BOOK OF PROCEEDINGS

XIV International Scientific Agriculture Symposium

"Agrosym 2023"

Jahorina, October 05-08, 2023

AgroSym 2023

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Impressum

XIV International Scientific Agriculture Symposium „AGROSYM 2023“

Book of Proceedings Published by

University of East Sarajevo, Faculty of Agriculture, Republic of Srpska, Bosnia
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Website:
http://agrosym.ues.rs.ba

CIP - Каталогизација у публикацији
Народна и универзитетска библиотека
Републике Српске, Бања Лука

631(082)(0.034.2)

INTERNATIONAL Scientific Agriculture Symposium "AGROSYM" (14 ; 2023 ; Jahorina)
Book of Proceedings [Електронски извор] / XIV International Scientific Agriculture Symposium "AGROSYM 2023", Jahorina, October 05 - 08, 2023 ; [editor in chief Dusan Kovacevic]. - Onlajn izd. - El. zbornik. - East Sarajevo : Faculty of Agriculture, 2023. - Ilustr.

Sistemski zahtjevi: Nisu navedeni. - Način pristupa (URL): https://agrosym.ues.rs.ba/article/showpdf/BOOK_OF_PROCEEDINGS_2023_FINAL.pdf. - El. publikacija u PDF formatu opsega 1377 str. - Nasl. sa naslovnog ekrana. - Opis izvora dana 15.12.2023. - Bibliografija uz svaki rad. - Registar.

ISBN 978-99976-816-1-4

COBISS.RS-ID 139524097

A SAMPLE PREPARATION METHODOLOGY USING DIFFERENT d-SPE SORBENT FOR AMITRAZ ANALYSIS IN HONEY

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Abstract

An analytical method was validated using gas chromatography with tandem mass spectrometry (GC/MS) for the precise analysis of amitraz and its metabolites 2, 4-dimethylaniline (2, 4 - DMA) in honey. The QuEChERS technique, which comes from the words Quick, Easy, Cheap, Effective, Rugged, and Safe extraction of the pesticides was used. Amitraz is both an acaricide and insecticide and is often used in beekeeping to protect honeybee colonies against *Varroa destructor*. The procedure was validated according to SANTE/11325/2021 and the values of recovery, precision, and linearity, limit of detection and limit of quantification were established. The average recoveries obtained for amitraz were ranged from 79.4 to 100.8% and for 2, 4 dimethylaniline from 78.1% to 103.6 % at the two spiked levels 0.01 and 0.02 mg/kg. Several clean-up approaches were tested: d-SPE with Enhanced Matrix Removal-Lipid (EMR-Lipid), combination of anhydrous magnesium sulfate, primary secondary amine (PSA) and C18 sorbents, and a third method using Z-Sep. The most precise recovery, i.e. the highest accuracy, was achieved using purification with EMR lipid. The linearity of the analytical response across the studied range of concentrations (0.010 - 0.10 mg/kg) was excellent, obtaining correlation coefficients higher than 0.99. Limit of quantification was 0.004 mg/kg for amitraz and 0.005 mg/kg for 2, 4-DMA. The method was used for the determination of amitraz and 2, 4-DMA in real samples of acacia and flower honey.

Key words: *honey, QuEChERS method, GC/MS, amitraz.*

Introduction

Amitraz is a synthetic pesticide that acts as an insecticide and acaricide. It is used primarily for the control of animal ectoparasites, i.e. an acaricide used against the parasite *Varroa destructor* in bees. It can be applied in the form of tablets, but it is much more often used as an organic solution of amitraz in a concentration of 20%, which is applied by smoking using self-burning smoke sheets. The effect of amitraz is reflected in its ability to kill the nervous system of the *Varroa destructor* parasite. It is most often sold in the form of a 20% solution under different trade names. Amitraz is readily hydrolyzed (under acidic conditions) to 2, 4-dimethylphenylformamide, which can be rapidly hydrolyzed under alkaline conditions to 2, 4-dimethylaniline. After use, amitraz molecules have the ability to hydrolyse through the intermediate metabolites N-2,4-dimethylphenyl-N-methylformamidine (DPMF), and N-2,4-dimethylphenylformamide (DMF) and 2,4-dimethylaniline (2,4- DMA). It is generally accepted that 2, 4-dimethylaniline is the stable endpoint of amitraz degradation (Caldow *et al*., 2007). The compound 2, 4-dimethylaniline is environmentally stable and toxic jedinjenje (Pohorecka et al., 2018). For this reason, the legal regulation defines amitraz which, in addition to the amount of amitraz, includes the metabolites containing the 2, 4-dimethylaniline moiety expressed as amitraz (EU, Annexes II Reg. 396/2005). The maximum allowed value of amitraz is regulated to a value of 0.2 mg/kg based on Legislation Reg. (EU) 2017/623 applicable from 26/04/2017.

This paper describes the validation of the QuEChERS method for determination of amitraz in honey samples. The QuEChERS method is an extraction method which has been developed for analysis pesticides from fruit, vegetables and cereals (Ananstassiades *et al.*, 2003). Therefore, various modifications of this method are used for the determination of pesticides in honey and other bee products (Česnik *et al*., 2019; Juan-Borras *et al*., 2016). One of the methods of preparation and extraction of 2,4-DMA involves acid extraction and heating in a water bath at - 80°C, and then extraction in a basic medium (Caldow et al., 2007).

Control of the quality and safety of honey is important, given that, in addition to its nutritional value, it also has therapeutic potential. Honey is the sweet product that has been used in medicine since ancient times due to its antioxidant, anti-inflammatory and antimicrobial activity. The use of honey in the diet is a significant combination of the carbohydrates, pollen, aromatic compounds, minerals, enzymes, vitamins, pigments, and various acids (Mititelu *et al.*, 2022). The chemical composition of honey is very variable and depends on the place where it is produced, due to the nature of the soil and the quality of water and air, which affect the quality of the raw materials used by the bees. The results of this study show that residues of pesticides, especially neonicotinoids, may occur in different regions and in greater frequency and concentration in areas where more technology is applied and more pesticides are used in agricultural production systems, such as southern Jalisco, Mexico (Ponce-Vejar *et al.*, 2022). In bee feeding, the use of products with added medicines or products purchased on the basis of honey and non-sterilized pollen is prohibited, as they contaminate bee families with spores and mycelia that cause bee-specific diseases. Artificial feeding is stopped 15 days before the beginning of the harvest. On the other hand, the quality of the environment has a significant impact on the degree of its pollution with various toxic pollutants (Mititelu *et al.*, 2022). In modern beekeeping, contamination of honey can be direct (i.e., in colonies treated for veterinary purposes) or indirect, as honeybees travel long distances for foraging and come into contact with contaminated pollen, nectar, and water (Panseri *et al.*, 2020).

The main objective of this study was to evaluate and optimize a modified method for the determination of amitraz and 2, 4 - DMA in honey samples using gas chromatography with mass spectrometry (GC-MS). Another specific objective was to develop and optimize a sample preparation procedure with a minimal solvent volume and high selectivity without the need for additional purification procedures. To achieve maximum efficiency for the target residues, a final extract dilution was applied to simplify the sample preparation procedure and reduce analyte loss during sample preparation. However, it should be emphasized that this approach could also be used for the determination of other pesticides. The proposed method was evaluated in terms of limit of detection (LOD) and limit of quantification (LOQ), linearity, specificity, precision and trueness.

Material and methods

Amitraz and 2, 4 -DMA were purchased from Dr Ehrenstorfer (LGC). The sample preparation was carried out using three methods of purification until the establishment of purification with satisfactory recovery. The first stage of preparation involved the homogenization of honey samples and the establishment of the method through the preparation and validation process. Then, a validated method with satisfactory performance was applied for the determination of amitraz and its metabolite in real samples. The analysis was carried out against the presence of pesticides in real samples of acacia and flower honey. For analysis, there were 17 samples of acacia honey and 28 samples of flower honey from the territory of the Republic of Serbia, i.e. the vicinity of Belgrade, a place called Batajnica. Honey samples were sampled in the amount of 500 g in a sterile plastic jar, closed and thus delivered to the laboratory. The samples were sampled and delivered to the laboratory in the period from the beginning of September to the middle of October 2022 and were from harvest 2022. All the samples were

from the territory of Batajnica, which is known for beekeepers and raising bees for many years. Batajnica is an urban settlement in the northwest of Belgrade, it is a lowland village that donated significant amounts of honey to hospitals in Serbia during the COVID-19 pandemic.

The methods for the determination of the pesticides residue and also amitraz and 2,4-DMA, was the already described for honey samples (Kubiak *et al.*, 2020). Before the analysis, the honey samples were homogenized and 5 g was weighed into a 50 ml plastic tube. Water (5 ml) and acetonitrile (10 ml) were added and then the samples were vortexed. Then, an internal standard was added (ISTD, triphenyl phosphate), and for the samples for validation and verification of the recovery rate and for the determination of the recovery rate, the standards amitraz and 2,4-DMA were added at 0.01 and 0.02 mg/kg, respectively, and amounts of 0.1 and 0.2 μg/kg were added for precision when the maximum residue level was exceeded. Prior to analysis of the recovery samples, the honey was tested to ensure that it did not contain amounts of the specified analytes.

In the first phase of preparation the samples are shaken and a salt and buffer mixture is added and the samples are shaken again. After centrifugation for 5 min (4000 rpm) the supernatants are put in -20 degree freezer. In the second stage for purification, three methods of checking recovery and purification were used: d-SPE with Enhanced Matrix Removal-Lipid (EMR-Lipid), combination of anhydrous magnesium sulfate, primary secondary amine (PSA) and C18 sorbents, and a third method using Z-Sep. A stock solution of amitraz and 2,4-dimethylaniline were prepared in acetonitrile and stored at −20 °C. Intermediate dilutions were prepared in hexane before analysis. The amitraz and 2, 4 -DMA compound are separated on a Elite-CLP column (PerkinElmer, 30 m; 0,25 mm ID; 0,25 umdf) and determinated by GC/MS operating in the multiple reaction monitoring mode (MRM). Quantification analysis was performed using an Clarus 680 GC gas chromatograph connected to a SQ8T mass detector (PerkinElmer, USA). The system is equipped with Electrin Impact (EI) ionisation and 255L/sec turbomolecular pump. The operation of the system is controlled and also data analyzed through the PerkinElmer TurboMassTM GC/M software system. The mass analyzer in the mass detector is a quadropole with pre-filter mass range: 1.0 – 1200 u (amu). The injection volume of calibration standards, recovery samples and analyzed samples was 2 μl. The temperature of splitless was set at 250°C and the detector temperature was set at 280°C. The quantifer ions monitored for 2,4- dimethylaniline were m/z 106, 120, 121,77 and for amitraz m/z 121, 132, 162, 293. Validations and performance validation parameters were carried out according to the SANTE 11312/2021 document (EC, 2022), which includes the criteria for pretreatment which is should be carried out during method validation in order for the results of the analysis to be considered satisfactory. The final extract is prepared for GC/MS analysis. Before sample analysis, samples were calibrated in a solvent (hexane:toluene, 1:1, v:v) and then calibrated in a honey matrix to reduce matrix interferences during quantification and matrix effect during calibration.

Results and discussion

The applied extraction procedure is shown in Figure 1. The chromatographic separation was carefully optimized. Five-point calibration curves were plotted using standard mixtures of the analytes at various concentrations added to the honey sample (Table 1). The limits of detection achieved were determined to be 0.004 mg/kg for amitraz and 0.005 mg/kg for 2, 4 – DMA. Linearity was checked by using matrix-matched standards (Table 1). Linearity and range were determined by linear regression using calibration with an internal standard, Method repeatability was calculated as the relative standard deviation (RSD) of five

replicates, whereas instrument repeatability was determined as the RSD of five consecutive injections of the same sample concentrations.

Table 1.GC-MS validation data for amitraz and metabolite 2, 4 -DMA in honey

Compound	Retention time, min	Calibration curve	Correlation coefficient, r^2	LOQ, mg/kg	Expanded Uncertainty (%)
2,4- DMA	5.07	y=1.05368 – 3.69617	0.999048	0.005	25
Amitraz	29.79	y=1.23725 – 35.7889	0.999052	0.004	28

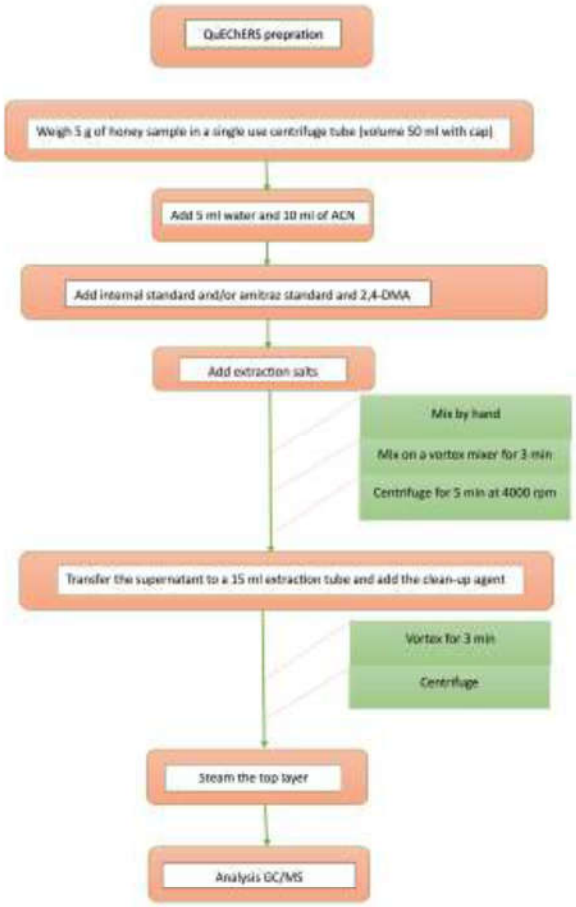


Figure 1. Extraction preparation scheme

The applied extraction scheme is shown in stages in Figure 1. The results of the RSD and recovery for spiked honey samples at four concentration levels are shown in Figure 2. Three different types of sorbents were used for pretreatment. Common sample preparation procedures include the post-extraction cleaning step, which is time-consuming and requires the use of solvents that are not environmentally friendly. The comparison of various SPE phases included Z-sep (ZrO_2-coated silica phase), $MgSO_4$/PSA/C18 and EMR-lipid. When a 15 ml EMR-Lipid tube was used after separating the supernatant after the extraction and before the purification phase the EMR-Lipid salts were preactivated with 3 ml of water. All tested samples were in compliance and with a value lower than the MDK prescribed by the European and the regulations of the Republic of Serbia (EC, 2005; Rulebook 91/2022).

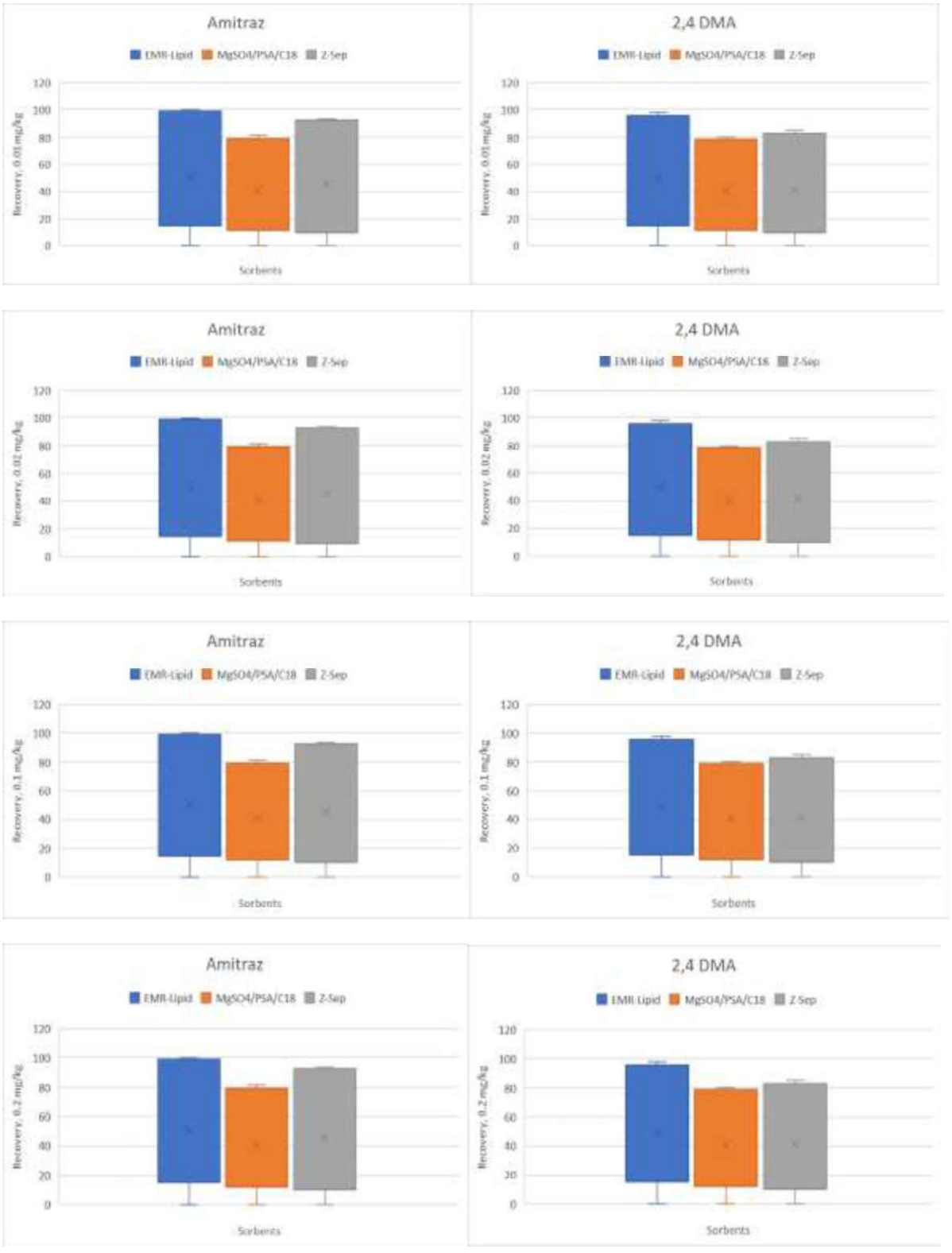
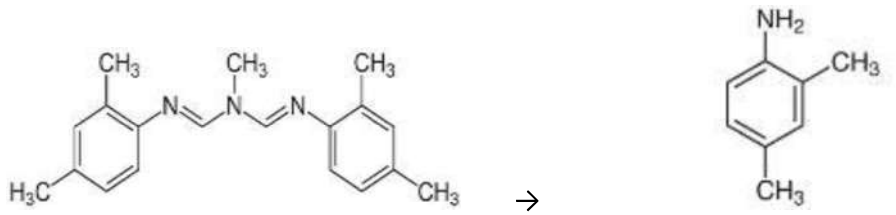


Figure 2. Recovery results using three type od sorbents at the four concentration levels

Table 2. Results obtained by testing real honey samples

Compound	Type of honey	Tested samples	Attendance percentage	Min value, mg/kg	Max value, mg/kg	Mean value, mg/kg
2, 4- DMA	acacia honey	17	11.8	0.015	0.052	0.034
Amitraz	acacia honey	17		n.d.	n.d.	
2, 4- DMA	flower honey	28	17.4	0.024	0.086	0.055
Amitraz	flower honey	28	17.4	0.028	0.036	0.032



Amitraz → 2, 4 –DMA

Figure 3. Structures of amitraz and its degradation product

Furthermore, results also showed that in the case of $MgSO_4$/PSA/C18 clean-up, the low recoveries with a pre-spiked standard solution originated mainly from the incomplete recovery in relation to other sorbents. As can be seen in Figure 2, the amount of sorbent and the type of sorbent have a small effect on the recovery of amitraz and metabolites. Similar recoveries (from 79.4% to 89%) were obtained for amitraz and 2,4-DMA when the sorbent amount was PSA, C18, and Z-sep. Better recoveries were obtained when EMR lipid purification was used (100.8% for amitraz and 103.6% for 2,4-DMA), and this sorbent was chosen for conducting further experiments with honey samples.

Investigations by other authors have confirmed that in most honey samples only the final products of amitraz decomposition such as 2,4-dimethylformanilide (DMF) and 2,4-DMA can be detected while amitraz was not present (Kubiak *et al.,* 2020). The structure of amitraz and the degradation product are shown in Figure 3. Their molar masses are important for the calculation of the conversion factor and calculation of the amitraze sum. Tests of Australian honey samples did not confirm the presence of acaricides and neonicotinoids, but the presence of polycyclic aromatic hydrocarbons was confirmed in some honey samples. From a total of 212 honey samples tested, PAHs were detected in 23 honeys, where only 4 of 33 PAHs were found, while the presence of tested pesticides (Cyhalothrin isomers) was confirmed (Hungerford *et al.*, 2021). The examination of samples from the territory of Batajnica is in agreement with other examinations, because the obtained amounts were below the MRL, and in a larger percentage of the examined samples below the LOQ (Table 2).

In the scientific literature, there are test results of honey samples treated with agents containing amitraz (eg Apiwarol). Colony treatment by Apiwarol single treatment or a four times every four days during a mount after the harvest, the presence of amitraz was not determined in the examined honey. While of the examined metabolites, the most frequently determined compound was 2, 4 DMA (Pohorecka *et al.,* 2018). After two long-lasting treatments with Apiwarol (strips with 500 mg amitraz each, contact action, 42 days), no residue of the parent compound was detected in honey. Tests of honey samples from conventional production in Slovenia, only the active substances amitraz, coumaphos and

thymol were found ≥LOQ, the amount of amitraz in the samples ranged from 0.01 to 0.12 mg/kg (Česnik *et al*., 2019).

Conclusion

In this study, the presence of amitraz and 2, 4-DMA was investigated by different analytical methods in honey samples from production areas in the Batajnica region. A rapid, simple and efficient GC-MS method for the determination of amitraz and 2, 4-DMA in honey samples was achieved using water and acetonitrile with an EMR lipid purification step. The QuEChERS extraction was modified and applied to the samples. A dilution approach was applied to the final extract, which was found to be sufficiently reliable for the selected matrices. In this way, the process is simplified and the possible loss of analytes during sample preparation is minimized. Chromatographic conditions, optimized sample preparation and minimization of matrix effects were optimized. The method was validated in terms of linearity, precision, accuracy, specificity, limit of detection, and limit of quantitation.
Satisfactory validation parameters were obtained for the determined residues according to the SANTE/11312/2021 requirements. The applicability of the method was tested on a real sample and proved to be suitable for routine analysis of the pesticides investigated in this study.

Acknowledgments

The study was funded by the Serbian Ministry of Science, Technological Development and Innovation (Contract No. 451-03-47/2023-01/200030).

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