Determination of selected pesticides in honey and mead by HPLC

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The aim of this study was to monitor selected pesticides (Carbendazim, Acetamiprid, Thiacloprid, Epoxiconazole, Metconazole, Prochloraz, Deltamethrin, and T-fluvalinate) in honey and mead samples by reversed-phase liquid chromatography with spectrophotometric detection after QuEChERS extraction. The final separation was performed on YMC triart C18 column with mobile phase consisting of water and acetonitrile. The identification of selected pesticides in samples of honey and mead was performed on the basis of retention times conformity with the standards and quantification using the calibration curve method.

Introduction

Pesticides are chemicals commonly used in agriculture for the protection of crops from pests which include insects, fungi, weeds, different animals, and prions [1]. Although the application of these substances brings benefits to agriculture, there is a risk of contamination of soil, water, and food [2]. The most common types of pesticides include fungicides, herbicides, and insecticides. Pesticides can be inorganic and organic compounds both being present in the different forms (e.g., dry and moistened powders, solids for preparing aqueous solutions, or prior-to-made concentrates for making up emulsions or sprays [3]).

Some pesticides have carcinogenic properties and may affect the function of the nervous and reproductive systems [4–6]. Therefore, the monitoring of pesticides in food and environment is important to protect consumer safety [2].

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For this reason, maximum residue limits (MRLs) are set for the content of pesticides in the individual raw materials. In case of exceeding the MRL value for the monitored sample, the Czech Agricultural and Food Inspection Authority will impose a ban on the sale and distribution of the inspected food. The food can be shipped after analyses are completed; otherwise, the food is ordered to be withdrawn from sale or use [7].

Honey is a product containing a mixture of sugars, especially fructose and glucose, but also maltose, sucrose, and other carbohydrates with more complex structure [8–10]. Honey also contains minerals, proteins, amino acids, vitamins, flavonoids, pigments, several organic acids, and antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrine [2]. Mead is an alcoholic beverage prepared by fermentation of honey solutions. Therefore, all compounds presented in honey are transferred to mead and the quality of mead is thus mainly affected by the original quality of honey [11,12]. In addition to compounds which positively influence health, the honey and mead may also contain harmful compounds, such as pesticides. The source of contamination can be the beekeeping itself, because various substances are often applied inside the hive in order to prevent and eliminate common pests [13]. Indirect contamination of honey can be caused by the application of pesticides in agriculture, soil, air, water, and flowers, where bees collect nectar to produce honey [14]. Pesticides are dangerous for both bees and human. Therefore, the use of the pesticides in environment should be monitored and the quality of honey controlled using well-established analytical methods [8].

Several methods are used to determine pesticides in honey. First and important step in their analysis is the sample preparation serving for isolation and the enrichment of monitored pesticides. The most frequently used extraction techniques for the determination of pesticides in honey are liquid-liquid extraction (LLE [15,16]), solid phase extraction (SPE) [17], solid supported liquid-liquid extraction (SLE [18]) and QuEChERS [19], which is an acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe. QuEChERS is nowadays a very popular method for the analysis of pesticides in different matrices. The advantage of this technique is simplicity and low-time consuming [2,18,19].

SPE is a simple, robust, and fast method with low solvent consumption [17,20] and it is also very popular. Also miniaturized techniques, such as dispersive liquid-liquid microextraction [21], microextraction by packed sorbent [22], solid phase microextraction [23], stir bar sorptive extraction [24], single drop microextraction [25], and magnetic solid phase extraction [26] were tested to enrich the sample for the determination of pesticides in honey. The choice of separation technique depends on the properties of pesticides. Volatile, semi-volatile, and thermally stable compounds are determined by gas chromatography (GC) coupled to mass spectrometer [17,27–29] or nitrogen phosphorus [30], electron capture [26,31], atomic-emission [32], and flame photometric detectors [17]. Nowadays the high-performance liquid chromatography (HPLC) is preferred in analysis of pesticides

because these compounds are mainly polar, thermal unstable and do not evaporate easily [18,27]. Detection can be performed spectrophotometrically [21,33] or fluorimetrically [28]; nevertheless, currently, the most widely used is a coupling HPLC with mass spectrometry [34–37].

Materials and methods

Chemicals and reagents

The mead and honey samples were received from the beekeepers or bought in local markets. The lists of honey and mead samples are shown in Tables 1 and 2, whereas technical samples (insecticidal and fungicidal preparations which serve to control a wide range of pests) are surveyed in Table 3.

Table 1 List of honey samples

No.	Honey type	Site	Region	Country	Source
1	Flower honey	Čestín	Central Bohemia	CZ	market
2	Meadow honey	Čestín	Central Bohemia	CZ	market
3	Mixed honey	Dlouhopolsko	Central Bohemia	CZ	beekeeper
4	Acacia honey	Záboří nad Labem	Central Bohemia	CZ	beekeeper
5	Meadow honey	Doubravice	Hradec Králové	CZ	beekeeper
6	Forest honey	Nové Město nad Metují	Hradec Králové	CZ	beekeeper
7	Meadow honey	Přibyslav	Hradec Králové	CZ	beekeeper
8	Meadow honey	Trutnov	Hradec Králové	CZ	beekeeper
9	Meadow honey	Zaječice	Pardubice	CZ	beekeeper
10	Flower honey	Pardubice	Pardubice	CZ	beekeeper
11	Meadow honey	Pardubice	Pardubice	CZ	beekeeper
12	Meadow honey	Horka	Pardubice	CZ	beekeeper
13	Meadow honey	Jezbořice	Pardubice	CZ	beekeeper
14	Flower honey	unspecified	South Bohemia	CZ	beekeeper
15	Dandelion honey	unspecified	South Moravia	CZ	beekeeper
16	Flower honey	unspecified	unspecified	IT	open-air market
17	Meadow honey	unspecified	unspecified	CR	open-air market
18	Flower honey	mixed	mixed	EU	market
19	Flower honey selective	mixed	mixed	EU	market
20	Bio honeydew	mixed	mixed	EU	market

Table 2 List of mead samples

No.	Name	Site	Region	Country	Source
1	Mead "Medvědí objetí"	Hlinsko	Pardubice	CZ	market
2	Mead "Tajemství noci"	Hlinsko	Pardubice	CZ	market
3	Gold mead	Hlinsko	Pardubice	CZ	market
4	Homemade mead	Orlické podhůří	Pardubice	CZ	beekeeper
5	Royal mead	Zábřeh	Olomouc	CZ	market
6	"Karpatská" mead	Zábřeh	Olomouc	CZ	market
7	Mead from "Podhradí"	Mezholezy	Plzeň	CZ	market
8	Honey wine	Domažlice	Plzeň	CZ	beekeeper
9	"Dolská" mead	Máslovice	Central Bohemia	CZ	market
10	"Staročeská" mead	Havlíčkův Brod	Vysočina	CZ	beekeeper
11	Bio mead	Havlíčkův Brod	Vysočina	CZ	beekeeper
12	"Staroslovanská" mead	Dolná Krupá	Trnava	SK	market

Table 3 List of technical samples and pesticides

Product	Pesticides
Mospilan 20 SP	Acetamiprid
Proteus 110 OD	Thiacloprid, Deltamethrin
Alert S	Carbendazim, Fusilazole
Bumper Super	Prochloraz, Propiconazole
Tango Super	Epoxiconazole, Fenpropimorph
Caramba	Metconazole
Decis mega	Deltamethrin
Mavric 2 F + citric acid	T-fluvalinate

All the standards of pesticides (Carbendazim, Acetamiprid, Thiacloprid, Epoxiconazole, Metconazole, Prochloraz, Deltamethrin, and T-fluvalinate) were 98 % or higher purity and purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared by a Milli-Q purification system (Merck Millipore, Darmstadt, Germany). Methanol, acetonitrile (both HPLC gradient grade), formic acid (98%), ammonium formate (98%), magnesium sulphate, sodium chloride (both p.a.), and SPE clean-up cartridge including primary-secondary amine plus magnesium sulphate were purchased again from Sigma-Aldrich.

Instruments and conditions

The liquid chromatograph LC-20AD combined two LC-20AD pumps, a DGU-3014 degasser, an SPD-20A spectrophotometric detector (all Shimadzu, Kyoto, Japan) plus an LCO 102 Single column thermostat (Ecom, Prague, Czech Republic). The optimization of reversed-phase separation of pesticides was performed on different octadecyl silica-gel columns: Ascentis Express C18 (150 × 3 mm, 2.7 μm, Supelco, Bellefonte, PA, USA), Kinetex C18 (150 × 3 mm, 2.6 μm, Phenomenex, Torrance, CA, USA) and Zorbax SB-Aqua (150 × 3 mm, 3.5 μm, Agilent, Santa Clara, CA, USA). The best resolution of the individual pesticides was achieved on YMC-Triart C18 column (150 × 3 mm, 3 μm, Supelco). The flow rate of the mobile phase was 0.7 mL min^{-1}, injection volume 2 μL, temperature 30 °C and the detection wavelength 220 nm. The mobile phase was composed of deionized water (A) and acetonitrile (B). The gradient-elution profile was programmed as follows: 0 min – 20% B, 8 min – 95% B, 10 min – 95% B.

Sample preparation

For QuEChERS extraction, an amount of 0.1 g of technical sample or 10 g of honey was dissolved in 10 mL water. In case of mead, a volume of 10 mL of the individual sample was used for analysis. The extraction procedure was taken from the literature [38] with slight modification. 10 mL of acetonitrile, 4 g of magnesium sulphate, and 1 g of sodium chloride were added to the sample. The mixture was shaken for 1 min and centrifuged (5000 rpm) for 5 min. Then, 1 mL of the upper organic layer was transferred to 2 mL SPE clean-up cartridges with 150 mg of magnesium sulphate and 25 mg of primary-secondary amine. Sample was shaken and centrifuged (10 000 rpm) for 1 min, filtered through 0.45 μm PTFE syringe filter and analysed using the optimized HPLC method.

Results and discussion

Optimization of separation

The separation of selected pesticides was optimized using the individual standards. The composition and flow rate of the mobile phase, the stationary phase and the wavelength were the parameters of choice to attain the separation of selected pesticides with resolution $R = 1$ or higher. Flow rates of the mobile phase were tested in the range of 0.4–0.7 mLmin^{-1} according to the dimension of the respective column. Four chemically bonded octadecyl silicagel columns (namely: Ascentis Express C18, Kinetex C18, Zorbax SB-Aq, and YMC C18) were

selected for optimization. The separation of first three compounds was very difficult and with using octadecyl silica gel-based columns (here: Ascentis Express C18, Kinetex C18) because of a high coelution and the tailing of peaks. The resolution of three corresponding peaks did not improve any change of the mobile phase composition even after addition of formic acid to the mobile phase or when using acetic buffer. Thus, the column Zorbax SB-Aq with modified octadecyl silica gel stationary phase was tested. The tailing of first three pesticides monitored was improved, however, the resolution was not sufficient. Successful separation of all the pesticides was achieved on the last column, YMC-Triart C18, with organic/inorganic hybrid silica-based stationary phase. Using appropriate gradient elution profile, the resolution more than one was achieved for all pesticides within 10 min (see Fig. 1). Due to the different UV spectrum of the compounds monitored, their response at wavelengths of 220, 240, 245, and 280 nm was tested to obtain maximal sensitivity; the highest value for monitoring all compounds being observed at 220 nm.
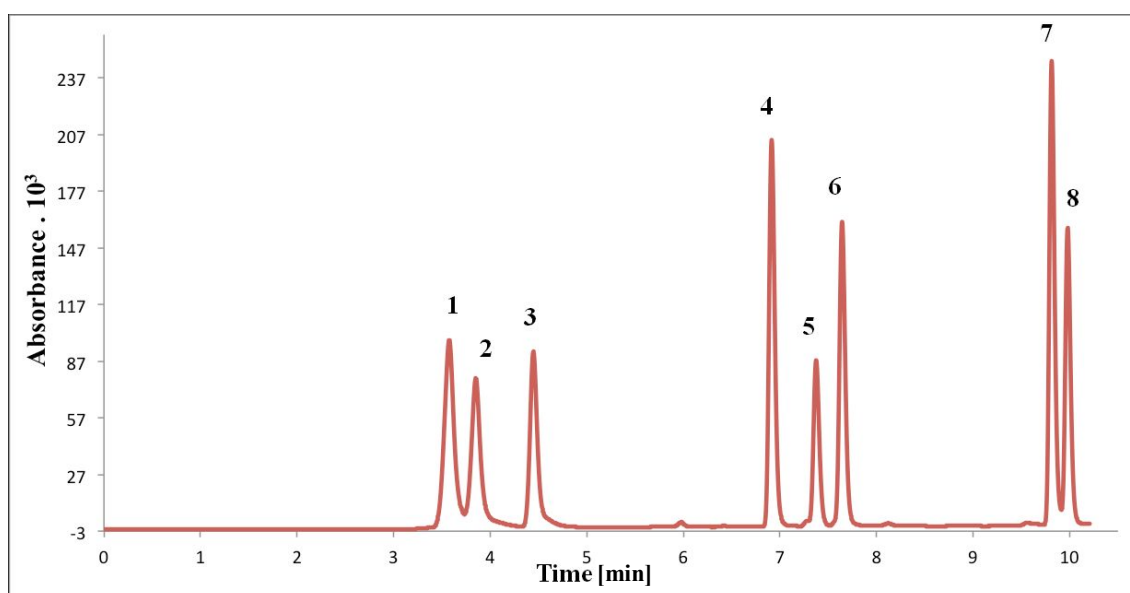

Fig. 1 HPLC separation of monitored pesticides

YMC - Triart C18 column, gradient elution: 0 min – 20% B, 8 min – 95% B, 10 min – 95% B (A – water, B – acetonitrile), $F = 0.7$ mL min^{-1}, injection 2 μL, temperature 30 °C, λ = 220 nm, 1 – Carbendazim, 2 – Acetamiprid, 3 – Thiacloprid, 4 – Epoxiconazole, 5 – Metconazole, 6 – Prochloraz, 7 – Deltamethrin, 8 – T-fluvalinate.

Optimization of extraction

According to the information from literature [38], the extraction efficiency of three different procedures was tested using a mixture of standards. Among the basic procedure with magnesium sulphate and sodium chloride, other two modifications with acetic acid or sodium citrate were tried. As found out, this

modification did not improve the extraction efficiency at all; therefore, the basic extraction procedure employing only acetonitrile as the solvent of choice and a mixture of magnesium sulphate with sodium chloride was used in all further experiments. Consequently, the cleaning of extract was accomplished using a mixture of magnesium sulphate and primary-secondary amine.

Quantification of pesticides

The individual pesticides were identified based on the retention-time conformity with the corresponding standards. Quantitative analysis was carried out using the calibration curve method; the respective data being measured at seven concentration levels, for each one three times ($n = 3$). The linearity of calibration curves was checked by inspecting the plots of residuals while the significance of the intercept of regression straight-lines was evaluated using the Student's t-test. For all the compounds, the coefficient of determination (Table 4) was higher than 0.99, demonstrating a satisfactory linearity. The limits of detection (LOD) estimated and calculated as the concentration via the signal-to-noise (S/N = 3) criterion were below 100 µg L^{-1} for all compounds analysed (see again Table 4).

Table 4 Parameters of linear regression, intercept together with confident interval, slope, limit of detection (LOD), limit of quantification (LOQ) and coefficient

Standards	Slope	Intercept	R^2	LOD [µg L^{-1}]	LOQ [µg L^{-1}]
Carbendazim	1.59 ± 0.018	-1.417 ± 0.78	0.9997	50.5	168
Acetamiprid	11.41 ± 0.574	2.284 ± 1.67	0.9950	71.3	237
Thiacloprid	18.54 ± 0.64	-11.74 ± 5.001	0.9962	96.2	321
Epoxiconazole	868.62 ± 21.9	$P = 0.2099*$	0.9934	74.0	247
Metconazole	520.99 ± 11.28	17.88 ± 12.12	0.9990	92.3	308
Prochloraz	11.801 ± 0.072	$P = 0.2269*$	0.9998	87.0	290
Deltamethrin	995.94 ± 22.72	44.856 ± 14.44	0.9990	56.2	187
T-fluvalinate	18.52 ± 0.48	50.03 ± 17.94	0.9983	82.8	276

* absolute value is insignificant, probability $P > 0.05$

Identification and quantification of pesticides in technical samples

Optimized method was used with extraction performed twice and each extract was measured in three replicates. Based on calibration data, the content of the individual pesticides in technical samples was calculated and the results are shown in Table 5. The results are in good agreement with declared value. The difference ascertained could be caused by different concentration units used in most formulations.

Table 5 Quantitative representation of pesticides in selected technical samples

Product	Pesticide	*c* declared	*c* [g kg^{-1}] measured
Alert S	Carbendazim	250 g L^{-1}	245.9 ± 3.2
Mospilan 20 SP	Acetamiprid	200 g kg^{-1}	195.1 ± 1.1
Proteus 110 OD	Thiacloprid	100 g L^{-1}	102.2 ± 1.3
Tango Super	Epoxiconazole	84 g L^{-1}	82.7 ± 1.3
Caramba	Metconazole	60 g L^{-1}	55.6 ± 1.6
Bumper Super	Prochloraz	400 g L^{-1}	394.5 ± 2.1
Decis mega	Deltamethrin	50 g L^{-1}	44.51 ± 1.2
Mavric 2 F + citric acid	T-fluvalinate	240 g L^{-1}	232.8 ± 2.2

Identification and quantification of pesticides in honeys and meads

Selected pesticides were analysed in twenty honey and twelve mead samples using the optimized extraction and the corresponding separation methods; the former one being performed twice and each extract analysed three times. Based on the calibration curves, the content of monitored pesticides in honey (Table 6) and mead samples (Table 7) was calculated. The results show that there is no difference whether the honey is bought in a shop or received from a beekeeper. The amount of pesticides that pollute honey from the environment cannot be affected. Among the pesticides monitored, Thiacloprid, Acetamiprid, and Prochloraz are present in many samples. Compared to this, Carbendazim and T-fluvalinate were found only scarcely. The highest concentration of Thiacloprid and T-fluvalinate was found in honey sample 7, although this specimen of honey was prepared by beekeeper. Probably, chemically treated field is located close to his beehive(s). Thiacloprid at a higher concentration was also contained in honey sample 5 from Hradec Králové region (identical to 7) and mead sample 12 from Slovakia, where the highest amount of Acetamiprid was also observed.

A higher amount of Acetamiprid was further found in honey sample 12 originating from the Pardubice region and, again, being prepared by beekeeper. Pesticides have been also founded in bio-products (honey sample 20 and mead sample 20), although, in the case of these special products, it should be guaranteed that the bees collect the pollen at places free of any pesticide treatment. Generally, the presence of pesticides was lower in the mead samples than that in the honey specimens. Carbendazim has been found in two samples, even though this substance has been banned since 2013.

Table 6 Quantitative representation of pesticides found in honey samples

No.	Carbendazim [mg kg^{-1}]	Acetamiprid [mg kg^{-1}]	Thiacloprid [mg kg^{-1}]	Prochloraz [mg kg^{-1}]	T-fluvalinate [mg kg^{-1}]
1	–	–	1.44 ± 0.19	–	–
2	–	2.78 ± 0.03	2.04 ± 0.03	–	–
3	–	–	1.67 ± 0.02	–	–
4	–	0.24 ± 0.02	1.25 ± 0.07	0.20 ± 0.01	–
5	5.91 ± 0.47	–	16.4 ± 0.4	–	–
6	–	0.22 ± 0.91	1.96 ± 0.32	–	0.02 ± 0.01
7	–	–	21.7± 0.3	–	76.3 ± 0.5
8	–	–	1.25 ± 0.07	0.50 ± 0.01	–
9	–	–	1.34 ± 0.05	–	–
10	–	–	–	0.63 ± 0.01	–
11	–	0.41 ± 0.06	0.85 ± 0.07	1.12 ± 0.01	–
12	–	17.0 ± 0.7	0.92± 0.08	–	–
13	–	–	1.91 ± 0.06	0.59 ± 0.01	–
14	–	–	–	0.42 ± 0.06	–
15	–	5.32 ± 0.02	–	–	–
16	–	1.32 ± 0.65	1.17 ± 0.84	–	–
17	–	–	0.90 ± 0.04	–	–
19	–	–	0.88 ± 0.03	–	5.46 ± 0.02
20	–	–	2.25 ± 0.04	–	3.22 ± 0.06

Table 7 Quantitative representation of pesticides found in mead samples

No.	Carbendazim [mg L^{-1}]	Acetamiprid [mg L^{-1}]	Thiacloprid [mg L^{-1}]
1	–	1.64 ± 0.01	–
2	–	2.39 ± 0.07	1.92 ± 0.03
10	–	0.68 ± 0.02	–
11	–	8.33 ± 0.51	–
12	0.19 ± 0.02	79.2 ± 0.4	17.8 ± 0.9

Conclusions

This article has dealt with the analysis of pesticides that can be used as biomarkers for the evaluation of environmental impact. Therefore, the aim of the work was to determine eight selected pesticides in the honey- and mead samples using optimized HPLC separation coupled with QuEChERS extraction. The chromatographic

separation was achieved using gradient elution with YMC-Triart C18 column and a mixture of acetonitrile with water as the mobile phase. The optimized procedure for HPLC separation was carried out in 10 min and the respective method had been applied to a set of 20 honey and 12 mead samples obtained from beekeepers or purchased in local markets. Furthermore, 8 technical samples of pesticide agents were analysed.

The results have shown that it does not matter whether the honey is from beekeepers or purchased in a store because the analyses performed confirmed the amount of pesticides that are contained in honey and come from the environment cannot be affected in any way. It is also interesting that all the samples (from Czech Republic, Slovakia, Italy, and Croatia) contain similar pesticides. Samples 5 and 7 exhibited a high concentration of the Thiacloprid pesticide and the sample 7 contained additionally a very high amount of T-fluvalinate. These samples came from the Náchod region and both from beekeepers. Pesticides were also found in bio-products, although products labelled as bio should not contain any substances of this kind. Generally, the concentration of pesticides in the mead samples has been found lower than those in honey samples due to the dilution during the production of mead. Rather interesting finding was the presence of Carbendazim pesticide in some samples, even though it has been banned already in 2013. Our results indicate that this pesticide is apparently still occurring in the environment. In case of mead, there is a risk of contamination of products by pesticides from the other materials which change the flavour of mead.

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