>72.0	46	30	
Deltamethrin	506.9	506.2>281.0	38	14	1.87
		506.2>92.9	38	62	
Permethrin	183.0	183.0>109.0	40	18	1.14
		183.0>155.0	40	8	
Bifenthrin	440.4	440.4>181.1	18	16	2.15
		440.4>165.9	18	54	
Amitraz	294.0	294.0>181.1	28	24	2.06
		294.0>211.0	28	12	
Coumaphos	363.2	363.2>227.0	46	26	7.42
		363.2>307.1	46	18	
Bromopropylate	341.4	341.4>57.0	40	24	7.52
		341.4>71.0	40	30	
Dichlorvos	221.0	221.0>79.0	36	32	4.96
		221.0>109.0	36	18	
Diazinone	305.2	305.2>153.1	44	20	7.89
		305.2>169.1	44	22	
Malathion	331.2	331.2>99.0	28	24	9.07
		331.2>127.0	28	12	
Parathion	292.1	292.1>181.0	26	12	2.05
		292.1>221.0	26	10	
Dimethoate	230.0	230.0>125.0	24	22	3.05
		230.0>199.0	24	10	
Omethoate	214.0	214.0>155.0	26	16	1.08
		214.0>183.0	26	12	
Thiametoxam	292.2	292.2>181.1	26	24	2.08
		292.2>211.0	26	12	
Methomyl	163.1	163.1>104.6	36	24	3.78
		163.1>122.6	36	16	

^aCV- cone voltage^bCE – collision energy^cRT – retention time

Method validation

To ensure that the optimized procedure was suitable for the application in routine analysis, the analytical performance parameters were determined and assessed. The obtained linearity (R²) in the range 5-250 ng/mL for all compounds was better than 0.99. The determined LODs and LOQ were lower than the reporting level 10 µg/kg, except in the case of coumaphos and thiametoxam where obtained LOQ was 11.4 and 12.2 µg/kg, respectively (Table

2). Furthermore, the obtained LOQ values were substantially lower than the established MRLs for pesticides in honey (EU Pesticide database; EC, 2010).

The recovery experiments exhibited values within the required range 70-120 %, laid down as acceptable in the SANCO document (2013), except for omethoate, whereas the obtained recovery was 152.7 and 181.2 % for 10 and 50 µg/kg, respectively. According to some previous investigations (Tomasini *et al.* 2012), a recovery value over 150 % is considered as high matrix effect. Such effect occurs when molecules co-eluting with the compounds of interest alter the ionization efficiency in reference to conditions in absence of matrix. Practically, it means that for accurate omethoate quantification we shall use matrix-matched calibration curve that will compensate the matrix effect. Optimized method exhibited satisfactory precision; the obtained RSD, for all compounds investigated, for both validation levels, was lower than 20 % (Table 2).

Table 2. Validation parameters for the pesticide compounds according to the SANCO requirements [9]

Pesticide	LOD/ µg/kg	LOQ/ µg/kg	Recovery at 10 µg/kg (RSD/%)	Recovery at 50 µg/kg (RSD/%)
Carbaryl	1.8	5.8	80.3 (8.5)	81.7 (8.4)
Carbofuran	2.5	8.1	79.7 (12.6)	89.4 (6.2)
Fenvalerate	1.4	4.7	91.1 (9.4)	84.6 (16.2)
Cypermethrin	1.9	6.2	81.3 (13.5)	91.8 (13.0)
Deltamethrin	0.5	1.7	86.9 (9.8)	77.6 (7.4)
Permethrin	1.6	5.2	87.0 (10.6)	85.0 (14.8)
Bifenthrin	2.3	7.6	76.9 (17.0)	75.0 (19.3)
Amitraz	1.8	6.0	91.7 (10.1)	80.7 (11.3)
Coumaphos	3.4	11.4	93.7 (13.2)	83.2 (7.3)
Bromopropylate	2.8	9.2	96.3 (12.4)	92.7 (7.6)
Dichlorvos	0.5	1.6	73.8 (9.5)	74.4 (6.9)
Diazinone	1.2	4.0	92.1 (13.7)	82.2 (5.8)
Malathion	0.9	3.9	97.1 (12.9)	104.1 (12.1)
Parathion	1.3	3.3	82.5 (8.8)	79.6 (5.7)
Dimethoate	2.4	7.8	84.1 (14.5)	81.0 (7.7)
Omethoate	3.0	9.7	152.7 (11.3)	181.2 (9.6)
Thiametoxam	3.7	12.2	100.2 (15.9)	102.4 (9.7)
Methomyl	2.0	6.6	75.7 (8.6)	83.4 (7.7)

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Honey samples analysis

When analyzing real honey samples, internal quality control was implemented to provide the correct working of the method and the UHPLC-MS/MS system. A duplicate analysis of honey samples was accompanied with a recovery experiment within each batch, to check the extraction efficiency. Positive samples were quantified with reference to the four - level calibration curves obtained for each batch of samples. Furthermore, it enables avoiding adverse effects impact on the quantification process coming from the instrument. Each positive result (over the analyte reporting level) was corrected for the recovery rate obtained for each batch of samples.

The method was applied to 50 multifloral honey samples collected at individual beekeepers from different geographical locations in Macedonia. In these samples five different pesticides, namely bromopropylate, methomyl, diazinone, fenvalerate, dichlorvos, cypermethrin and omethoate were detected in concentration range between 6.9 and 26.5 $\mu\text{g}/\text{kg}$ in 16 honey samples (32 %) (Table 3). The positive findings were confirmed by acquiring two transitions for quantification and identification in compliance with the EU guidelines (SANCO, 2013). The findings demonstrated that the method could be useful for analyzing pesticides in real samples of honey. Carbaryl, carbofuran, fenvalerate, deltamethrin, permethrin, bifenthrin, amitraz, coumaphos, malathion, parathion, dimethoate and thiametoxam were present in concentrations lower than the LOQ (Table 3).

Table 3. Positive findings of pesticides in 50 honey samples, using the proposed method.

Pesticide	Min ($\mu\text{g}/\text{kg}$)	Max ($\mu\text{g}/\text{kg}$)	Mean ^a ($\mu\text{g}/\text{kg}$)	MRL ($\mu\text{g}/\text{kg}$)
Cypermethrin	<6.2	24.6	16.5 (2) ^b	50
Bromopropylate	<9.2	21.5	15.8 (3) ^b	10
Dichlorvos	<1.6	11.5	9.2 (2) ^b	10
Diazinone	<4.0	26.5	24.0 (3) ^b	10
Fenvalerate	<7.8	10.6	-- (1) ^b	10
Omethoate	<9.7	16.3	14.0 (2) ^b	10
Methomyl	<6.6	24.1	18.5 (3) ^b	10

^amean value of positive findings

^bnumber of positive findings

Bromopropylate, diazinone and methomyl were detected and confirmed over the limits of quantification at three samples (6 % positivity), cypermethrin, dichlorvos and omethoate at two samples (4 % positivity) and fenvalerate at one honey sample (2 % positivity). The determined concentrations for all positive findings for bromopropylate, diazinone, omethoate and methomyl (Table 3), exceeded the required reporting concentration level according to EU pesticide database, being 10 $\mu\text{g}/\text{kg}$. For dichlorvos the respective level (10 $\mu\text{g}/\text{kg}$) was exceeded only at one sample, while for cypermethrin, the detected concentration levels for both positive samples were below the level of interest (50 $\mu\text{g}/\text{kg}$).

The presence of methomyl, fenvalerate and omethoate in the analyzed honey samples can be attributed to environment contamination since there are no known beekeeping practices using these pesticides (Bogdanov, 2006). On the other hand, bromopropylate and diazinone are known as used against parasites and diseases in the conventional beekeeping practice (Bogdanov, 2006; Fernandez-Garsia *et al.* 1994).

Likewise, other researchers reported no significant residues of insecticides in honey (Bogdanov, 2006; Naccari *et al.* 2014). Several investigations conducted on different types of honey and applying various analytical methods showed, instead, the presence of pesticides in significant extent (Rodríguez-López *et al.* 2014; Herrera *et al.* 2005; Mukherjee, 2009). In another study of honey with origin from Spain and Portugal (Blasco *et al.* 2003) most of the compounds found were chlorinated pesticides.

According to some previous publications (Bogdanov, 2006) the relatively low concentration of pesticides in honey seems to be due to a filtering effect of bees. Initially high pesticides concentrations in nectars are decreased approximately by factor of 1000. In addition, many of the modern pesticides used today are unstable and disintegrate quickly after use. Considering the fact that pesticide residues concentrations found in honey are comparable to the ones found in other foodstuffs (Kuchen *et al.* 1998) and the low honey inclusion in the diet, it can be concluded that pesticide residues found in honey within this study, are safe from toxicological point of view.

CONCLUSION

The QuEChERS in combination with UHPL-MS/MS method provides high-quality results, minimizes the number of analytical steps, and uses reagents in small quantities. The method showed good performance, which was verified through the method validation procedure. The achieved recoveries and precision were acceptable, and in line with the legislation requirements. Matrix-matched calibration curves should be used for accurate quantification of the positive findings. The data for pesticide findings from this study and the frequency of pesticide detection are an indication for the presence of both direct and indirect honey contamination. However, no significant amounts of investigated pesticides are detected, that could pose health risk to the consumers. This research is the first insight on the monitoring of residues of pesticides in honey samples from different geographical parts of Macedonia.

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