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ORIGINAL PAPER

Liquid–liquid extraction procedure for nonvolatile pesticides determination in acacia honey as environmental biomonitor

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Abstract

In recent years, bees' products (nectar, honey, beeswax, pollen) have been considered as potential biomonitors of air pollution. The efficacy of these matrices, especially honey, in environmental biomonitoring has been demonstrated in many studies to be due to its medicinal properties. A multiresidue method based on liquid–liquid extraction using ethyl acetate, followed by an analysis using liquid chromatography coupled with tandem mass spectrometry for the analysis of pesticides, was developed in this paper. Afterwards, the method was validated, and results showed that the intraday and interday relative standard deviation was below 5%, and the recoveries obtained generally ranged from 68% to 104%. Furthermore, the method showed high precision and sensitivity for all target compounds, with detection and quantification limits lower than 3 and 9 ng g^{-1}, respectively. Residues from a range of pesticides were detected in each of the samples collected. Pesticide contamination was highest in samples collected from the Akkar and Koura area, which are both well known for their agricultural production. In contrast, samples obtained from Bcharre showed the lowest pesticide contamination. Finally, analysis of real honey samples collected from Lebanon shows the potentiality of honey as a biomonitor for assessing air pollution.

Keywords Honey · Biomonitoring · Organic pollutants · Sample extraction · Liquid chromatography–tandem mass spectrometry

Introduction

Industrialization, transportation, agricultural practices, and increasing population have contributed to pollution of the global environment, with changes in its composition and structure negatively impacting biodiversity, leading to behavioral and physiological damage to living organisms such as bees (Hendry et al. 2017; Lee et al. 2015).

Biomonitoring, a valuable tool for assessing air pollution that has gained increasing attention, is defined as the detection of pollutants in the air by its effects on ecosystems and organisms (Baroudi et al. 2020a, b). Air quality bioindication is the use of bioindicators (lichens, conifers, mosses, insects, etc.) that provide quantitative information on the contamination of the air and can evaluate directly the environmental impacts of pollutants. These study organisms also make it possible to control also the spatiotemporal distribution of air pollutants (Baroudi et al. 2021, 2020a, b; Al Alam et al. 2019; Dołęgowska and Migaszewski 2014).

In fact, as biological indicators, honeybees and their products can contribute greatly to environmental

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biomonitoring procedure (Bargańska et al. 2016). Although bee populations are increasing worldwide, multiple factors such as climate change, poisoning by chemical compounds, reduced flower diversity, and infection with pathogens have caused colony losses (Belsky and Joshi 2019; Li et al. 2018). Bees are essential pollinators for worldwide agriculture and have been widely considered as biomonitors of pollutants from the air (Patel et al. 2020). During their foraging activities, these organisms are exposed to pollutants, including pesticides and metals associated with particles of various sizes in the air, soil, vegetation, and water (Sáez et al. 2019; Klein et al. 2019). Contaminants are transferred to the hives and can also be present in apiary products, including wax and honey (Al-Waili et al. 2012).

Honey has long been known as a significant source of energy, but it has also been considered for its antioxidant and antibacterial properties. Several researches have shown that honey is a potential source of natural antioxidants, which can help to prevent heart disease, immune system deficiency, cancer, and various inflammatory responses (Boussaid et al. 2018). Indeed, honey, widely used for therapeutic and nutritional purposes, is subject to various types of contamination. The indirect contamination of honey by air, water, soil, and flowers may occur during pesticide application in agriculture during bees' foraging activities (Sánchez-Bayo and Goka 2016).Therefore, pesticides can be transferred into the hive where they can cause high mortality among bees and contaminate the honey, making it unsuitable for human consumption (Gregorc et al. 2018; Ravoet et al. 2015). Recent surveys show that bees are highly exposed to pesticides used in crops (Zawislak et al. 2019), including organophosphate insecticides, pyrethroids, and fungicides that are the most common agrochemical residues collected by bees from treated crops (Moniruzzaman et al. 2014). Like any pollutant, the exposure of bees to sublethal doses of pesticides over long periods of time has the potential to harm their immune system, making them much more sensitive to parasitic fungi and other pathogens, and may also affect their products (Williamson et al. 2014; Wood and Goulson 2017).

Pesticides are commonly employed to manage agricultural and domestic pests, but they are widely dispersed in water, soil, and air, presenting a direct risk to the environment and human health (Lehmann et al. 2018). However, several enzymatic reactions can lead to their degradation. A molecule enters the body of the microorganism in a particular way, and then, via a series of biochemical and physiological reactions mediated by various enzymes, the pesticide is divided into smaller molecules that are nontoxic or have low toxicity (Chen et al. 2011). Bacterial degradation methods include reduction, oxidation, dehydrogenation, hydrolysis, decarboxylation, dehalogenation, and condensation, which allow the bacteria to degrade organic macromolecules into small nontoxic molecules, therefore preventing secondary contamination (Huang et al. 2018; Ye et al. 2018).

Several extraction methods have been used to investigate the contamination of honey, such as supercritical fluid extraction (Messina et al. 2020), solid phase extraction (Ruiz et al. 2020), liquid–liquid extraction (LLE) (Zhu et al. 2019), matrix solid phase dispersion (Balsebre et al. 2018), pressurized solvent extraction (Chiesa et al. 2016), and QuEChERS (quick, easy, cheap, effective, rugged, and safe) (Zhang et al. 2019). Among all these currently used extraction procedures, LLE extraction is one of the oldest methods and most commonly used for the qualitative and quantitative survey of honey pesticides (Souza Tette et al. 2016).

For those reasons, the aim of this manuscript was to develop and validate a simple procedure for the assessment of 32 nonvolatile pesticides in acacia honey based on liquid–liquid extraction followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. This validated method was applied to five real samples of honey collected from several regions in northern Lebanon.

Experimentation

Chemicals and reagents

A solution of certified standard pesticides (1 g L^{-1}) including pymetrozine, foramsulfuron, fluroxypyr, spinosad-A, terbutryn, spinosad-D, sulcotrione, chloridazone, chlortoluron, isoproturon, metalaxyl-M, diuron, epoxiconazole, thiacloprid, triflusulfuron-methyl, boscalid, anilazine, carbendazim, tebuconazole, diflubenzuron, nicosulfuron, penconazole, propiconazole, chlorfenvinphos, cyazofamid, carbetamide, isoxadifen, pyraclostrobin, lufenuron, acetamiprid, flufenoxuron, and pendimethalin was prepared in acetonitrile (ACN).

Standard pesticides, internal standards (carbendazim-d^4, pendimethalin-d^5, diuron-d^6, and nicosulfuron-d^6), acetonitrile, and polytetrafluoroethylene (PTFE) membranes were obtained from Sigma Aldrich, St, Quentin Fallavier, France.

LC–MS/MS grade water and acetonitrile, ethyl acetate, and hydrochloric acid were obtained from VWR Prolabo, France.

Ultrapure water was purchased through a Milli-Q system (18 MΩ cm) from Elga Veolia, France.

Sample collection

Organic acacia honey was purchased from a Lebanese local market for calibration and method development. For the real sample study, five honey samples were obtained from beekeepers in northern Lebanon (Bcharre, Akkar Valley, Koura, and Batroun). Samples were collected and frozen at −18 °C in propylene tubes until analysis. The geographical partition

of the five sampling sites is presented in Supplementary Information (Supplementary Fig. 1).

Method development

Preparation of spiked samples

One gram of the organic honey, weighed in a plastic centrifuge tube (50 mL), was heated at 25 °C for 15 min in a water bath to reduce its viscosity and then fortify the solution with specific concentrations of each mixture of pesticides (5, 10, 25, 50, 100, 200, 300, and 500 ng g^{-1}). Spiked honey was kept in centrifuge tubes at 4 °C for 24 h until analysis to best fit the pesticide to the matrix.

Extraction procedure

Organic and fortified samples underwent the modified liquid–liquid extraction method developed by Bernal et al. (1997) for the extraction of carbendazim and benomyl in honey.

The procedure used was the following: 5 mL of ethyl acetate and 1 mL of hydrochloric acid (0.05 M) were added to 1 g of honey. The organic layer was collected after mechanical shaking (15 min) and centrifugation (10 min at 4000 rpm). Then, 5 mL of ethyl acetate was added to the remaining solid layer in the tube, which was then centrifuged to collect the organic layer combined with the previous one. Next, 5 mL of ethyl acetate and 1 mL of sodium hydroxide (0.1 M) were added to the remaining solid layer, and the tube was mechanically shaken and then centrifuged for another 10 min to collect the organic layer. The three organic layers were combined and evaporated under a hood to obtain 1 mL as a final solution. Then, the mixture was filtered through a PTFE membrane of 0.50 µm pore size (Whatman syringe filter, 25 mm diameter) prior to chromatographic analysis.

Chromatography analysis

A Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Surveyor pump and autosampler (Accela Autosampler) operating in positive electrospray ionization mode (ESI) was used. Chromatographic separation was performed on a Macherey–Nagel Nucleodur C_{18} pyramid column (150 × 3 mm; 3 µm). The mobile phases consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. The flow rate of the mobile phase was maintained at 0.3 mL min^{-1}, the injection volume was 20 µL, and the column was kept at 15 °C. Samples were separated using a 36 min gradient (30/70 for 5 min, 50/50 for 6 min, 80/20 for 7 min, 95/5 for 10 min, and 30/70 for 8 min).

Method validation

For all parameters, including linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and reproducibility standard deviation (%RSD), the method developed has been validated. First, for linearity, matrix-matched calibration curves were done in triplicate using samples spiked with 5, 10, 25, 50, 100, 200, 300, and 500 ng g^{-1}. LOD and LOQ were defined as the lowest concentrations where accuracy and precision corresponded respectively to the signal-to-noise ratio ≥ 3 and ≥ 10.

The method was validated for its repeatability (intraday), which was determined by analyzing the fortification of five samples with three different levels of pesticide concentration (10, 100, and 300 ng g^{-1}), and reproducibility (interday), which was determined by analyzing the fortification of five samples with the same pesticide concentrations on three consecutive days.

The recoveries of spiked honey were calculated according to Eq. 1.

$$\text{Recovery (\%)} = (\text{Sample concentration} / \text{Standard solution concentration}) * 100. \quad (1)$$

Results and discussion

The proposed LLE extraction procedure followed by liquid chromatographic analysis to determine pesticides in honey is of great importance in the assessment of air pollution. Results showed that the method has been validated for its linearity, limits of quantification and detection, reproducibility, and repeatability. The regression coefficient was higher than 0.99 for all analyzed nonvolatile pesticides, and limit of detection ranged from 0.02 to 2.5 ng g^{-1}, while limit of quantification ranged from 0.07 to 8.33 ng g^{-1}. Furthermore, results showed that, for repeatability (intraday) and reproducibility (interday), all the pesticides were detected with high precision with %RSD lower than 5% except fluroxypyr. Moreover, the method showed good recoveries between 68% and 104% for nonvolatile pesticides. Calibration curves of some analyzed pesticides are shown in Supplementary Information (Supplementary Fig. 2).

Table 1 presents the validation parameters for nonvolatile pesticides analyzed by LC–MS/MS.

The use of an LLE is governed by various physicochemical parameters depending on the solutions to be extracted, which provides information on the pH, choice of solvent, type and concentration of reagents, and how those choices

Table 1 Validation parameters for non-volatile pesticides analyzed by LC–MS/MS

Pesticide	Regression line equation	Regression coefficient	Limit of detection (ng g^{-1})	Limit of quantification (ng g^{-1})	%RSD Intraday (Repeatability)	%RSD Interday (Reproducibility)	Recovery (%)
Pymetrozine	$Y=0.000160239 \times X$	0.9951	0.57	1.90	1.52	1.16	93.24
Carbendazim	$Y=0.0328428 \times X$	0.9978	0.27	0.90	0.94	1.02	98.80
Chloridazone	$Y=0.00131559 \times X$	0.9987	0.07	0.23	0.83	1.21	96.70
Acetamiprid	$Y=0.00172956 \times X$	0.9957	2.30	7.67	1.51	2.25	86.99
Nicosulfuron	$Y=0.000155547 \times X$	0.9966	0.75	2.50	0.82	0.61	74.41
Thiacloprid	$Y=0.033226 \times X$	0.9990	0.65	2.17	2.75	4.73	83.05
Carbetamide	$Y=0.0577388 \times X$	0.9997	0.19	0.63	0.31	0.42	72.06
Foramsulfuron	$Y=0.000570191 \times X$	0.9976	0.39	1.30	0.94	2.23	92.69
Fluroxypyr	$Y=0.00806344 \times X$	0.9914	0.78	2.60	6.38	2.65	84.59
Spinosad-A	$Y=0.000839845 \times X$	0.9953	0.05	0.17	0.77	0.74	85.12
Terbutryn	$Y=0.00455538 \times X$	0.9980	1.87	6.23	0.84	1.23	104.51
Spinosad-D	$Y=2.93436e-006 \times X$	0.9915	2.14	7.13	1.12	1.85	90.96
Sulcotrione	$Y=0.00202121 \times X$	0.9985	1.76	5.87	0.93	4.71	69.44
Chlortoluron	$Y=0.0146021 \times X$	0.9945	0.93	3.10	1.68	4.89	74.10
Isoproturon	$Y=0.0422272 \times X$	0.9984	0.02	0.07	2.24	3.21	80.06
Metalaxyl-M	$Y=0.000765717 \times X$	0.9973	0.51	1.70	1.47	3.43	68.30
Diuron	$Y=0.0165449 \times X$	0.9969	0.64	2.13	3.39	4.47	84.47
Epoxiconazole	$Y=0.127481 \times X$	0.9972	0.20	0.67	0.76	3.81	81.4
Triflusulfuron-Methyl	$Y=0.0557256 \times X$	0.9984	0.05	0.17	3.15	3.23	70.54
Boscalid	$Y=0.0505686 \times X$	0.9988	0.04	0.13	0.85	4.11	94.01
Anilazine	$Y=0.00104083 \times X$	0.9993	0.37	1.23	1.37	2.51	93.48
Tebuconazole	$Y=0.0586595 \times X$	0.9978	0.60	2.00	0.99	2.68	70.74
Diflubenzuron	$Y=0.00471512 \times X$	0.9995	0.24	0.80	1.49	3.92	72.03
Penconazole	$Y=0.139982 \times X$	0.9985	0.10	0.33	2.41	4.23	92.72
Propiconazole	$Y=0.454928 \times X$	0.9990	0.04	0.13	4.87	3.25	82.90
Chlorfenvinphos	$Y=0.0755802 \times X$	0.9989	2.50	8.33	0.75	2.33	81.31
Cyazofamid	$Y=0.000428225 \times X$	0.9905	2.14	7.13	2.85	3.27	nd
Isoxadifen	$Y=0.00638442 \times X$	0.9966	1.60	5.33	1.51	4.63	72.31
Pyraclostrobin	$Y=0.000600441 \times X$	0.9982	0.65	2.17	2.37	3.99	90.72
Lufenuron	$Y=0.000330173 \times X$	0.9961	0.53	1.77	3.21	4.33	83.18
Flufenoxuron	$Y=0.00713239 \times X$	0.9969	0.26	0.87	1.72	1.54	72.23
Pendimethalin	$Y=0.00673314 \times X$	0.9958	1.87	6.23	1.99	3.58	95.87

nd not detected

affect the selectivity needed for sample cleanup (Chemat et al. 2019; Daso and Okonkwo 2015). Several solvents such as acetonitrile, ethyl acetate, and methanol were used for the analysis of pesticide in honey that depend on the physicochemical characteristics of each pesticide (Panseri et al. 2014; Salami and Queiroz 2013). In this work, the nonvolatile pesticides were extracted using ethyl acetate, which yielded acceptable quantitative results. During extraction, hydrochloric acid was used to increase the solubility of pesticides while sodium hydroxide was used to avoid the persistence of these compounds in the aqueous phase after the last extraction (Leng et al. 2014).

In fact, LLE has been a technique of sample extraction for many years, involving the direct preparation of the honey matrix with a water-immiscible solvent (Kuś and Jerkovic 2018). Among other multiresidue processes, the method developed has proved its effectiveness. Several studies using LLE extraction followed by liquid chromatography coupled with tandem mass spectrometry showed an improvement in the method's sensitivity (Souza Tette et al. 2016). For instance, compared with the reference method based on the study of Bernal et al. (1997) for the analysis of benomyl and carbendazim in honey by reversed-phase high-performance liquid chromatography, the extraction protocol used

followed by liquid chromatography coupled with tandem mass spectrometry analysis resulted in a greater number of extracted pesticides with better limits and recoveries. For 1 g of honey fortified with 1000 ng g^{-1}, the percentage for recovery and precision for carbendazim respectively was 97.4% and 4.1%, while by the presented developed method for the fortification of 1 g by 100 ng g^{-1} the results were 98.8% and 1.52%, respectively. Furthermore, comparison of our results with those provided by the LLE extraction using acetonitrile containing 1% of formic acid followed by ultra-high-performance liquid chromatography showed improvement in limits of detection of some compounds. For example, the LOD of boscalid and fluroxypyr analyzed using the developed method was respectively 0.04 and 0.78 ng g^{-1}, while these limits were respectively 50 and 25 ng g^{-1} with the extraction using acetonitrile as solvent (Gómez-Pérez et al. 2012). The use of different solvents may also affect extraction efficiency and the interferences, including pigments and carbohydrates, can be co-extracted and influence the recovery of the pesticides depending on the nature and properties of the solvent (Souza Tette et al. 2016). Ethyl acetate seems to be the appropriate and effective solvent for the extraction of pesticides in honey, and in all extractions the %RSD obtained was lower than 5%.

Moreover, all RSDs of repeatability and intermediate precision obtained within this developed method respect the validation norms for the honey matrix (Tiwari and Tiwari 2010), while the RSD% for some pesticides was higher than 20% in the study using the QuEChERS method following LC–MS/MS. For example, the interday RSD% obtained from honey fortified at 10 ng g^{-1} for these two methods was for carbendazim 3.33% and 10%, for penconazole 4.82% and 11%, for propiconazole 2.43% and 4%, and for tebuconazole 4.34% and 22% (Wiest et al. 2011).

LC–MS/MS has also been used widely for the analysis of thermally labile pesticides in honey owing to the possibility of separating several components based on molecular weight, polarity, and ionic mobility (Stachniuk and Fornal 2016) and allows their detection in complex matrices at low concentrations by improving the sensitivity and the reduction of matrix interferences (Sampaio et al. 2012).

Application to real samples

The five real samples purchased from four regions of northern Lebanon underwent the same method of extraction described above. Results showed that acetamiprid and sulcotrione residues were observed in all honey samples. Residues of nonvolatile pesticides detected in the samples analyzed are shown in Fig. 1 and Table 2.

The chromatogram corresponding to the sampling sites as well as the individual MRM traces provided as an example for MRM fragmentation of pesticide residues is shown in Supplementary Information (Supplementary Fig. 3).

Bees and their products have been widely studied, with the life cycle and geographical distribution of the bees as well as the activities and properties of honey being well documented (Thakur and Nanda 2020; Zięba et al. 2020; Bodó et al. 2020). Honey is increasingly used as a biomonitor of air pollution and has been employed for a wide range of environmental pollutants, ranging from pesticides (Sgargi et al. 2020; El-Nahhal 2020; Al Alam et al. 2017) and persistent organic pollutant (Wang et al. 2020; Villalba et al. 2020)

Table 2 Levels of nonvolatile pesticides detected in honey samples

Pesticide	Average concentration (ng g^{-1})	Detection (%)
Acetamiprid	62	100
Sulcotrione	33	100
Cyazofamid	42	60
Penconazole	51	80
Epoxiconazole	34	80
Fluroxypyr	7	40
Metalaxyl-M	10	40

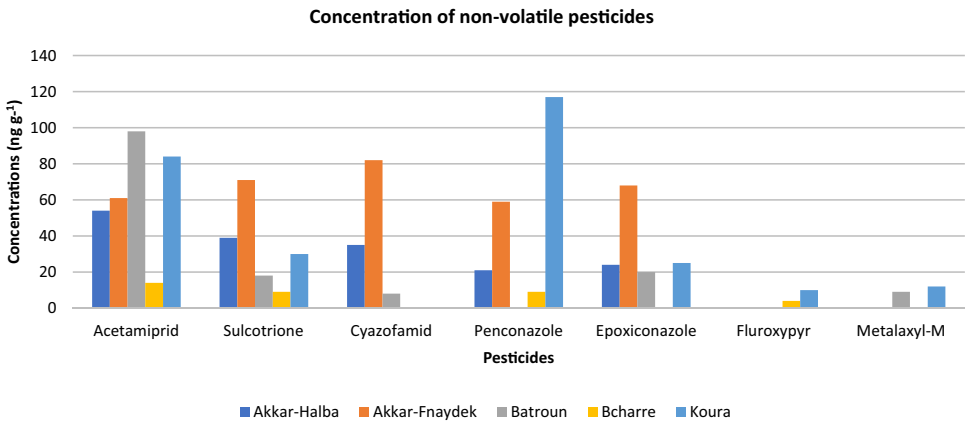

Fig. 1 Concentration of nonvolatile pesticides (ng g^{-1}) detected in real samples

to heavy metals (Ragab et al. 2020). Each of the apiaries investigated was in a rural region known for crop yields. As a result, these areas are bordered by cultivations, making them near to the application site of pesticides.

The results showed that Akkar Valley appears to be the most polluted with pesticide residues. In this region, widely recognized for its crop yields, the researchers noticed the presence of pesticides in the groundwater, verifying the use of these chemicals in this agricultural region (El-Osmani et al. 2014).

These analysis revealed that samples collected from the Bcharre were the least polluted with pesticide residues. This area, which is typically devoted to organic agriculture, has the lowest pesticide exposure. In contrast, penconazole and acetamiprid, used to treat many different types of vegetable and fruit diseases, were found to have the highest concentrations in Koura and Batroun. The prevalence of these pesticides is justified by the agricultural yields in these two regions that are widely known for their vegetable and fruit production.

Conclusion

The current study shows that agricultural activities in the Akkar area have affected honey quality, and high pesticide contamination was detected in the collected samples. Honey sample from Bcharre was shown to be less polluted than samples from Akkar and Koura region, where pollution levels can reach relatively high concentrations, particularly in Fnaydek. Farmers' lack of awareness about pesticide safety is caused by poverty and illiteracy, but other factors such as environmental quality, pesticide application frequency, and the presence of air pollution from various sources in the sampling sites all contribute to pesticide accumulation in the honey matrix. The method developed proved its efficiency, and the validation proved its good performance in terms of linearity, accuracy, precision, and limit of detection and quantitation. Tandem mass spectrometry detector fulfills such criteria in terms of high sensitivity and selectivity, as well as reliable analyte identification at very low detection limits.

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Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval Not applicable.

Consent to participate Not applicable.

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