Detection of Residual Pesticides in Foods

Edited by
Roberto Romero-González

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Editor

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Preface to "Detection of Residual Pesticides in Foods"

Despite the fact that analytical methods focused on the determination of pesticide residues in food have been developed for fifty years ago, there are new challenges scientist have to face out. Thus, multiresidue methods that covers a huge number of pesticides with different physic-chemical properties are developing, metabolites are being monitored and the use of mass spectrometry increases the reliability of the identification process. This book covers the main issues related to pesticide residue analysis, including valuable examples of the capabilities of current analytical methods in this field.

Roberto Romero-González
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Editorial

Detection of Residual Pesticides in Foods

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Pesticides are used worldwide. Despite the fact that organic farming is increasingly popular, pesticides are still widely applied in many countries under different pesticide regulations and monitoring programs. To provide reliable results, robust and sensitive analytical methodologies based on chromatographic techniques coupled to mass spectrometry (MS) are widely used. Another important issue is the application of generic extraction methods, allowing for the extraction of pesticides with different physico-chemical properties. The aim of the Special Issue "Detection of Residual Pesticides in Foods" was to gather original research papers focused on the development, validation and application of analytical methods, based on hyphenated techniques (chromatography-MS), in pesticide residue analyses, bearing in mind that they combine the separation capacity of the chromatographic techniques with the identification power of MS. This Special Issue is comprised of nine valuable scientific contributions, covering different topics related to pesticide residue monitoring.

One of the main aims in the study of pesticide residue is increasing the scope of the analysis; therefore, multiresidue methods have been proposed. Almeida et al. [1] developed a methodology for the determination of 168 pesticide residues in honey by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (127 compounds) and gas chromatography (GC)-MS/MS (41 pesticides), performing an extraction procedure based on the QuEChERS approach. Suitable validation parameters were achieved, and the method was applied to more than 30 honey samples; it was observed that carbendazim (20 samples), thiabendazole (20 samples), azoxystrobin (15 samples), chlorpyrifos (12 samples) and imidacloprid (12 samples) were the compounds most frequently detected, indicating that pesticide residues should be monitored in this sensitive matrix. In addition to the number of compounds, sample throughput is another key point to be considered when analytical methods are being developed. Grande-Martinez et al. [2] developed a 7 min multifamily residue method for the simultaneous quantification and confirmation of 8 phytohormones and 27 acidic herbicides in fruit and vegetables using ultra high-performance liquid chromatography (UHPLC) coupled to MS/MS. The method, which was validated according to SANTE 12682/2019, was also accredited (UNE-EN-ISO/IEC 17025:2017). Although sample treatment was based on the QuEChERS approach, because of the special characteristics of these kinds of compounds, a previous step of alkaline hydrolysis was needed. The proposed method was applied to the analysis of more than 450 samples of cucumber, orange, tomato, watermelon, and zucchini, and several compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 4-(3-indolyl)butyric acid (IBA), dichlorprop (2,4-DP), 2-methyl-4-chlorophenoxy acetic acid (MCPA), and triclopyr were detected, but at concentrations below the maximum residue level (MRL) regulated by the European Union (EU). Despite to the fact that QuEChERS can be widely used for the extraction of pesticide residues from vegetables, some compounds, such as sulfonylurea herbicides, are poorly recovered, and different modifications should be applied. Thus, dispersive solid phase extraction (d-SPE) sorbents should be carefully evaluated, considering that C18 is the most

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suitable sorbent for the determination of these compounds in strawberries by LC-MS/MS, achieving suitable precision and recovery [3].

The determination of highly polar pesticides is still a problem, and laboratories are looking for a pluri-residue method that encompasses the largest number of polar pesticides. In this sense, different stationary phases have been tested, and Manzano-Sánchez et al. [4] observed that the stationary phase Torus DEA provided the best separation of ethephon, 2-hydroxyethylphosphonic acid (HEPA), fosetyl aluminum, glyphosate, aminomethylphosphonic acid (AMPA), N-acetyl-glyphosate, and N-acetyl-AMPA. Previous LC separation, QuPPe method was used for the extraction of the targeted compounds, but slight modifications were necessary depending on the tested matrix (tomato, orange, aubergine, grape). For the detection of the compounds, a high-resolution single mass spectrometer, such as an Exactive-Orbitrap analyzer, provided reliable identification, considering that in addition to the characteristic ion, at least two fragments were monitored per compound.

Nowadays, it is also relevant to understand the dissipation of parent compounds to determine their persistence in different matrices. Thus, in a first study, aryloxyphenoxy-propionates and cyclohexanediones herbicide dissipation in vegetables was evaluated using LC-MS/MS after QuEChERS extraction. Non-linear models were applied, and it was observed that aryloxyphenoxy-propionates can contaminate vegetables with a short growing season and vegetables treated with fluazifop may not be suitable for baby food; however, propaquizafop and cycloxidim were found to be prospective herbicides for non-residual (baby food) vegetable production [5]. In a second study related to this topic, the degradation of 32 active substances (15 fungicides and 17 insecticides) was analyzed in different matrices, such as iceberg lettuce, onion, leek, carrot, and parsley. A first-order kinetic model was used, allowing the determination of an action pre-harvest interval based on an action threshold of 0.01 mg kg^{-1} to produce vegetables intended for zero-residue production. It was observed that the highest amount of pesticide residues was found in carrot and parsley leaves, and pesticide dissipation was generally slow. Lower amounts were found in leeks and lettuce. The authors indicate that it seems feasible to apply reduced pesticide amounts to stay below unwanted residue levels [6].

Related to this issue, in addition to the determination of parent compounds, metabolites are also being monitored in food and environmental matrices, bearing in mind that they can be more toxic or persistent than parent compounds. In a research article focused on this topic, three pyrethroid metabolites—3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), and cis-3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid (TFA)—were determined in tea by applying QuEChERS and UHPLC-MS/MS. Because of the complexity of the matrix, a clean-up step based on d-SPE was necessary, and a mixture of graphitized carbon black (GCB), florisil, and C18 was needed. Lower limits of quantification (LOQs) were achieved, from 2 to 10 μg kg^{-1}, and the validated method was applied to different types of tea, detecting 3-PBA and TFA in two samples [7].

Finally, monitoring activities are also important to achieve a full overview of the presence of these contaminants in foods of different origins. The study carried out by Panseri et al. [8] investigated the presence of contaminant residues (persistent organic pollutants (POPs) and pesticides, including glyphosate and metabolites) in organic honey samples from different production areas to confirm their incidence and possible impact on the food safety traits of organic production. Whereas GC-MS/MS was used for the determination of non-polar compounds, ionic chromatography coupled to Q-Exactive Orbitrap was used for glyphosate, glufosinate, and AMPA monitoring. Traces of organochlorine and organophosphate pesticides were detected in honey samples, in addition to other persistent organic pollutants. According to these results, it would seem mandatory to intensify the safety monitoring of this foodstuff and to keep improving good beekeeping practices, as suggested by the EU framework. In addition to vegetables, pesticides can also be detected in other matrices, such as meat. In the study performed by Kartalovic et al. [9], 19 organochlorine pesticides (OCPs) and other compounds were monitored in smoked meat

by GC-MS; α-HCH, lindane, PCB 28, PCB 52, and PCB 153 were detected in the analyzed samples, highlighting that the concentrations of OCPs and PCBs were not significantly affected by product type and by conditions of production.

In summary, this Special Issue, Detection of Residual Pesticides in Foods, highlights that even though much work has been done in pesticide residue analysis, there are still some gaps that should be covered (e.g., dissipation studies, metabolites, determination of orphan compounds). For this purpose, hyphenated techniques are valuable tools to achieve sound results and improve the information related to the presence of pesticides in foods of different origins.

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Article

Optimization of Method for Pesticide Detection in Honey by Using Liquid and Gas Chromatography Coupled with Mass Spectrometric Detection

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Abstract: This study aimed to optimize and validate a multi-residue method for identifying and quantifying pesticides in honey by using both gas and liquid chromatographic separation followed by mass spectrometric detection. The proposed method was validated to detect 168 compounds, 127 of them by LC-MS/MS (liquid chromatography tandem mass spectrometric detection) and 41 by GC-MS/MS (gas chromatography tandem mass spectrometric detection). The limit of detection (LOD) and limit of quantification (LOQ) values for the analytes determined by LC-MS/MS were 0.0001–0.0004 mg/kg and 0.0002–0.0008 mg/kg, respectively. For GC-MS/MS analyses, the LOD and LOQ values were 0.001–0.004 mg/kg and 0.002–0.008 mg/kg. In total, 33 samples of commercial honey produced by apiaries in six Brazilian states were analyzed with the validated method. Residual amounts of 15 analytes were detected in 31 samples (93.9%). The method described in the present study was able to detect an extensive and broad range of pesticides with very high sensitivity.

Keywords: residues in food; pesticides; LC-MS/MS; GC-MS/MS; QuEChERS; honey

1. Introduction

Honey is consumed by humans worldwide because of its characteristic sweet flavor and as a medicinal food. It is produced by honeybees, mainly from nectar collected from flowers. However, honey may be contaminated with pesticides used on crops foraged by bees. Contamination may occur through direct contact of the bee body to the pesticide or by bee consumption of the contaminated nectar, pollen, and guttation fluid (an exudate eliminated through the tips or edges of leaves of some plants) [1–3]. Furthermore, some pesticides are used to treat beehives against diseases [4].

The consumption of residual pesticides in contaminated foods has been linked to several toxic effects in humans, such as carcinogenesis, immunological disorders, and neurological disturbances [5]. Maximum residue levels (MRLs) have been established for pesticides in honey to ensure consumers' safety [6–9]. It is mandatory to avoid the commercialization of honey containing residual pesticides at levels above the MRLs. To determine residual pesticide levels, precise and sensitive analytical methods must be able to detect an extensive and broad range of compounds.

Several analytical methods have been developed for detecting single compounds to a few dozen pesticides in honey. In these methods, detection and quantification are performed using techniques such as liquid chromatography (LC) with diode array [10], ultraviolet [11,12],

fluorescence [13], and electrochemical [11] detectors, gas chromatography (GC) with electron capture [14], flame ionization [15], nitrogen–phosphorus [16], flame photometric [17], thermionic-specific [18], and atomic emission [19] detectors, and excitation–emission matrix fluorescence data [20].

The performance of chromatographic analysis depends on adequate sample extraction and cleanup procedures. Matrix compounds are concentrated at the extraction procedure, whereas interfering substances are removed by the cleanup procedure [21]. An innovative technique developed for sample extraction and cleanup procedures is the QuECHERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method [22]. Compared to earlier procedures, this method reduces the volume of solvents, and offers practical performance. Modifications of the QuECHERS method have been used for the detection of pesticides in different matrices such as meat [23], fish [24], milk [25], and honey [3,26–31].

Simultaneous detection of the residual levels of several pesticides in honey is mandatory in several countries to inspect this food before commercialization. Multi-residue analysis of at least one hundred pesticides in honey has been achieved using LC and GC coupled to mass spectrometric (MS) or tandem mass spectrometric (MS/MS) detection [1,3,26–32].

This study aimed to develop and validate a multi-residue method for identifying and quantifying pesticides in honey by using both gas and liquid chromatographic separation followed by mass spectrometric detection.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile, ethyl acetate (both high performance liquid chromatography [HPLC] grade), and formic acid (for analysis) were supplied by Merck (Darmstadt, Germany). Methanol (HPLC grade) was obtained from Honeywell (Charlotte, NC, USA). Ammonium formate (>99%) was purchased from Vetec (Rio de Janeiro, Brazil). A DisQuE™ CEN sample preparation kit in pouch format (each pouch containing 4.0 g of anhydrous magnesium sulfate, 1.0 g of sodium chloride, 1.0 g of trisodium citrate dihydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate; all > 99%) was supplied by Waters (Milford, CT, USA). An ExtraBond® QuEChERS Dispersive kit EN (each tube containing 900 mg of anhydrous magnesium sulfate and 150 mg of primary and secondary amine (PSA); both > 99%) was obtained from Scharlab (Barcelona, Spain). D-Sorbitol (≥98%) and gluconolactone (>99%) were purchased from Sigma–Aldrich (Darmstadt, Germany). Ultrapure water was generated with a Millipore Milli-Q system (Milford, CT, USA). All reference standards were of high purity grade (>98.0%) and were obtained from Dr. Ehrenstorfer (Augsburg, Germany) or AccuStandard (New Haven, CT, USA). Individual stock solutions were prepared at an approximate concentration of 1000 ng/µL in acetonitrile or acetone and stored in a freezer at −20 °C. Working solutions were prepared through appropriate dilutions of the stock solutions.

2.2. Samples

Blank samples of honey were obtained from apiaries managed under an organic system, and repeated analyses confirmed the absence of residual pesticides. These blank samples were fortified with target analytes for the validation of the analytical method. Furthermore, 33 samples of commercial honey produced by apiaries in six Brazilian states (Distrito Federal, Goias, Minas Gerais, Rio Grande do Norte, Rio Grande do Sul, and São Paulo) were analyzed using the validated method.

2.3. Sample Preparation

The modified QuEChERS method for extraction and cleanup was optimized from previously described procedures [22,28,32,33]. Each honey sample (5.0 g) was placed into a 50 mL polypropylene tube and spiked with appropriate amounts of pesticides in working solutions. Next, 10.0 mL of ultrapure water was added, and the mixture was agitated at 1750 rpm for 2 min. Exactly 10.0 mL of a solution of acetonitrile and ethyl acetate (70:30, *v/v*) was added, and each tube was agitated again

at 1750 rpm for 2 min. Then, 4.0 g of anhydrous magnesium sulfate, 1.0 g of sodium chloride, 1 g of trisodium citrate dehydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate were added, and the tubes were agitated at 1750 rpm for another 2 min and centrifuged at 4000 rpm for 5 min. The whole organic layer was transferred to a 15 mL polypropylene tube, and the mixture was kept at −40 °C for at least 2 h. The supernatant (6.0 mL) was mixed with 900 mg of anhydrous magnesium sulfate and 150 mg of PSA, and the mixture was agitated at 1750 rpm for 1 min and centrifuged at 3600 rpm for 5 min. The extract (4.0 mL) was transferred to two 13 × 100 mm glass tubes, with 2.0 mL in each tube. The solution was dried in an evaporator with a water bath maintained at 45 °C and nitrogen pressure of 15 psi.

The procedural internal standard (P-IS) [34] for the LC analysis was Propoxur, and the P-IS for GC analysis was 4,4′-dichlorodiphenyldichloroethylene (4,4′-DDE). After weighing the honey sample, 10 µL of the P-IS solution containing 4.0 ng/µL of Propoxur and 4.0 ng/µL of DDE 4,4 was added. Propoxur and 4,4′-DDE were then validated following the validation method described in Section 2.3.

For LC analysis, the dried residue was reconstituted with 200 µL of methanol:water (1:1), with both solvents containing 5 mM ammonium formate and 0.01% formic acid. After 30 min, the tube was vortexed for 1 min, and the solution was transferred to a vial containing a conical insert of 250 µL.

For GC analysis, the dried residue was reconstituted with 200 µL of acetonitrile:ethyl acetate (7:3) and 6 µL of analyte protectant solution, composed of 10 mg/mL gluconolactone and 5 mg/mL D-sorbitol in acetonitrile:water (7:3). The tube was then immediately vortexed for 0.5 min, and the solution was transferred to a vial containing a conical insert of 250 µL.

2.4. Liquid Chromatography

LC-MS/MS (liquid chromatography tandem mass spectrometric detection) analysis was performed using an Agilent 6495 Triple Quadrupole LC/MS system. Chromatographic separations were carried out on a Zorbax SB-C18 Rapid Resolution HT column (4.6 × 150 mm, 1.8 µm) at a 40 °C column temperature. The mobile phases were water containing 5 mM ammonium formate and 0.01% formic acid (phase A) and methanol containing 5 mM ammonium formate and 0.01% formic acid (phase B), with gradient elution at a flow rate of 0.6 mL/min. The gradient elution program was as follows: 0 min, 90% B; 2.0 min, 50% B; 20 min, 100% B. The total chromatographic run time was 25 min. The injection volume was 5 µL.

For mass spectrometric analysis, an electrospray ionization (ESI) source was used in both negative (ESI-) and positive (ESI+) modes. Source parameters were set as follows: gas temperature 120 °C, gas flow 15 L/min, nebulizer 45 psi, sheath gas flow 12 L/min, sheath gas temperature 300 °C, capillary voltage 3500 V (+ and −), nozzle voltage 300 V (+)/500 V (−), iFunnel RF high pressure 150 V (+)/90 V (−), and iFunnel RF low pressure 60 V (+ and −). The retention times, delta retention times, polarities, ion transitions, and collision energies are presented in Table 1. Two transitions were chosen for almost all pesticides, but an extra confirmatory transition was included for four pesticides to avoid false-positives at trace pesticide levels. The analysis was run according to all requirements for identifying analytes by MS/MS established by European Union SANTE/12682/2019 [34].

Table 1. Chromatographic parameters and MS/MS (tandem mass spectrometric) detection for compounds analyzed by LC-MS/MS (liquid chromatography tandem mass spectrometric detection).

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
2,4-D	9.60	1.5	ESI−	QI [3] 218.9 > 161. 1st CI [4] 218.9 > 124.9 2nd CI 220.9 > 162.9	11 35 11
Acephate	4.56	1.5	ESI+	QI 184.0 > 143.0 1st CI 184.0 > 125.0	5 15
Acetamiprid	6.56	1.0	ESI+	QI 223.1 > 126.0 1st CI 223.1 > 56.0	15 15
Aldicarb	8.05	1.5	ESI+	QI 208.1 > 116.2 1st CI 208.1 > 89.1	10 24
Aldicarb-Sulfone	4.99	1.5	ESI+	QI 223.1 > 148.0 1st CI 223.1 > 76.0	5 5
Aldicarb-Sulfoxide	4.88	1.5	ESI+	QI 207.1 > 131.9 1st CI 207.1 > 89.1	0 8
Allethrin	19.36	1.5	ESI+	QI 303.2 > 135.0 1st CI 303.2 > 123	10 20
Ametryn	13.15	1.5	ESI+	QI 228.1 > 186.1 1st CI 228.1 > 116.1 2nd CI 228.1 > 96.0	20 28 25
Aminocarb	7.32	1.5	ESI+	QI 209.1 > 152.2 1st CI 209.1 > 137.2	12 20
Atrazine	11.34	1.5	ESI+	QI 216.1 > 174.1 1st CI 216.1 > 68.0	16 40
Avermectin B1a	21.71	1.5	ESI+	QI 890.5 > 567.4 1st CI 890.5 > 305.1	8 16
Azaconazole	11.03	1.5	ESI+	QI 300.0 > 231.1 1st CI 300.0 > 159.0	16 28
Azinphos-Ethyl	14.74	1.5	ESI+	QI 346.1 > 132.1 1st CI 346.1 > 97.0	12 32
Azinphos-Methyl	12.15	1.5	ESI+	QI 318.0 > 261.0 1st CI 318.0 > 132.1	0 8
Azoxystrobin	12.90	1.5	ESI+	QI 404.1 > 372.1 1st CI 404.1 > 344.1 2nd CI 404.1 > 329.1	12 28 36
Benalaxyl	16.86	1.5	ESI+	QI 326.2 > 294.1 1st CI 326.2 > 148.1	4 27
Bitertanol	16.98	1.5	ESI+	QI 338.2 > 99.1 1st CI 338.2 > 70.0	10 4
Boscalid	13.34	1.5	ESI+	QI 343.0 > 307.1 1st CI 343.0 > 271.2	16 32
Bromacil	9.29	1.5	ESI+	QI 261.0 > 205.0 1st CI 261.0 > 187.9	20 40
Bromuconazole	15.50	3.0	ESI+	QI 378.0 > 159.0 1st CI 378.0 > 70.0	32 35
Buprofezin	19.13	1.5	ESI+	QI 306.2 > 201.1 1st CI 306.2 > 116.1	5 10
Cadusafos	17.96	1.5	ESI+	QI 271.1 > 130.9 1st CI 271.1 > 97.0	20 40
Carbaryl	9.80	1.5	ESI+	QI 202.1 > 145.1 1st CI 202.1 > 127.1	4 28
Carbendazim	7.07	1.5	ESI+	QI 192.1 > 160.1 1st CI 192.1 > 132.1	16 32
Carbofuran	9.35	1.5	ESI+	QI 222.1 > 165.1 1st CI 222.1 > 123.1	20 30
3-Hydroxycarbofuran	6.35	1.5	ESI+	QI 238.1 > 220.1 1st CI 238.1 > 163.1	0 8

Table 1. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
Carboxin	9.88	1.5	ESI+	QI 236.1 > 143.1 1st CI 236.1 > 93.1	12 36
Chlorfenvinphos	17.20	1.5	ESI+	QI 358.9 > 155.0 1st CI 358.9 > 99.2	8 28
Chlorfluazuron	20.00	1.5	ESI+	QI 539.9 > 383.0 1st CI 539.9 > 158.0	44 36
Chlorpyrifos	20.20	1.5	ESI+	QI 349.9 > 198.0 1st CI 349.9 > 97.0	20 20
Chlorpyrifos-Methyl-Oxon	15.89	1.5	ESI+	QI 334.0 > 306.0 1st CI 334.0 > 278.0	8 8
Clofentezine	16.99	1.5	ESI+	QI 303.0 > 138.0 1st CI 303.0 > 102.0	12 40
Clomazone	12.70	1.5	ESI+	QI 242.1 > 127.0 1st CI 240.1 > 125.0 2nd CI 240.1 > 89.1	20 20 56
Clothianidin	6.08	1.5	ESI+	QI 250.0 > 169.0 1st CI 250.0 > 131.9	8 8
Cyanazine	8.43	1.5	ESI+	QI 241.1 > 214.1 1st CI 241.1 > 104.0	18 44
Cyanofenphos	16.60	1.0	ESI+	QI 304.1 > 276.0 1st CI 304.1 > 157.0	12 24
Cyazofamid	15.43	1.5	ESI+	QI 325.0 > 261.0 1st CI 325.0 > 108.0	4 8
Cymoxanil	6.97	1.5	ESI+	QI 199.1 > 128.0 1st CI 199.1 > 110.9	4 12
Cyproconazole	14.80	2.0	ESI+	QI 292.1 > 125.0 1st CI 292.1 > 70.0	32 16
Cyprodinil	17.10	1.5	ESI+	QI 226.1 > 108.0 1st CI 226.1 > 93.0	30 40
Cyromazine	4.48	1.5	ESI+	QI 167.1 > 125.0 1st CI 167.1 > 85.0	16 16
Diafenthiuron	20.82	1.5	ESI+	QI 385.2 > 329.2 1st CI 385.2 > 278.2	16 32
Diazinon	17.10	1.5	ESI+	QI 305.1 > 169.1 1st CI 305.1 > 97.0	32 40
Dichlorvos	9.11	1.5	ESI+	QI 221.0 > 109.0 1st CI 221.0 > 79.0	12 24
Dicrotophos	5.83	1.5	ESI+	QI 238.0 > 127.0 1st CI 238.0 > 112.1	12 8
Difenoconazole	17.80	1.5	ESI+	QI 406.1 > 337.0 1st CI 406.1 > 251.0	18 28
Diflubenzuron	14.96	1.5	ESI+	QI 311.0 > 158.0 1st CI 311.0 > 141.0	8 32
Dimethoate	6.53	1.5	ESI+	QI 230.0 > 198.8 1st CI 230.0 > 125.0	0 16
Dimethomorph	13.80	3.0	ESI+	QI 388.1 > 301.1 1st CI 388.1 > 165.1	20 32
Diniconazole	18.00	1.5	ESI+	QI 326.1 > 159.0 1st CI 326.1 > 70.0	28 28
Disulfoton	17.69	1.5	ESI+	QI 275.0 > 89.0 1st CI 275.0 > 61.0	12 44
Disulfoton-Sulfone	10.72	1.5	ESI+	QI 307.0 > 125.0 1st CI 307.0 > 97.0	10 30
Disulfoton-Sulfoxide	10.78	1.5	ESI+	QI 291.0 > 185.0 1st CI 291.0 > 157.0	10 20

Table 1. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
Diuron	11.38	1.5	ESI+	QI 2350. > 72.0 1st CI 233.0 > 160.0 2nd CI 233.03 > 72.1	20 24 20
Emamectin B1a	20.89	2.0	ESI+	QI 886.5 > 158.0 1st CI 886.5 > 82.1	44 64
Emamectin B1b	20.34	2.0	ESI+	QI 872.5 > 158.3 1st CI 872.5 > 82.3	40 68
Epoxiconazole	15.21	1.5	ESI+	QI 330.1 > 121.0 1st CI 330.1 > 101.2	16 52
Ethion	19.65	1.5	ESI+	QI 385.0 > 199.1 1st CI 385.0 > 142.8	4 24
Etofenprox	22.82	1.5	ESI+	QI 394.2 > 359.0 1st CI 394.2 > 177.0	5 5
Ethoprophos	15.72	1.5	ESI+	QI 243.1 > 130.9 1st CI 243.1 > 97.0	15 30
Etrimfos	16.80	1.5	ESI+	QI 293.1 > 265.0 1st CI 293.1 > 125.0	26 28
Famoxadone	16.93	1.5	ESI+	QI 392.1 > 330.9 1st CI 392.1 > 238.0	4 12
Fenamiphos	15.92	1.5	ESI+	QI 304.1 > 234.0 1st CI 304.1 > 217.1	12 20
Fenbuconazole	15.11	1.5	ESI+	QI 337.1 > 125.1 1st CI 337.1 > 70.0	40 33
Fenpyroximate	20.91	1.5	ESI+	QI 422.2 > 366.2 1st CI 422.2 > 135.0	12 36
Fenthion	16.86	1.5	ESI+	QI 279.0 > 247.1 1st CI 279.0 > 169.1	8 12
Fipronil	15.50	1.5	ESI+	QI 437.0 > 368.0 1st CI 437.0 > 255.0	18 26
Flazasulfuron	11.24	1.5	ESI+	QI 408.1 > 182.1 1st CI 408.1 > 83.0	28 40
Fluazifop-Butyl	18.80	1.5	ESI+	QI 384.1 > 328.1 1st CI 384.1 > 282.2	12 20
Flufenoxuron	19.88	1.5	ESI+	QI 489.1 > 158.0 1st CI 489.1 > 140.9	20 56
Fluquinconazole	15.11	1.5	ESI+	QI 376.0 > 349.0 1st CI 376.0 > 307.1	20 24
Flutriafol	11.17	1.5	ESI+	QI 302.1 > 122.9 1st CI 302.1 > 70.1	28 16
Furathiocarb	19.30	1.5	ESI+	QI 383.2 > 251.9 1st CI 383.2 > 195.0	8 16
Heptenophos	11.79	1.5	ESI+	QI 251.0 > 127.0 1st CI 251.0 > 125.0	15 25
Hexaconazole	17.40	1.5	ESI+	QI 314.1 > 159.0 1st CI 314.1 > 70.1	30 20
Hexythiazox	19.90	1.5	ESI+	QI 353.1 > 227.9 1st CI 353.1 > 168.1	8 24
Imazalil	14.30	3.0	ESI+	QI 297.1 > 201.0 1st CI 297.1 > 159.0	15 20
Imazapyr	5.48	3.0	ESI+	QI 262.1 > 217.1 1st CI 262.1 > 131.0	20 40
Imazethapyr	7.42	1.5	ESI+	QI 290.1 > 245.1 1st CI 290.1 > 177.0	24 29
Imibenconazole	19.60	1.5	ESI+	QI 411.0 > 171.0 1st CI 411.0 > 125.0	20 40

Table 1. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
Imidacloprid	5.97	1.5	ESI+	QI 258.0 > 210.9 1st CI 256.0 > 208.9 2nd CI 256 > 175	12 12 12
Indoxacarb	17.88	1.5	ESI+	QI 528.1 > 203.0 1st CI 528.1 > 150.0	45 20
Iprodione	15.98	1.5	ESI+	QI 330.0 > 287.9 1st CI 330.0 > 244.9	10 14
Iprovalicarb	15.20	1.5	ESI+	QI 321.2 > 202.9 1st CI 321.2 > 119	5 16
Kresoxim-Methyl	16.50	1.5	ESI+	QI 314.1 > 267.0 1st CI 314.1 > 222.1	0 10
Linuron	12.67	1.5	ESI+	QI 249.0 > 160.1 1st CI 249.0 > 133.0	20 36
Lufenuron	19.03	1.5	ESI+	QI 510.9 > 158.0 1st CI 510.9 > 141.0	20 57
Malaoxon	9.37	1.5	ESI+	QI 315.0 > 127.1 1st CI 315.0 > 99.2	20 4
Malathion	14.30	1.5	ESI+	QI 331.0 > 126.9 1st CI 331.0 > 99.0	5 10
Metalaxyl	11.93	1.5	ESI+	QI 280.2 > 220.1 1st CI 280.2 > 160.1	10 20
Metconazole	17.30	1.5	ESI+	QI 320.1 > 125.0 1st CI 320.1 > 70.1	48 24
Methamidophos	4.31	1.5	ESI+	QI 142.0 > 125.0 1st CI 142.0 > 94.0	10 10
Methidathion	11.95	1.5	ESI+	QI 302.9 > 145.0 1st CI 302.9 > 85.1	0 15
Methiocarb	13.14	1.5	ESI+	QI 226.1 > 169.0 1st CI 226.1 > 121.1	4 12
Methomyl	5.44	1.5	ESI+	QI 163.1 > 106.0 1st CI 163.1 > 88.0	4 0
Methoxyfenozide	14.08	1.5	ESI+	QI 369.2 > 313.1 1st CI 369.2 > 149.0	0 10
Metolachlor	16.30	1.5	ESI+	QI 284.1 > 252.1 1st CI 284.1 > 176.1	8 24
Metribuzin	9.38	1.5	ESI+	QI 215.1 > 187.1 1st CI 215.1 > 84.0	15 30
Mevinphos	7.35	3.0	ESI+	QI 225.0 > 193.1 1st CI 225.0 > 127.0	0 12
Monocrotophos	5.52	1.5	ESI+	QI 224.1 > 193.0 1st CI 224.1 > 127.0	0 10
Myclobutanil	14.00	1.5	ESI+	QI 289.1 > 125.1 1st CI 289.1 > 70.1	32 16
Naled	11.80	1.5	ESI+	QI 380.7 > 127.0 1st CI 380.7 > 109.0	8 24
Omethoate	4.77	1.5	ESI+	QI 214.0 > 125.0 1st CI 214.0 > 109.0	16 24
Oxamyl	5.09	1.5	ESI+	QI 237.1 > 90.0 1st CI 237.1 > 72.0	10 12
Paclobutrazol	13.95	1.2	ESI+	QI 294.1 > 125.2 1st CI 294.1 > 70.1	40 20
Paraoxon	10.88	1.5	ESI+	QI 276.1 > 220.0 1st CI 276.1 > 94.0	10 40
Paraoxon-Methyl	8.05	1.5	ESI+	QI 248.0 > 201.9 1st CI 248.0 > 90.0	20 25

Table 1. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
Parathion	16.40	1.5	ESI+	QI 292.0 > 236.1 1st CI 292.0 > 94.1	8 40
Penconazole	16.70	1.5	ESI+	QI 284.1 > 159 1st CI 284.1 > 70.1	30 15
Pencycuron	18.00	1.5	ESI+	QI 329.1 > 125.1 1st CI 329.1 > 89.1	24 60
Pendimethalin	20.20	1.5	ESI+	QI 282.1 > 212.1 1st CI 282.1 > 194.1	4 16
Phenthoate	16.20	1.5	ESI+	QI 321.0 > 163.1 1st CI 321.0 > 79.1	8 44
Phorate	17.50	1.5	ESI+	QI 261.0 > 199.0 1st CI 261.0 > 75.1	2 5
Phosmet	12.80	1.5	ESI+	QI 317.9 > 160.0 1st CI 317.9 > 133.0	8 36
Phosphamidon	8.30	1.5	ESI+	QI 300.0 > 174.1 1st CI 300.0 > 127.1	8 16
Picloram	4.71	1.5	ESI+	QI 243.0 > 196.8 1st CI 243.0 > 169.8 2nd CI 241 > 222.8	22 34 10
Picoxystrobin	16.03	1.5	ESI+	QI 368.1 > 205.2 1st CI 368.1 > 145.0	4 20
Pirimicarb	11.08	1.5	ESI+	QI 239.1 > 182.1 1st CI 239.1 > 72.1	12 20
Pirimiphos-Ethyl	19.39	1.5	ESI+	QI 334.1 > 198.1 1st CI 334.1 > 182.1	22 24
Pirimiphos-Methyl	18.00	1.5	ESI+	QI 306.2 > 164.1 1st CI 306.2 > 108.1	20 30
Prochloraz	18.03	1.5	ESI+	QI 376.0 > 308.0 1st CI 376.0 > 265.9	4 12
Profenofos	18.83	1.5	ESI+	QI 374.9 > 347.0 1st CI 374.9 > 304.9	12 19
Propamocarb	4.72	1.5	ESI+	QI 189.2 > 144.0 1st CI 189.2 > 102.0	8 12
Propargite	20.26	1.5	ESI+	QI 368.1 > 231.2 1st CI 368.1 > 175.2	0 8
Propiconazole	17.50	1.5	ESI+	QI 342.1 > 159.0 1st CI 342.1 > 69.1	32 16
Propoxur	9.33	1.5	ESI+	QI 210.11 > 168.1 1st CI 210.11 > 111.1	5 8
Pyraclostrobin	17.50	1.5	ESI+	QI 388.11 > 193.8 1st CI 388.11 > 163.1	8 20
Pyrazophos	17.26	1.5	ESI+	QI 374.1 > 222.1 1st CI 374.1 > 194.1	16 32
Pyridaben	21.90	1.5	ESI+	QI 365.1 > 309.1 1st CI 365.1 > 147.2	4 20
Pyridaphenthion	14.36	1.5	ESI+	QI 341.0 > 205.1 1st CI 341.0 > 189.0	10 20
Pyrimethanil	13.88	1.5	ESI+	QI 200.1 > 106.9 1st CI 200.1 > 82.0	20 25
Pyriproxyfen	19.90	1.5	ESI+	QI 322.2 > 227.2 1st CI 322.2 > 185.0 2nd CI 322.2 > 96	12 20 12
Quinalphos	16.48	1.5	ESI+	QI 299.0 > 163.0 1st CI 299.0 > 147.0	20 20
Quizalofop-Ethyl	19.10	1.5	ESI+	QI 373.0 > 271.2 1st CI 373.0 > 255.1	24 36

Table 1. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Polarity	Transitions	Collision Energy
Simazine	9.45	1.5	ESI+	QI 202.1 > 132.0 1st CI 202.1 > 124.1	22 26
Spinosyn A	19.87	2.0	ESI+	QI 732.5 > 142.1 1st CI 732.5 > 98.1	28 60
Spinosyn D	20.78	2.0	ESI+	QI 746.5 > 142.1 1st CI 746.5 > 98.0	35 55
Spirodiclofen	21.25	1.5	ESI+	QI 411.1 > 313 1st CI 411.1 > 71.2	8 15
Spiromesifen	20.67	1.5	ESI+	QI 371.2 > 273.1 1st CI 371.2 > 255.1	12 24
Sulfentrazone	9.38	1.5	ESI+	QI 404.0 > 306.9 1st CI 404.0 > 273.0	28 40
Tebuconazole	16.80	1.5	ESI+	QI 308.1 > 124.9 1st CI 308.1 > 70.0	47 40
Tebufenozide	16.08	1.5	ESI+	QI 353.2 > 297.1 1st CI 353.2 > 133.0	4 20
Teflubenzuron	19.00	1.5	ESI+	QI 381.0 > 158.0 1st CI 381.0 > 141.0	12 48
Temephos	18.90	1.5	ESI+	QI 467.0 > 419.0 1st CI 467 > 124.9	20 44
Terbufos	19.60	1.5	ESI+	QI 289.1 > 233.0 1st CI 289.1 > 57.1	0 16
Tetraconazole	15.09	1.5	ESI+	QI 372.0 > 159.0 1st CI 372.0 > 70.0	36 20
Thiabendazole	8.22	1.5	ESI+	QI 202.0 > 175.0 1st CI 202.0 > 131.0	24 36
Thiacloprid	7.10	1.5	ESI+	QI 253.0 > 126.0 1st CI 253.0 > 90.0	16 40
Thiamethoxam	5.42	1.5	ESI+	QI 292.0 > 211.1 1st CI 292.0 > 181.1	8 20
Thiobencarb	18.03	1.5	ESI+	QI 258.0 > 125.1 1st CI 258.07 > 100.1	25 5
Thiodicarb	10.59	1.5	ESI+	QI 355.0 > 108.1 1st CI 355.0 > 88.1	8 8
Thiophanate-Methyl	8.65	1.5	ESI+	QI 343.0 > 151.0 1st CI 343.0 > 93.0	20 56
Tolylfluanid	15.99	1.5	ESI+	QI 346.9 > 238.1 1st CI 346.9 > 137.0	12 25
Triadimefon	14.80	1.5	ESI+	QI 294.1 > 197.2 1st CI 294.1 > 69.1	8 16
Triadimenol	14.70	1.5	ESI+	QI 296.1 > 99.1 1st CI 296.1 > 70.0	16 12
Triazophos	14.30	1.5	ESI+	QI 314.1 > 162.1 1st CI 314.1 > 119.1	16 36
Trichlorfon	6.55	1.5	ESI+	QI 256.9 > 221.0 1st CI 256.9 > 109.0	4 12
Trifloxystrobin	18.35	1.5	ESI+	QI 409.1 > 186.0 1st CI 409.1 > 145.0	12 52
Triflumizole	18.50	1.5	ESI+	QI 346.1 > 278.0 1st CI 346.1 > 43.1	4 20
Vamidothion	6.39	1.5	ESI+	QI 288.0 > 146.1 1st CI 288.0 > 58.0	6 44
Zoxamide	16.96	1.5	ESI+	QI 336.0 > 187.0 1st CI 336.0 > 159.0	16 44

[1] RT: retention time. [2] DRT: delta retention time. [3] QI: quantification ions. [4] CI: confirmation ions.

2.5. Gas Chromatography

GC-MS/MS (gas chromatography tandem mass spectrometric detection) analysis was performed using an Agilent 7000C Triple Quadrupole GC/MS system with a multimode inlet. The temperature of the injector was maintained at 150 °C (0.1 min), ramped up to 300 °C at 600 °C/min (20 min hold), and then ramped down to 200 °C at 20 °C/min until the end of the analysis. The injection volume was 2 µL. The pulsed splitless injection was at 50 psi for 0.5 min with a split flow of 50 mL/min for 0.6 min. The gas saver was set to 20 L/min and started after 5 min. The carrier gas was helium, and the inlet pressure was 5.59 psi (constant pressure mode) during the run and 2.0 psi during the backflush. From the inlet, two Agilent HP-5ms Ultra Inert (5%-phenyl)-methylpolysiloxane columns (0.25 mm, 0.25 µm) were coupled to each other through a purged ultimate union for post-run backflushing; the first column was 30 m, and the second column was 2 m. The total chromatographic run time was 29.5 min, and backflushing started after 25.5 min with 8.92 psi. The column oven temperature was maintained at 60 °C for 1.0 min, ramped up to 180 °C at 30 °C/min, and then ramped up to 300 °C at 5 °C/min.

For the mass spectrometric analysis, an electron ionization source was used with an ionization voltage of 70 eV, ion source temperature of 290 °C, and interface temperature of 280 °C. The retention times, delta retention times, polarities, ion transitions, and collision energies are presented in Table 2. Two transitions were chosen for almost all pesticides, but an extra confirmatory transition was included for seven pesticides to avoid false-positives at trace pesticide levels. The analysis was run according to all requirements for identifying analytes by MS/MS established by European Union SANTE/12682/2019 [34].

Table 2. Chromatographic parameters and MS/MS detection for compounds analyzed by GC-MS/MS (gas chromatography tandem mass spectrometric detection).

Name	RT [1] (min)	DRT [2] (min)	Quantification Transition	Collision Energy
Alachlor	11.33	1	QI [3] 188.1 > 160.1 1st CI [4] 188.1 > 130.1	10 40
Aldrin	12.62	1	QI 263.0 > 193.0 1st CI 298.0 > 263.0	30 8
Bifenthrin	19.78	2	QI 182.0 > 167.0 1st CI 181.0 > 165.0	12 25
Bromophos-Methyl	13.06	1	QI 330.9 > 315.9 1st CI 329.0 > 314.0	16 16
Bromopropylate	19.82	2	QI 341.0 > 185.0 1st CI 341.0 > 183.0	5 20
Carbophenothion	17.71	1	QI 153.0 > 96.9 1st CI 153.0 > 79.0 2nd CI 157.0 > 75.1	10 30 40
Cyfluthrin	24.39	2	QI 162.9 > 127.0 1st CI 226.9 > 77.1	5 30
Cypermethrin	25.01	2	QI 162.9 > 127.0 1st CI 181.1 > 127.1	5 35
Clordane Gama (Trans)	14.42	2	QI 272.0 > 237.0 1st CI 375.0 > 266.0	16 25
Chlorfenapyr	16	1	QI 247.0 > 227.0 1st CI 247.0 > 200.0 2nd CI 247.0 > 197.0	15 25 5
Chlorothalonil	10.17	1	QI 265.9 > 230.9 1st CI 263.8 > 229.0 2nd CI 263.8 > 168.0	20 20 25
Chlorpyrifos-Methyl	11.14	2	QI 288.0 > 93.0 1st CI 288.0 > 273.0 2nd CI 286.0 > 271.0	26 15 16

Table 2. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Quantification Transition	Collision Energy
Chlorthiophos	16.93	1	QI 297.0 > 269.0 1st CI 269.0 > 205.0	14 16
2,4′-DDD	15.7	1	QI 237.0 > 165.0 1st CI 235.0 > 165.0	20 20
2,4′-DDE	14.47	2	QI 246.0 > 176.0 1st CI 248.0 > 211.0	30 20
4,4′-DDE	15.92	1	QI 246.0 > 176.0 1st CI 248.0 > 176.0	30 20
2,4′-DDT	16.84	1	QI 237.0 > 165.0 1st CI 235.0 > 165.0	20 20
4,4′-DDT	18	1	QI 237.0 > 165.0 1st CI 235.0 > 165.0	20 20
Deltamethrin	27.94	1	QI 253.0 > 93.0 1st CI 253.0 > 174.0	20 15
Dicofol	18.46	2	QI 253.0 > 141.0 1st CI 249.9 > 139.1	15 10
Dieldrin	15.69	1	QI 263.0 > 191.0 1st CI 263.0 > 193.0	35 35
Endosulfan Alpha	14.84	2	QI 238.8 > 204.0 1st CI 241.0 > 206.0	15 15
Endosulfan Beta	16.35	1	QI 241.0 > 206.0 1st CI 195.0 > 159.0	15 15
Endosulfan Sulfate	17.94	1	QI 271.9 > 236.9 1st CI 240.8 > 205.9	15 15
Endrin	16.06	1	QI 263.0 > 191.0 1st CI 263.0 > 193.0	35 35
Esfenvalerate	26.91	2	QI 225.0 > 119.0 1st CI 167.0 > 125.0	15 10
Fenpropathrin	20.1	1	QI 265.0 > 210.0 1st CI 265.0 > 89.0 2nd CI 181.0 > 152.0	15 35 26
Fenarimol	21.94	1	QI 139.0 > 111.0 1st CI 219.0 > 107.0	15 10
Fenitrothion	11.94	1	QI 277.0 > 260.0 1st CI 277.1 > 109.0 2nd CI 276.8 > 125.0	5 20 15
Phosalone	20.91	1	QI 182.0 > 111.0 1st CI 182.0 > 75.1	15 40
HCH Alpha	9.1	2	QI 180.9 > 145.0 1st CI 218.8 > 183.0	12 5
HCH Beta	9.57	2	QI 180.9 > 145.0 1st CI 218.8 > 183.0	12 5
HCH Delta	10.38	1	QI 180.9 > 145.0 1st CI 218.8 > 183.0	12 5
HCH Gamma	9.81	2	QI 180.9 > 145.0 1st CI 218.8 > 183.0	12 5
Heptachlor	11.63	1	QI 271.9 > 236.8 1st CI 274.0 > 239.0	25 20
Heptacloro Exo Epoxid	13.71	1	QI 353.0 > 263.0 1st CI 353.0 > 282.0	15 15
Hexachlorobenzene (HCB)	9.23	1	QI 283.9 > 213.9 1st CI 283.9 > 248.8	35 25
Lambda Cyhalothrin	21.65	1	QI 181.1 > 152.1 1st CI 197.0 > 161.0	30 10
Methoxychlor	20	2	QI 227.0 > 141.1 1st CI 227.0 > 169.0	40 20

Table 2. *Cont.*

Name	RT [1] (min)	DRT [2] (min)	Quantification Transition	Collision Energy
Mirex	21.68	1	QI 271.9 > 235.0 1st CI 272.0 > 237.0	25 20
Ovex (Clorfenson)	15.11	1	QI 174.8 > 111.1 1st CI 177.0 > 113.0 2nd CI 302.0 > 175.0	10 12 4
Oxyfluorfen	15.64	1	QI 252.0 > 146.0 1st CI 252.0 > 170.0	32 32
Parathion-Methyl	11.28	2	QI 263.0 > 109.1 1st CI 263.0 > 79.1	15 30
Permethrin	23.38	2	QI 183.1 > 153.1 1st CI 183.0 > 115.2	15 25
Procymidone	13.97	2	QI 283.0 > 96.0 1st CI 283.0 > 67.1	10 40
Prothiofos	15.21	1	QI 162.0 > 63.1 1st CI 267.0 > 239.0	40 5
Quintozene	9.73	1	QI 249.0 > 214.0 1st CI 295.0 > 237.0	20 20
Tetradifon	20.71	1	QI 226.9 > 199.0 1st CI 355.7 > 159.0	10 10
Trifluralin	8.48	1	QI 306.0 > 264.0 1st CI 263.9 > 160.1	10 15
Vinclozolin	11.22	2	QI 212.0 > 172.0 1st CI 212.0 > 109.0	15 40

[1] RT: retention time. [2] DRT: delta retention time. [3] QI: quantification ions. [4] CI: confirmation ions.

2.6. Method Validation

Validation was performed following the European Union SANTE/12682/2019 [34] and Codex Alimentarius CXG90-2017 [35] guidelines. The following analytical performance parameters were assessed: linearity, selectivity, trueness, precision (repeatability and within-lab reproducibility), limit of detection (LOD), and limit of quantification (LOQ). A total of 209 different analytes were tested, 159 of them by LC-MS/MS and 50 by GC-MS/MS.

Matrix-matched calibration (MMC) was used to minimize the matrix effect. For the preparation of analytical MMC curves, blank honey extracts were spiked with appropriate amounts of standard solutions at the six final concentrations. Three independent solutions were prepared for each level of the curve (n = 18), and the samples were injected randomly. The difference between the calculated concentration and the theoretical concentration must be less than or equal to 20% for the curve's best fit. The selectivity was determined by identifying the pesticide in the presence of the matrix and other analytes. If interfering peaks were detected at the same retention time as some pesticides, the interfering agents' areas had to be less than or equal to 30% of the analyte LOQs.

The trueness and precision (repeatability and within-lab reproducibility) were determined from the recovery assay results of blank samples spiked with all of the analytes at two distinct levels (LOQ and 10× LOQ) for GC-MS/MS and three distinct levels (LOQ, 2× LOQ, and 10× LOQ) for LC-MS/MS. Repeatability was evaluated using data from replicate samples (n = 6) analyzed on the same day for each level. The within-lab reproducibility was evaluated using replicate data (n = 12) from two different days and two analysts for each level. Repeatability and within-lab reproducibility are expressed by the relative standard deviation (RSD in %), whereas average recovery values express trueness. The expanded measurement uncertainty (U) was estimated by the top-down approach. All results are reported in Tables 3 and 4. Average recovery ranging from 70% to 120% was considered adequate. Precision deviations of up to 20% were considered acceptable [34].

Table 3. Linearity, recovery (in %), repeatability relative standard deviation (RSD; in %), expanded measurement uncertainty (U; in %), limit of detection (LOD; in mg/kg), and limit of quantification (LOQ; in mg/kg) for each analyte of the LC-MS/MS method for analysis of pesticides in honey.

Compound	Linearity			Average Recovery			RSD			U			LOD	LOQ
	Type of Adjust	Ponderation	LR [1] (µg/kg)	Pt [2] 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	(mg/kg)	(mg/kg)
3-Hydroxycarbofuran	Linear		1–10	110	115	92	6	6	8	6	6	8	0.00010	0.00020
Acephate	Linear	1/x	1–10	109	91	81	4	10	5	8	10	10	0.00010	0.00020
Acetamiprid	Linear	1/x	1–10	101	96	101	10	7	4	20	19	8	0.00010	0.00020
Aldicarb	Linear	1/x	1–10	95	98	86	8	11	12	12	15	12	0.00010	0.00020
Aldicarb-sulfone	Linear		1–10	118	104	90	5	11	6	5	11	6	0.00010	0.00020
Aldicarb-sulfoxide	Linear		1–10	118	111	100	7	11	9	8	14	17	0.00010	0.00020
Allethrin	Linear		1–10	92	80	83	10	18	20	10	18	20	0.00010	0.00020
Ametryn	Linear		1–10	119	106	99	3	4	4	6	4	9	0.00010	0.00020
Aminocarb	Linear	1/x	1–10	97	100	90	13	9	4	14	9	12	0.00010	0.00020
Atrazine	Linear		1–10	107	104	103	4	5	4	14	10	6	0.00010	0.00020
Azaconazole	Linear		1–10	108	107	108	12	10	4	12	11	10	0.00010	0.00020
Azinphos-ethyl	Linear		1–10	104	106	96	8	7	7	8	8	9	0.00010	0.00020
Azinphos-methyl	Linear		1–10	119	98	88	9	3	4	9	14	14	0.00010	0.00020
Azoxystrobin	Linear	1/x	1–10	105	93	99	7	3	4	14	20	16	0.00010	0.00020
Benalaxyl	Linear		1–10	119	109	97	6	7	8	10	7	8	0.00010	0.00020
Bitertanol	Linear		1–10	111	103	94	4	8	5	7	8	5	0.00010	0.00020
Boscalid	Linear		1–10	119	97	88	10	4	6	12	20	14	0.00010	0.00020
Bromacil	Linear	1/x	1–10	100	95	92	6	6	4	16	19	12	0.00010	0.00020
Bromuconazole	Linear		2–20	118	108	101	4	5	4	7	5	4	0.00020	0.00040
Buprofezin	Linear	1/x	1–10	102	103	88	18	17	14	19	17	14	0.00010	0.00020
Cadusafos	Linear		1–10	110	102	92	5	7	11	9	7	11	0.00010	0.00020
Carbaryl	Linear		1–10	116	95	95	10	7	4	10	12	11	0.00010	0.00020
Carbendazim	Linear	1/x	1–10	114	108	114	6	8	3	7	12	12	0.00010	0.00020
Carbofuran	Linear	1/x	1–10	106	99	101	4	2	3	9	18	15	0.00010	0.00020
Chlorfenvinphos	Linear	1/x	1–10	111	110	108	4	6	5	10	6	5	0.00010	0.00020
Chlorpyrifos	Linear		1–10	91	79	74	14	18	16	16	18	16	0.00010	0.00020
Chlorpyrifos-methyl-oxon	Linear		1–10	113	108	100	4	6	9	6	6	9	0.00010	0.00020
Clofentezine	Linear		1–10	117	105	92	6	9	9	9	12	9	0.00010	0.00020
Clomazone	Linear	1/x	1–10	105	88	99	8	5	5	12	19	15	0.00010	0.00020
Clothianidin	Linear		1–10	119	105	100	5	6	6	10	8	6	0.00010	0.00020
Cyanazine	Linear		1–10	118	95	90	9	4	7	9	19	19	0.00010	0.00020

Table 3. *Cont.*

Compound	Linearity			Average Recovery			RSD			U			LOD	LOQ
	Type of Adjust	Ponderation	LR [1] (µg/kg)	Pt [2] 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	(mg/kg)	(mg/kg)
Cyanofenphos	Linear	1/x	1–10	105	102	96	9	15	11	10	15	11	0.00010	0.00020
Cyazofamid	Linear		1–10	105	99	94	7	4	5	19	18	9	0.00010	0.00020
Cyproconazole	Linear	1/x2	2–20	105	110	115	3	4	2	15	7	4	0.00020	0.00040
Cyprodinil	Linear		1–10	100	106	95	7	18	11	13	18	11	0.00020	0.00040
Dicrotophos	Linear	1/x	1–10	104	92	93	5	5	4	13	19	16	0.00010	0.00020
Difenoconazole	Linear		1–10	113	105	100	5	7	8	5	8	9	0.00010	0.00020
Diflubenzuron	Linear		1–10	113	106	101	3	9	10	5	9	10	0.00010	0.00020
Dimethoate	Linear		1–10	100	100	99	8	8	4	10	12	4	0.00010	0.00020
Dimethomorph	Linear		1–10	119	109	102	5	3	5	6	8	5	0.00010	0.00020
Diniconazole	Linear	1/x	1–10	97	101	97	7	8	10	10	12	11	0.00010	0.00020
Disulfoton-sulfone	Linear		1–10	110	109	106	3	4	5	10	8	6	0.00010	0.00020
Diuron	Linear	1/x	1–10	112	104	110	4	10	3	13	11	4	0.00010	0.00020
Emamectin B1a	Linear		1–10	114	118	106	6	7	5	8	8	5	0.00010	0.00020
Emamectin B1b	Linear		1–10	113	112	111	8	9	6	13	10	6	0.00010	0.00020
Epoxiconazole	Linear		1–10	109	110	102	8	5	5	8	6	8	0.00010	0.00020
Ethion	Linear	1/x2	1–10	84	88	87	9	19	20	16	19	20	0.00010	0.00020
Ethoprophos	Linear		1–10	107	101	92	6	7	8	6	7	9	0.00010	0.00020
Etrimfos	Linear	1/x	1–10	106	100	97	7	5	7	8	6	9	0.00010	0.00020
Famoxadone	Linear	1/x	1–10	106	98	88	6	11	12	16	11	12	0.00010	0.00020
Fenbuconazole	Linear	1/x	1–10	98	100	108	5	6	5	20	17	8	0.00010	0.00020
Fenpyroximate	Linear		1–10	113	103	95	6	9	4	6	13	20	0.00010	0.00020
Fenthion	Linear	1/x	1–10	106	94	90	5	10	8	19	12	11	0.00010	0.00020
Fipronil	Linear	1/x2	2–20	106	101	99	5	11	8	6	11	8	0.00020	0.00040
Fluazifop-P-butyl	Linear	1/x	1–10	106	87	77	8	12	16	9	12	16	0.00010	0.00020
Fluquinconazole	Linear		1–10	106	106	110	7	6	6	10	8	6	0.00010	0.00020
Furathiocarb	Linear		1–10	107	96	88	4	6	10	6	6	10	0.00010	0.00020
Heptenophos	Linear		1–10	104	97	94	7	7	8	8	9	12	0.00010	0.00020
Hexaconazole	Linear	1/x	1–10	105	108	100	6	9	6	14	11	9	0.00010	0.00020
Hexythiazox	Linear		1–10	97	87	77	5	16	15	20	16	15	0.00010	0.00020
Imazalil	Linear	1/x	1–10	106	102	103	9	4	6	17	16	8	0.00010	0.00020
Imibenconazole	Linear	1/x	1–10	89.5	85.8	79.9	4.8	9.9	13.6	20.0	12.9	13.6	0.00010	0.00020
Imidacloprid	Linear		1–10	99	91	92	10	10	4	10	18	18	0.00010	0.00020
Indoxacarb	Linear		1–10	105	95	87	5	11	11	6	11	11	0.00010	0.00020

Table 3. *Cont.*

Compound	Linearity			Average Recovery			RSD			U			LOD	LOQ
	Type of Adjust	Ponderation	LR [1] (µg/kg)	Pt [2] 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	(mg/kg)	(mg/kg)
Iprodione	Linear	1/x2	1–10	94	101	95	14	10	15	14	11	16	0.00010	0.00020
Iprovalicarb	Linear		1–10	103	106	105	4	7	4	16	14	7	0.00010	0.00020
Kresoxim-methyl	Linear		1–10	112	104	96	6	6	4	6	9	8	0.00010	0.00020
Linuron	Linear		1–10	117	91	90	18	11	3	18	19	10	0.00010	0.00020
Malaoxon	Linear		1–10	118	117	100	19	10	7	19	11	8	0.00010	0.00020
Malathion	Linear		1–10	102	104	109	4	3	4	20	19	16	0.00010	0.00020
Metalaxyl	Linear	1/x	1–10	108	105	107	2	4	4	14	14	11	0.00010	0.00020
Metconazole	Linear		1–10	111	106	94	4	7	9	5	7	9	0.00010	0.00020
Methidathion	Linear	1/x	1–10	108	100	96	9	4	5	10	12	11	0.00010	0.00020
Methiocarb	Linear		1–10	120	100	91	7	6	3	10	20	19	0.00010	0.00020
Methomyl	Linear	1/x	1–10	92	85	88	10	10	6	19	20	20	0.00010	0.00020
Methoxyfenozide	Linear		1–10	110	108	99	3	6	7	6	6	7	0.00010	0.00020
Metolachlor	Linear	1/x	1–10	105	105	100	5	4	9	20	12	9	0.00010	0.00020
Metribuzin	Linear	1/x	1–10	96	97	98	15	9	5	20	10	12	0.00010	0.00020
Monocrotophos	Linear		1–10	108	90	82	13	15	6	15	16	17	0.00010	0.00020
Myclobutanil	Linear		1–10	110	108	103	5	3	5	11	5	5	0.00010	0.00020
Omethoate	Linear		1–10	95	84	78	7	5	3	8	10	6	0.00010	0.00020
Oxamyl	Linear		1–10	99	101	100	10	10	3	19	17	10	0.00010	0.00020
Paclobutrazol	Linear		1–10	107	98	97	6	3	3	10	9	10	0.00010	0.00020
Paraoxon	Linear		1–10	111	119	117	10	8	5	10	15	15	0.00010	0.00020
Parathion	Linear	1/x	4–40	94	99	93	16	11	14	16	11	14	0.00040	0.00080
Penconazole	Linear		1–10	107	113	103	8	6	4	10	8	6	0.00010	0.00020
Pencycuron	Linear	1/x	1–10	92	86	89	7	12	15	12	12	15	0.00010	0.00020
Pendimethalin	Linear		1–10	98	82	80	12	15	13	14	15	13	0.00010	0.00020
Phenthoate	Linear		1–10	109	101	93	5	10	12	7	10	15	0.00010	0.00020
Phosmet	Linear		1–10	107	104	108	5	4	4	14	16	10	0.00010	0.00020
Phosphamidon	Linear		1–10	118	108	100	4	3	4	7	6	5	0.00010	0.00020
Picoxystrobin	Linear		1–10	110	107	110	6	5	7	15	9	7	0.00010	0.00020
Pirimicarb	Linear	1/x	1–10	115	110	111	3	2	4	9	4	4	0.00010	0.00020
Pirimiphos-ethyl	Linear		1-10	102	90	89	9	8	13	16	8	13	0.00010	0.00020
Pirimiphos-methyl	Linear	1/x	1–10	104	101	95	7	10	11	10	14	13	0.00010	0.00020
Prochloraz	Linear	1/x	1–10	108	106	106	3	7	6	12	10	11	0.00010	0.00020
Profenofos	Linear	1/x2	2–20	103	98	95	5	10	10	10	10	12	0.00020	0.00040

Table 3. *Cont.*

Compound	Linearity			Average Recovery			RSD			U			LOD	LOQ
	Type of Adjust	Ponderation	LR [1] (µg/kg)	Pt [2] 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	Pt 1	Pt 2	Pt 6	(mg/kg)	(mg/kg)
Propargite	Linear		1–10	104	88	73	12	18	19	12	18	19	0.00010	0.00020
Propoxur	Linear	1/x	1–10	108	98	95	10	4	5	15	20	19	0.00010	0.00020
Pyraclostrobin	Linear		1–10	110	99	96	7	10	11	10	12	13	0.00010	0.00020
Pyrazophos	Linear		1–10	111	104	96	4	7	7	6	7	7	0.00010	0.00020
Pyridaphenthion	Linear		1–10	117	111	102	5	6	4	6	6	4	0.00010	0.00020
Pyriproxyfen	Quadratic	1/x2	1–10	98	92	93	18	15	17	20	15	17	0.00010	0.00020
Quinalphos	Linear		1–10	110	105	98	5	7	8	6	7	10	0.00010	0.00020
Quizalofop-P-ethyl	Linear		1–10	96	88	82	8	10	15	8	10	17	0.00010	0.00020
Simazine	Linear	1/x	1–10	104	103	96	5	6	5	15	18	14	0.00010	0.00020
Spinosyn A	Linear		1–10	105	106	101	4	5	6	5	5	6	0.00010	0.00020
Spinosyn D	Linear		1–10	106	101	97	13	17	14	17	17	16	0.00010	0.00020
Spirodiclofen	Linear		1–10	99	84	84	11	19	16	11	19	20	0.00010	0.00020
Spiromesifen	Linear		1–10	86	81	73	10	12	17	10	12	17	0.00010	0.00020
Tebuconazole	Linear		1–10	96	102	100	7	4	8	12	9	8	0.00010	0.00020
Tebufenozide	Linear		1–10	106	100	84	11	10	16	11	10	16	0.00010	0.00020
Teflubenzuron	Linear	1/x	2–20	104	90	81	9	13	18	10	13	18	0.00020	0.00040
Terbufos	Linear	1/x2	2–20	86	74	71	13	12	11	19	13	11	0.00020	0.00040
Tetraconazole	Linear	1/x	1–10	102	105	105	5	6	6	9	11	10	0.00010	0.00020
Thiabendazole	Linear	1/x	1–10	111	114	95	17	18	5	19	18	5	0.00010	0.00020
Thiacloprid	Linear		1–10	117	103	98	6	5	3	6	6	3	0.00010	0.00020
Thiamethoxam	Linear	1/x	1–10	106	94	91	8	7	6	8	9	8	0.00010	0.00020
Thiobencarb	Linear	1/x	1–10	106	94	91	11	7	8	13	9	9	0.00010	0.00020
Thiodicarb	Linear		1–10	111	98	97	10	3	4	10	14	9	0.00010	0.00020
Tolylfluanid	Linear		1–10	100	95	96	8	5	7	12	8	8	0.00010	0.00020
Triadimefon	Linear	1/x	1–10	103	106	104	6	8	5	14	13	11	0.00010	0.00020
Triadimenol	Linear		1–10	116	116	106	4	3	5	6	9	11	0.00010	0.00020
Triazophos	Linear	1/x	1–10	113	104	109	4	5	3	14	13	11	0.00010	0.00020
Trichlorfon	Linear	1/x2	1–10	107	101	97	9	7	5	9	9	9	0.00010	0.00020
Trifloxystrobin	Linear		1–10	105	96	93	5	11	12	9	11	12	0.00010	0.00020
Triflumizole	Linear		1–10	93	94	92	9	7	13	10	7	13	0.00010	0.00020
Vamidothion	Linear	1/x	1–10	105	92	90	6	7	4	12	15	6	0.00010	0.00020
Zoxamide	Linear		1–10	104	92	88	6	9	8	9	10	10	0.00010	0.00020

[1] LR: linearity range. [2] Pt: point.

Table 4. Linearity, recovery (in %), repeatability relative standard deviation (RSD; in %), expanded measurement uncertainty (U; in %), limit of detection (LOD; in mg/kg), and limit of quantification (LO Q; in mg/kg) for each analyte of the GC-MS/MS method for analysis of pesticides in honey.

Compound	Linearity			Average Recovery		RSD		U		LOD	LOQ
	Type of Adjust	Ponderation	FT (μg/kg)	Pt 1	Pt 6	Pt 1	Pt 6	Pt 1	Pt 6	(mg/kg)	(mg/kg)
DDE 4,4	Linear	1/x	10–100	119	94	8	7	20	7	0.001	0.002
Alachlor	Linear		10–100	103	109	7	5	19	12	0.001	0.002
Aldrin	Linear	1/x	20–200	110	99	10	9	18	11	0.002	0.004
Azoxystrobin	Linear		10–100	98	78	9	7	13	8	0.001	0.002
Bifenthrin	Linear		20–200	117	91	3	4	11	4	0.002	0.004
Bromophos-methyl	Linear		20–200	119	100	6	7	15	16	0.002	0.004
Bromopropylate	Linear	1/x	20–200	113	91	6	6	14	7	0.002	0.004
Carbophenothion	Linear	1/x	20–200	115	93	5	5	17	5	0.002	0.004
Cyfluthrin	Linear	1/x	40–400	106	92	5	8	13	8	0.004	0.008
Cypermethrin	Linear		20–200	102	89	7	8	16	8	0.002	0.004
Clordane gama (trans)	Linear	1/x	20–200	112	99	7	5	18	10	0.002	0.004
Chlorfenapyr	Linear	1/x	20–200	103	101	8	5	20	6	0.002	0.004
Chlorfenvinphos	Linear		10–100	120	98	10	4	16	13	0.001	0.002
Chlorpyrifos-methyl	Linear	1/x	10–100	113	101	15	9	20	18	0.001	0.002
Chlorthiophos	Linear	1/x	20–200	118	94	9	6	18	6	0.002	0.004
DDD 2,4	Linear	1/x	10–100	115	95	10	7	14	7	0.001	0.002
DDT 2,4	Linear	1/x	10–100	112	98	5	7	20	8	0.001	0.002
DDT 4,4	Linear	1/x	20–200	109	98	5	5	19	7	0.002	0.004
Deltamethrin	Linear	1/x2	10–100	96	119	13	4	20	10	0.001	0.002
Dieldrin	Linear	1/x	20–200	113	96	10	7	20	8	0.002	0.004
Difenoconazole	Linear	1/x	10–100	99	85	9	7	16	8	0.001	0.002
Endosulfan alpha	Linear	1/x	20–200	116	98	8	5	17	8	0.002	0.004
Endosulfan beta	Linear	1/x	20–200	111	95	5	6	18	7	0.002	0.004
Endosulfan sulfate	Linear	1/x	20–200	108	103	7	5	18	6	0.002	0.004
Endrin	Linear	1/x	20–200	111	98	10	5	19	6	0.002	0.004
Esfenvalerate	Linear	1/x	20–200	100	105	8	6	19	9	0.002	0.004
Fenpropathrin	Linear	1/x	20–200	109	92	5	5	16	5	0.002	0.004
Fenarimol	Linear	1/x	20–200	105	85	6	7	12	10	0.002	0.004
Fipronil	Linear	1/x	20–200	109	104	11	4	19	15	0.002	0.004
Fluquinconazole	Linear	1/x	10–100	108	90	7	5	13	6	0.001	0.002
Phosalone	Linear	1/x	20–200	102	92	8	7	18	7	0.002	0.004
HCH alpha	Linear	1/x	20–200	107	84	8	11	13	11	0.002	0.004

Table 4. *Cont.*

Compound	Linearity			Average Recovery		RSD		U		LOD	LOQ
	Type of Adjust	Ponderation	FT (μg/kg)	Pt 1	Pt 6	Pt 1	Pt 6	Pt 1	Pt 6	(mg/kg)	(mg/kg)
Heptachlor	Linear	1/x	20–200	108	90	12	11	18	16	0.002	0.004
Hexachlorobenzene (HCB)	Linear	1/x	10–100	98	80	12	9	13	13	0.001	0.002
Iprodione	Linear	1/x	20–200	108	89	13	8	15	8	0.002	0.004
Lambda cyhalothrin	Linear	1/x	20–200	110	101	7	6	16	6	0.002	0.004
Methoxychlor	Linear	1/x	20–200	108	97	5	6	19	6	0.002	0.004
Mirex	Linear	1/x	10–100	113	108	5	9	18	18	0.001	0.002
Chlorfenson	Linear	1/x	20–200	109	93	9	6	17	8	0.002	0.004
Oxyfluorfen	Linear	1/x2	20–200	108	113	5	6	19	15	0.002	0.004
Pendimethalin	Linear	1/x	10–100	99	104	6	6	18	18	0.001	0.002
Permethrin	Linear	1/x	20–200	99	86	11	5	18	6	0.002	0.004
Pirimicarb	Linear	1/x	10–100	115	104	13	6	14	16	0.001	0.002
Pirimiphos-ethyl	Linear	1/x	10–100	102	105	7	5	18	16	0.001	0.002
Procymidone	Linear	1/x	20–200	103	96	6	7	10	10	0.002	0.004
Profenofos	Linear	1/x	20–200	112	102	4	5	14	13	0.002	0.004
Prothiofos	Linear	1/x	20–200	113	97	8	5	15	8	0.002	0.004
Quintozene	Linear	1/x	10–100	106	87	9	14	18	14	0.001	0.002
Tetradifon	Linear	1/x	40–400	107	90	4	7	15	8	0.004	0.008
Trifluralin	Linear	1/x	20–200	112	94	9	11	15	14	0.002	0.004
Vinclozolin	Linear	1/x	20–200	111	101	6	7	8	14	0.002	0.004

The LOQ was determined as the lowest concentration level of the calibration curve with acceptable accuracy. The LOD corresponded to 50% of the estimated value for the quantification limit, provided that the recoveries presented an area greater than or equal to 50% of the point in the matrix solution injected and that the signal/noise ratio was higher than or equal to 3.

3. Results and Discussion

3.1. Extraction Method

The extraction procedure is a crucial step for detecting pesticides, and it can be challenging for a complicated matrix such as honey. Extraction procedures that have been developed for honey samples include solvent extraction, supercritical fluid extraction, solid-phase extraction, matrix solid-phase dispersion, solid-phase microextraction, stir bar sorptive extraction [36], purge and trap, dispersive liquid–liquid microextraction, microextraction by packed sorbent, single-drop microextraction, magnetic solid-phase extraction [37], and solvent floatation [38]. In the present method, the QuEChERS method was optimized for the extraction and cleanup of honey samples from the original method [22] with modifications for honey [28,33] and bee pollen samples [32]. The original QuEChERS method consists of an extraction step with acetonitrile and separation using extraction salts, followed by a cleanup step with purification salts [22].

Different extraction and cleanup conditions were evaluated for this method. Honey samples were diluted in water prior to extraction. Acetonitrile:ethyl acetate (70:30, *v/v*) solution provided better extraction efficiency, similar to Souza Tette et al. [28]. On the other hand, Mitchell et al. [33] used acetonitrile:water (50:50, *v/v*) solution without the sample's previous dilution. In the present study, the extracted solution was subjected to freeze-out before the dispersive solid phase extraction (d-SPE) cleanup, following the method developed for bee pollen by Vázquez et al. [32]. The extraction recoveries for most pesticides were improved by keeping the extract in the freezer at −40 °C for at least 2 h (Supplementary Materials Table S1). Furthermore, extracted solutions that were subjected to freeze-out were visually more translucid than solutions that were not subjected to freeze-out.

The cleanup procedure of the present study was performed with magnesium sulfate and PSA. The same purification salts were also used by Mitchell et al. [33], but at different amounts (150 mg magnesium sulfate and 100 mg PSA); in contrast, Souza Tette et al. [28] also included Florisil (50 mg) to magnesium sulfate (150 mg) and PSA (50 mg). The extract was concentrated ten times after cleanup to achieve lower LOD and LOQ values, similarly to an earlier study [33]. The effectiveness of the modifications to the QuEChERS method in the present study was confirmed by the wide range of pesticides successfully detected and the high sensitivity evidenced by the low LOD and LOQ values.

3.2. Validation Assay

The proposed method was validated to detect 168 compounds, 127 of them by LC-MS/MS and 41 by GC-MS/MS. The matrix effect was minimized by using MMC. The method's selectivity was determined by identifying the pesticide in the presence of the matrix and other analytes. All validated compounds showed average recoveries ranging from 70% to 120%. The mean repeatability relative standard deviation (RSD) for all samples in the LC-MS/MS method was 7.75%, ranging from 2% to 20%, and in the GC-MS/MS method the RSD was 7.24%, ranging from 3% to 15%. The expanded measurement uncertainty (U) for all samples in the LC-MS/MS method was 11.4%, ranging from 3% to 20%, and in the GC-MS/MS method was 13.1%, ranging from 4% to 20%. Average recoveries ranging from 70% to 120% and precision RSD of up to 20% were considered adequate [34]. The estimation of the uncertainty of an analytical method can be performed in different ways, including empirical, practical, or top-down approaches [39]. In the present study, the uncertainty was estimated using the top-down approach. In this way, the experimental design to estimate the RSD under conditions of partial reproducibility varied the day and the analysts to reproduce the variations.

Tables 3 and 4 show the linearity, recovery, RSD, expanded measurement uncertainty (U), LOD, and LOQ results for analytes determined using LC-MS/MS and GC-MS/MS, respectively. The LOD and LOQ values for 119 analytes determined by LC-MS/MS were 0.0001 mg/kg and 0.0002 mg/kg, respectively, whereas seven analytes showed LOD and LOQ values of 0.0002 mg/kg and 0.0004 mg/kg, and the values for one analyte were 0.0004 mg/kg and 0.0008 mg/kg. For GC-MS/MS analyses, the LOD and LOQ values were 0.001 mg/kg and 0.002 mg/kg for nine analytes, 0.002 mg/kg and 0.004 mg/kg for 30 analytes, and 0.004.0 mg/kg and 0.008 mg/kg for two analytes.

A total of 41 analytes could not be validated, 32 of which were analyzed by LC-MS/MS and 9 by GC-MS/MS (Supplementary Materials Tables S2 and S3). These compounds were detected, but the obtained values for linearity, recovery rate, RSD, and U were not following the European Union SANTE/12682/2019 [34] and Codex Alimentarius CXG90-2017 [35] guidelines.

Pacífico da Silva et al. [1] developed an analytical method with an LC-MS/MS system for the simultaneous detection of 152 pesticides in honey after extraction with ethyl acetate and cleanup using Florisil. The LOD and LOQ values for all the tested pesticides were 0.005 and 0.01 mg/kg, respectively [1]. Paoloni et al. [40] used Florisil for sample cleanup after extraction with n-Hexane for determining 13 pesticides in honey using GC-MS/MS. The LOQ for all tested pesticides was 0.01 mg/kg, and the LOD was not provided [40]. Česnik et al. [31] used a GC-MS method for detecting 75 pesticides and an LC-MS/MS method for detecting 60 pesticides in honey after extraction with a mixture of petroleum ether and dichloromethane. The LOQ ranged from 0.01 to 0.05 mg/kg with the GC-MS method and from 0.003 to 0.01 mg/kg with the LC-MS/MS method [31].

The QuEChERS method was applied for pesticide extraction in honey by other authors [26–30,41,42]. The LC-MS/MS method described by Souza Tette et al. [28] was validated to measure 116 pesticides in honey, but 11 compounds showed recoveries at 0.010 mg/kg out of the 70–120% range. The LOD was 0.005 mg/kg and the LOQ varied between 0.01 and 0.025 mg/kg [28]. The LC-ESI-MS/MS method of Kasiotis et al. [26] detected 115 pesticides, but some analytes showed recoveries below 70%. The LOD ranged from 0.00003 to 0.0233 mg/kg, and the LOQ ranged from 0.0001 to 0.078 mg/kg [26]. Another LC-MS/MS method for analyzing honey samples was described for 207 pesticides [30], with LOQ values ranging from 0.001 to 0.01 mg/kg. However, the LOD was not reported, and some pesticides showed recoveries out of the 70–120% range [30]. In another LC-MS/MS method [29], 132 tested compounds were measured in honey, obtaining recoveries ranging from 70% to 120% for 116 compounds. However, the LOD and LOQ were not provided in the manuscript nor supplementary material [29]. The GC-MS/MS method described by Zheng et al. [41] was validated to measure six pesticides in honey. The LOD ranged from 0.0004 to 0.002 mg/kg and the LOQ varied between 0.001 and 0.005 mg/kg [41]. Another GC-MS/MS method was developed by Shendy et al. [27] for the detection of 200 pesticides in honey. The LOD ranged from 0.001 to 0.003 mg/kg and the LOQ was 0.005 to 0.01 mg/kg, but the recoveries ranged from 51.13–126.55% [27]. Both LC-MS/MS and GC-MS/MS analysis of residual pesticides in honey was described by Bargańska et al. [42]. This method was validated for 51 compounds, 18 of them determined by LC-MS/MS, 21 compounds by GC-MS/MS, and 12 compounds by both methods. The LOD ranged from 0.0028 to 0.09 mg/kg with the LC method and from 0.0023 to 0.027 mg/kg with the GC method [42]. Compared with these above articles, the method described in the present study was able to detect extensive and broad-spectrum pesticides (168) with very high sensitivity.

3.3. Real Samples

Of the 33 honey samples analyzed, 31 (93.9%) showed residual levels of pesticides (Table 5). Each sample contained up to 15 detected analytes. The most frequently detected compounds were carbendazim (20 samples), thiabendazole (20 samples), azoxystrobin (15 samples), chlorpyrifos (12 samples), and imidacloprid (12 samples). Carbendazim is a fungicide that is widely used in agriculture. Its toxic effects include liver damage, disruption of endocrine and hematological functions, and reproductive toxicity [43]. Thiabendazole is a fungicide and anthelmintic compound

with hepatotoxic and teratogenic effects, and it is probably a carcinogen [44]. Azoxystrobin is also a fungicide, and its toxicity includes lesions in the liver and kidneys [45]. Chlorpyrifos is an organophosphate pesticide that is used as an insecticide and acaricide. It is considered moderately toxic and can cause disruption of neuronal, reproductive, immune, and endocrine systems, cancer, and chromosome damage [46]. Imidacloprid is a neonicotinoid insecticide that is highly toxic to honeybees [1,2], with neurotoxic, immunotoxic, teratogenic, and mutagenic effects in mammals [47]. The presence of pesticides in a considerable percentage of the analyzed samples is indicative of widespread environmental contamination by these compounds. However, the consumption of the analyzed honey may not be considered unsafe because the residual levels of all detected pesticides were below the MRLs established for Brazil [9] and the European Union [6–8].

Table 5. Detected pesticides (in mg/kg) in 33 samples of honey using the developed LC-MS/MS and GC-MS/MS method.

Compound	Positive Samples	Maximum Levels	LOD [1]	LOQ [2]	MRL [3]
Acephate	8	0.00779	0.0001	0.0002	0.020
Acetamiprid	1	<LQ	0.0001	0.0002	0.050
Azoxystrobin	15	0.00019	0.0001	0.0002	0.050
Bifenthrin	3	<LQ	0.002	0.004	0.010
Boscalid	1	<LQ	0.0001	0.0002	0.050
Carbaryl	2	0.00050	0.0001	0.0002	0.050
Carbendazim	20	0.00350	0.0001	0.0002	1.0
Clomazone	5	<LQ	0.0001	0.0002	-
Chlorpyrifos	12	0.00034	0.0001	0.0002	0.010
Clothianidin	2	0.00063	0.0001	0.0002	-
Diflubenzuron	3	0.00026	0.0001	0.0002	0.050
Dimethoate	6	0.00194	0.0001	0.0002	0.010
Diuron	5	<LQ	0.0001	0.0002	0.050
Imidacloprid	12	0.00618	0.0001	0.0002	0.050
Metoxyphenazide	1	<LQ	0.0001	0.0002	0.050
Omethoate	2	<LQ	0.0001	0.0002	0.010
Pyraclostrobin	2	<LQ	0.0001	0.0002	0.050
Pyrimethanil	3	0.00040	0.0001	0.0002	-
Pyriproxyfen	3	<LQ	0.0001	0.0002	0.050
Tebuconazole	10	0.00045	0.0001	0.0002	0.050
Thiabendazole	20	0.00130	0.0001	0.0002	0.010
Thiamethoxam	9	0.00209	0.0001	0.0002	0.050
Triazophos	1	<LQ	0.0001	0.0002	0.010
Trifloxystrobin	5	0.00030	0.0001	0.0002	0.050

[1] LOD: limit of detection (in mg/kg). [2] LOQ: limit of quantification (in mg/kg). [3] MRL: maximum residue level (in mg/kg) [9].

Few studies have been aimed at determining the presence of residual pesticides in honey in Brazil. Organophosphorus trichlorfon was detected in just one sample from one hundred commercial honey samples from five states of Brazil [28]. A total of 19 pesticides were found in 53 honey samples collected directly from colonies in the Rio Grande do Norte state, northeastern Brazil. Thirteen of these pesticides were detected in honey produced by honeybees pollinating melon crops (23 samples); however, only six were found in honey from honeybees foraging in the forest (20 samples), and four in honey produced by the stingless bee *Melipona subnitida* (10 samples) [1]. In another study, honey produced by *M. subnitida* from the Rio Grande do Norte state was tested for residual pesticides. Of the 35 analyzed samples, 25 showed residual pesticides, and the detected compounds were chlorpyrifos-methyl, monocrotophos, and trichlorfon [3]. These data support the requirement for testing honey for the presence of pesticides to avoid commercialization of batches containing residual levels above the MRLs.

4. Conclusions

The proposed method was successfully optimized and validated for multi-residue identification and quantification of pesticides in honey. It was able to detect an extensive and broad range of pesticides with remarkably high sensitivity and precision. The developed method was successfully applied to Brazilian commercial honey, showing the analyzed honey was considered safe for consumption.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/10/1368/s1, Table S1. Modified QuEChERS method optimization. Extraction with acetonitrile, or a solution of acetonitrile and ethyl acetate (70:30, v/v), and inclusion of a freezing out step prior to clean up (900 mg of anhydrous magnesium sulfate and 150 mg of PSA). Results are presented as recovery (in %) for each analyte of the LC-MS/MS; Table S2. Non-approved analytes. Linearity, recovery (in %), repeatability relative standard deviation (RSD; in %), expanded measurement uncertainty (U; in %), limit of detection (LOD; in mg/kg), and limit of quantification (LOQ; in mg/kg) for each analyte of the LC-MS/MS method for analysis of pesticides in honey; Table S3: Non-approved analytes. Linearity, recovery (in %), repeatability relative standard deviation (RSD; in %), expanded measurement uncertainty (U; in %), limit of detection (LOD; in mg/kg), and limit of quantification (LOQ; in mg/kg) for each analyte of the GC-MS/MS method for analysis of pesticides in honey.

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Article

Multifamily Determination of Phytohormones and Acidic Herbicides in Fruits and Vegetables by Liquid Chromatography–Tandem Mass Spectrometry under Accredited Conditions

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Abstract: A 7-min multifamily residue method for the simultaneous quantification and confirmation of 8 phytohormones and 27 acidic herbicides in fruit and vegetables using ultra high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) was developed, validated according to SANTE 12682/2019, and accredited according to UNE-EN-ISO/IEC 17025:2017. Due to the special characteristics of these kinds of compounds, a previous step of alkaline hydrolysis was carried out for breaking conjugates that were potentially formed due to the interactions of the analytes with other components present in the matrix. Sample treatment was based on QuEChERS extraction and optimum detection conditions were individually optimized for each analyte. Cucumber (for high water content commodities) and orange (for high acid and high water content samples) were selected as representative matrices. Matrix-matched calibration was used, and all the validation criteria established in the SANTE guidelines were satisfied. Uncertainty estimation for each target compound was included in the validation process. The proposed method was applied to the analysis of more than 450 samples of cucumber, orange, tomato, watermelon, and zucchini during one year. Several compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 4-(3-indolyl)butyric acid (IBA), dichlorprop (2,4-DP), 2-methyl-4-chlorophenoxy acetic acid (MCPA), and triclopyr were found, but always at concentrations lower than the maximum residue level (MRL) regulated by the EU.

Keywords: phytohormones; acidic herbicides; fruits and vegetables; multifamily method; UHPLC–MS/MS

1. Introduction

Phytohormones play essential roles in the regulation of physiological processes in plants and vegetables, most of them involved in the plants' growth, development, defense, and response to environmental stimuli [1,2]. In consequence, plant hormones have an influence on plant development and crop yield, directly or indirectly. Therefore, research into the hormone physiology of plants has become an important target for agriculture development [3,4]. Plant hormones can be considered one of the cornerstones of molecular breeding and a key to opening the door of modern agriculture. They may be generated by plants in a natural way. However, in the last few years, the artificial synthesis of plant hormones and compounds with similar functions (plant growth regulators, PGRs) in vegetables

has increased [5] because these compounds are capable of increasing harvest grain productivity and improving agricultural production. However, special care and control must be taken over them since it has been shown that, in some cases, they have carcinogenic or teratogenic effects on animals and humans. For these reasons, the control and analysis of these compounds have a special interest in food safety [6]. Like other chemicals used in farming, their usage is restricted and must be carried out responsibly, assuring that the limits established by the mandatory regulations are not exceeded [7,8].

On the other hand, pesticides are used worldwide to control pests and diseases in agriculture, allowing an improvement in product quality and crop productivity. The presence of pesticide residues in food has become common and necessary to maintain the supply needs of the current population. In consequence, the control of these substances acquires relevant importance, even more so when adverse health effects that this type of residue can have in the long term have been demonstrated. In the case of acidic herbicides, their acid properties make them possess special characteristics of reactivity, increasing their possibility of interacting with other natural components present in plants and being transformed into conjugated residues by biological reactions. Conjugate formation is conditioned by a large number of factors, such as the specific stage during crop development in which these compounds are applied, if applied as free acids, salts, or esters, and the climatic conditions. For these reasons, the European Union (EU) has included them in residue definitions and, therefore, it regulates them for maximum residue levels (MRLs), not only for the active ingredients but also for other possible structures that can be formed due to chemical interactions with natural compounds or secondary reactions. Hence, it is important to control these analytes and their related compounds because, on some occasions, they can be more harmful than the parent compound [9]. The control of their residues in fruits and vegetables minimizes the health risks associated with their consumption or the environmental damage that may result.

It is easy to find monofamily methods for the determination of phytohormones (auxins [10,11], cytokinins [12,13], gibberellins [14,15]) or methods specifically developed for the analysis of acidic herbicides [16–18] in the literature. Some scientific publications have even jointly analyzed various families of phytohormones [19–24]. It is rare to find studies in which phytohormones and acidic herbicides are grouped together under one method [25,26] The issue of the residue determination of analytes that belong to different functional families is the broad range of physical–chemical properties and structures that these compounds present. Often, specific characteristics of the target analytes cause the extraction procedure to be modified to make it adequate for those particular properties. In this particular case of study, a previous alkaline treatment is necessary due to the trend of conjugate formation as a consequence of the interaction of the target compounds with matrix components. The goal of the alkaline hydrolysis is to convert the conjugates into the parent compound by breaking residues through hydrolysis of the sample [27]. Many of the existing methods hardly manage to gather each and every one of the principles on which “standard” QuEChERS methodology is based [24,28]. Most of the methods found require a high volume of solvents [29] or complex sample treatments such as derivatization [30] or solid-phase extraction (SPE) [31]. The present work achieves the processing of a large number of samples in a short time (quick), to be carried out in a simple way (easy), without using large quantities of solvents and reagents (cheap), completely and quantitatively extracting the amount of target analytes present in the samples (effective), being able to withstand procedural variations (rugged), and being reliable (safe).

The high separation efficiency of chromatographic systems and its ability to be combined with different detectors make it the most useful technique for the analysis of phytohormones and acidic herbicides [30,31]. The analysis of these compounds is carried out almost exclusively by liquid chromatography (LC), although some references proposing the use of gas chromatography (GC) [32] can also be found. LC coupled to mass spectrometry (MS) provides a powerful tool to analyze both phytohormones [4,22] and acidic herbicides [33] in food matrices. Furthermore, LC can be coupled to other detection systems due to the presence of chromophores of the analytes under UV–vis conditions,

such as diode arrays (DADs) ultraviolet (UV) [34,35] and fluorescence detectors (FLDs), [36], but these have less applicability in this field due to their technical limitations.

The developed analysis method allows the simultaneous determination of phytohormones and acidic herbicides in the same "run" and in only 7 min. Cucumber and orange have been taken as representative commodities of fruits and vegetables containing high water content and high acid and high water content, respectively, because they are the groups of matrices more commonly cultivated in southeastern Spain. In addition, with the aim of increasing the applicability of the method, matrices such as tomato, watermelon, and zucchini were also checked, obtaining results that meet those validation criteria established by the European SANTE guidelines [37]. The method was developed in order to reach quantification limits that were sufficiently low enough to allow the determination of concentrations of such compounds at trace levels in order to evaluate the compliance with current food safety regulations. The validation of the method has been carried out, including the uncertainty of the method [37,38], an important parameter when a result is utilized to decide whether it indicates compliance or noncompliance with a specification of regulatory limits. In most publications, it is a parameter that is not estimated.

The aim of the present study is the development, optimization, and validation of a method for the simultaneous determination of 27 acidic herbicides and 8 phytohormones in cucumber and orange matrices at trace levels, applying a modified QuEChERS extraction method and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) determination. The proposed method has demonstrated proper reliability and robustness in order to fulfill accreditation criteria under UNE-EN-ISO/IEC 17025:2017 and its application to routine conditions in a laboratory for food safety monitoring.

2. Materials and Methods

2.1. Reagents and Chemicals

A commercial phytohormone standard of gibberellic acid (GA) was purchased from Riedel de Haën (Seelze-Hannover, Germany). Indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthylacetic acid (NAA) and naphthylacetamide (NA) were supplied by Fluka (Sleeze-Hannover, Germany), while 4-chlorophenoxy acetic acid (4-CPA), 4-chloro-2-methyl-phenoxy acetic acid (MCPA), 4-chloro-2-methyl-phenoxy butyric acid (MCPB), and N^6-benzyladenine (BA) were obtained from Sigma-Aldrich (Steinheim, Germany).

Acidic herbicide reference standards were purchased from Dr. Enhrenstofer (Augsburg, Germany), Riedel-de-Haën (Sigma-Aldrich), Fluka, Chem Service (West Chester, PA, USA), and HPC Standards GmbH (Borsdorf, Germany). Triphenyl-phosphate (TPP), used as an internal standard, was purchased from Supelco (Bellefonte, PA, USA).

Individual analyte stock standard solutions of each compound were prepared in the range of 200 to 300 mg/L of concentrations, considering standard purity, by accurately weighing powder or liquid of individual analytical standards into 50 mL volumetric flasks and dissolving them with acetone (LC–MS gradient grade solvent from Sigma-Aldrich), except for fenoprop [2,4,5-TP] in acetonitrile (HPLC grade, Sigma Aldrich). For that, an analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) and a vortex mixer Heidolph (Kelheim, Germany) Model Reax 2000 were used. These solutions were stored at −20 °C in the dark. All the stock solutions were not stored for more than 6 months in order to avoid stability problems. From these solutions, various working standard solutions at a concentration of 10 mg/L of each compound were prepared weekly by appropriate dilution with acetonitrile and stored in screw-capped glass tubes at −20 °C in the dark.

Methanol (LC–MS gradient grade), sodium citrate dihydrate, sodium citrate dibasic sesquihydrate, and sodium hydroxide were supplied by Sigma-Aldrich. Anhydrous sodium chloride (99.5%) and magnesium sulfate (97%) were purchased from Panreac (Barcelona, Spain). Methanol and highly purified water (Millipore, Bedford, MA, USA) were used for sample preparation and mobile phases.

Formic acid (>99.0% Optima, LC–MS grade), acetic acid (purity higher than 99.8% for HPLC) and sulfuric acid (96% solution in water, extra pure) were obtained from Fluka.

2.2. Sample Preparation

Samples were obtained from supermarkets in Almería (southeastern Spain). Sampling was made in accordance with Directive 2002/63/EC. A representative portion of the sample was homogenized using a Sammic SK-3 kitchen blender and a Reax 2 rotary agitator from Heildoph (Schwabach, Alemania), processed, and the analysis was completed on the day of sample reception. If samples were not analyzed immediately, they were stored for the shortest possible time in a freezer (−20 °C) until analysis. Organic samples of cucumber and orange were purchased from specialized stores in Almería (Spain). These samples, showing the absence of the target analytes, were used as blanks for the preparation of calibration standards and for recovery and precision studies during the method validation.

2.3. Extraction Procedure and Sample Analysis

Briefly, 10 ± 0.1 g of homogenized fruit or vegetable sample was weighed into a 50-mL polypropylene tube, and 5 mL of Milli-Q water was added in high water content samples (or 10 mL in high acid content and high water content samples). The mixture was shaken and homogenized for 2 min. Then, 10 mL of acetonitrile acidified with 1% formic acid and containing TPP at 0.05 mg/L as a surrogated internal standard was added. Afterwards, alkaline hydrolysis was performed, adding 300 μL of sodium hydroxide 5 N for 30 min and heating at 80 °C in a thermostated water bath. The mixture was shaken in a vortex for 2 min and neutralized with 300 μL of sulphuric acid 5 N. Then, 4 g of anhydrous $MgSO_4$, 1 g of NaCl, 1 g of dehydrated sodium citrate, and 0.5 g of sodium citrate dibasic sesquihydrate were added. The mixture was shaken vigorously for 2 min and centrifuged (centrifuge from Orto Alresa, Mod. Cónsul, Madrid, Spain) at 3060 × *g* for 10 min. Finally, 750 μL of the supernatant was transferred to a chromatographic vial and diluted with 750 μL of Milli-Q water. If necessary, the extract was filtered through a 0.20-μm nylon filter for the removal of potential solid interferents before injection into the UHPLC system.

On the other hand, internal quality control was used in each analysis sequence in order to ensure the method's suitability and the truthfulness of the results obtained. For this purpose, the following samples were analyzed in each batch: (i) a reagent blank to check that there were no reagent interferences, and a blank matrix to test if the matrix was free of target compounds, (ii) a matrix-matched calibration, (iii) a spiked blank sample at the LOQ (10 μg/Kg) to evaluate if recoveries were between 70% and 120%, and (iv) a control sample at 5x LOQ concentration (50 μg/Kg), injected every 25 samples, in order to check the precision of the method and to verify that the instrument was stable throughout the sequence. The maximum admissible error for the control sample was ±20% of spiked concentration.

2.4. UHPLC–MS/MS

Instrumental determination was done using an Agilent 1290 Infinity UPLC system (Santa Clara, CA, USA) coupled with an AB Sciex Triple QuadTM 5500 mass spectrometer (Foster City, CA, USA). The chromatographic separation was performed using an Acquity UPLCTM BEH C-18 column (100 × 2.1 mm id, 1.7 μm particle size) from Waters (Mildford, MS, USA). Analyst software version 1.6 (AB Sciex, USA) was used for data acquisition and processing.

A total of 10 μL of the sample was injected at a flow rate of 0.35 mL/min. The temperature of the chromatographic column was set at 30 °C. Chromatographic analyses were carried out using 1% acetic acid and 5% methanol in water (eluent A) and 1% acetic acid in methanol (eluent B) as mobile phases. The gradient elution started with 10% eluent B, which was linearly increased up to 90% in 4.0 min. This composition was held for further 0.5 min before returning to the initial conditions in 0.5 min, followed by a re-equilibration time of 2 min, to give a total run time of 7 min.

The mass analyses were performed with an ESI source using scheduled multiple reaction monitoring (MRM), with rapid switching between positive (ESI^+) and negative (ESI^-) modes and with

N_2 as the nebulizer. The switching time was 50 ms. The MRM detection window was 40 s, with a 5.0 ms pause. The target scan time was 0.25 s, and the mass spectrometric resolution was 1 Da.

The parameters of the MS source employed were electrospray ionization voltage (IS) ± 4500 V (depending on the ESI+ or ESI− mode), source temperature 500 °C, air curtain gas pressure (CUR) 40 psi, ion source gas 1 (GS1) and 2 (GS2) 55 psi, and collision gas pressure (CAD) 7 psi. Curtain gas was nitrogen (>95% purity), and the gas used to fragment the precursor ions (collision gas) was argon (99.9999%).

2.5. Validation

The developed method was validated according to the European SANTE guidelines (SANTE 12682/2019). The calculated validation parameters were retention time window (RTW), specificity, linearity, trueness, precision, limit of quantification (LOQ), and uncertainty.

1. RTW: Defined as the average retention time ± six standard deviations of the retention time (RT ± 6SD), with a tolerance of ±0.1 min [37]). RTW values were calculated by analyzing 10 blank samples spiked at 50 µg/kg.
2. Specificity: Responses for reagent blanks and blank control samples had to be less than 30% LOQ.
3. Linearity and working range: Linearity was studied in the range of 10 to 150 µg/Kg using matrix-matched standard calibration to overcome the matrix effect. The determination coefficient (r2) must be higher than 0.98 for all the studied compounds, and deviation of the residuals of each calibration point must be in the range of ±20%.
4. Trueness: Expressed as the mean recovery in %, it was evaluated by spiking blank samples (n = 10) at two different spiking levels (10 and 50 µg/Kg); values must be in the range 70–120%. Recovery of TPP was also checked, and values must be in the range 70–130%.
5. Precision study: Repeatability (intraday precision) and intermediate precision (interday precision) data were calculated at the same concentrations tested for trueness (10 and 50 µg/Kg). Intraday precision data were obtained from the analysis of spiked blank samples (n = 10) on the same day and by the same analyst, while interday precision values were obtained over ten different days by three different analysts. In both cases, the obtained values must be lower than or equal to 20%, expressed as relative standard deviation (RSD).
6. Limit of Quantification: LOQ was established as the lowest spike level meeting recoveries in the range 70–120% and precision values lower or equal to 20%.
7. Expanded uncertainty: Expanded uncertainty (U) was estimated based on intralaboratory validation data for individual analytes contained in the target matrices (cucumber and orange) at two concentration levels (10 and 50 µg/Kg, respectively); n = 10. In order to simplify the uncertainty estimation (u′), $u'_{Precision}$ and u'_{bias} were considered as main contributor variabilities. They included the uncertainty associated with the precision method and the uncertainty associated with the preparation of standards and the trueness of the method, respectively. Calculations were based on Equation (1).

$$u' = \sqrt{{u'_{Precision}}^2 + {u'_{bias}}^2} \quad (1)$$

A coverage factor of 2 (k = 2, level of confidence = 99.54%) was applied to calculate the expanded uncertainty (U = ku′). U-values must be ≤50% for the LOQ concentration and ≤40% for concentrations higher than or equal to 50 µg/kg.

3. Results and Discussion

3.1. Optimization of UHPLC–MS/MS

The first step considered in the method development was the optimization of the mass spectrometer parameters. Standard solutions of each individual analyte were infused using negative and positive ionization modes (ESI$^-$ and ESI$^+$, respectively). The possibility of using the two ionization modes during the execution of the method resulted in an advantage due to the differences between the families of the analyzed compounds (phenoxy acid, benzoic acid, imidazolinones), obtaining better sensitivity using the optimum ionization mode for each analyte. During this optimization, the goal was to find a compromise between the highest abundance precursor/product ion combinations and the *m*/*z* ratio in order to obtain high sensitivity but, at the same time, high selectivity. The rest of the experimental parameters of the ionization source were set up to obtain adequate ionization of the compounds and the proper volatilization of the mobile phase. The mass analyzer parameters were adjusted in order to achieve the optimal ion transitions that allow the detection of the compounds at low concentrations but, at the same time, avoid potential interferences of the studied matrices. The mass spectrometric parameters for each compound are listed in Table 1.

For the chromatographic separation of the compounds, methanol was present in both proposed phases. Due to the characteristics of the target analytes, the addition of methanol was necessary to achieve an adequate chromatographic profile. In addition, acetic acid was added in both phases because low pH (apparent values ≤5.5) values prevent the dissociation of the acid compounds and the ionization of residual silanol groups in the stationary phase, avoiding peak tailing and slightly improving the resolution between chromatographic peaks. The total chromatographic resolution between the compounds was not reached (Figure 1), although the observed coelutions were solved through the spectral resolution provided by the mass analyzer.

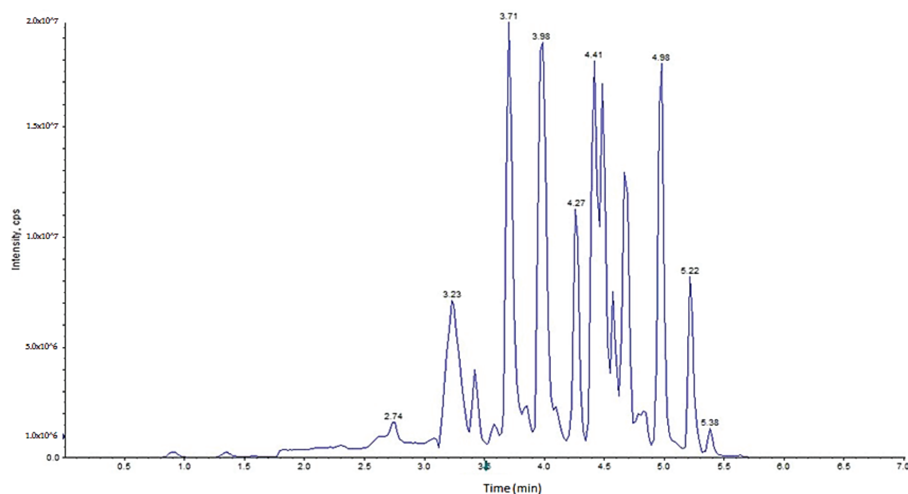

Figure 1. Total ion chromatogram (TIC) of a spiked cucumber sample at 150 µg/kg.

In these conditions, the total run time to determine the target analytes was 7 min. This analysis time is lower than the published methods for a lower or similar number of these compounds [27,30]. Specifically, in [27], 14 analytes were determined in 8 min, while in [30], the separation of 28 analytes needed 22 min.

Table 1. Shows the specific detection conditions for each analyte.

	Precursor Ion (m/z)	Product Ion (m/z)		Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)
Naphthylacetamide	186.1	141.2	a	3.5	1	10	29
	186.1	115.2	b	3.5	1	10	10
2,4,5-trichlorophenoxyacetic acid [2,4,5-T]	253.2	158.6	a	4.6	−55	−10	−46
	253.2	194.4	b	4.6	−55	−10	−13
2,4-Dichlorophenoxyacetic acid [2,4-D]	218.9	161.0	a	4.3	−65	−10	−20
	220.9	163.0	b	4.3	−65	−10	−14
2,4-dichlorophenoxy butyric acid [2,4-DB]	247.0	160.9	a	4.7	−35	−10	−14
	247.0	124.8	b	4.7	−35	−10	−9
2-(4-Chlorophenoxy)acetic acid [4-CPA]	185.0	127.0	a	3.8	−55	−10	−20
	187.0	128.8	b	3.8	−100	−10	−7
4-(3-indolyl)butyric acid [IBA]	203.0	186.0	a	3.8	41	10	19
	203.0	130.0	b	3.8	41	10	12
Gibberellic acid	345.1	239.0	a	2.8	−60	−10	−20
	345.1	227.2	b	2.8	−150	−10	−10
Bentazon	239.0	132.0	a	3.8	−100	−10	−38
	239.0	175.0	b	3.8	−100	−10	−4
2-naphthyloxyacetic acid (BNOA)	201.0	142.9	a	4.1	−100	−10	−40
	201.0	115.0	b	4.1	−100	−10	−17
Bromoxynil	275.7	80.9	a	4.1	−40	−10	−30
	275.7	78.9	b	4.1	−40	−10	−9
Clomazone	240.0	125.0	a	4.3	66	12	30
	240.0	89.0	b	4.3	66	10	6,5

Table 1. *Cont.*

	Precursor Ion (m/z)	Product Ion (m/z)	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)
Dicamba	219.0	174.8 [a]	3.6	−5	−10	−8
	221.0	177.0 [b]	3.6	−5	−10	−9
Dichlorprop [2,4-DP]	232.9	160.9 [a]	4.5	−25	−10	−18
	232.9	124.9 [b]	4.5	−25	−10	−13
Fenoprop [2,4,5-TP]	269.0	196.8 [a]	4.8	−70	−10	−14
	269.0	160.9 [b]	4.8	−70	−10	−15
Fenoxaprop P	362.0	288.0 [a]	5.2	126	10	25
	362.0	119.0 [b]	5.2	126	10	18
Flamprop	320.1	121.0 [a]	4.4	−75	−10	−22
	320.1	247.7 [b]	4.4	−75	−10	−45
Fluazifop	328.0	254.0 [a]	4.5	126	10	35
	328.0	282.0 [b]	4.5	126	10	16
Fluroxypyr	253.1	194.8 [a]	3.6	−120	−10	−18
	253.1	232.8 [b]	3.6	−120	−10	−13
Haloxyfop	360.1	287.8 [a]	4.9	−95	−10	−20
	360.1	195.8 [b]	4.9	−95	−10	−13
Haloxyfop-etoxyl	434.1	315.9 [a]	5.2	11	12	25
	434.1	288.0 [b]	5.2	121	10	6,5
Haloxyfop-methyl	376.0	316.0 [a]	5.1	131	10	30
	376.0	288.0 [b]	5.1	126	10	12
Imazamox	306.1	261.1 [a]	3.0	71	10	25
	306.1	245.9 [b]	3.0	71	10	28

Table 1. *Cont.*

	Precursor Ion (m/z)	Product Ion (m/z)		Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)
Imazapyr	274.1	186.9	a	3.0	−30	−10	−18
	274.1	230.0	b	3.0	−30	−10	−13
Imazethapyr	287.6	243.9	a	3.4	−75	−10	−18
	287.6	186.1	b	3.4	−75	−10	−21
Ioxynil	369.9	126.9	a	4.3	−90	−5	−25
	369.9	116.0	b	4.3	−90	−5	−10
2-methyl−4-chlorophenoxy acetic acid (MCPA)	199.0	141.0	a	4.3	−65	−10	−20
	201.0	143.0	b	4.3	−65	−10	−12
2-methyl−4-chlorophenoxy butyric acid (MCPB)	227.0	141.0	a	4.7	−55	−10	−20
	229.0	143.0	b	4.7	−55	−10	−12
Mecoprop (MCPP)	212.9	140.9	a	4.6	−45	−10	−18
	212.9	70.9	b	4.6	−45	−10	−9
Quimerac	222.1	141.1	a	2.9	36	10	45
	222.1	114.1	b	2.9	36	10	8
Quinclorac	241.9	223.9	a	3.5	26	10	21
	241.9	161.0	b	3.5	26	10	16
Sulcotrione	328.9	139.1	a	3.5	111	10	25
	328.9	111.1	b	3.5	130	10	10
Triclopyr	255.7	197.7	a	4.5	−15	−10	−14
	255.7	217.8	b	4.5	−15	−10	−11

[a] Quantification ion. [b] Confirmation ion.

3.2. Optimization of Extraction Method

In the present study, the QuEChERS extraction method was adapted to extract the target analytes. The main modifications with regard to other methodologies previously described were (i) acidification of the extraction solvent for avoiding potential dissociation of the analytes and improving the method precision, (ii) customized addition of water to samples (5 mL of water for cucumber or 10 mL of water for orange) in order to adapt water content to the original acidity of the samples, (iii) addition of a lower amount of both sodium hydroxide 5 N (pH ≈ 12) and sulfuric acid 5 N (pH ≈ 1) to reduce potential contamination of the samples, reduce costs, and miniaturize the method, and (iv) carrying out hydrolysis for 30 min but increasing temperature from 40 to 80 °C in order to improve the robustness of the method and the reliability of the results. In addition, a reduced matrix effect was observed due to the 2-fold dilution of the obtained extracts. It represented an improvement with regard to alternative clean-up steps with sorbents (better selectivity) and avoided analyte losses by increasing extraction efficiency for most of the target analytes (enhanced robustness).

With the optimal extraction conditions described in the experimental section, the proposed method was tested, analyzing 10 replicates of spiked blank samples at 50 μg/Kg of target compounds. Tables 2 and 3 show the results obtained for cucumber and orange, respectively. All the studied compounds were recovered with rates between 70% to 120%, and precision data were always ≤20%. The results complied with the limit values set by the European SANTE.

Table 2. Retention time windows and validation parameters for the target compounds in cucumber.

Compound	Repeatability				Intermediate Precision				Uncertainty		RTW
	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	(%) [a]	(%) [b]	(min.)
Gibberellic acid	109	6	75	4	93	17	85	6	28	14	2.75–2.85
Imazamox	101	3	116	3	97	19	103	15	46	27	2.95–3.15
Imazapyr	115	6	71	2	100	14	82	8	25	18	2.98–3.08
Quimerac	109	3	94	4	108	13	81	18	25	32	3.14–3.24
Imazethapyr	114	9	117	6	107	16	103	15	29	27	3.38–3.48
Quinclorac	104	4	99	4	103	19	86	12	37	21	3.46–3.56
Naphthylacetamide	92	5	120	2	90	18	111	7	35	14	3.47–3.57
Sulcotrione	95	4	102	2	94	12	90	10	24	18	3.7–3.57
Fluroxypyr	114	6	91	3	94	17	86	9	28	17	3.52–3.62
Dicamba	97	6	86	5	82	17	82	14	30	27	3.56–3.66
2-(4-Chlorophenoxy)acetic acid (4-CPA)	102	4	82	4	88	13	82	11	22	22	3.73–3.83
Bentazon	102	6	108	1	89	10	93	9	18	16	3.73–3.83
4-(3-indolyl)butyric acid (IBA)	86	14	120	4	93	20	92	20	42	33	3.78–3.88
2-naphthyloxyacetic acid (BNOA)	104	6	89	3	91	13	85	7	23	12	4.02–4.12
Bromoxynil	108	6	109	2	94	13	92	10	23	16	4.02–4.12
Clomazone	96	6	118	4	84	17	116	8	30	10	4.22–4.32
2-methyl-4-chlorophenoxy acetic acid (MCPA)	107	8	92	3	85	18	86	15	28	28	4.24–4.34

Table 2. *Cont.*

Compound	Repeatability				Intermediate Precision				Uncertainty		RTW
	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	(%) [a]	(%) [b]	(min.)
Ioxynil	110	10	83	4	87	19	86	8	30	17	4.26–4.36
Flamprop	101	14	103	11	92	18	96	14	32	26	4.28–4.38
2,4-Dichlorophenoxyacetic acid (2,4-D)	99	7	99	3	92	13	88	11	24	19	4.34–4.44
Triclopyr	105	9	101	6	92	13	89	13	23	24	4.37–4.47
Fluazifop	107	7	94	2	104	20	99	14	38	29	4.38–4.48
Dichlorprop (2,4-DP)	103	9	103	4	94	15	95	12	28	22	4. 45–4.55
Mecoprop (MCPP)	105	7	101	4	87	15	93	11	25	20	4.48–4.58
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)	113	10	102	4	103	20	89	12	36	22	4.54–4.64
2,4-dichlorophenoxy butyric acid (2,4-DB)	110	11	102	16	113	18	85	20	38	33	4.63–4.73
2-methyl-4-chlorophenoxy butyric acid (MCPB)	106	12	107	11	105	16	90	14	33	23	4.66–4.76
Fenoprop (2,4,5-TP)	113	7	105	4	93	14	91	11	24	20	4.75–4.85
Haloxyfop	102	11	79	7	98	20	89	17	39	38	4.79–4.89
Haloxyfop-methyl	107	14	101	11	92	14	84	20	24	33	4.95–5.05
Haloxyfop-etoxyl	104	19	118	13	85	19	76	19	32	24	5.07–5.17
Fenoxaprop P	97	20	119	10	87	20	92	19	35	34	5.10–5.20

Rec: Recovery. RSD: Relative standard deviation. [a] Level concentration in the validation study of 10 μg/kg. [b] Level concentration in the validation study of 50 μg/kg.

Table 3. Retention time windows and validation parameters for the target compounds in orange.

Compound	Repeatability				Intermediate Precision				Uncertainty		RTW
	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	(%) [a]	(%) [b]	(min.)
Imazamox	90	1	109	2	86	8	98	9	15	16	2.96–3.06
Quimerac	73	2	81	2	80	14	77	14	30	27	3.12–3.24
Naphthylacetamide	108	2	120	2	101	10	107	6	20	10	3.47–3.57
Quinclorac	85	1	83	1	91	10	78	12	21	23	3.47–3.57
Sulcotrione	95	4	106	1	93	12	91	9	24	16	3.47–3.57
Fluroxypyr	101	6	112	6	99	10	98	8	19	14	3.53–3.63
Bentazon	96	6	120	11	100	10	97	4	21	7	3.74–3.84
2-(4-Chlorophenoxy)acetic acid (4-CPA)	88	3	104	6	89	15	85	13	30	21	3.75–3.85
4-(3-indolyl)butyric acid (IBA)	101	2	120	3	106	13	105	9	27	16	3.79–3.89
2-naphthyloxyacetic acid (BNOA)	80	4	112	8	91	19	94	6	44	10	4.03–4.13
Bromoxynil	103	4	117	7	106	13	104	5	27	9	4.03–4.13
Clomazone	119	1	119	2	114	8	109	6	15	11	4.23–4.33
2-methyl-4-chlorophenoxy acetic acid (MCPA)	83	7	109	4	91	19	94	9	42	16	4.25–4.35
Ioxynil	117	4	114	6	118	11	108	8	22	16	4.28–4.38
Flamprop	107	9	106	3	113	12	111	6	25	13	4.29–4.39

Table 3. *Cont.*

Compound	Repeatability				Intermediate Precision				Uncertainty		RTW
	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	Rec (%) [a]	RSD (%) [a]	Rec (%) [b]	RSD (%) [b]	(%) [a]	(%) [b]	(min.)
2,4-Dichlorophenoxyacetic acid (2,4-D)	78	8	107	8	91	17	93	5	39	9	4.36–4.46
Triclopyr	94	9	98	4	102	13	103	8	29	17	4.38–4.48
Dichlorprop (2,4-DP)	84	8	115	5	91	16	99	8	35	13	4.46–4.56
Mecoprop (MCPP)	91	8	114	4	88	20	98	8	40	15	4.49–4.59
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)	92	5	115	7	99	12	98	8	26	14	4.55–4.65
2,4-dichlorophenoxy butyric acid (2,4-DB)	106	2	111	9	102	18	107	19	35	37	4.64–4.74
2-methyl-4-chlorophenoxy butyric acid (MCPB)	90	6	120	7	100	14	106	12	31	21	4.66–4.76
Fenoprop (2,4,5-TP)	110	5	117	5	107	10	94	10	19	15	4.76–4.86
Haloxyfop	119	17	107	8	119	11	109	9	20	18	4.90–5.00
Haloxyfop-methyl	120	3	113	1	107	11	92	11	19	17	4.95–5.05
Haloxyfop-etoxyl	107	14	106	12	112	14	86	13	30	22	5.08–5.18
Fenoxaprop P	105	14	118	11	110	19	93	18	40	28	5.10–5.20

Rec: Recovery. RSD: Relative standard deviation. [a] Level concentration in the validation study of 10 µg/kg. [b] Level concentration in the validation study of 50 µg/kg.

3.3. Method Validation

The validation protocol was designed in order to fulfill the requirements and obtain accreditation according to UNE-EN-ISO/IEC 17025:2017 by ENAC (National Accreditation Body in Spain) and SANTE guidelines for reliable identification and quantification of phytohormones and acidic herbicides. Methods found in the literature were generally validated, but it is very unusual to find a method accredited by an international quality standard.

The identification of target compounds was based on RTW values. Tables 2 and 3 show the obtained values, meeting the threshold (±0.1 min) established by SANTE guidelines for all compounds. Analytes were confirmed by mass spectrometry by comparing the ion intensity ratios of their most characteristic ions with those obtained for standards analyzed at similar concentrations. In all cases, the obtained values were in the permitted tolerance range (±30%) for confirmation as a compound.

3.3.1. Specificity

Specificity was investigated by analyzing ten blank samples and checking that no interfering chromatographic peaks were observed at the same RTWs of the analytes. Figures 2 and 3 show the absence of interferences in the blanks of cucumber and orange, respectively. Hence, no matrix interferences or other analytes that would cause a false-positive signal were observed at the RTW of each analyte.

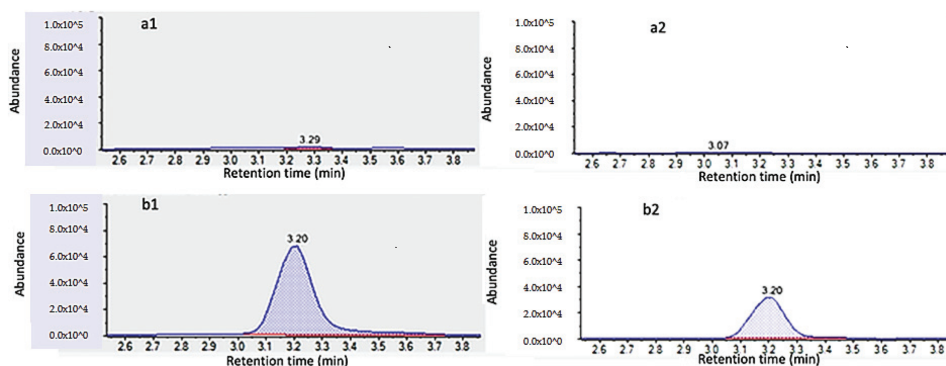

Figure 2. UHPLC–MS/MS chromatogram of (**a**) blank cucumber sample, monitoring transitions at *m/z* 222/141 (**a1**) and *m/z* 222/114 (**a2**), and (b) blank cucumber sample spiked with 10 µg/kg of quinmerac, monitoring transitions at m/z 222/141 (**b1**) and m/z 222/114 (**b2**).

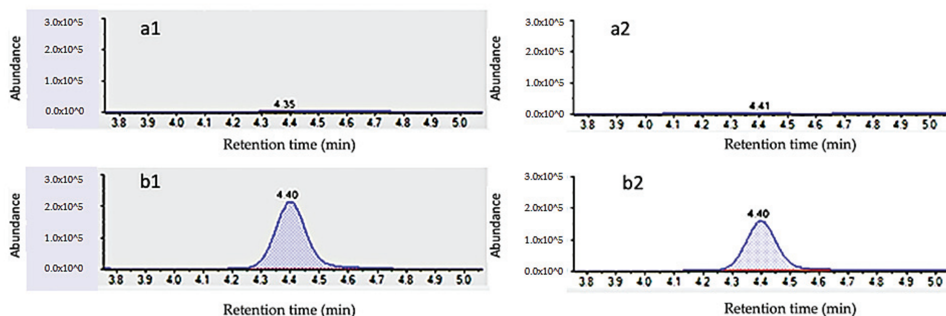

Figure 3. UHPLC–MS/MS chromatogram of (**a**) blank orange sample, monitoring transitions at m/z 219/161 (**a1**) and m/z 220/163 (**a2**), and (**b**) blank orange sample spiked with 10 µg/kg of 2,4-D, monitoring transitions at m/z 220/161 (**b1**) and m/z 220/163 (**b2**).

3.3.2. Linearity and Working Range

The matrix-matched calibration curves were prepared at 10, 25, 50, 100, and 150 μg/kg. This working range was established for all compounds, taking into account that there was good linearity, and it included the MRLs in the target matrices. Weighting least-squares regression was used, plotting peak area versus concentration of the calibration standards, with weighting factor 1/x, and not forcing curves to pass through the origin. A weighted fit of the calibration line was used to compensate for the observed homoscedasticity and to improve the accuracy of the analytical results.

The experimentally obtained correlation coefficient (r^2) was always higher than 0.98 for all target analytes. The individual residual of each point of the calibration curve did not deviate more than ±20% from their values predicted, complying with the requirements established by EU legislation. With the fit of both parameters, the capacity of the calibration function could be assured as adequate within the concentration range studied.

3.3.3. Trueness (Trueness Assessment)

To ensure that the developed method provided truthful results, the % recovery for each analyte at the concentration of 10 μg/Kg was also calculated. Recoveries ranged from 86% to 115% in cucumber samples (Table 2), and from 73% to 120% in orange commodities (Table 3), according to the requirements of the UNE-EN-ISO/IEC 17025:2017 accreditation and SANTE guidelines.

3.3.4. Precision study

Intraday precision values, ranging between 1–20% for cucumber (Table 2) and between 1–17% for orange (Table 3) samples, were obtained. Interday precision values were in the range 5–20% for cucumber (Table 2) and 4–20% for orange (Table 3) samples. It can be observed that RSDs for intra- and interday precision studies were always equal or lower than 20%.

3.3.5. Limit of Quantification

LOQs were set at 10 μg/kg for all analytes. Additionally, at this concentration level, the signal-to-noise ratio (S/N) of the chromatographic peaks was calculated, verifying that the target analytes originated an S/N higher than 10 for the smallest transition ion (qualifier). It is remarkable that all the analytes had a LOQ lower or equal to the lowest MRL established in each matrix.

3.3.6. Uncertainty

The U-values obtained for the lowest level of concentration studied (10 μg/kg) in cucumber samples ranged from 18% to 46%, with an average of 30%, while for the higher studied level (50 μg/kg), it ranged from 10% to 38% (Table 2), with an average of 23%. For orange samples (Table 3), the U-values were between 15% to 44% (28% average) and 7% to 37% (17% average) for the lowest and highest concentration levels, respectively. These values were in agreement with EU requirements, where a generalized U-budget of ±50% is applicable as the default value [37,39].

3.4. Sample Analysis

To improve the scope of the method, new matrices such as tomato, melon, and zucchini were also verified. Studies (n = 3) of trueness and precision at 10 μg/Kg were carried out. Tomato and watermelon were checked with calibration curves prepared with cucumber, whereas for zucchini, orange was used as the representative matrix.

A total of 457 samples were analyzed with this method during the last year. Real samples of cucumber (67), orange (150), tomato (120), watermelon (60), and zucchini (60) were analyzed. A total of 32 positive samples were found in tomato and orange samples; no positives were detected in cucumber, melon, or zucchini samples (Table 4). Tomato (6 positive samples—1.3% of the total samples) was the only food commodity with target analytes above their MRL in the group of matrices with high water

content, whereas, for the high acid and high water content samples, positive results were only found in the representative matrix orange (26 positive samples—5.7% of the total sample analyzed). In all cases, the presence of target compounds was detected as below the MRLs established by the EU.

2,4-dichlorophenoxyacetic acid (2,4-D), which can act as herbicide or phytohormone, and 4-(3-indolyl)butyric acid (IBA), a phytohormone, were detected in the positive samples of tomato. In orange, four analytes were detected. Two of those compounds can be used as herbicides or phytohormones: 2,4-D and dichlorprop (2,4-DP) (Figure 4). The other two detected compounds can be used as herbicides: 2-methyl-4-chlorophenoxy acetic acid (MCPA) and triclopyr. In tomato, the analyte most found was IBA, detected in four of the studied samples (66.7% of the total positive samples) at a concentration that ranged from 12 to 52 μg/Kg. The most commonly detected analyte in orange was MCPA, being determined in 10 of the studied samples (38.5% of the total positive samples) at a concentration that ranged from 13 to 74 μg/Kg.

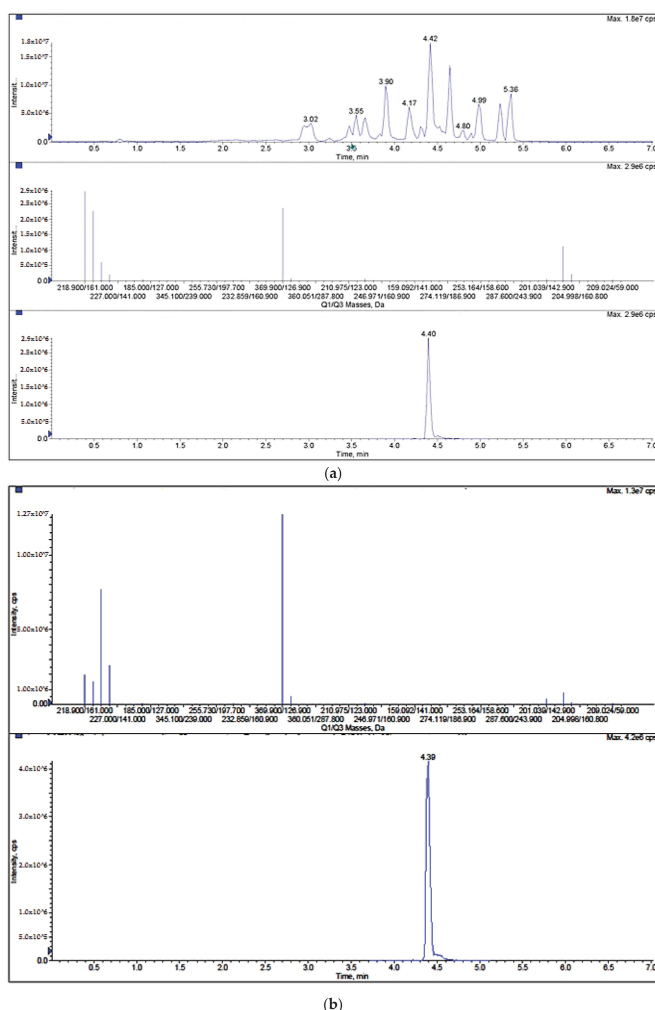

Figure 4. (**a**) Positive orange sample containing 2,4 D (total ion chromatogram, relation ions, and UHPLC–MS/MS chromatogram), and (**b**) relation ions and UHPLC–MS/MS chromatogram of a matrix-matched standard of 2,D.

Table 4. Compounds in analyzed samples above the LOQ of the method expressed as mg/kg.

Compound Detected	Tomato		Orange	
	MRL (mg/kg)	Concentration (mg/kg)	MRL (mg/kg)	Concentration (mg/kg)
2,4-Dichlorophenoxyacetic acid (2,4-D)	0.01	0.012 to 0.016	1	0.014 to 0.670
Dichlorprop (2.4-DP)	0.05	Not detected	0.3	0.014 to 0.097
2-methyl-4-chlorophenoxy acetic acid (MCPA)	0.05	Not detected	0.05	0.013 to 0.074
Triclopyr	0.01	Not detected	0.1	0.017 to 0.066
4-(3-indolyl)butyric acid (IBA)	0.1	0.012 to 0.052	0.1	Not detected

The compounds with values higher than the LOQ (positives samples) were identified and confirmed as described in Section 3.3, fulfilling the requirements based on RTW values and the ratio between quantification ion and confirmation ion intensity.

4. Conclusions

The developed method allows us to group in a single "run" the phytohormones and acidic pesticides widely used in cucumber (a matrix with high water content) and orange (a matrix with high acid and water content) by UHPLC–MS/MS. The extraction procedure was based on the QuEChERS method but with some modifications to adapt the extraction to the broad physicochemical properties of the target analytes. The instrumental analysis time was 7 min. The proposed method has been designed to be successfully implemented in testing laboratories to perform routine analyses, thanks to its simple sample treatment and rapid chromatographic analysis.

To ensure method suitability, a validation (linearity, specificity, trueness, precision, LOQs, and uncertainty) was performed in compliance with the SANTE 2019 guidelines. Uncertainty is not a very common parameter found in the bibliography about organic contaminants in fruits and vegetables. It has been included in the validation process in order to carry out an evaluation of compliance with the requirements of current legislation about MRLs established by the EU.

Subsequently, 457 real samples were analyzed. Compounds such as 2.4-D, IBA, 2.4-DP, MCPA, and triclopyr were the only five compounds found, often at concentrations lower than their MRLs. It should be noted that most of the previous publications did not carry out an extensive application of the method to real samples, and the detection of positive cases have rarely been reported. The results obtained show evidence of its applicability to the analysis of real samples in routine residue monitoring programs and that it is fit-for-purpose.

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Dispersive Solid–Liquid Extraction Coupled with LC-MS/MS for the Determination of Sulfonylurea Herbicides in Strawberries

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Abstract: The monitoring of food quality and safety requires a suitable analytical method with simultaneous detection in order to control pesticide and herbicide residues. In this study, a novel analytical method, referred to as "dispersive solid–liquid extraction", was applied to monitor seven sulfonylurea herbicides in strawberries. This method was optimized in terms of the amount of C_{18} and the volume of added water, and it was validated through satisfactory linearities ($R^2 > 0.99$), recoveries of 70% to 84% with acceptable precisions, and limits of quantification lower than the maximum residue limits for the seven sulfonylurea herbicides in strawberries. The cleanup efficiency of the dispersive solid–liquid extraction technique was compared to that of the QuEChERS- ("quick, easy, cheap, effective, rugged and safe") based method with dispersive solid phase extraction. The recoveries of the former were found to be comparable to those involving QuEChERS C_{18} cleanup (recoveries of 74%–87%). The method was used to determine sulfonylurea herbicide residues in ten strawberry samples. None of the samples had herbicide residues higher than that of limit of quantifications (LOQs) or maximum residue limits (MRLs). The results suggest that the dispersive solid–liquid extraction method combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) is effective for the analysis of sulfonylurea herbicide residues in strawberries.

Keywords: dispersive solid-liquid extraction; sulfonylurea herbicides; QuEChERS; strawberry; cleanup

1. Introduction

With ever-tightening regulations that govern the maximum residue limits (MRLs) of pesticides and herbicides in foods, issues associated with their residues and food safety have received significant attention. Multi-residue analysis techniques are widely used to monitor food quality and safety [1]. While the multi-residue analysis of pesticides is capable of determining trace components in food matrices, it requires effective sample preparation, including extraction and cleanup steps, in order to eliminate interference (pigments, lipids, etc.) present in real samples [2,3]. The fact that pesticide multi-residues are associated with a broad spectrum of chemical and physical properties, provides challenges that require suitable extraction and cleanup procedures in order to detect these analytes with satisfactory accuracies.

Sulfonylurea herbicides are used to control broadleaf weeds and annual grasses in agricultural crops. The European Union (EU) [4] and Korea [5] have set MRLs for most sulfonylurea herbicide residues in strawberries at 0.01 mg/kg; however MRLs have not been set for all sulfonylurea herbicides. Therefore, controlling these herbicide residues in foods through monitoring is important for consumer safety. The QuEChERS ("quick, easy, cheap, effective, rugged, and safe") sample preparation technique coupled with MS and MS/MS is a widely accepted methodology for most pesticides, with the exception of nonpolar pesticides in food materials [6]. Unfortunately, the original QuEChERS method provided

relatively weak recovery values (<70%) for sulfonylurea herbicides [7,8]. To overcome this issue, several sample preparation techniques, including solid phase extraction (SPE) using Chem Elut SPE cartridges [9] or a mini-column packed with oxidized carbon nanotubes [10], magnetic-SPE using multiwalled carbon nanotubes [11], dispersive SPE (d-SPE) using C_{18} and graphitized carbon black (GCB) [12], dispersive liquid–liquid microextraction (DLLME) [13], matrix solid phase dispersion followed by DLLME [14], and stir bar sorption extraction [15], have been studied and validated. Lee et al. [8] reported that a modified QuEChERS method involving C_{18} cleanup after extraction with a citrate buffer provided the best recoveries for some sulfonylurea herbicides in brown rice and rice straw. Kaczyński and Łozowicka [7] introduced a one-step QuEChERS extraction and cleanup protocol for 23 sulfonylurea herbicides in cereals using chitin followed by LC-MS, with satisfactory recoveries (70%–120%) reported.

Dispersive solid–liquid extraction (d-SLE), an environmentally friendly cleanup procedure, was introduced by Sun et al. [16]. According to their d-SLE procedure, analytes are adsorbed by sorbents such as C_{18}, after which the analytes are eluted from these sorbents using organic solvents. This cleanup procedure was successfully applied to determine the E/Z-fluoxastrobins (broad-spectrum fungicides) in fruits and vegetables, which demonstrated better cleanup and lower matrix effects than the d-SPE. Yao et al. [17] reported that the d-SLE procedure showed lower matrix effects than the d-SPE; however, sensitivity toward some fungicides and insecticides (carbendazim, clothianidin, imidacloprid, prochloraz, thiamethoxam, etc.) following the d-SLE cleanup were not satisfactory due to dilution and the lack of nitrogen blowing and evaporation procedures. However, the cleanup efficacy of d-SLE for the multi-residue analysis of sulfonylurea herbicides has not been studied as this novel technique has only recently appeared.

Herein, we propose that the d-SLE cleanup method can be applied to determine sulfonylurea herbicide residues in strawberries. In this study, we developed and validated a multi-residue analysis technique for the seven sulfonylurea herbicides found in strawberries, namely, azimsulfuron, chlorsulfuron, ethoxysulfuron, flucetosulfuron, halosulfuron-methyl, imazosulfuron, and metazosulfuron, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in combination with d-SLE. The cleanup efficiency of d-SLE was compared to that of the QuEChERS method.

2. Materials and Methods

2.1. Chemicals and Reagents

Ammonium acetate (>99.0%), triphenyl phosphate (TPP), formic acid, and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). A QuEChERS AOAC extraction kit (P/N 5982-7755), QuEChERS d-SPE kits for general fruits and vegetables (P/N 5982-5022), fatty samples (P/N 5982-5122), pigments (P/N 5982-5222), pigments and fats (P/N 5982-5421), and a C_{18} adsorbent (end-capped) were purchased from Agilent Technologies (Santa Clara, CA, USA). All solvents used were of analytical or HPLC grade. The standard herbicide solutions listed in Table 1 were obtained from AccuStandard (New Haven, CT, USA). The purities of all standards exceeded 95%. Herbicide standard stock solutions (each 100 μg mL^{-1}) were prepared in acetonitrile. The combined working standard solutions were prepared by serial dilutions of the stock solutions with the same solvent. The stock and working solutions were stored at −20 °C until analyzed.

Table 1. LC-MS/MS parameter values used during the detection of selected sulfonylurea herbicides.

Sulfonylurea Herbicides [a]	R.T. (min)	Precursor Ion (*m*/*z*)	Quantification Transition (*m*/*z*)	D.P. (V)	C.E. (V)	Confirmatory Transition (*m*/*z*)	D.P. (V)	C.E. (V)
Azimsulfuron	4.6	425.0	182.1	61	23	156.1	61	45
Chlorsulfuron	4.3	410.9	149.0	66	27	119.1	66	53
Ethoxysulfuron	5.3	388.0	167.1	71	21	204.9	71	33
Flucetosulfuron	4.9	358.0	141.0	71	23	167.1	71	25
Halosulfuron-methyl	5.6	435.1	182.1	51	35	138.9	51	71
Imazosulfuron	5.2	399.0	217.9	66	33	260.9	66	21
Metazosulfuron	5.0	476.0	181.8	91	33	295.1	91	25

[a] The mass spectra for all of the pesticides tested was obtained using the positive ion mode. R.T., retention time; D.P., declustering potential; and C.E., collision energies.

2.2. Sample Preparation

2.2.1. Extraction and d-SLE

For d-SLE, strawberry samples collected from local markets were stored at 4 °C and analyzed within 3 days. Frozen strawberries were homogenized using a commercial grinder and each sample (1 g) was placed in a 15 mL centrifuge tube to which 2 mL of acetonitrile containing 1% formic acid was added as the extraction solvent. The centrifuge tube was vigorously shaken on vortex for 2 min. Then, 150 mg of magnesium sulfate ($MgSO_4$) was added and the tube was shaken for 1 min, after which it was centrifuged at 5000 *g* for 10 min at 4 °C. Aliquots (0.2 mL) of the supernatant extract was transferred to 2 mL centrifuge tubes containing different amounts of C_{18} (50, 100, 150, 200, 250, and 300 mg) to which different volumes of water (0.5, 1, 1.5, and 2 mL) were added to the selected amount of C_{18}. The tubes were shaken for 1 min and then centrifuged at 15,294 *g* for 3 min. Each supernatant was removed with a 10 mL disposable syringe and 50 mg anhydrous $MgSO_4$ was added to remove residual water. Acetonitrile (1 mL) was then added and the tube shaken for 1 min and centrifuged at 15,294 *g* for 3 min. Each supernatant was filtered through a 0.22 μm nylon membrane filter and analyzed by LC-MS/MS.

2.2.2. QuEChERS Extraction and d-SPE

The QuEChERS extraction and d-SPE (QuEChERS-d-SPE) were carried out using the QuEChERS AOAC extraction kit containing 6 g of $MgSO_4$ and 1.5 g of sodium acetate. Each sample (15 g) was placed in a 50 mL centrifuge tube to which a 15 mL solution of 1% acetic acid in acetonitrile was added as the extraction solvent. Triphenyl phosphate was spiked directly into the centrifuge tube (as the internal standard) to a concentration of 1 μg mL^{-1}. The centrifuge tube was shaken for 1 min. The QuEChERS AOAC extraction kit was then added and the tube was shaken strongly for 10 min, after which the sample was centrifuged at 4000 × *g* for 10 min at 4 °C. Cleanup was performed by d-SPE following analyte extraction. A 1 mL aliquot of the upper layer was transferred to a 2 mL d-SPE tube containing various sorbent mixtures. Five different cleanup procedures were tested, namely, cleanup 1:50 mg primary secondary amine (PSA), and 150 mg $MgSO_4$; cleanup 2: 50 mg PSA, 50 mg C_{18}, and 150 mg $MgSO_4$; cleanup 3: 50 mg PSA, 50 mg GCB, and 150 mg $MgSO_4$; cleanup 4: 50 mg PSA, 50 mg C_{18}, 50 mg GCB, and 150 mg $MgSO_4$; cleanup 5: 50 mg C_{18} and 150 mg $MgSO_4$. The tube was tightly closed and vortexed for 1 min, after which it was centrifuged at 15,294 × *g* for 5 min at 4 °C. The extract was filtered through a syringe with a 0.22 μm nylon membrane filter and transferred into an autosampler vial for LC-MS/MS analysis.

2.3. LC-MS/MS

Analyses were performed on an Agilent LC 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 4000 QTRAP mass spectrometer equipped with a turbo ion-spray ionization source (AB SCIEX, Foster City, CA, USA). Chromatographic separations were achieved using a reversed-phase Cadenza CD-C_{18} HT column (50 × 2.0 mm, 3 μm; Imtakt Company, Kyoto, Japan). The mobile phase consisted of: (A) 5 mM ammonium acetate and 0.1% (*v*/*v*) formic acid in water, and

(B) 5 mM ammonium acetate and 0.1% (*v*/*v*) formic acid in methanol. A linear binary mobile phase solvent gradient was used as follows: 95% A at 0 min, 60% A at 0.5 min, 40% A at 1.5 min, 30% A at 2 min, 20% A at 5 min, 0% A at 6–7.5 min, and 95% A at 8–12 min. The flow rate, column temperature and injection volume were 0.3 mL min^{-1}, 40 °C, and 2 μL, respectively. The mass spectrometer was operated in the positive-ion ESI mode. The MS/MS was performed using scheduled multiple reaction monitoring (MRM) with the following general settings: curtain gas, 30 psi; ion-source gas (1), 50 psi; ion-source gas (2), 55 psi; source temperature, 400 °C; and ion-spray voltage, 5500 V. The MRM transitions, retention times, collision energies, and declustering potentials of the analytes are summarized in Table 1. The contents of individual herbicides were calculated using the matrix-matched calibration curve.

2.4. Validation Study and Matrix Effects

The method was validated following the European Commission SANTE/11813/2017 [18] and ICH/2005/Q2/R1 [19] protocols. To assess linearity, blank extracts were spiked with a multistandard solution at concentrations of 0.005, 0.01, 0.02, 0.05, and 0.1 mg/kg. A coefficient of determination $R^2 > 0.99$ was acceptable.

The recovery (%) and precision, in terms of repeatability and reproducibility, were determined by repeated analysis of fortified blank samples at three concentrations (0.01, 0.05, and 0.1 mg/kg). The recovery (%) is expressed as: Recovery (%) = (measured concentration)/(spiked concentration) × 100. Repeatability and reproducibility were determined by at least six-replicate analyses on the same day and on different days. Precision is expressed as the relative standard deviation (RSD) of replicate measurements.

The limit of detections (LODs) and limit of quantifications (LOQs) were determined from five independently spiked concentrations of herbicides (0.005, 0.01, 0.02, 0.05, and 0.1 mg/kg). The LODs and LOQs were calculated based on the standard deviations of response and slope, and they are expressed as: LOD = 3.3 σ/s, LOQ = 10 σ/s, where, σ is the standard deviation of the response, and s is the slope of the matrix-matched calibration curve.

To compensate for matrix effects (MEs), we compared the slopes of the pesticide standards in the solvent and in the extracts. The ME (%) is expressed as:

$$\text{ME (\%)} = (\text{slope of the calibration curve of the extract/slope of the calibration curve of solvent} - 1) \times 100. \quad (1)$$

3. Results and Discussion

3.1. Optimizing the LC-MS/MS Parameters

The ionizations and fragmentations of the seven sulfonylurea herbicides were studied prior to method validation. The seven sulfonylurea herbicides in strawberries were identified on the basis of the retention times and ion abundances of qualitative and quantitative ions. The positive ESI technique was used for LC-MS/MS. The most intense product ion (*m*/*z*) was used to quantify each herbicide. The selected sulfonylurea herbicides were successfully determined, both qualitatively and quantitatively, using multiple reaction monitoring (MRM). The optimized LC-MS/MS parameters, namely retention times, quantified and qualified ion transitions, declustering potentials, and collision energies for data acquisition, were used to obtain the best MRM transitions (Table 1).

Since the seven sulfonylurea herbicides, which contain sulfonic functional groups, are weak acids according to their pKa values (approximately 3–5) [20], acidic mobile phases provided better retentions and chromatographic separations. The selected herbicides were analyzed using ammonium acetate and formic acid as the mobile phase additives, which improved peak intensities, and promoted the ionization and separation of the analytes [21]. To ensure the highest resolution for all analytes, the gradient conditions were optimized by adjusting the flow rate to 0.3 mL min^{-1} which resulted in an

analysis time of 12 min. Well-resolved and separated peaks with good shapes were achieved, as shown in Supplemental Figure S1.

3.2. Optimizing the d-SLE Method

We investigated the efficacy of the d-SLE cleanup method, which is capable of enriching analytes and removing the co-eluent through adsorption and desorption procedures. To optimize this method, the recoveries (%) of the seven sulfonylurea herbicides were compared when different amounts of C_{18} and volumes of water were added to the extraction solvent. Acetonitrile containing 1% formic acid has been previously reported to provide the best recoveries for 23 sulfonylurea herbicides [7] and was selected as the extraction solvent in this study. Strawberries contain water-soluble pigments and vitamins, and over 90% moisture. The use of $MgSO_4$ in the partition step reduced the volume of the aqueous phase, resulting in the removal of the water-soluble co-eluents.

3.2.1. The Effect of the Amounts of C_{18} on Extraction Efficacy

In order to achieve satisfactory cleanup, we optimized the amount of adsorbent used. The effect of C_{18} in the 50–300 mg range was examined at a fortified level of 0.05 mg/kg. Water (1 mL) was added to promote the adsorption of the analytes in the acetonitrile onto the C_{18}. As shown in Figure 1A, the recoveries depend on the sulfonylurea herbicide. It is evident that less than 200 mg of C_{18} provides lower recoveries for all tested herbicides (Figure 1A). Lower recoveries were observed when the amount of C_{18} was increased from 200 to 300 mg, with the lowest recoveries observed with 300 mg of C_{18} (Figure 1A). The optimized amount of C_{18} used in the cleanup step was sufficient to remove matrix interferences, and thus led to an improvement of target analytes detection and their recoveries [22]. Consequently, 200 mg of C_{18} was selected for the adsorption of the target herbicides in this study. Leandro et al. [23] reported that the addition of 200 mg of the C_{18} sorbent led to an effective reduction of nonpolar analytes.

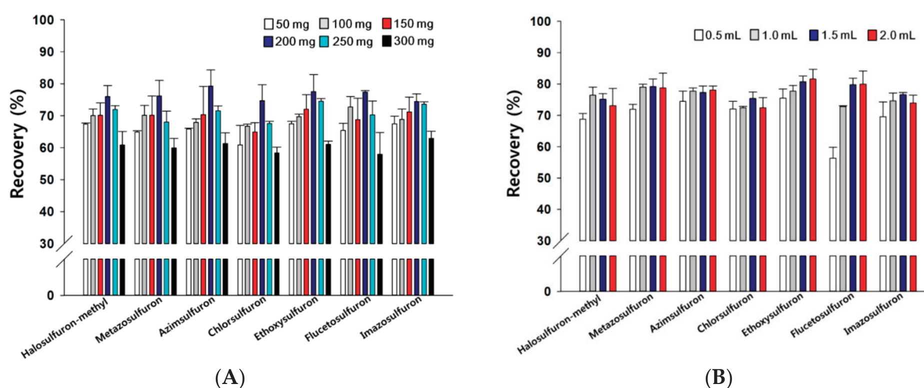

Figure 1. Recoveries as functions of (**A**) the amount of added C_{18} and (**B**) the volume of added water for selected sulfonylurea herbicides in strawberries fortified at 0.05 mg/kg using the dispersive solid–liquid extraction (d-SLE) method. In (**A**), 1.0 mL of water was added to facilitate the adsorption of the analytes in acetonitrile onto the C_{18}. In (**B**), 200 mg of C_{18} was used to adsorb the target herbicides.

3.2.2. The Volume of Added Water

The volume of the added water, which reduces the proportion of acetonitrile, is a critical factor that significantly affects the retention or adsorption performance of adsorbents toward analytes in a reversed-phase system. Various volumes of water (0.5–2.0 mL) were added to the extract with 200 mg of C_{18}. As shown in Figure 1B, the volumes of added water examined do not appear to significantly affect the recoveries (%) of azimsulfuron, chlorsulfuron, and ethoxysulfuron. Increasing the volume of

water to 1.5 mL led to increases in the recoveries of some herbicide (Figure 1B); however, the addition of more than 1.5 mL of water resulted in no further changes in the observed recoveries, which is in agreement with the study by Sun et al. [16]. Therefore, adding 1.5 mL of water was used to promote the adsorption of herbicides in further experiments. After removal of the water, 1 mL of acetonitrile was used as the desorption solvent and $MgSO_4$ was added to remove trace amounts of residual water.

3.3. Method Validation and Matrix Effects

Validation experiments that assess linearities, accuracies, precisions, LODs, and LOQs, were used to evaluate the extraction and cleanup procedure under the optimized d-SLE conditions. Calibration curves were constructed by the matrix-matched standard calibration method at concentrations of 0.005, 0.01, 0.02, 0.05, and 0.1 mg/kg in blank strawberry extracts. As shown in Table 2, satisfactory correlation coefficients (R^2) of 0.9985–0.9994 were obtained for the seven sulfonylurea herbicides, while LODs between 0.001 mg/kg and 0.002 mg/kg were observed. In addition, LOQs between 0.004 mg/kg and 0.005 mg/kg were obtained for the target analytes, with the majority lower than the lowest points of the respective linear ranges. Moreover, the d-SLE method provided a lower LOQ than the MRLs established by the EU and Korea for each sulfonylurea herbicide studied. On the basis of the EU requirements, each LOQ should be lower or equal to the MRL. Recoveries were determined at three fortification levels (0.01, 0.05, and 0.1 mg/kg), with recoveries of 70%–84%, repeatability RSDs of less than 14%, and reproducibility RSDs of less than 14% observed for all herbicides, which are within the quality control criteria prescribed by the SANTE guidelines.

MEs (%) were determined in order to evaluate ion suppression and/or enhancement, which play crucial roles in analyte quantification [24]. An ME of between −20% and 20% is regarded to indicate no matrix effect, while a value outside of this range indicates enhancement (>20%) or suppression (<−20%) [25]. Regarding the strawberry matrix, all tested sulfonylurea herbicides that were cleaned up using d-SLE exhibited no matrix effects (Table 2). Minimizing the ME obtained by removing co-eluted matrix interference can improve chromatographic selectivity. The d-SLE method exhibited a better ME than the d-SPE method, which is due to the high dilution factor [17]. An appropriate dilution factor can reduce the ME to less than 20% [26]. In this study, the d-SLE method provided good MEs with acceptable recoveries at significantly higher dilutions (more than ten times) than those used for the d-SPE method (Table 2).

Table 2. Matrix-effect and method-validation data for selected sulfonylurea herbicides in strawberries using the d-SLE method with LC-MS/MS.

Sulfonylurea Herbicides	Matrix Effect (%)	Linearity (R^2)	LOD (mg/kg)	LOQ (mg/kg)	Recovery (%)			Repeatability, %RSD			Within-laboratory Reproducibility, %RSD		
					0.01	0.05	0.1	0.01	0.05	0.1	0.01	0.05	0.1
					mg/kg			mg/kg			mg/kg		
Azimsulfuron	−16.0	0.9988	0.001	0.004	79.7	76.6	75.8	4.5	2.0	3.7	7.3	4.1	11.1
Chlorsulfuron	1.0	0.9991	0.001	0.005	77.5	76.0	83.2	12.2	0.2	0.8	13.6	2.4	8.6
Ethoxysulfuron	−4.5	0.9994	0.001	0.004	79.8	73.7	82.0	8.6	0.9	4.0	6.1	2.2	8.3
Flucetosulfuron	0.9	0.9985	0.002	0.005	75.7	83.7	70.4	13.8	13.0	0.6	12.5	10.0	13.8
Halosulfuron-methyl	−11.2	0.9993	0.002	0.005	77.9	74.9	70.4	7.0	1.4	2.7	8.0	1.0	9.2
Imazosulfuron	−9.3	0.9988	0.002	0.005	81.8	76.2	75.3	8.6	2.4	0.4	8.2	1.8	10.0
Metazosulfuron	−14.1	0.9989	0.001	0.004	78.2	77.0	79.9	8.4	3.7	2.1	8.6	3.0	10.8

LOD, limit of detection. LOQ, limit of quantification. RSD, relative standard deviation.

3.4. Comparing d-SLE and QuEChERS-d-SPE

The d-SLE and QuEChERS-d-SPE methods were compared in order to evaluate analyte-extraction and cleanup procedure performance. Acetate-buffered extraction was used in the QuEChERS-d-SPE method because this method has been shown to provide higher and more consistent recoveries for pH-dependent pesticides in fruit and vegetable matrices [27]. PSA, GCB, and C_{18} are the sorbents most commonly used to remove co-extracts from fruits and vegetables during cleanup [28–30]; however, the optimal cleanup sorbent depends on the characteristics of the pesticide components and may vary as a consequence. PSA and GCB can adsorb some weakly acidic herbicides, including sulfonylureas [31]. As summarized in Table 3, the recovery rates of most sulfonylurea herbicides using d-SPE with PSA (cleanup 1–4) were found to be less than 70%. The recovery rates were lower using cleanup 3, which uses both GCB and PSA, than cleanup 1 which uses only PSA; similar results were also observed for cleanups 2 (PSA + C_{18}) and 4 (PSA + GCB + C_{18}). GCB is known to adsorb and retain pesticides with planar structures [27,32,33]. Moreover, the addition of C_{18} (cleanup 4) tended to further reduce the recovery rates of some of the herbicides as compared with d-SPE without C_{18} (Cleanup 3). Lower extraction efficacies were observed with C_{18} when the aqueous phase was not completely removed by phase separation [22]. The reduced recoveries (%) observed with the d-SPE methods (Table 3) is partially ascribable to the use of GCB and PSA, and/or a combination of GCB, PSA, and C_{18}. On the other hand, cleanup 5, which only used C_{18}, met the requirements of accuracy (74.1%–87.2%), which is ascribable to the C_{18} sorbent having the lowest affinity for acidic herbicides as compared with PSA and GCB, and excellent performance during extract purification [31]. Kaczyński et al. [7] reported a method for extracting acidic herbicides with C_{18} cleanup; their method exhibited recoveries of between 65% and 89% for sulfonylurea herbicides, which are consistent with our results (Table 3). Lee et al. [8] also reported that C_{18} cleanup afforded the best recoveries for some sulfonylurea herbicides in brown rice and rice straw.

Table 3. Comparing selected sulfonylurea herbicide recoveries from strawberries using five dispersive solid phase extraction (d-SPE) cleanup procedures and the optimized d-SLE method.

Sulfonylurea Herbicides	Fortification Level (mg/kg)	Mean Recovery (%)					
		Cleanup 1	Cleanup 2	Cleanup 3	Cleanup 4	Cleanup 5	d-SLE [a]
Azimsulfuron	0.01	72.3	56.9	28.8	29.9	82.1	79.7
	0.05	62.0	62.2	29.1	22.3	78.2	76.6
	0.1	58.1	66.0	44.3	24.3	85.9	75.8
Chlorsulfuron	0.01	38.8	43.6	33.4	33.1	79.9	77.5
	0.05	47.8	48.5	36.9	27.3	74.8	76.0
	0.1	42.7	49.8	46.2	31.4	79.8	83.2
Ethoxysulfuron	0.01	64.1	52.0	3.8	3.2	86.5	79.8
	0.05	50.2	56.7	3.7	2.8	81.7	73.7
	0.1	41.7	54.8	9.0	2.7	86.3	82.0
Flucetosulfuron	0.01	72.0	62.4	24.4	23.2	82.1	75.7
	0.05	64.5	67.9	22.6	23.9	75.0	83.7
	0.1	55.7	70.8	39.7	20.5	82.9	70.4
Halosulfuron-methyl	0.01	54.5	53.4	36.0	33.1	74.1	77.9
	0.05	50.3	55.1	42.5	27.6	75.3	74.9
	0.1	42.0	54.7	57.4	28.8	80.8	70.4
Imazosulfuron	0.01	68.4	57.3	20.5	19.2	87.2	81.8
	0.05	57.3	60.4	18.8	15.0	83.8	76.2
	0.1	52.3	61.2	31.7	15.4	87.1	75.3
Metazosulfuron	0.01	42.7	37.8	24.0	21.1	87.0	78.2
	0.05	37.2	41.1	30.3	17.2	83.7	77.0
	0.1	29.2	42.4	46.9	17.7	84.4	79.9

[a] C_{18} (200 mg) and water (1.5 mL) were added during the determination of sulfonylurea herbicides in strawberries by d-SLE.

The d-SLE method effectively removed impurities and pigments. The strawberry samples purified by d-SLE were visually colorless as compared with those purified by d-SPE. The recoveries using the d-SLE method were 70.4%–83.7%, which are comparable with the C_{18} cleanup recoveries of between 74.1% and 87.2% at fortification levels of 0.01, 0.05, and 0.5 mg/kg. The results in this study demonstrate that the seven sulfonylurea herbicides meet the recovery and %RSD requirements using d-SLE with C_{18} as an adsorbing material in the absence of an extraction salt. Therefore, this study suggests that d-SLE is a comparable cleanup method for the effective analysis of sulfonylurea herbicides.

QuEChERS extraction was carried out using the AOAC (6 g $MgSO_4$ and 1.5 g sodium acetate) extraction kit. Cleanups were performed as follows: Cleanup 1, 50 mg PSA; cleanup 2, 50 mg PSA + 50 mg C_{18}; cleanup 3, 50 mg PSA + 50 mg GCB; cleanup 4, 50 mg PSA + 50 mg C_{18} + 50 mg GCB; and cleanup 5, 50 mg C_{18}.

3.5. Applying the Analytical Method

Table 4 lists the analysis data for sulfonylurea herbicides in ten real strawberry samples collected from local markets in Wanju, Korea. Although the selected herbicides exhibited no MEs after d-SLE cleanup, matrix-matched calibration curves were prepared for more accurate quantification. All tested herbicides were present below the LODs and MRL levels recommended by Korea [5] and the EU [4] using d-SLE cleanup followed by LC-MS/MS. According to these results, the optimized d-SLE method combined with LC-MS/MS is suitable for determining multiple sulfonylurea herbicide residues in strawberry samples.

Table 4. Applying the d-SLE method to ten strawberry samples.

Sulfonylurea Herbicides	Korea MRLs [a] (mg/kg)	EU-MRLs [b] (mg/kg)	No. of Samples	
			>LOQ	>LOD
Azimsulfuron	0.01	0.01		
Chlorsulfuron	0.01	0.05		
Ethoxysulfuron	0.01	0.01		
Flucetosulfuron	0.01	NA		
Halosulfuron-methyl	0.01	0.01		
Imazosulfuron	0.01	0.01		
Metazosulfuron	0.01	NA		

[a] Maximum residue limit (MRL) on Food Code [5]. [b] European Union maximum residue level [4]. NA, MRL not currently available for strawberry analyzed. LOD, limit of detection. LOQ, limit of quantification.

4. Conclusions

We demonstrated that an optimized d-SLE cleanup procedure in combination with LC-MS/MS can be used to determine multiple sulfonylurea herbicides residues in strawberries. The d-SLE method positively influenced strawberry-extract cleanup, providing good linearity, precision, and accuracy for each sulfonylurea herbicide examined. The LOQs are compliant with Korea and EU MRLs. The d-SLE method in combination with LC-MS/MS was subsequently applied to monitor herbicide residues in ten strawberry samples, with no MRL exceedances observed for any tested sample. We suggest that d-SLE using C_{18} as the adsorbent is an alternative method for quantitatively analyzing sulfonylurea herbicides that remain in strawberries. However, further study is needed in order to determine the applicability of the d-SLE method to other crops, such as rice and wheat, among others, where sulfonylurea herbicides are mainly used.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/8/7/273/s1, Figure S1: Dispersive solid–liquid extraction coupled with LC-MS/MS for the determination of sulfonylurea herbicides in strawberries.

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Development and Application of a Novel Pluri-Residue Method to Determine Polar Pesticides in Fruits and Vegetables through Liquid Chromatography High Resolution Mass Spectrometry

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Abstract: Nowadays, highly polar pesticides are not included in multiresidue methods due to their physico-chemical characteristics and therefore, specific analytical methodologies are required for their analysis. Laboratories are still looking for a pluri-residue method that encompasses the largest number of polar pesticides. The aim of this work was the simultaneous determination of ethephon, 2-hydroxyethylphosphonic acid (HEPA), fosetyl aluminum, glyphosate, aminomethylphosphonic acid (AMPA), N-acetyl-glyphosate and N-acetyl-AMPA in tomatoes, oranges, aubergines and grapes. For that purpose, an ultra high performance liquid chromatography (UHPLC) coupled to a high resolution single mass spectrometer Orbitrap-MS were used. Different stationary phases were evaluated for chromatographic separation, and among them, the stationary phase Torus DEA provided the best separation of the selected compounds. The QuPPe method was used for the extraction of the analytes, but slight modifications were needed depending on the matrix. The developed method was validated, observing matrix effect in all matrices. Intra- and inter-day precision were estimated, and relative standard deviation were lower than 19%. Recoveries were satisfactory, and mean values ranged from 70% to 110%. Limits of quantification were between 25 and 100 μg kg^{-1}. Finally, the analytical method was applied to different fruits and vegetables (oranges, tomatoes, aubergines and grapes).

Keywords: high polar pesticides; UHPLC-Orbitrap-MS; QuPPe; pluri-residue analysis

1. Introduction

High polar pesticides have different physico-chemical characteristics compared with other pesticides, and therefore, they are not included in multiresidue methods, and a pluri-residue method is required for their simultaneous analysis [1]. The main problems are unsuitable extraction, due to low or null affinity for the organic phase, and the incompatibility with conventional reverse phases because these compounds are poorly retained, and therefore, bad peak shapes are obtained. In order to sort out these analytical problems, ion pairing agents [2,3] or derivatization processes [4–6] are used. However, in the last few years, the most common strategy is based on using new stationary phases, due to the fact that derivatization increases the manipulation of the sample, increasing errors associated to this step.

The European Reference Laboratory (EURL, EU Reference Laboratory) developed a generic method, named Quick Polar Pesticides Methods (QuPPe) [7], based on the extraction of polar pesticides

from the sample with acidified methanol and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). This was used for the determination of polar pesticides in different food [8] or biological matrices [9]. The QuPPE method proposes the use of different stationary phases such as graphitized porous carbon (Hypercarb), hydrophilic interaction liquid chromatography (HILIC) or ionic exchange. However, the authors themselves [7] pointed out numerous issues that require additional studies, such as: (i) interaction of the analytes with the active sites of the column and deterioration of the stationary phase; (ii) similar *m/z* transitions of several compounds; (iii) degradation of fosetyl-Al and ethephon to phosphonic acid and (iv) possible "matrix effects" that could affect the quality of the results, among others.

The QuPPe method was modified by several authors. Some of them used additional cleaning steps with OASIS cartridges [10,11] or carbon nanotubes [12]. Other authors utilized alternative methods, such as extraction with acetonitrile and *n*-hexane for the extraction of ethephon from tomatoes [13], or the use of an aqueous solution of ethylenediaminetetraacetic acid (EDTA) acidified with acetic acid to improve the extraction of glyphosate, glufosinate and AMPA from grapes [11].

In relation to chromatographic separation, the current bibliography provides various alternatives. For the determination of glyphosate, glufosinate and their metabolites, Hypercarb [14,15] or HILIC [16,17] stationary phases were used. Alternatively, for glyphosate, ethephon and fosetyl-Al, mixed mode columns were tested, such as Acclaim Trinity Q1 [7], due to its versatile retention mechanism, and Obelisc N [18], obtaining better retention time reproducibility and robustness than those obtained with HILIC stationary phases. However, for the simultaneous determination of different polar pesticides, including glyphosate and glufosinate metabolites among others, HILIC provided the best results, while for glyphosate and AMPA, the mixed-mode separation column Obelisc N [16] offered the best values in terms of retention times and peak shape reproducibility. Other authors eliminated the chromatographic separation stage for the simultaneous analysis of different polar pesticides (ethephon, fosetyl-Al, glyphosate, glufosinate and metabolites), although a strong matrix effect was observed and this required a high dilution of the extract, and therefore, analytical sensitivity was affected [19]. In recent years, the use of supercritical fluid chromatography has also been proposed for the simultaneous separation of compounds with a wide polarity range, including quats and fosetyl-Al, using typical reverse phase columns [20]. Other approaches such as the use of parallel columns (HILIC and C18) [21] or ion chromatography [8,22,23] were also tested, as well as the use of isotopic labelled internal standards for each analyte, but this approach increases the cost of the analysis [24].

The aim of this study was the development of a pluri-residue method for the simultaneous determination of polar pesticides (ethephon, fosetyl, glyphosate) and metabolites (2-hydroxyethylphosphonic acid (HEPA), aminomethylphosphonic acid (AMPA), n-acetyl-AMPA, n-acetyl-glyphosate) in different matrices (fruits and vegetables), testing different stationary phases as well as introducing some modifications to the QuPPe method. For the detection of the compounds, a high resolution mass spectrometry (HRMS) analyzer was used bearing in mind that high mass accuracy monitorization of the characteristic ion and fragments can be performed [15,25], increasing the reliability of the identification process.

2. Materials and Methods

2.1. Reagents and chemicals

Ethephon, HEPA, fosetyl-Al, glyphosate, N-acetyl-glyphosate, AMPA, and N-acetyl-AMPA reference standards were purchased from Dr Ehrenstorfer GmbH (Schlosser, Augsburg, Germany). N-Acetyl-d3-glufosinate, used as the internal standard, was acquired from Sigma-Aldrich (Saint Louis, MO, USA). Purity of all compounds was ≥99.7%.

Stock standard solutions of each compound (1 mg mL^{-1}) were prepared by exact weighing of the solid substances and dissolved in 50 mL of solvent (methanol or a mixture of methanol:water),

according to the instructions provided by EURL [7], and they were stored at −18 °C without being exposed to light. Then, a working standard solution (at 10 mg L^{-1}), containing the polar pesticides, was prepared in an aqueous solution (10% acetonitrile) and was stored as the stock standard solutions. The stock standard solutions were stable up to one year and working standard solutions were prepared every two months.

LC-MS grade methanol, acetonitrile and water were purchased from Honeywell (LC-MS grade, Morrison, NJ, USA) while ultrapure water was obtained by a Milli-Q water gradient system (Millipore, Bedford, MA, USA). Formic acid was purchased from Fisher Scientific (Erembodegem, Belgium).

Finally, 0.22 µm nylon syringe filters were used and they were acquired from Agilent Technologies (Santa Clara, CA, USA).

2.2. Apparatus and Instrument

A Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was used to extract polar pesticides from the samples. WX vortex from Velp Scientifica (Usmate, Italy) and a Polytron PT 2100 from Kinematica (Luzern, Switzerland) were utilized for the homogenization of the samples. To centrifuge the extracts, a Centronic-PL II centrifuge from JP Selecta (Barcelona, Spain) was used.

For the analysis of the targeted compounds, Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was utilized. LC system was coupled to a high resolution single mass spectrometer Exactive-Orbitrap analyzer (Thermo Fisher Scientific, Bremen, Germany) and ionization was performed using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA).

The chromatographic separation was carried out with a Torus DEA column (100 × 2.1 mm, 1.7 µm particle size) (Waters, Milford, MA, USA). Moreover, four columns were also tested during the optimization of the method: Obelisc N (100 × 2.1 mm, 5 µm particle size) (Sielc, Wheeling, IL, USA), HILIC-A (250 × 4.6 mm, 3 µm particle size) (ACE, Aberdeen, Scotland), HILIC-B (250 × 4.6 mm, 3 µm particle size) (ACE) and Zorbax HILIC Plus (100 × 2.1 mm, 3.5 µm particle size) (Agilent, Santa Clara, CA, USA).

2.3. Samples Collection

Samples were obtained from local supermarkets located in Almeria (Spain). The analyzed samples were tomato (n = 10), orange (n = 10), aubergine (n = 10) and grapes (n = 10). The total number of analyzed samples was 40.

2.4. Sample Preparation

Extraction method is based on QuPPe method [7] with some modifications. Briefly, 10 g of sample was weighed in 50-mL polypropylene centrifuge tubes. For orange and aubergine, 1.5 mL and 1 mL of water were added respectively before the addition of 10 mL of acidified methanol (1 % formic acid). The tubes were homogenized with polytron for 1 min, and then, in a rotatory agitator for 10 min. After that, the mixture was centrifuged at 4000 rpm for 5 min. Finally, 1 mL of the supernatant was filtered into a 0.22 µm nylon syringe filter and injected into the LC system.

2.5. UHPLC-Orbitrap-MS Analyses

The chromatographic separation was performed using a mobile phase that comprises water (0.9% formic acid) as eluent A, and acidified acetonitrile (0.9% formic acid) as eluent B. The gradient elution started at 10% of A and raised to 90% A in 6 min. This composition was held for 16 min, then decreased to 50 % for 2 min and came back to initial conditions (10% A) for 2 min. Finally, this composition was held for 1 min. Column temperature was kept at 50 °C, injection volume was 10 µL, the flow rate was set at 0.5 mL min^{-1} and the analysis time was 27 min.

The ESI parameters for the spectrometric detection were as follows: spray voltage, 4 kV; sheath gas (N_2, >95%), 10 (adimensional); capillary voltage, −35 V; skimmer voltage, 18 V; tune lens voltage,

95 V; capillary temperature, 300 °C; heater temperature, 305 °C. Two alternating acquisition functions were used: (1) full MS, ESI^-, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 full width at half maximum (FWHM); scan time = 0.25 s and (2) all-ions fragmentation (AIF), ESI^-, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s. Mass range in the full scan experiment was set at *m/z* 50–500.

The chromatograms and spectra were processed using Xcalibur™ 7.0 (Thermo Fisher Scientific, Les Ulis, France).

2.6. Validation

Validation of the optimized method was carried out using the SANTE guidelines [26]. Linearity, matrix effect, limit of quantification (LOQ), trueness, intra- and inter-day precision were evaluated.

To study the linearity of the proposed method, matrix-matched calibration was built, and blank extracted samples were spiked at several concentrations: 25, 50, 100, 50, 1000 $\mu g\ kg^{-1}$.

Equation (1) was used to calculate the percentage of matrix enhancement or suppression:

$$\text{Matrix effect } (\%) = \left[\frac{\text{slope in matrix}}{\text{slope in solvent}} - 1\right] \quad (1)$$

Matrix effect was considered negligible if it is equal to or lower than ±20%, while values higher than 20% indicate strong matrix enhancement and values lower than −20% indicate considerable matrix suppression.

Indications described in the SANTE guidelines [26] were followed for the estimation of the LOQ, defining this parameter as the lowest concentration of the analyte that has been validated with acceptable trueness (recovery ranging from 70–120%) and precision (RSD lower than 20%). Thus, spiked samples at low concentrations, from 10 to 1000 $\mu g\ kg^{-1}$, were extracted and LOQs were estimated in the four matrices evaluated.

Trueness was investigated through recovery studies spiking blank samples at two concentration levels (LOQ and 10 × LOQ), and each concentration level was analyzed five times.

Precision was evaluated by means of repeatability (intra-day precision) and reproducibility (inter-day precision). The results were expressed as relative standard deviation (RSD, %). Five replicates at two concentration levels (LOQ and 10 × LOQ) were evaluated for intra-day precision. For inter-day precision, five replicates at the same concentration levels (LOQ and 10 × LOQ) were tested for 5 days.

3. Results and Discussion

3.1. Optimization of High Resolution Mass Spectrometry

For the spectrometric characterization of the analytes, a solution of 1 $mg\ L^{-1}$ in water:acetonitrile (90:10 *v/v*) of each compound was injected into the LC-HRMS system. This analysis was performed in negative ionization mode. The flow rate was 0.2 $mL\ min^{-1}$ and the mobile phase was composed of a mixture of water:acetonitrile (50:50, *v/v*) both with 0.9% formic acid for 2 min without a chromatographic column due to the fact that separation is not required for this step.

The exact mass of the characteristic ion was selected in the full scan mass spectrum from molecular formulae. Then, fragments were selected in the pseudo MS/MS spectrum, known as All Ion Fragmentation (AIF). The fragments have to fit the retention time of the characteristic ion, as well as the peak shape should be similar to that obtained by the characteristic ion. Figure 1 shows the high resolution mass spectrum that was obtained for glyphosate.

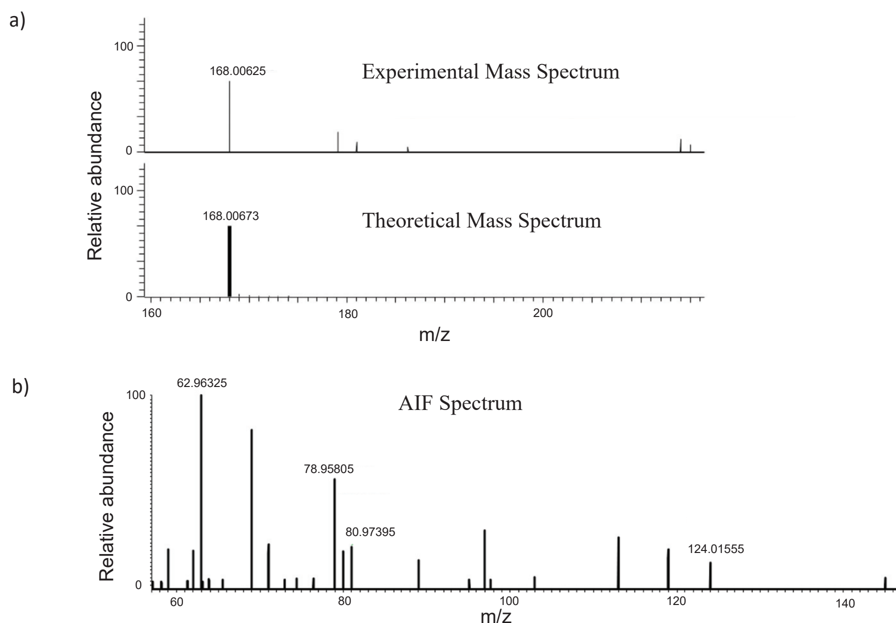

Figure 1. (**a**) Experimental and theoretical MS spectrum of glyphosate. (**b**) Pseudo MS/MS (all ion fragmentation spectrum) of glyphosate.

Spectrometric parameters for all the studied compounds are shown in Table 1, where it can be observed that at least two fragments were monitored for each compound and mass errors were always lower than 5 ppm.

Table 1. HRMS parameters for the polar pesticides.

Analyte	Characteristic Ion		Fragment Ion		
	Exact Mass	Mass Error (ppm)	Exact Mass	Molecular Formule	Mass Error (ppm)
Ethephon	142.96616	1.576	78.95795 106.98926	$[O_3P]^-$ $[C_2H_6O_4P]^-$	−0.087 −0.533
HEPA	124.99982	1.827	59.01276 78.95795 94.98926	$[C_2H_3O_2]^-$ $[O_3P]^-$ $[CH_4O_3P]^-$	2.611 0.293 −1.232
Fosetyl-Al	109.00491	0.027	62.96304 78.95795 80.97361	$[O_2P]^-$ $[O_3P]^-$ $[H_2PO_3]^-$	0.281 −0.087 0.161
Glyphosate	168.00673	3.243	62.96304 78.95795 80.97361 124.01581	$[O_2P]^-$ $[O_3P]^-$ $[H_2PO_3]^-$ $[C_2H_7O_3NP]^-$	−1.307 −2.367 3.360 −2.065
AMPA	110.00125	−0.419	62.96304 78.95795	$[O_2P]^-$ $[O_3P]^-$	0.281 −0.721
N-acetyl-AMPA	152.01182	2.364	62.96304 78.95795	$[O_2P]^-$ $[O_3P]^-$	1.393 0.926
N-acetyl-glyphosate	210.01730	2.000	62.96304 124.01581 148.01581	$[O_2P]^-$ $[C_2H_7O_3NP]^-$ $[C_4H_7O_3NP]^-$	1.711 −0.936 3.329
N-acetyl-d3-glufosinate (ILIS)	225.07196	1.832			

The fragmentation of the different compounds was evaluated and for instance, ethephon's fragments are due to the loss of a molecule of hydrochloric acid (106.98926 *m/z*) followed by loss of ethene (78.95795 *m/z*). HEPA loses a molecule of water as well as ethene to give the metaphosphate ion (78.95795 *m/z*). In addition, two fragments were monitored for HEPA, corresponding to the loss of formaldehyde and metaphosphoric ion, 94.98926 and 59.01276 *m/z* respectively. For fosetyl aluminum,

metaphosphite ion (62.96304 *m/z*) is obtained by loss of ethanol, whereas the ion corresponding to metaphosphate (78.95795 *m/z*) was monitored because the loss of ethane; moreover, the characteristic ion of phosphonic acid (80.97360 *m/z*) is obtained by the loss of an ethene molecule.

A similar fragmentation pathway was obtained for these compounds because they belong to the same family of organophosphate pesticides. Therefore, there are common fragments as 62.96304 or 78.95795 *m/z*, which were observed for fosetyl aluminum, glyphosate, AMPA and N-acetyl-AMPA fragmentation (Table 1).

Additionally, spectrometric parameters were optimized, such as spray voltage 2.5, 3, 3.5 and 4 kV; capillary temperature 150, 200 and 300 °C and capillary voltage: −20, −25 and −35 V. The optimal parameters were 4 kV, 300 °C and −35 V, respectively.

3.2. Optimization of Chromatographic Separation

Orbitrap allows the identification of coeluting compounds with high accuracy based on the exact masses, but optimal chromatographic separation is necessary due to the presence of common fragments and an unequivocal identification of each compound is mandatory.

In this study, five different stationary phases (described in Section 2.2) were evaluated according to bibliography and a summary of the tested conditions are shown in Table S1 (see supplementary material). For optimization purposes, 10 µL of a mix solution of the compounds (1 mg L^{-1}) were injected.

The first chromatographic column tested was Obelisc N, which was used for the analysis of polar pesticides. According to Botero-Coy et al. [15], water (0.1% formic acid) and acetonitrile were used as the mobile phase, and an isocratic mode proposed previously [16] with the aqueous phase (water 0.1% formic acid) and acetonitrile (20:80, *v/v*) was checked. First, the flow rate was set at 0.3 mL min^{-1} for 4.5 min, and then it was increased to 0.8 mL min^{-1} for 15 min. The temperature of the chromatographic column was 50 ºC. The obtained chromatogram is shown in Figure S1 (see supplementary material) and it can be observed that the signal of glyphosate was not sensitive and peak shape of AMPA was not acceptable, as well as high noise was observed for ethephon.

Then, the HILIC-A stationary phase was tested. The same mobile phase checked before was used (see Table S1). In this case, an elution gradient was tested, starting at 100% of acetonitrile, which was kept constant for 5 min, and then decreased to 60% for 1 min and it was held for 17 min before coming back to the initial conditions in 1 min. Finally, 20 min was used as post-equilibration time to allow the column to equilibrate prior to the next injection [27]. The flow rate was 0.3 mL min^{-1}. According to the results shown in Figure S2, this column provided better peak shape and sensitivity, but an adequate separation of the analytes was not achieved, since some compounds as HEPA, glyphosate and N-acetyl-glyphosate coeluted. Other gradient profiles were tested (data not shown) but similar results were obtained.

Then, the HILIC-B stationary phase was evaluated as well as Zorbax HILIC Plus. The same mobile phases, elution gradient and post-equilibration time tested for HILIC-A, were used. Applying these conditions, no chromatographic peaks were obtained for the analytes of interest when HILIC-B was used, as these may have eluted without interaction with the active sites of the column. When the Zorbax HILIC Plus column was checked, chromatograms were obtained for the analytes but there was no chromatographic separation and broad peaks were observed for some of them (Figure S3). It can be observed that different results were obtained when different HILIC stationary phases were tested indicating that a different mechanism could be involved in the polar analyte partitioning [28].

Finally, the Torus DEA stationary phase from Waters was tested. It was developed for the separation of polar compounds, as those included in this study, and two elution gradients were tested. On the one hand, Method A comprises of (A) 50 mM ammonium formate aqueous solution (0.9% formic acid), and (B) acetonitrile (0.9% formic acid) as the mobile phase. The gradient elution started at 10% of A and increased to 60 % in 4.5 min. This composition was held for 11 min before coming back to initial conditions (10% A) in 1 min. Finally, this composition was held for 1 min. On the other hand, Method B uses (A) water (0.9% formic acid) and (B) acetonitrile (0.9% formic acid) as

components of the mobile phase. The gradient elution started at 10 % of A and increased to 85 % A in 4 min. This composition was held for 14 min, before coming back to the initial conditions (10% A) in 1 min. Finally, this composition was held for 1 min. In both cases, column temperature was kept at 50 °C as it was recommended by Waters [29].

When Method A was tested, an adequate chromatographic separation was not achieved for all the tested compounds (see Figure S4), although narrow peaks were obtained for most of them. When Method B was tested, the chromatographic separation was achieved for the targeted analytes (Figure S5) but glyphosate and N-acetyl-glyphosate show neither suitable sensitivity nor peak shape. In order to improve the elution of these two compounds, Method B was slightly modified, and the gradient profile described in Section 2.5 was used. As it can be observed in Figure 2, suitable peak shapes and elution of the target compounds were achieved. Additionally, retention times were reduced, and for instance, for ethephon, it was decreased from 12.06 to 9.10 min. Therefore, the Torus DEA stationary phase and chromatographic conditions described in Section 2.5 were used for further analysis.

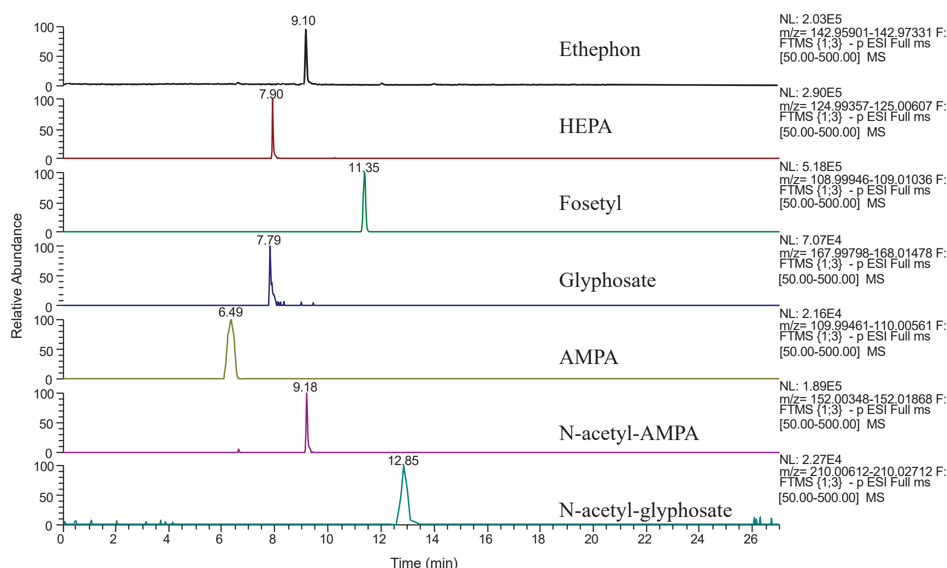

Figure 2. Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using Torus DEA and the optimized chromatographic conditions described in Section 2.5.

3.3. Optimization of Extraction Method

The extraction method was optimized using the QuPPe procedure [7] as the starting point. This procedure was tested in the four selected matrices and suitable recoveries were achieved in tomato and grape (Table 2), whereas recoveries lower than 70% were obtained in orange and aubergine. However, it was observed that for aubergine and orange, the addition of water was needed to achieve suitable results. This was also observed by previous researchers [16], as well as it is recommended by EURL laboratory [7]. Thus, 1 mL should be added to aubergine and 1.5 mL of water to orange to minimize volumetric errors and make easier the homogenization step.

Table 2. Recovery values obtained after the application of several extraction procedures.

Compound	Matrix	QuPPe	Water Addition [a]	Polytron	Internal Standard [b]
Ethephon	Tomato	119 (6)[c]		111 (2)	
	Grape	83 (12)		85 (7)	
	Orange	43 (13)	82 (6)	100 (4)	
	Aubergine	<30	<30	42 (14)	78 (10)
HEPA	Tomato	88 (8)		113 (2)	
	Grape	58 (20)		72 (14)	
	Orange	68 (14)	82 (5)	105 (5)	
	Aubergine	<30	<30	56 (23)	82 (12)
Fosetyl-Al	Tomato	92 (7)		90 (1)	
	Grape	70 (18)		79 (10)	
	Orange	58 (18)	79 (5)	95 (4)	
	Aubergine	<30	<30	56 (20)	78 (13)
Glyphosate	Tomato	90 (12)		92 (8)	
	Grape	80 (13)		84 (5)	
	Orange	65 (13)	75 (12)	73 (8)	
	Aubergine	<30	<30	39 (15)	85 (12)
AMPA	Tomato	73 (15)		76 (10)	
	Grape	71 (9)		73 (4)	
	Orange	45 (20)	84 (18)	89 (5)	
	Aubergine	<30	<30	41 (20)	79 (9)
N-acetyl-AMPA	Tomato	92 (17)		96 (11)	
	Grape	89 (6)		91 (4)	
	Orange	65 (12)	81 (23)	79 (11)	
	Aubergine	<30	<30	51 (23)	94 (8)
N-acetyl-glyphosate	Tomato	82 (14)		84 (11)	
	Grape	72 (9)		75 (6)	
	Orange	45 (29)	65 (25)	72 (13)	
	Aubergine	<30	<30	52 (19)	80 (10)

[a] Evaluated only in orange (1.5 mL) and aubergine (1 mL); [b] Evaluated only in aubergine using n-acetyl-d3-glufosinate; [c] Relative standard deviation in brackets (n = 3).

Bearing in mind that some analytes, such as fosetyl-Al, are systemic pesticides, it is essential to quickly break down plant tissues. Therefore, the use of polytron was evaluated, and the four matrices were spiked with the targeted compounds at 250 μg kg^{-1} (three replicates) and polytron was used for 1 min, showing the results in Table 2. It can be observed that recoveries and repeatability were better when polytron was used, so it was applied for further experiments. Although recoveries for the targeted compounds were improved, they were still lower (39–71%) in aubergine, so n-acetyl-d_3-glufosinate was used as the internal standard. Thus, for this matrix, 500 μg kg^{-1} was added to the sample before the extraction and it can be observed (see Table 2) that recoveries considerably improved for the target compounds (from 78% to 94%). Therefore, in addition to polytron, the use of an internal standard was needed for the analysis of these pesticides in aubergine, whereas in the other matrices, it was not necessary.

3.4. Method Validation

The optimized method for each matrix was validated for the target compounds using current SANTE Guidelines (SANTE 12682/2019) [26].

Firstly, linearity was evaluated throughout determination coefficients (R^2) and they were >0.999 in all the cases. In addition, the standard deviation of the residuals was lower than 20%.

Regarding matrix effect (see Table 3), which was calculated using Equation (1), significant enhancement (positive) matrix effect (>20%) was observed for the tested compounds in tomato and grape matrices, whereas suppression (negative) effect (<−20%) was obtained in orange. Nevertheless, matrix effect was not significant (−20 < matrix effect < 20%) in aubergine because the addition of the internal standard. Therefore, matrix matched calibration was used for quantification of the target

compounds in tomato, grape and orange, whereas in aubergine, the addition of the internal standard was also needed.

Table 3. Validation results.

Matrix	Compound	Matrix Effect	LOQ (μg kg^{-1})	Recovery (%) [a]	Precision [b]
Tomato	Ethephon	46	25	100–70	6.7 (9.5)
	HEPA	51	25	94–97	5.2 (10.9)
	Fosetyl-Al	37	25	78–70	8.9 (9.9)
	Glyphosate	38	25	73–85	7.4 (15.6)
	AMPA	36	50	82–98	8.6 (11.3)
	N-acetyl-AMPA	20	50	103–75	9.4 (10.8)
	N-acetyl-glyphosate	34	50	98–91	5.8 (13.5)
Grape	Ethephon	36	25	97–79	7.0 (13.9)
	HEPA	44	25	82–90	8.9 (15.3)
	Fosetyl-Al	31	25	76–87	7.8 (9.7)
	Glyphosate	75	25	74–83	12.8 (15.2)
	AMPA	69	50	86–81	4.6 (8.4)
	N-acetyl-AMPA	48	50	94–89	8.2 (11.3)
	N-acetyl-glyphosate	−86	50	81–83	12.4 (15.4)
Orange	Ethephon	−33	50	77–102	11.6 (15.8)
	HEPA	−21	25	86–105	15.4 (18.3)
	Fosetyl-Al	−27	25	91–88	9.9 (11.3)
	Glyphosate	−26	50	102–79	9.9 (14.4)
	AMPA	−40	100	90–74	6.7 (10.8)
	N-acetyl-AMPA	−31	100	77–80	7.2 (10.3)
	N-acetyl-glyphosate	−43	100	86–88	7.8 (10.9)
Aubergine	Ethephon	18	50	92–108	9.7 (13.5)
	HEPA	14	25	97–86	9.6 (15.3)
	Fosetyl-Al	21	25	90–105	8.4 (13.5)
	Glyphosate	25	50	95–85	12.4 (15.2)
	AMPA	14	100	102–91	5.6 (12.0)
	N-acetyl-AMPA	18	100	110–104	9.1 (16.3)
	N-acetyl-glyphosate	17	100	93–100	4.6 (8.4)

[a] Recovery values at LOQ and 10 times LOQ.; [b] Intraday precision at LOQ. Inter-day precision at LOQ is given in parenthesis. In both cases, n = 5.

LOQs ranged between 25 and 100 μg kg^{-1}, depending on the combination of compound/matrix evaluated. The highest values were obtained for AMPA, n-acetyl AMPA and n-acetyl glyphosate in orange and aubergine. Nevertheless, these values are equal to or lower than the MRLs set by the EU [30] for these matrices. For instance, the lowest MRL set by the EU for this type of compounds is 50 μg kg^{-1}, which was established for ethephon in orange and aubergine.

The average recoveries ranged between 70–103% in tomato, 74–97% in grape, 74–105% in orange and 85–110% in aubergine (Table 3). Overall, recoveries were suitable for all matrices due to the fact that these values are between 70–110%.

Intra-day precision was always equal to or below 9.4% in tomato, 12.8% in grape, 15.4% in orange and 12.4% in aubergine, whereas inter-day precision was always below 19.0% for the tested compounds in the four matrices evaluated (Table 3). Although these values are slightly higher than those obtained by ion chromatography [19], they are lower than 20%, which is the maximum level set by SANTE guidelines [26] and similar to those obtained in previous studies [15].

3.5. Sample Analysis

The analytical method was applied to the analysis of the target compounds in 40 samples (10 samples from each matrix). In order to ensure the reliability of the results, an internal quality control was used. Thus, a reagent blank, a matrix blank, a spiked sample at the LOQ of the target

compounds, and a matrix matched calibration were injected in every sequence of samples in order to check the stability of the proposed method.

The compounds were not detected in the analyzed samples. Figure 3 shows the extracted ion chromatograms of a spiked aubergine sample at LOQ, and it can be observed that suitable peak shape was obtained for the target compounds and no interferences were detected.

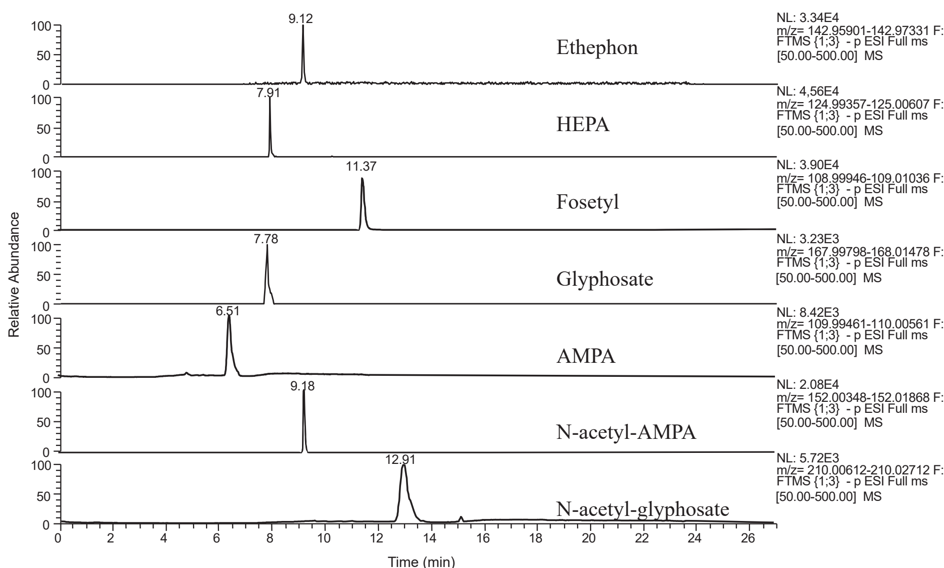

Figure 3. Extracted ion chromatograms from a spiked aubergine sample (100 μg kg^{-1}) of the targeted compounds.

Finally, and comparing the proposed methodology with previous methods, it must be highlighted that the derivatization procedure is not needed [6], simplifying sample handling. Additionally, similar sensitivity than that obtained by ion chromatography can be achieved [23] with a shorter running time, and a higher number of compounds can be analyzed simultaneously than using other conventional columns as Hypercarb [15].

4. Conclusions

A pluri-residue method was developed and validated for the simultaneous determination of polar pesticides in fruits and vegetables. After evaluation of different stationary phases, TORUS DEA column shows an adequate separation of the analytes. Despite the fact that QuPPe is a well-established procedure for the extraction of the targeted compounds from fruits and vegetables, several modifications, such as the use of polytron, were performed in order to improve the recovery of polar pesticides from different matrices. Additionally, it was noted that different amounts of water should be added to the sample depending on the type of matrix. For the detection of the compounds, high resolution single mass spectrometer, such as an Exactive-Orbitrap analyzer, provided a reliable identification, taking into account that in addition to the characteristic ion, at least two fragments were monitored per compound. Additionally, retrospective analysis can be performed in order to detect suspect compounds. The proposed method is an interesting alternative to previous methodologies, considering that shorter running times were achieved for the analyses of a higher number of compounds at concentrations equal to or below the MRL set by EU.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/5/553/s1, Table S1. Chromatographic conditions tested during the optimization of the LC method; Figure S1: Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using Obelisc N as stationary phase; Figure S2: Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using HILIC-A as stationary phase; Figure S3: Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using Zorbax HILIC Plus as stationary phase; Figure S4: Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using TORUS DEA as stationary phase and applying method A as gradient profile; Figure S5: Extracted ion chromatograms of a standard solution of the targeted compounds (1000 μg L^{-1}) using TORUS DEA as stationary phase and applying method B as gradient profile.

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foods

Article

Dynamics of the Degradation of Acetyl-CoA Carboxylase Herbicides in Vegetables

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Abstract: (1) Background: Aryloxyphenoxy-propionates and cyclohexanediones are herbicides most widely used in dicot crops worldwide. The main objective of the study was to determine the dynamics of herbicide residues in carrot, lettuce, cauliflower, and onion in order to suggest a low level of residues in harvested vegetables. (2) Methods: Small plot field trials were carried out in four vegetables in the Czech Republic. The samples of vegetables were collected continuously during the growing season. Multiresidue methods for the determination of herbicide residues by LC-MS/MS were used. Non-linear models of degradation of individual herbicides in vegetables were calculated using the exponential decay formula. Action GAP pre-harvest intervals for the 25% and 50% maximum residue limit (MRL) and 10 μg kg^{-1} limit (baby food) were established for all tested herbicides. (3) Results: The degradation dynamics of fluazifop in carrot, onion, and cauliflower was significantly slower compared to quizalofop and haloxyfop. The highest amount (2796 μg kg^{-1}) of fluazifop residues was detected in cauliflower 11 days after application. No residue of propaquizafop and cycloxydim was detected in any vegetable samples. (4) Conclusions: Aryloxyphenoxy-propionate herbicide (except propaquizafop) could contaminate vegetables easily, especially vegetables with a short growing season. Vegetables treated with fluazifop are not suitable for baby food. Lettuce and cauliflower treated by quizalofop are not suitable for baby food, but in onion and carrot, quizalofop could be used. Propaquizafop and cycloxydim are prospective herbicides for non-residual (baby food) vegetable production.

Keywords: herbicide residues; non-residual production; low-residual production; pesticide degradation in vegetables

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1. Introduction

Aryloxyphenoxy-propionates (FOPs) and cyclohexanediones (DIMs) are the most commonly used leaf graminicides (herbicides against grass weeds) in dicotyledonous (dicot) crops [1–3]. Herbicides of both chemical groups block the conversion of acetyl-CoA to malonyl-CoA by inhibiting the activity of the enzyme acetyl-CoA carboxylase (ACCase). This inhibition of fatty acid synthesis blocks the production of phospholipids used in building new cell membranes required for cell growth [4]. Dicot plants are naturally tolerant of these herbicides because of an insensitive ACCase, but tank-mix combinations with other herbicides could cause great damage to treated crops, especially under unsuitable weather conditions [5].

FOPs are formulated and applied as an ester of their acids. Emulsifiable concentrate is a common formulation for these herbicides. After application, esters are rapidly converted to acids by carboxyesterase activity. Acid forms are readily translocated to the meristematic tissues through the phloem. This process inhibits the growth of young developing leaves of susceptible grass weeds [6]. Necroses of the growing points are visible two weeks after application depending on the temperature.

FOPs are applied post-emergently because they are taken up by the leaves of plant, but some herbicide will also fall to the soil [7]. The soil fate of FOPs will depend on soil pH, with slower degradation in alkaline soils [8]. There is also a concern of water being contamination with these herbicides, as documented in Greek surface waters with quizalofop [9] and Brazilian rivers with haloxyfop [10]. On the other hand, Mantzos et al. [11] showed minimal risk of the contamination of the soil and adjacent water by quizalofop. These herbicides had transient, harmful effects on most of the soil's microbiological parameters [12].

Conventional farming practices include the use of pesticides within an integrated pest management program for crop protection against diseases, pests, and weeds. However, pesticides are potentially toxic to humans and can have both acute and chronic health effects, depending on the quantity and ways in which a person is exposed [13]. For this reason, it is necessary to model pesticide distribution in harvested crops [14]. Maximum residue limits (MRLs) for pesticides in crops were established by the European Union in a regulation of the European Commission [15] as the highest level of pesticide residues that are tolerated in food. Low-residual production is a designation for agricultural production where residues of used pesticides in harvested products are below the limit for a predetermined action threshold, for example 25% MRL or 50% MRL [13,16]. Furthermore, non-residue production is a classification for products with pesticide residues below the limit of 10 μg kg^{-1}. This limit is currently used worldwide for baby foods [17]. Some FOPs (diclofop, haloxyfop, quizalofop) contain chlorine, and these herbicides could metabolize to chlorate, especially in carrots and potatoes. These would be unacceptable for baby food products [18].

Accumulation of pesticide residues in vegetables is less frequent than in fruits [19]. Within vegetables, pesticide residues are most widely detected in pepper and cucumber, while samples of lettuce, cauliflower, and carrots seldom contained pesticide residues [20,21]. This is not to say that residues are never detected in these vegetables. Elgueta et al. [22] and Skovgaard et al. [23] detected pesticide residues in 50% of lettuce samples with 16–20% of samples above the MRL. Santareli et al. [24] also detected many lettuce samples over the MRL in Italy. Even organically managed vegetables could be occasionally contaminated by pesticide residues, especially in countries where the control of pesticide use is less strict. For example, in Brazil, a large number of organic carrot samples contain pesticide residues [25].

Most of above-mentioned studies detected insecticides or fungicides in vegetables, but few studies have monitored the contamination of vegetables by herbicides. For example, Sing et al. [26] monitored pre-emergent herbicides in carrots, but no residue was detected. Similar results published by Saritha et al. [27] did not detect any residues of metribuzin in tomato. Khan et al. [28] detected residues of linuron in onion, carrot, and lettuce. There are a few cases where pendimethalin was detected in vegetables [29,30], especially in vegetables with a short growing season like lettuce [31] or kohlrabi [32]. Risk of contamination by FOP residues is quite high because late post-emergence application is on the label, but to our knowledge, no study has focused on the degradation dynamics of FOPs yet.

The main objective of this study was to determine the degradation dynamics of herbicide residues in carrot, lettuce, cauliflower, and onion to provide suggestions for low-residue production of harvested vegetables. The specific objectives of this work were (1) to quantify the concentration of herbicide residues in tested vegetables, (2) to develop recommendations for herbicide weed control for low-residual and non-residual vegetable production, and (3) to recommend the safest leaf graminicide for each tested vegetable.

2. Materials and Methods

Small plot field trials were carried out in carrot (variety Grivola), onion (variety Wellington), lettuce (variety Elenas), and cauliflower (variety Chamborg) in a field of the University of Life Sciences, Prague, the Czech Republic (300 m a.s.l., 50°7′ N, 14°22′ E) in 2012–2016. The region has a temperate climate with an annual mean air temperature of about 9 °C and a mean annual precipitation of about 500 mm. None of the tested herbicides were used in previous crops (potatoes). Each vegetable was grown in a separate growing area

with specific agrotechnological requirements (soil preparation, fertilization, and irrigation). Common agricultural practices of the European and Mediterranean Plant Protection Organization were used. Experimental plots were arranged in randomized blocks. Plot size was 16 m^2 (2 m × 8 m) for each vegetable. Crop density, row spacing, and planting/sowing times are given in Table 1.

Table 1. Crop and plot arrangements.

Vegetable	Crop Density (Plant m^{-2})	Inter-Row Spacing (m)	Date of Planting/Sowing				
			2012	2013	2014	2015	2016
carrot	90	0.5	25.3	20.4	31.3	21.4	28.4
onion	70	0.3	29.3	15.4	12.3	30.3	-
lettuce	10	0.3	28.3	9.4	27.3	-	-
cauliflower	4	0.5	9.5	6.5	5.5	-	-

The samples of vegetable (roots of carrot, bulbs of onion, leaves of lettuce and florets of cauliflower) were collected continuously during the growing season from the central part of each plot. There was a two-week interval between the first and second sampling and between the second and third sampling. A minimum of four plants was collected from one plot during each sampling term. The samples were stored at −20 °C until the extraction procedure.

All tested herbicides (Table 2) were formulated as emulsifiable concentrates. A small-plot sprayer, fitted with a Lurmark 015F110 nozzle, was used to apply the herbicides. The application pressure was 0.25 MPa, and the water volume applied was 300 L/ha. The maximum registered rates of all tested herbicides were used. Herbicides were applied post-emergently in two terms (Table 3).

Table 2. Description of tested herbicides.

Active Ingredient (ai)	Trade Name	Concentration of ai (g L^{-1})	Application Rate (g ha^{-1} ai)	Manufacturer
cycloxydim	Stratos Ultra	100	200	BASF
fluazifop	Fusilade Forte	150	300	Syngenta
haloxyfop	Gallant Super	104	104	Corteva
propaquizafop	Agil	100	150	ADAMA
quizalofop	Targa Super	100	250	Chemtura

Table 3. Term of herbicide application in experimental years.

Vegetable	Growth Stage	Date of Herbicide Application				
		2012	2013	2014	2015	2016
carrot	5 TL [1]	27.6	24.6	16.6	17.6	16.6
	7 TL	9.7	7.7	23.6	30.6	28.6
	9 TL	-	15.7	7.7	-	-
onion	6 L [2]	11.6	7.7	11.6	17.6.	-
	9 L	27.6	22.7	7.7	13.7.	-
lettuce	4 WAP [3]	15.5	13.5	23.5	-	-
	6 WAP	25.5	29.5	3.6	-	-
cauliflower	6 WAP	19.6	24.6	23.6	-	-
	8 WAP	27.6	7.7	7.7	-	-

[1] true leaves, [2] leaves, [3] weeks after planting.

Analyses of pesticide residues were performed by the testing laboratory of the University of Chemistry and Technology using the LC-MS/MS method accredited according to

the EN ISO/IEC 17025 standard [33]. The analytical method used in this study is based on EN standards [34].

In brief, the following steps were performed: (i) alkaline hydrolysis (10 mL of acetonitrile and 2 mL of 5 M NaOH added to 10 g of homogenized sample, shaking 2 h at 40 °C); (ii) acidification (2 mL of 2.5 M H_2SO_4) and addition of 100 µL formic acid); (iii) QuEChERS like extraction (addition of 4 g $MgSO_4$ and 1 g of NaCl; and internal standard, triphenylphosphate, then, intensive shaking; centrifugation to separate acetonitrile phase for further analysis). An aliquot of the upper organic layer was transferred to a vial for LC-MS/MS. An Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 µm particle size, Waters, USA) and mobile phases consisting of (A) water with 5 mM ammonium formate/0.1% (*v*/*v*) formic acid and (B) methanol were used for ultra-high performance liquid chromatography (U-HPLC) in extract separation. A triple quadrupole mass spectrometer (Xevo TQ-S, Waters, Milford, MA, USA) with electrospray ionization in a positive ion mode (ESI+) was used for the final identification and quantification of herbicide residues (Table 4). The method used for residues analysis was fully validated in line with the requirements stated in the European Commission's guidance document SANTE/12682/2019 [35]. Limits of quantification together with maximum residue limits (MRLs) established by Regulation EC 396/2005, are summarized in Table 5. The extended uncertainty of measurement at 0.01 mg/kg level was 15%. To avoid results bias due to matrix effects, matrix-matched calibration was used.

Table 4. Mass spectrometric detector setting.

Analyte	Quantification Transition (*m*/*z*)	Cone (V)	Collision (V)	Confirmation Transition (*m*/*z*)	Cone (V)	Collision (V)
Cycloxydim	326.3 > 280.2	30	13	326.3 > 180.4	30	25
Fluazifop	328.2 > 282.1	35	20	328.2 > 91.2	35	30
Haloxyfop	362 > 315.8	27	18	362 > 91	27	30
Propaquizafop	444.2 > 100.04	30	20	444.2 > 56.2	30	15
Quizalofop	344.46 > 298.83	54	18	346.46 > 300.83	54	18

Table 5. Limits of quantification and maximum residue limit for tested herbicides and vegetables.

Herbicide	Carrot		Onion		Lettuce		Cauliflower	
	LOQ [1]	MRL [2]	LOQ	MRL	LOQ	MRL	LOQ	MRL
	µg kg^{-1}							
cycloxydim	2	5000	-	3000	2	1500	2	5000
fluazifop	2	400	1	300	1	20	1	10
haloxyfop	2	90	2	200	-	10	-	10
propaquizafop	2	200	2	40	2	400	2	200
quizalofop	2	200	1	40	1	400	1	200

[1] limit of quantification, [2] maximum residue limit.

The generated data was processed using MassLynx software version 4.1 (Waters Corporation, Milford, USA). External quality control was ensured by regular participation in proficiency tests of the European Commission's Proficiency Testing Program.

The obtained data were processed in R project version 3.6.1 (R Core Team, 2019) and subjected to the comparison analysis (*t*-test) to reflect the differences in experimental years. Non-linear models of degradation of individual herbicides in crops were calculated using the exponential decay formula in drc package using the following equation:

$$y = a(\exp(-x/b)) \quad (1)$$

where y was the amount of active ingredient (μg kg^{-1}), x was number of days after herbicide application, parameter b > 0 determined the steepness of decay, and a was the upper limit. Goodness of fit was assessed by F-test. All tests were performed using a significance level of 0.05. Parameters of models and the analytical results are shown in Table 6.

Table 6. Parameters of the exponential decay model and analytical results.

Vegetable	Active Ingredient	Parameter					
		a [1]	SE [2]	b [1]	SE	F-Test [3]	*p*-Value
carrot	fluazifop	804.09	121.91	18.82	3.39	0.55	0.91
	quizalofop	78.56	13.19	32.89	7.85	1.67	0.16
	haloxyfop	153.15	19.57	17.66	2.43	0.94	0.64
onion	fluazifop	458.81	67.95	10.30	1.18	2.28	0.12
	quizalofop	108.76	25.03	9.24	1.73	0.40	0.90
	haloxyfop	81.98	12.08	18.57	2.94	7.57	0.13
lettuce	fluazifop	1194.64	617.99	9.45	3.77	8.97	0.30
	quizalofop	262.89	120.05	29.10	21.72	3.91	0.14
cauliflower	fluazifop	5905.47	800.93	12.4	1.31	7.77	0.12
	quizalofop	107.99	33.25	26.77	10.17	4.95	0.18

[1] parameters of model - *a* represents upper limit of the curve, *b* is the steepness of the decay, [2] standard error, [3] significance $p = 0.05$.

Action pre-harvest intervals for the 50% MRL ($APHI_{50}$), 25% MRL ($APHI_{25}$), and 10 μg kg^{-1} limit ($APHI_{BF}$) were established for fluazifop and quizalofop in all four vegetables and for haloxyfop in onion and carrot. The above-mentioned equation was used to calculate the APHI (as variable x). For non-residue production, a concentration of 10 μg kg^{-1} was used. A given percentage of the MRL (50 and 25%) was calculated for low-residue production. The calculated value of APHIs was extended by one-third depending on a confidence interval of the model for each herbicide with the aim of increasing the reliability of APHIs, i.e.,

$$APHI = t + (1/3t). \quad (2)$$

The MRL parameter differs according to the active ingredient of the herbicide and the vegetable. The PHI was used when the computed APHI value extended by one-third was lower than the compulsory PHI as indicated in the list of registered products [36].

3. Results

3.1. Carrot

The degradation dynamics of fluazifop in carrot were significantly slower compared to quizalofop and haloxyfop. Relatively high differences in the degradation dynamics of fluazifop were recorded among the experimental growing season and application term, nevertheless, the differences were not statistically significant. The highest concentrations of fluazifop (above 500 μg kg^{-1}) in carrot were detected in the first two weeks after application. The degradation dynamics of quizalofop and haloxyfop were similar and any analyzed samples of onion did not contain more than 150 μg kg^{-1} (Figure 1). For fluazifop, quizalofop, and haloxyfop, the values of $APHI_{50}$ were shorter than PHI. Similarly, the values of $APHI_{25}$ for quizalofop and haloxyfop (25 and 45 days, respectively) were shorter than PHI (45 and 56 days, respectively). In such cases, the APHI was not relevant. While modeling the quizalofop curve, the negative value appeared. It was due to low observed values in the experiment and high MRL. The longest $APHI_{BF}$ (110 days) was calculated for fluazifop (Table 7). Any residue of propaquizafop and cycloxydim was not detected in any sample of carrot, regardless of the application term and year of sampling.

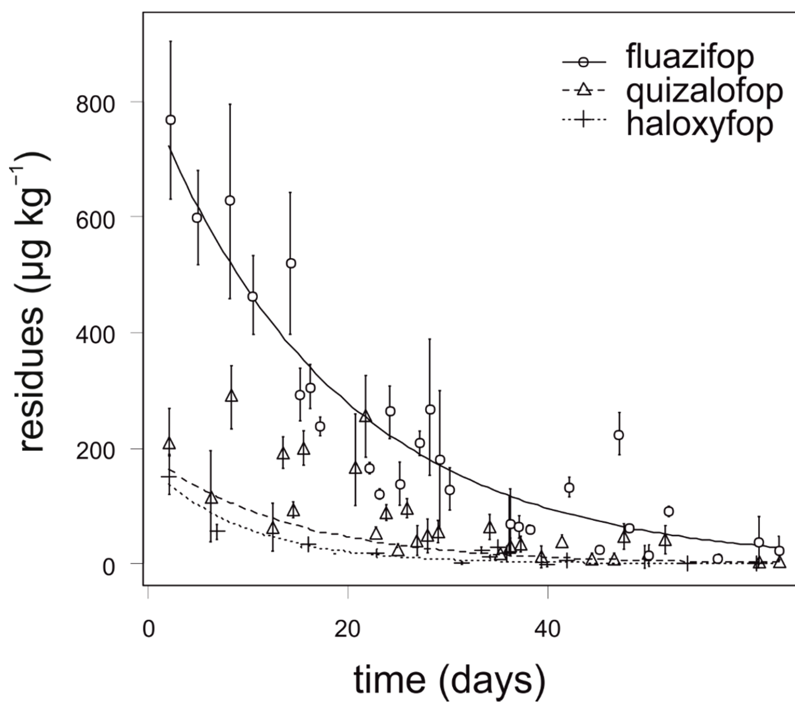

Figure 1. Degradation dynamics of fluazifop, quizalofop, and haloxyfop in carrot. Mean values with error bars representing standard error of the mean.

Table 7. Active pre-harvest interval for tested herbicides in tested vegetables for current MRL.

Vegetable	Active Ingredient	MRL [1] (µg kg^{-1})	Model (µg kg^{-1})	PHI [2] (Days)	$APHI_{BF}$ [3] (Days)	$APHI_{25}$ [4] (Days)	$APHI_{50}$ [4] (Days)
carrot	fluazifop	400	13.14	49	110	52	35
	quizalofop	200	−0.81 [5]	45	55	25	12
	haloxyfop	90	9.38	56	64	45	29
onion	fluazifop	300	4.38	28	53	25	15
	quizalofop	40	9.24	42	29	29	21
	haloxyfop	200	−9.29 [5]	28	47	15	2
lettuce	fluazifop	20	38.64	42	60	69	60
	quizalofop	400	5.22	30	71	31	19
cauliflower	fluazifop	10	79.13	56	105	128 [5]	117 [5]
	quizalofop	200	2.57	70	68	33	18

[1] maximum residue limit, [2] pre-harvest interval, [3] active pre-harvest interval for baby food (10 µg kg^{-1}), [4] active pre-harvest interval for low-residual production (25, resp. 50% MRL, [5] value is less than active pre-harvest interval for baby food (10 µg kg^{1}) due to low MRL, [5] Due to the low observed values in experiment and high MRL value, the model results in some negative predictions for quizalofop (carrot) and haloxyfop (onion).

3.2. Onion

Fluazifop showed the slowest degradation in onion, especially during the first four weeks after application. The highest concentration of fluazifop (235 µg kg^{-1}) in onion was detected 7 days after application. The degradation dynamics of quizalofop and haloxyfop was similar and all analyzed samples of onion contained no more than 70 µg kg^{-1} (Figure 2). For quizalofop, the calculated $APHI_{25}$ was equal to the $APHI_{BF}$ due to a low

MRL (40 μg kg^{-1}). For fluazifop and haloxyfop, the $APHI_{50}$ and $APHI_{25}$ were shorter than PHI. Modeled values of haloxyfop reached a negative value (Table 6); this is due to two facts. First, the MRL established by authorities is quite high and, second, the observed values did not reach such a high value. For quizalofop, the $APHI_{BF}$ (29 days) was shorter than PHI (42 days). In such cases, the APHI was not relevant (Table 7). Incidence of propaquizafop residue was not detected in any tested onion sample. Cycloxydim was not tested in onion.

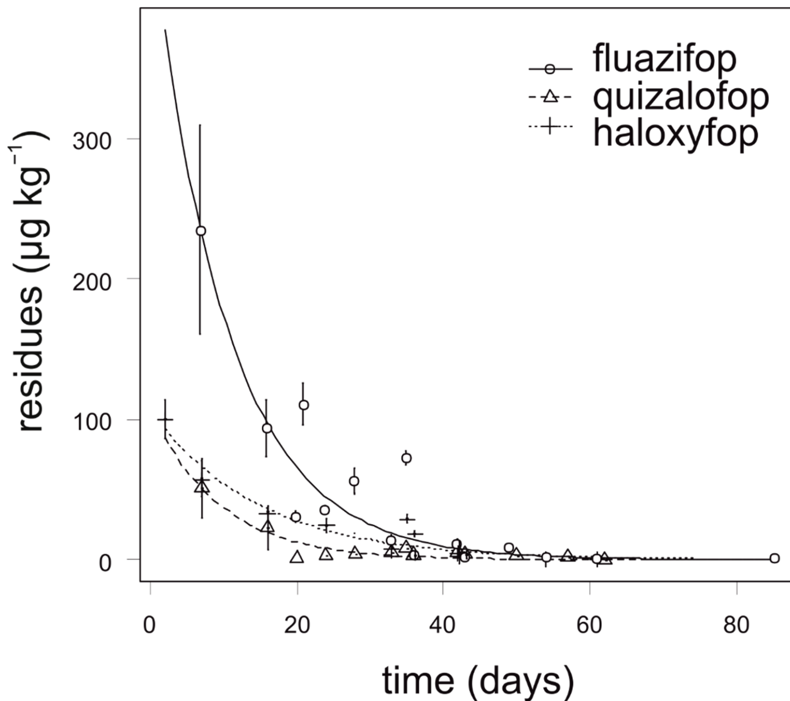

Figure 2. Degradation dynamics of fluazifop, quizalofop, and haloxyfop in onion. Mean values with error bars representing standard error of the mean.

3.3. Lettuce

During the first two weeks after application, fluazifop showed slower degradation in lettuce compared to quizalofop (Figure 3). The degradation dynamics of fluazifop were affected by the lettuce head size at the time of application. The highest concentration of fluazifop (350–550 μg kg^{-1}) in lettuce was detected when herbicide was applied on almost ripened heads. In contrast, lettuce head size had no effect on quizalofop degradation. For fluazifop, the calculated $APHI_{50}$ and $APHI_{25}$ were equal and longer than $APHI_{BF}$ (60 days) due to a low MRL (20 μg kg^{-1}). For quizalofop, the $APHI_{50}$ was not relevant because it is shorter than PHI. $APHI_{BF}$ for quizalofop was 71 days (Table 7). Residues of propaquizafop and cycloxydim were not detected in any tested lettuce sample. Haloxyfop was not tested in lettuce.

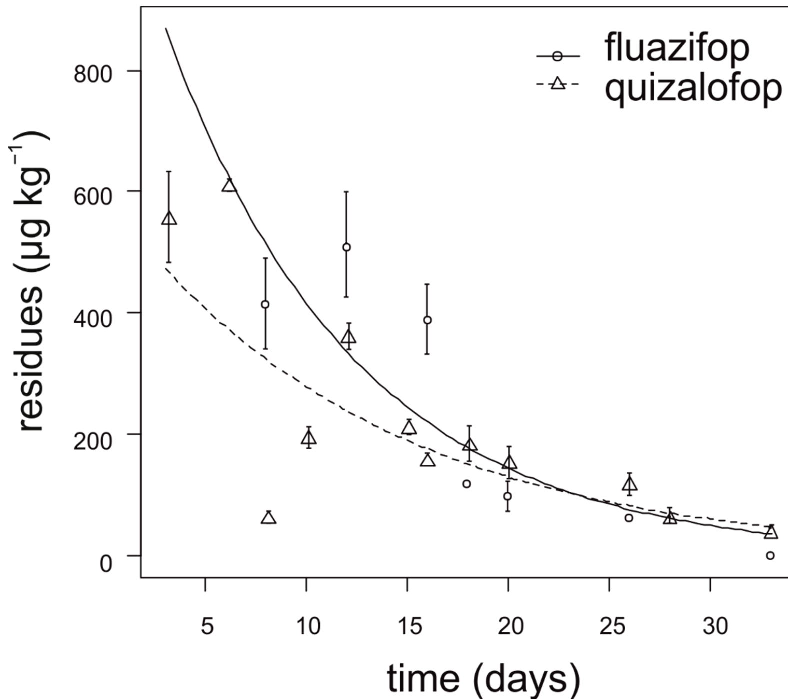

Figure 3. Degradation dynamics of fluazifop and quizalofop in lettuce. Mean values with error bars representing standard error of the mean.

3.4. Cauliflower

The degradation dynamics of fluazifop in cauliflower were significantly slower compared to quizalofop in all growing seasons (Figure 4). The concentration of fluazifop in all analyzed cauliflower samples was 18–220 times higher than MRL (10 μg kg^{-1}). In contrast, the concentration of quizalofop in cauliflower did not exceed 100 μg kg^{-1} in any tested sample. For fluazifop, the calculated $APHI_{50}$ and $APHI_{25}$ were not relevant due to a low MRL; APHI was 105 days (Table 7). For quizalofop, 18 (50% MRL), 33 (25% MRL), and 68 days (for baby food) APHIs were calculated, while the PHI is prescribed as 70 days for brassica vegetables. No residues of propaquizafop and cycloxydim were found in any tested cauliflower sample. Haloxyfop was not tested for in cauliflower.

Days needed to reach the hypothetically set-up MRL values to 10, 20, 50, 100, 200, and 500 μg kg^{1}, were calculated (Table 8). The aim here was to establish the baseline for a case in which authorities re-establish MRLs at new levels. Out of three active ingredients tested, the longest time was observed in fluazifop, regardless of the vegetable. In most cases, the model crops used in this study will obtain an MRL equal to 100 μg kg^{-1}.

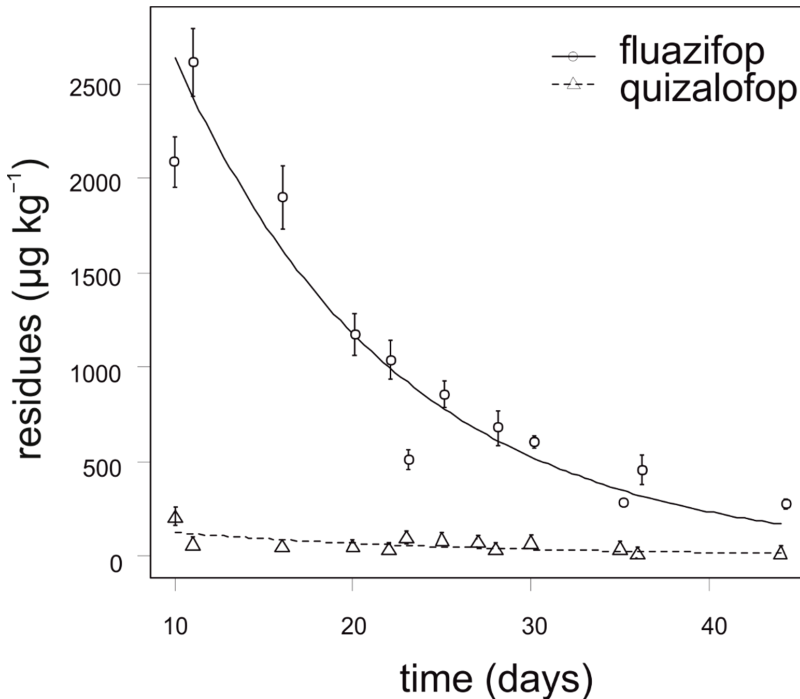

Figure 4. Degradation dynamics of fluazifop and quizalofop in cauliflower. Mean values with error bars representing standard error of the mean.

Table 8. Hypothetical pre-harvest interval for different MRL.

Vegetable	Active Ingredient	MRL 10 [1] (Days)	MRL 20 [2] (Days)	MRL 50 [3] (Days)	MRL 100 [4] (Days)	MRL 200 [5] (Days)	MRL 500 [6] (Days)
carrot	fluazifop	110	70	57	52	26	9
	quizalofop	55	45	15	12	x	x
	haloxyfop	64	36	20	10	x	x
onion	fluazifop	53	32	23	21	9	x
	quizalofop	29	16	7	1	x	x
	haloxyfop	47	26	9	1.26	x	x
lettuce	fluazifop	60	39	34	31	17	8
	quizalofop	71	65	48	31	8	x
cauliflower	fluazifop	105	83	75	67	42	31
	quizalofop	68	45	21	18	x	x

[1] maximum residue limit 10 µg kg^{-1}; [2] 20 µg kg^{-1}; [3] 50 µg kg^{-1}; [4] 100 µg kg^{-1}; [5] 200 µg kg^{-1}; [6] 500 µg kg^{-1}. In some cases, the calculated parameters reached value < 0, this is not meaningful from practical point of view, so these are marked as x.

4. Discussion

Of the tested leaf graminicides, **fluazifop** exhibited the slowest degradation dynamics in all tested vegetables. The highest amount (2796 µg kg^{-1}) of fluazifop residues was detected in cauliflower 11 days after application. Similar degradation dynamics of fluazifop were recorded by Doohan et al. in strawberry [37]. In their study, residues of fluazifop ranged between 50 and 3240 µg kg^{-1} within 12–28 days after application. The half-life of fluazifop in vegetable leaf (lettuce and spinach) was relatively low and ranged from

1.11 to 2.27 days [38]. Balinova and Lalova detected no residues of fluazifop in soybean seeds after harvest [39]; however, Sondhia detected a relatively high concentration of fluazifop both in straw (472–702 μg kg^{-1}) and seeds (297–312 μg kg^{-1}) of soybean after post-emergence application [40]. Risk of contamination of cauliflower and lettuce by fluazifop residue is relatively high due to the low MRL (10, resp. 20 μg kg^{-1}) and slow dissipation. Moreover, the growing period of these vegetables is short (less than 60 days for lettuce and less than 90 days for cauliflower) and leaf graminicides are usually used 3–5 weeks after planting crops. The MRL for fluazifop in onion and carrot is considerably higher (300, resp. 400 μg kg^{-1}), therefore, fluazifop could be used in these vegetables, along with canopies, for low-residual production (up to 25% MRL). Vegetables treated by fluazifop are not suitable for baby food due to the long APHI (53–110 days).

Concentration of **quizalofop** residues in all tested vegetable samples were below the MRL and did not exceed 400 μg kg^{-1} in lettuce and 100 μg kg^{-1} in carrot, onion, and cauliflower. Relatively low quizalofop residues in groundnut plants (104 μg kg^{-1} 30 days after application) were detected by Poonia et al. [41]. In their study, quizalofop residues decreased below detection limit (10 μg kg^{-1}) 60 days after application. In blueberry fruit, no residues were detected two weeks after split application of quizalofop [42]. The half-life of quizalofop in potato leaves ranged from 0.04 to 13.1 days in study of Wang et al., and no residues were detected in leaves and tubers at harvest [7]. Similar results were presented by Mantzos et al., who detected quizalofop residues in stems and leaves of sunflower 18 days after application, but no residues in inflorescences and seeds at harvest time [11]. In addition, in our study, the degradation dynamics of quizalofop was slower in leaves of lettuce compared to roots of carrot, tubers of onion, or florets of cauliflower. This theory confirms the study of Sahoo et al., where they reported a very short half-life of quizalofop in onion (0.85 day) and no residue was detected at harvest time [43]. The risk of contamination of tested vegetables by quizalofop residues is low because of the fast degradation dynamics. The calculated APHI for 25% MRL did not exceed PHI in any tested vegetable. Lettuce and cauliflower treated by quizalofop are not suitable for baby food due to long APHI (60, resp. 105 days), while onion and carrot could be treated by quizalofop (APHI for baby food 42, resp. 55 days).

A remarkably low quantity of residue was detected after application of **haloxyfop**. Only one sample of carrot (119 μg kg^{-1} 4 days after application) exceeded the MRL. The degradation dynamics of haloxyfop in onion was fast and no onion samples contained more than 60 μg kg^{-1}. However, concentration of haloxyfop residues in onion leaves could be significantly higher (100 and 800 μg kg^{-1} 10 days after application) [44]. Our suggested APHIs for 25% MRL did not exceed PHIs in onion and carrot. APHIs for baby food in these vegetables were relatively long (47 and 64 days, respectively), but possible.

No residues of **propaquizafop** were detected in any tested vegetable sample. Moreover, Duhan and Sing did not detect any residues of propaquizafop (detection limit 3 μg kg^{-1}) in cotton seeds and lint at harvest time [45]. No other relevant studies about the degradation dynamics of propaquizafop in vegetables have been published yet, such as the degradation dynamics of cycloxydim. Both herbicides seem to be prospective for non-residual (baby food) vegetable production.

In comparison to other herbicides in vegetables, only residues of the soil active herbicide pendimethalin have frequently contaminated lettuce [31]. Contamination of cauliflower by herbicide clomazone, clopyralid, picloram, quinmerac, metazachlor, pyridate, dimethachlor, dimethenamid-P, S-metolachlor, napropamide, and pendimethalin was not detected in the study of Suk et al. [46]. This herbicide is usually applied shortly before or after planting/sowing and, therefore, a longer PHI could be achieved. Applications of leaf graminicides were carried out later, resulting in a PHI that was actually shorter. The main result of this study is that aryloxyphenoxy-propionate herbicides (except propaquizafop) could contaminate vegetables easily, especially vegetables with a short growing season.

5. Conclusions

Aryloxyphenoxy-propionate herbicide (except propaquizafop) could contaminate vegetables easily, especially vegetables with a short growing season. Vegetables treated with fluazifop are not suitable for baby food. Lettuce and cauliflower treated by quizalofop are not suitable for baby food, but in onion and carrot, quizalofop could be used. Propaquizafop and cycloxydim are prospective herbicides for non-residual (baby food) vegetable production.

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Evaluation of Pesticide Residue Dynamics in Lettuce, Onion, Leek, Carrot and Parsley

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Abstract: The dynamics of 32 active substances contained in pesticide formulations (15 fungicides and 17 insecticides) were analyzed in iceberg lettuce, onion, leek, carrot, and parsley. Pesticide residues were monitored from the time of application until harvest. In total, 114 mathematical models of residue dissipation were developed using a first-order kinetic equation. Based on these models, it was possible to predict the action pre-harvest interval (the time between the last pesticide application and crop harvest) needed to attain a targeted action threshold (value significantly lower than the maximum limit) for low-residue vegetable production. In addition, it was possible to determine an action pre-harvest interval based on an action threshold of 0.01 mg kg^{-1} to produce vegetables intended for zero-residue production. The highest amount of pesticide residues were found in carrot and parsley leaves several days after treatment, and pesticide dissipation was generally slow. Lower amounts were found in leeks and lettuce, but pesticide dissipation was faster in lettuce. According to our findings, it seems feasible to apply reduced pesticide amounts to stay below unwanted residue levels. However, understanding the effectivity of reduced pesticide application for controlling relevant pest organisms requires further research.

Keywords: pesticide residues; low-residue production; zero-residue production; half-lives; pre-harvest interval; lettuce; onion; leek; carrot; parsley

1. Introduction

Conventional farming and integrated pest management (IPM) regimes relate to the utilization of pesticides for the protection of agricultural crops against diseases and pests. However, pesticides are potentially toxic to humans and can have both acute and chronic health effects, depending on the quantity and ways in which a person is exposed [1]. Hence, controlled use of agricultural pesticides is important, modelling pesticide distribution in crops as a tool in limiting the excessive use of pesticides [2]. Maximum residue limits (MRLs) for pesticide/commodity combinations have been established by the European Union in the Regulation of European Commission [3] as the highest level of pesticide residues that are legally tolerated in food or feed. MRLs are based on Good Agricultural Practice (GAP) data and must meet requirements for pesticide registration. A uniform MRL as low as 0.01 mg·kg^{-1} has been established by Commission Directives [4,5] for any pesticide residue in baby foods and processed cereal-based foods [6]. To meet the legal limits for pesticide residues in various food crops at a given harvest time, a pre-harvest interval (PHI) is officially established for particular pesticides defined as the time between the last pesticide application and the harvest of

the treated crop. This value is based on FAO (Food and Agriculture Organization of the United Nations) recommendation [7] and is usually fixed at a country level [8]. The aim of "zero-residue" vegetable production ("pesticide residue free production") is achieving pesticide residues in respective crops as low as 0.01 mg·kg^{-1} (or even lower). This limit corresponds to the limit used for baby food production [4]. Adopting MRLs and PHIs helps to keep residue-related health risks below an "acceptable risk level".

The most recent EU report summarizes the study of pesticide residue contamination of food carried out in the European Union (EU) Member States, Iceland, and Norway [9]. It summarizes the results of both the 2017 EU-coordinated control program (EUCP) and the national control programs (NP). To allow the assessment of representative consumer exposure to pesticide residues by food commodity, the same pattern of commodities is monitored for the presence of pesticide residues in 3-year cycles in the same countries. Regarding the 2017 EUCP, results were compared with the ones of 2014 for those commodities sampled in both years. The results of this showed some improvement in the overall situation in comparison with the results of 2014. No quantifiable residues were reported in 54.1% and 53.6% of the samples in 2017 and 2014, respectively. Quantified residues at or below the MRLs were reported in 41.8% and 43.4% of the samples in 2017 and 2014, respectively [9]. Overall, 95.9% of the samples analyzed in 2017 fell within the legal limits; nevertheless, 41.8% of the samples contained quantified residues at or below the MRLs. Nevertheless, the dietary risk assessment indicated that, for the samples analyzed, the probability of European citizens being exposed to pesticide residue levels that could lead to negative health outcomes is relatively low [9].

However, supermarkets, and thus also food crop producers, are continuously under pressure due to consumer concern about the health risks associated with the potential occurrence of pesticide residues in their diet. When seeking to rationalize pesticide use, both government [10] and supermarkets have tended to make the implicit assumption that any rationalization in this field is primarily an issue of decreasing the quantity of pesticides used, coupled with the banning of certain persistent or highly toxic substances [11,12]. Some retail chains apply for the assessment of contamination of products, mainly fruits and vegetables, originating from low-residue production. So called "action thresholds" declare the highest acceptable concentration of pesticide residues in the product corresponding to a percentage of the respective MRL, e.g., 25%. The required action thresholds commonly range from 25% to 75% MRL depending on the retail chain. In all cases, the action thresholds are fairly below the MRL. Achieving action thresholds for low-residue production or zero-residue production is based on a setting of an action pre-harvest interval (APHI), which is the minimal time between the last pesticide application and crop harvest within which the required drop in pesticide residue levels occurs. The knowledge of APHIs, which is necessary for setting relevant pesticide treatment regimes in low-residue and/or zero-residue production, needs the development of dissipation models of the active substances in pesticide preparations, based on which calculations are performed. In this context, information on the dissipation kinetics of pesticide residues in food crops and other plants is an additional key aspect of current risk and impact assessment practices. Considering more than 740 published studies, Fantke and Juraske [13] identified nine different calculation models, corresponding residual pesticide concentration curves C_t and related half-lives $t_{1/2}$. The authors concluded that pesticide dissipation in plants generally follows first-order kinetics, although some exceptions exist. Currently, experimental data are not available for all relevant pesticide−plant combinations; nevertheless, Fantke et al. [14] created a list of corrected geometric means of dissipation half-lives for 333 pesticides applied to an average plant under reference air temperature (20 °C). Half-lives $t_{1/2\ \mathrm{ref,i}}$ were calculated using model II based on dissipation data obtained for 346 pesticide residues in 183 treated plant species [13,14]. Model II also provides estimates for the influence of specific plants, which can be used to correct $t_{1/2\ \mathrm{ref,i}}$ for pesticides applied to a particular crop under given study conditions and temperature. Model III was designed to estimate dissipation half-lives from physico-chemical properties of individual pesticides of 14 substance classes for which no reference half-lives are available, i.e., they are not listed

in $t_{1/2\ ref,i}$ [14]. The model III is more uncertain as it tries to predict half-lives for chemicals not included in the fitting dataset.

Pesticide residues were analyzed in Brassica vegetables with application until harvest in order to establish mathematical models of residue dissipation and forecast the action pre-harvest interval for a given action threshold as a percentage of MRL for low-residue production in Brassica vegetables [15]. This study describes the dissipation of 22 active substances of pesticide formulations applied on Chinese cabbage, head cabbage, and cauliflower.

Experimental studies usually present aggregated dissipation data estimated from measuring the changes of the overall residual pesticide concentration in the respective plant over time. However, the overall dissipation of residues involves several processes. Jacobsen et al. [2] showed that degradation of a pesticide molecule is, in many cases, the most relevant dissipation component, followed by growth dilution and volatilization. Dissipation rate and half-lives of pesticides vary according to vegetable species and depend on plant characteristics, environmental conditions, and substance physico-chemical properties [7]. Hence, the models of pesticide dissipation are applicable in regions with similar climatic conditions and under comparable conditions of vegetable growing [2].

The main objective of the study was to evaluate dynamics of pesticide residues in iceberg lettuce, onion, leek, carrot, and parsley, and, based on generated data, to suggest a procedure enabling targeted (low) levels of pesticide residues in respective vegetable at harvest. The specific objectives of this work were (1) to quantify the dissipation rates of pesticide residues in tested vegetables, (2) to determine the $APHI_{25}$ and $APHI_{0.01}$ that would guarantee achieving "action thresholds" of 25% MRL and 0.01 mg residue kg^{-1}, respectively, for a low-residue and zero-residue production of tested vegetables, and (3) to critically assess differences in the dissipation rates and dissipation half-lives of particular pesticide residues in tested vegetable species and compare the results with available literature data.

2. Materials and Methods

Pesticide residues were analyzed in samples collected from semi-field experiments on iceberg lettuce (*Lactuca sativa* L.), leek (*Allium ampeloprasum* L.), carrot (*Daucus carota* L. subsp. sativus (Hoffm.) Arcang), and parsley (*Petroselinum crispum* (Mill.) Fuss) at the Crop Research Institute in Prague (GPS: 50.0864428N, 14.2985553E, soil type: illimerized luvisol, soil pH: 6.8) and on onion (*Allium cepa* L.) at the Czech University of Life Sciences Prague (GPS: 50.1267258N, 14.3770625E, soil type: black earth, pH: 7.3). The specific information of the geographic coordinates, soil type and soil pH are presented according to current reporting recommendations [16]. The studied vegetables were usually treated with pesticides registered for the control of vegetable diseases and pests in the Czech Republic. However, some additional pesticides permitted in neighboring countries were also selected for the case of their future use in the Czech Republic. Iceberg lettuce and both root vegetables were planted in a single season, while onion and leek were cultivated in two consecutive years (Table 1). Cultivation technologies (row and in-row plant spacing, fertilization) respected general field practices. The same pesticide application and sampling methodology were used for all tested vegetable crops. The average air temperature (min/max range) from the first chemical treatment until the last harvest was 20 °C (13.8/26.8 °C) in lettuce 2011; 18.3 °C (13.7/29.7 °C) and 17.3 °C (12.8/24.7 °C) in onion in 2012 and 2013, respectively; 9 °C (1.7/13.9 °C) in leek in 2008; 10.5 °C (1.7/19.3 °C) in leek as well as in carrot in 2009; and 12 °C (6.4/15.2 °C) in parsley in 2010. The crops were cultivated in three plots (A–C); three replications per plot were set up in a randomized complete block design. Each plot was treated with three different pesticide mixtures (Supplementary Table S1 online) on three different dates (Table 1). Thus, pesticide mixture 1 was sprayed in plot A on the first date, in plot B on the second date and in plot C on the third date of experiment. Pesticides in each mixture are marked with a superscript in Supplementary Table S1 online. The pesticide mixtures were different for each crop. The mixture composition depended on the efficacy of the pesticides in each crop. Pesticides were applied from the dates selected based on plant phenophase and the estimated harvest date. Plant stages at the time of application of pesticides were BBCH 42, 45, 45, 45, and 45 for iceberg lettuce, leek, carrot, parsley,

and onion, respectively [17]. All the pesticides were administered at maximum label application rates. In all variants, 500 L·ha^{-1} spraying with 0.1 L·ha^{-1} Silwet was used. Sample collection started three days after the last spraying. In each plot, the samples were collected on four different dates; thus, twelve samples of each vegetable were analyzed per year except for onion, where only three sample collections were performed in 2012. Crops, planting dates, varieties, pesticide application dates, and harvest dates are listed in Table 1.

Analyses of pesticide residues were performed by the testing laboratory of the University of Chemistry and Technology accredited according to the EN ISO/IEC 17025 standard. The standardized analytical method used in this study is based on the EN 12393-2 standard (Foods of plant origin-Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS-Part 2: Methods for extraction and clean-up) and EN 12393-2 (Foods of plant origin-Multiresidue methods for the determination of pesticide residues by GC or LC-MS/MS-Part 3: Determination and confirmatory tests).

Approximately 1 kg of representative vegetable samples from field experiments were delivered to the laboratory at the University of Chemistry and Technology in Prague. The edible part of the crop and the top leaves were separated and homogenized using a Retsch GM200 blender (Retsch GmbH, Haan, Germany) and stored in a freezer (−18 °C) until analysis. An ISO 17025 [18] accredited multiresidue method based on QuEChERS extraction followed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was employed, encompassing most of the examined pesticides (abamectin, acetamiprid, azoxystrobin, boscalid, chlorantraniliprole, chlorpyrifos, cymoxanil, cyprodinil, cypermethrin, deltamethrin, difenoconazole, dimethoate, dimethomorph, fludioxonil, fluoxastrobin, indoxacarb, mandipropamid, metalaxyl-M, methoxyfenozide, pirimicarb, propamocarb-hydrochloride, prothioconazole, pyraclostrobin, pyridaben, spinosad, tebuconazole, thiacloprid, thiamethoxam); for pymetrozine, a single residue method with pH adjustment of the sample was performed. Sample preparation and HPLC-MS/MS analysis were described in detail in our previous study [19], and the multiple reaction monitoring (MRM) conditions optimized for the pesticides evaluated in this study are summarized in Supplementary Table S2 online. These analytical procedures were introduced earlier by Ticha et al. [20] and subsequent validation protocol as well as the Internal Quality Control (IQC) measures followed document SANTE/11945/2015 (latest consolidated version SANTE/12682/2019) "Method Validation & Quality Control Procedures for Pesticide Residues Analysis in Food & Feed" [21], obligatorily used by the EU official control laboratories. MS measurement parameters of the optimized method are given in Table S2 (LC-based) and Table S3 (GC-based, confirmatory for GC amenable pesticides) in Supplementary Materials. The performance characteristics for all pesticides involved in this study are summarized in Supplementary Table S4 online. The mean recovery, repeatability standard deviation, and limit of quantitation (LOQ) were evaluated for each pesticide. This means that the entire analytical procedure (started by the handling of the analytical portion) is represented by the metrological characteristics given in Table S4. The performance of the accredited laboratory was verified (external quality control, QC) through participation of the laboratory in regular proficiency tests: (i) Food Analysis Performance Assessment Scheme (FAPAS®) and (ii) European Commission's Proficiency Testing Program (EU–PT).

Table 1. Crops, planting dates, varieties, as well as the dates of pesticide application and crop harvest in semi-field experiments.

Crop	Planting Date	Variety	Application Date			Harvest Date			
Lettuce [a]	6 July 2011	Diamantinus	2 August	11 August	16 August	19 August	23 August	29 August	2 September
Onion [b]	4 April 2012	Wellington	7 August	13 August	17 August	20 August	27 August	*	14 September
Onion [b]	17 April 2013	Wellington	6 August	12 August	16 August	19 August	26 August	2 September	9 September
Leek [a]	19 June 12008	Prelina	30 September	6 October	10 October	13 October	20 October	29 October	3 November
Leek [a]	20 April 2009	Bandit	21 September	28 September	2 October	5 October	12 October	19 October	26 October
Carrot [a]	15 April 2009	Nerac F1	21 September	28 September	2 October	5 October	10 October	19 October	26 October
Parsley [a]	18 April 2010	Eagle	3 September	10 September	13 September	16 September	23 September	29 September	7 October

a: semi-field experiments at the Crop Research Institute; b: semi-field experiments at the Czech University of Life Sciences Prague, *: crops not harvested in this term.

The following first-order kinetic equation was used to characterize the dissipation rate of active substances of the pesticides in crop products:

$$C_t = C_0 \times \exp(-k^{diss} \times t) \quad (1)$$

where C_t is the residual pesticide concentration (mg·kg^{-1}) at time t (days) after application; C_0 is the initial pesticide concentration; and k^{diss} is the pesticide dissipation rate constant (day^{-1}). ANCOVA was used to analyze the effect of the year and time after application on the residual pesticide concentration. If there was no significant difference between the years, the data from two years were combined. The corresponding dissipation half-life equation is:

$$t_{1/2} = \ln(2)/k^{diss} \quad (2)$$

where $t_{1/2}$ is the pesticide dissipation half-life (days), which was used for all pesticides with significant models ($R^2 > 0.5$). The model parameters of C_0 and k^{diss} were calculated from all experimentally determined residue values of each active pesticide substance in the products (C_t) and the corresponding number of days after pesticide application (t). Calculations were performed by Kruskal–Wallis test in the XLSTAT 2009 program (Addinsoft, New York, NY, USA). Our $t_{1/2}$ values were compared with corrected geometric means of estimated half-lives applied to specific plants in each set of production conditions and temperature using model II [14]. Two active substances (fluoxastrobin [22] and prothioconazole [23]) were not involved in the pesticide list needed for the $t_{1/2}$ calculation according to model II, so we used model III, which Fantke et al. [14] designed to predict the half-lives of individual pesticides for which no reference half-lives are available.

Maximum residue limits were obtained in EU Pesticides database [24]. Because the MRL of active substances used for carrot and parsley roots was the same, the missing MRL for carrot leaves was replaced by the MRL of parsley leaves. MRLs for carrot leaves are not established because carrot is grown almost exclusively for the root. However, raw young carrot leaves have recently been used not only as food for animals but also to enrich dishes such as salads or soups. "Action thresholds" for the 25% MRL ($APHI_{25}$) and 0.01 mg kg^{-1} limit ($APHI_{0.01}$) were established for 31 active substances in the pesticide formulations. To calculate the APHI, the following equation can be used:

$$t = (\ln C_t - \ln C_0)/k^{diss} \quad (3)$$

where C_t is the residual pesticide concentration in mg·kg^{-1} at time t (days) after application; C_0 is the initial pesticide concentration; and k^{diss} is the pesticide dissipation rate constant (day^{-1}). For zero-residue production, C_t = 0.01 mg·kg^{-1}; for low-residue production, C_t corresponds to a given percentage of the MRL (e.g., 25% MRL). The APHIs calculated from the pesticide dissipation models were extended by one-third based on a confidence interval of the model for active substances with the highest variability of dissipation rate to increase the reliability of APHIs, i.e.,

$$\text{APHI} = t + (1/3t) \quad (4)$$

The parameters C_0 and k^{diss} differ according to the active pesticide substance and the agricultural commodity. When the calculated APHI prolonged by one-third was shorter than the obligatory PHI according to a list of registered products [25], the PHI was used.

3. Results

3.1. Pesticide Dissipation Models, Dissipation Half-Lives and "Action Pre-Harvest Intervals"

The residues of 21 active substances were determined in lettuce, 29 in onion, 13 in leek, 9 in carrot, and 10 in parsley. The mathematical models could not be established for three pesticide active substances in onion, one on leek, two in carrot roots, and five in parsley roots because the concentration of residues in vegetables rapidly decreased after the application to very low levels. The parameters of 114 pesticide dissipation model equations and dissipation half-lives are shown in Supplementary Table S5A–E online. For seven active substances on onion, five on leek and parsley, and two on carrot, the calculation of model parameters yielded non-significant values ($R^2 < 0.5$) due to a high data variability. The results of residue analyses (mg·kg^{-1}) corresponding to the number of days in the obligatory PHI are shown in the following tables (Tables 2–6), as are $APHI_{25}$ and $APHI_{0.01}$ calculated for low-residue and zero-residue vegetable production, respectively. In cases where the models were not established because of rapid residue dissipation, $APHI_{25}$ and $APHI_{0.01}$ were identical to the PHI. Otherwise, the calculated $APHI_{25}$ and $APHI_{0.01}$ were used when their values were lower than the obligatory PHI. In several cases in which the MRL was very low (0.01–0.03 mg·kg^{-1}), the 25% MRL used to calculate $APHI_{25}$ was lower than 0.01 mg·kg^{-1}, so the calculated $APHI_{0.01}$ was shorter. At present, zero-residue production requires a pesticide residue limit of 0.01 mg·kg^{-1}, so that in the cases in which the calculated $APHI_{25}$ is longer, $APHI_{0.01}$ must be used instead of $APHI_{25}$.

The half-lives of active substances were shortest in iceberg lettuce where the mean half-life of all tested active substances was 1.43 (± 0.48), (Figure 1a). In onion, the mean half-life of all tested active substances was 4.15 (± 2.29), indicating the third fastest dissipation from all crops (Figure 1b). Leek belonged to the crops with higher half-life values. The mean half-life of all tested active substances was 6.83 (± 3.01) (Figure 1c). The mean half-lives of all tested active substances in carrot roots and leaves were 2.91 (± 1.50) and 5.87 (± 2.97), respectively (Figure 1d). The half-lives of active substances in carrot roots belonged to the shorter ones, whilst the half-lives of active substances in leaves exceeded six days in five cases. The mean half-lives of all tested active substances in parsley roots and leaves were 8.37 (± 2.63) and 5.99 (± 2.63), respectively (Figure 1e). The mean half-life of active substances in parsley root was the longest, however the concentration of pesticides detected in parsley roots was lower than 0.09 mg·kg^{-1} (Supplementary Table S7 online) and the dissipation of other five tested substances was very fast, the determination of their half-lives was impossible. The half-lives of active substances in parsley leaves were comparable to those determined for carrot leaves.

3.1.1. Iceberg Lettuce

In iceberg lettuce, 21 significant ($R^2 = 0.761-1.000$) pesticide dissipation models were generated (Supplementary Table S5A online). The half-lives in iceberg lettuce were in range 0.86 days (dimethomorph) to 2.65 days (thiamethoxam), as shown in Table S5A. The application of all active substances before PHI will allow residues below MRL (see slope model in Table 2). Dissipation models produce $APHI_{25}$ values lower or equal to the PHI. In the case of chlorpyrifos, iprodione and tebuconazole, the PHI was extended for a few days (Table 2). The calculated $APHI_{0.01}$ times for acetamiprid, chlorpyrifos, iprodione, lambda-cyhalothrin, mandipropamid, pirimicarb, pymetrozine, and thiacloprid were two to six days longer than the PHI. In tebuconazole, the limit for baby food exceeded nine days.

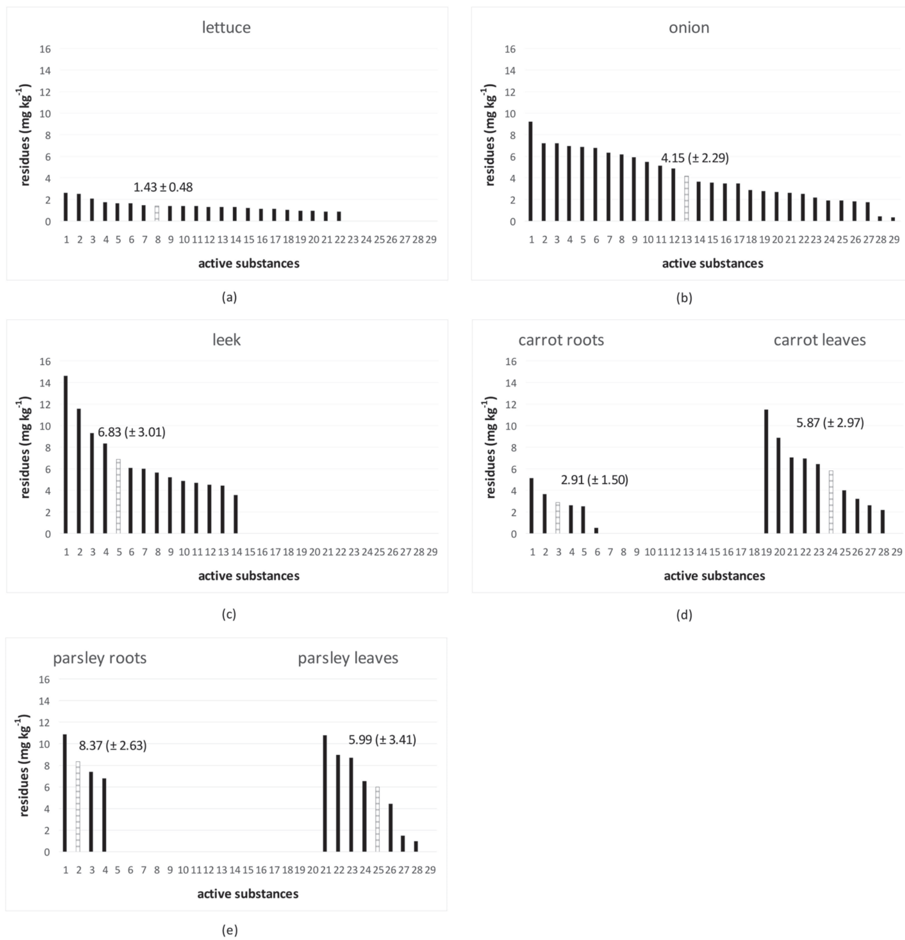

Figure 1. Half-lives of active substances (black columns) in decreasing order (**a**) lettuce; (**b**) onion; (**c**) leek; (**d**) carrot; (**e**) parsley. Mean half-live ± SD (hatched column). Units on the x axis represent the active substances listed in Supplementary Table S6 online.

Table 2. Pesticide residues in iceberg lettuce modelled in terms of the corresponding pre-harvest interval (PHI) according to a list of registered products ([25] the action pre-harvest interval (APHI) was calculated for 25% maximum residue limit (MRL)($APHI_{25}$)) and a 0.01 mg kg^{-1} limit ($APHI_{0.01}$). The MRL is cited according to the EU Pesticides Database [24].

Active Substance	MRL (mg kg^{-1})	Model (mg kg^{-1})	PHI (Days)	$APHI_{25}$ (Days) [e]	Suggested PHI for $APHI_{25}$	$APHI_{0.01}$ (Days) [e]	Suggested PHI for $APHI_{0.01}$
Acetamiprid	3	0.0342	3–7 [b]	0	7	13.2	
Azoxystrobin	15	0.0006	14	2.7	14	13.6	14
Beta-Cyfluthrin	1	0.0005	7 [b]	0	7	2.9	7
Cypermethrin	2	0.0012	14 [b]	2.9	14	13.2	14
Deltamethrin	0.5	0.0001	7–14 [b]	0.5	14	6.7	14
Difenoconazole	4	0.0007	3–14 [b]	2.1	14	12.5	14
Dimethoate	0.01 [a]	0.0006	21	21.4 [d]	21	14.7	21
Dimethomorph	15	<0.0001	21	4.3	21	11.9	21
Chlorpyrifos	0.01 [a]	0.0044	14 [b]	20.2 [d]		16.4	
Indoxacarb	3	0.0028	14	0	14	13.5	14
Iprodione	0.01 [a,f]	0.0045	14	20.3 [d]		16.5	
Lambda-Cyhalothrin	0.15	0.0093	7	4.7	7	9.1	
Mandipropamid	25	0.0067	3–14 [b]	0	14	17.4	
Metalaxyl-M	3	0.0004	14	0.1	14	10.6	14
Methoxyfenozide	4	0.0002	14 [c]	4.3	14	12.2	14
Pirimicarb	1.5	0.0095	7	0	7	9.2	
Pymetrozine	3	0.0217	7	2.9	7	10.7	
Spinosad	10	0.0001	14	0	14	8.6	14
Tebuconazole	0.5	0.1449	7 [c]	9.7		16.1	
Thiacloprid	1	0.0398	7	5.6	7	12.1	
Thiamethoxam	5	0.0056	3 [b]	0	3	1.0	3

[a] Limit corresponds to the practical limit of quantification (LOQ) of the analytical method; [b] PHI listed for pesticide application to another vegetable; for the APHI25 and APHI0.01 calculation, a longer PHI was used; [c] Currently only allowed on fruit trees (methoxyfenozide, tebuconazole) or cereals (fluoxastrobin, prothioconazole); [d] APHI25 is longer than APHI0.01; in this case, APHI0.01 (zero-residue production) must be used instead of APHI25; [e] In cases when the calculated APHI was shorter than the PHI, the recommended PHI should be followed by farmers; [f] Iprodione: MRL applicable from 31 July 2019 [26].

3.1.2. Onion

In onion, 29 active substances were analyzed, 27 of which were sprayed in two seasons, 2012 and 2013, and chlorantraniliprole and propamocarb-hydrochloride were tested in the second year only (Supplementary Table S1 online). In total, 52 significant (R^2 = 0.500–1.000) and 7 non-significant models were established (Supplementary Table S5B online). In 12 active substances, significant models were established from years as well as from both years analyzed together and only difenoconazole and fludioxonil showed differences between the years. Therefore they were evaluated in both years separately. No models were generated for three active substances (abamectin, deltamethrin, and lambda-cyhalothrin) in onion due to the low concentration of their residues in terms of harvest in both years. Similarly, no models were established for cymoxanil, prothioconazole and thiamethoxam in one year (Table S5B). Residue half-lives in onion ranged from 0.36 days (cymoxanil 2012) to 9.24 days (boscalid 2012), as shown in Table S5B. The application of active substances before the PHI will allow residues below MRL, except for iprodione and methoxyfenozide, which slightly exceeded the MRL in one year (see slope model in Table 3). In 9 active substances of pesticides, the calculated $APHI_{25}$ was longer than the $APHI_{0.01}$ due to a low MRL of 0.01–0.02 mg·kg^{-1}. In such cases, the $APHI_{25}$ was not relevant. For example, the 25% MRL "action threshold" for acetamiprid in 2013 was 13 days and the $APHI_{0.01}$ was 8 days (Table 3). In most cases, the $APHI_{25}$ was shorter or the same as the PHI. In the case of tebuconazole in 2013, the $APHI_{25}$ was longer than the PHI for 5 days (Table 3). The calculated $APHI_{0.01}$ values for 10 substances were longer than the PHI. In some cases, the difference in $APHI_{0.01}$ and PHI was even more than 20 days (boscalid 2012, tebuconazole 2013).

Table 3. Pesticide residues in onion.

Active Substance	MRL (mg kg^{-1})	Model (mg kg^{-1})	PHI (Days)	APHI$_{25}$ (Days) [e]	Suggested PHI for APHI$_{25}$	APHI$_{0.01}$ (Days) [e]	Suggested PHI for APHI$_{0.01}$
Abamectin 2012–13	0.01 [a]	x	3-7 [b]		7		7
Acetamiprid 2012	0.02 [a]	0.0013	3-7 [b]	2.3 [d]	7	0	7
Acetamiprid 2013	0.02 [a]	0.0081	3-7 [b]	13.1 [d]		7.7	
Acetamiprid 2012–13	0.02 [a]	0.0046	3–7 [b]	8.8 [d]		4.2	7
Azoxystrobin 2012	10	0.0040	14	0	14	12.7	14
Azoxystrobin 2013	10	0.0093	14	0	14	18.3	
Azoxystrobin 2012–13	10	0.0067	14	0	14	16.6	
Boscalid 2012	5	0.0360	14	0	14	41.4	
Boscalid 2013	5	0.0673 [n.s.]	14		14		14
Chlorantraniliprole 2013	0.01 [a]	0.0001	14[b]	8.6[d]	14	3.7	14
Cymoxanil 2012	0.01 [a]	<0.0001	14[b]	4.2[d]	14	3.2	14
Cymoxanil 2013	0.01 [a]	x	14[b]		14		14
Cypermethrin 2012	0.1	0.0013	14[b]	0	14	0	14
Cypermethrin 2013	0.1	0.0031 [n.s.]	14[b]		14		14
Cyprodinil 2012	0.3	0.0061	14	0	14	14.1	
Cyprodinil 2013	0.3	0.0051	14	7.9	14	16.0	
Cyprodinil 2012–13	0.3	0.0054	14	6.0	14	15.7	
Deltamethrin 2012–13	0.06	x	10		10		10
Difenoconazole 2012	0.5	0.0006	3–14 [b]	0	14	0	14
Difenoconazole 2013	0.5	0.0023	3–14 [b]	0	14	0	14
Dimethoate 2012	0.01 [a]	0.0005	14	10.5[d]	14	3.2	14
Dimethoate 2013	0.01 [a]	0.0007 [n.s.]	14		14		14
Dimethomorph 2012	0.6	0.0011	14	0	14	7.3	14
Dimethomorph 2013	0.6	0.0028	14	2.8	14	13.6	14
Dimethomorph 2012–13	0.6	0.0019	14	0.5	14	11.7	14
Fludioxonil 2012	0.5	0.0004	14	0	14	8.2	14
Fludioxonil 2013	0.5	0.0183	14	5.1	14	22.9	
Fluoxastrobin 2012	0.04	0.0002	35 [c]	2.3	35	2.3	35
Fluoxastrobin 2013	0.04	0.0007	35 [c]	10.5	35	10.5	35
Fluoxastrobin 2012–13	0.04	0.0006	35 [c]	6.6	35	6.6	35
Chlorpyrifos 2012	0.2	0.0018	14 [b]	0	14	0	14
Chlorpyrifos 2013	0.2	0.0020	14 [b]	0	14	4.7	14
Chlorpyrifos 2012–13	0.2	0.0019	14 [b]	0	14	2.0	14
Indoxacarb 2012	0.02 [a]	0.0016	1–14 [b]	3.3 [d]	14	0	14
Indoxacarb 2013	0.02 [a]	0.006 [n.s.]	1–14 [b]		14		14
Iprodione 2012	0.01 [a,f]	0.0012	3–28 [b]	32.9 [d]		24.3	28
Iprodione 2013	0.01 [a,f]	0.0108	3–28 [b]	53.3 [d]		38.2	
Iprodione 2012–13	0.01 [a,f]	0.0105	3–28 [b]	54.7 [d]		37.9	
Lambda-cyhalothrin 2012–13	0.2	x	7–14 [b]		14		14
Mandipropamid 2012	0.1	0.0023	3–14 [b]	2.4	14	8.7	14
Mandipropamid 2013	0.1	0.0052	3–14 [b]	11.5	14	15.7	
Mandipropamid 2012–13	0.1	0.0039	3–14 [b]	9.3	14	13.9	14
Metalaxyl-M 2012	0.5	0.0049 [n.s.]	7		7		7
Metalaxyl-M 2013	0.5	0.0083	7	0	7	8.1	
Methoxyfenozide 2012	0.01 [a]	0.0094	14 [c]	40.8 [d]		17.5	
Methoxyfenozide 2013	0.01 [a]	0.0156	14 [c]	40.3 [d]		24.0	
Methoxyfenozide 2012–13	0.01 [a]	0.0124	14 [c]	39.9 [d]		21.5	
Pirimicarb 2012	0.1	0.0005	14	0	14	4.1	14
Pirimicarb 2013	0.1	0.0009	14	7.2	14	10.4	14
Pirimicarb 2012–13	0.1	0.0007	14	5.4	14	8.7	14
Propamocarb-hydrochloride 2013	2	0.2499	7	5.5	7	27.1	
Prothioconazole 2012	0.05	x	35 [c]		35		35
Prothioconazole 2013	0.05	0.0008	35 [c]	10.2	35	13.1	35
Pyraclostrobin 2012	1.5	0.0037	14	0	14	8.3	14
Pyraclostrobin 2013	1.5	0.0168 [n.s.]	14		14		14
Spinosad 2012	0.07	0.0038 [n.s.]	7		7		7
Spinosad 2013	0.07	0.0050	7	0.7	7	4.6	7
Tebuconazole 2012	0.15	0.0209	7 [c]	4.2	7	15.8	
Tebuconazole 2013	0.15	0.0471	7 [c]	12.3		29.1	
Tebuconazole 2012–13	0.15	0.0342	7 [c]	8.3		23.3	
Thiacloprid 2012	0.01 [a]	0.0100	21 [b]	9.2 [d]	21	0.8	21
Thiacloprid 2013	0.01 [a]	0.0003	21 [b]	15.9 [d]	21	11.1	21
Thiacloprid 2012–13	0.01 [a]	0.0006	21 [b]	14.5 [d]	21	9.3	21
Thiamethoxam 2012	0.01 [a]	x	3 [b]		3		3
Thiamethoxam 2013	0.01 [a]	0.0040	3 [b]	4.4 [d]	3	3.2	

× the model was not established due to the rapid dissipation of the active substance in the crop; [n.s.]: non-significant model ($R^2 < 0.5$); [a] Limit corresponds to the practical limit of quantification (LOQ) of the analytical method; [b] PHI listed for pesticide application to another vegetable; for the APHI25 and APHI0.01 calculation, a longer PHI was used; [c] Currently only allowed on fruit trees (methoxyfenozide, tebuconazole) or cereals (fluoxastrobin, prothioconazole); [d] APHI25 is longer than APHI0.01; in this case, APHI0.01 (zero-residue production) must be used instead of APHI25; [e] In cases when the calculated APHI was shorter than the PHI, the recommended PHI should be followed by farmers; [f] Iprodione: MRL applicable from 31 July 2019 [26].

3.1.3. Leek

In leek, 13 active substances were evaluated; seven of them were sprayed in two seasons, 2008 and 2009 (Supplementary Table S1 online). Seventeen significant (R^2 = 0.568–0.939) and five non-significant pesticide dissipation models were established. In azoxystrobin and thiacloprid, significant models were established from years as well as from both years analyzed together. As the data obtained for deltamethrin showed a difference between the years, evaluation of this insecticide was performed separately in both years No model was generated for abamectin due to its rapid dissipation in leek (Supplementary Table S5C online). The ranges of the half-lives in leek were 2.16 days (thiacloprid 2009) to 14.65 days (deltamethrin 2008) (Table S5C). The application of these active substances before the PHI did not exceed the MRL, except for acetamiprid, chlorpyrifos, and thiamethoxam (see slope model in Table 4). For acetamiprid, chlorpyrifos, and thiamethoxam, the calculated $APHI_{25}$ was longer than the $APHI_{0.01}$ due to the low MRL of 0.01 mg kg^{-1}. In such cases, the $APHI_{25}$ was not relevant. For pyridaben and tebuconazole, the $APHI_{25}$ was longer than the PHI by 18 and 8 days, respectively. The calculated $APHI_{0.01}$ time was longer for all tested active substances than the PHI. The longest extension time of PHI was 34 days to 55 days for azoxystrobin 2008 (Table 4).

Table 4. Pesticide residues in leek.

Active Substance	MRL (mg kg^{-1})	Model (mg kg^{-1})	PHI (Days)	$APHI_{25}$ (Days) [e]	Suggested PHI for $APHI_{25}$	$APHI_{0.01}$ (Days) [e]	Suggested PHI for $APHI_{0.01}$
Abamectin 2009	0.01 [a]	x	3–7 [b]		7		7
Acetamiprid 2008	0.01 [a]	0.0152	3–7 [b]	30.1 [d]		14.1	
Acetamiprid 2009	0.01 [a]	0.0130 [n.s.]	3–7 [b]		7		7
Azoxystrobin 2008	10	0.0969	21	0	21	54.8	
Azoxystrobin 2009	10	0.0247	21	0	21	33.7	
Azoxystrobin 2008–09	10	0.0636	21	0	21	44.7	
Cypermethrin Nurelle D 2008	0.5	0.0431 [n.s.]	14 [b]		14		14
Cypermethrin Vaztak 2009	0.5	0.0413	7–14 [b]	0	14	50.1	
Deltamethrin 2008	0.3	0.0250	10	0	10	39.2	
Deltamethrin 2009	0.3	0.0129	10	0	10	15.5	
Difenoconazole 2009	0.6	0.0508	3–14 [b]	8.5	14	33.9	
Chlorpyrifos 2008	0.01 [a]	0.0484	14 [b]	50.9 [d]		35.8	
Lambda-Cyhalothrin 2008	0.07	0.0070 [n.s.]	7–14 [b]		14		14
Lambda-Cyhalothrin 2009	0.07	0.0107	7–14 [b]	9.8	14	19.8	
Pyridaben 2008	0.05 [a]	0.0241	21 [g]	38.6		42.2	
Pyridaben 2009	0.05 [a]	0.0647 [n.s.]	21 [g]		21		21
Spinosad 2009	0.2	0.0489	7	9.1		25.3	
Tebuconazole 2009	0.6	0.3535	7 [c]	15.2		33.8	
Thiacloprid 2008	0.1	0.0233	3–21 [b]	27.1		38.8	
Thiacloprid 2009	0.1	0.0006	3–21 [b]	12.1	21	15.9	21
Thiacloprid 2008–09	0.1	0.0099	3–21 [b]	20.0	21	27.9	
Thiamethoxam 2008	0.01 [a]	0.0727	3 [b]	43.6 [d]		27.3	
Thiamethoxam 2009	0.01 [a]	0.0221 [n.s.]	3 [b]		3		3

× the model was not established due to the rapid dissipation of the active substance in the crop; [n.s.]: non-significant model (R^2 < 0.5); [a] Limit corresponds to the practical limit of quantification (LOQ) of the analytical method; [b] PHI listed for pesticide application to another vegetable; for the APHI25 and APHI0.01 calculation, a longer PHI was used; [c] Currently only allowed on fruit trees (methoxyfenozide, tebuconazole) or cereals (fluoxastrobin, prothioconazole); [d] APHI25 is longer than APHI0.01; in this case, APHI0.01 (zero-residue production) must be used instead of APHI25; [e] In cases when the calculated APHI was shorter than the PHI, the recommended PHI should be followed by farmers; [g]: currently only allowed on ornamentals.

3.1.4. Carrot

In carrot, residues of nine active substances were evaluated in the root and leaves separately. Five (root; R^2 = 0.516–1.000) and nine (leaves; R^2 = 0.713–0.986) significant pesticide dissipation models were established. No models were generated for two active substances (deltamethrin and lambda-cyhalothrin) due to their rapid dissipation in carrot roots (Supplementary Table S5D online). The half-lives of pesticide residues in carrot roots and leaves were in the range of 0.56 days (acetamiprid) to 5.13 days (cypermethrin) and 2.14 days (spinosad) to 11.50 days (cypermethrin), respectively, as shown in Table S5D. The application of active substances before the PHI will allow residue below MRL except for acetamiprid and tebuconazole in leaves (see slope model in Table 5). In carrot

roots, dissipation models produce $APHI_{25}$ and $APHI_{0.01}$ values that are equal to the PHI, except for azoxystrobin, where the $APHI_{0.01}$ time exceeded the PHI by 8 days. In carrot leaves, the calculated $APHI_{25}$ time exceeded the PHI in cypermethrin and tebuconazole by more than 1.5 and 3 times, respectively. The $APHI_{0.01}$ limits prolonged the PHI in all active substances by a minimum of 10 days in spinosad and a maximum of 111 days in cypermethrin (Table 5).

Table 5. Pesticide residues in carrot.

Active Substance Root (R)/Leaves (L)	MRL (mg kg^{-1})	Model (mg kg^{-1})	PHI (Days)	$APHI_{25}$ (Days) [e]	Suggested PHI for $APHI_{25}$	$APHI_{0.01}$ (Days) [e]	Suggested PHI for $APHI_{0.01}$
Acetamiprid (R)	0.01 [a]	0.0001	7 [b]	5.3 [d]	7	3.8	7
Acetamiprid (L)	3	0.3350	7 [b]	3.8	7	33.2	
Cypermethrin (R)	0.05 [a]	0.0026	14 [b]	3.3	14	5.5	14
Cypermethrin (L)	2	0.5593	14 [b]	21.6		124.8	
Azoxystrobin (R)	1	0.0177	14	5.2	14	21.6	
Azoxystrobin (L)	70	2.1616	14	4.8	14	54.3	
Deltamethrin (R)	0.02 [a]	x	14 [b]		14		14
Deltamethrin (L)	2	0.1072	14 [b]	0	14	57.8	
Difenoconazole (R)	0.4	0.0208 [n.s.]	14 [b]		14		14
Difenoconazole (L)	10	0.6157	14 [b]	0	14	74.8	
Lambda-Cyhalothrin (R)	0.04	x	14 [b]		14		14
Lambda-Cyhalothrin (L)	0.7	0.0808	14 [b]	8.7	14	45.6	
Spinosad (R)	0.02 [a]	0.0013	14 [b]	12.0 [d]	14	8.6	14
Spinosad (L)	60	0.0333	14 [b]	0	14	23.6	
Tebuconazole (R)	0.4	0.0764 [n.s.]	14 [c]		14		14
Tebuconazole (L)	2	4.4736	14 [c]	42.1		100.7	
Thiacloprid (R)	0.05	0.0106	14	8.2	14	9.7	14
Thiacloprid (L)	5	0.9069	14	6.3	14	51.7	

× the model was not established due to the rapid dissipation of the active substance in the crop; [n.s.]: non-significant model (R^2 < 0.5); [a] Limit corresponds to the practical limit of quantification (LOQ) of the analytical method; [b] PHI listed for pesticide application to another vegetable; for the APHI25 and APHI0.01 calculation, a longer PHI was used; [c] Currently only allowed on fruit trees (methoxyfenozide, tebuconazole) or cereals (fluoxastrobin, prothioconazole); [d] APHI25 is longer than APHI0.01; in this case, APHI0.01 (zero-residue production) must be used instead of APHI25; [e] In cases when the calculated APHI was shorter than the PHI, the recommended PHI should be followed by farmers; Root (R); Leaves (L).

3.1.5. Parsley

In parsley, residues of ten active substances were evaluated in the root and leaves separately. Three (root; R^2 = 0.532–0.778) and seven (leaves; R^2 = 0.707–0.985) significant pesticide dissipation models were established. No models were generated for five active substances (cypermethrin, deltamethrin, lambda-cyhalothrin, metalaxyl-M, and pirimicarb) due to their rapid dissipation in parsley roots (Supplementary Table S5E online). The half-lives in parsley roots were in the range 6.81 days (thiacloprid) to 10.88 days (tebuconazole). In parsley leaves, the half-lives ranged from 0.97 days (azoxystrobin) to 10.89 days (lambda-cyhalothrin), as shown in Table S5E. The application of all active substances before PHI will allow residue below MRL. In parsley roots and leaves, dissipation models produce $APHI_{25}$ values that are equal to the PHI. In parsley roots, the calculated $APHI_{0.01}$ time was, for azoxystrobin and tebuconazole, more than two and six times longer than the PHI, respectively. In parsley leaves, the limit for zero-residue production was highly exceeded in five active substances by a minimum of 23 days in spinosad and a maximum of 52 days in cypermethrin (Table 6).

Table 6. Pesticide residues in parsley.

Active Substance	MRL (mg kg^{-1})	Model (mg kg^{-1})	PHI (Days)	$APHI_{25}$ (Days) [e]	Suggested PHI for $APHI_{25}$	$APHI_{0.01}$ (Days) [e]	Suggested PHI for $APHI_{0.01}$
Azoxystrobin (R)	1	0.0272	14	0	14	32.9	
Azoxystrobin (L)	70	0.0029	14	2.5	14	16.4	
Cypermethrin (R)	0.05 [a]	x	14 [b]		14		14
Cypermethrin (L)	2	0.1573	14 [b]	0	14	66.2	
Deltamethrin (R)	0.02 [a]	x	14 [b]		14		14
Deltamethrin (L)	2	0.0701	14 [b]	0	14	51.3	
Difenoconazole (R)	0.4	0.0490 [n.s.]	14 [b]		14		14
Difenoconazole (L)	10	0.7473 [n.s.]	14 [b]		14		14
Lambda-Cyhalothrin (R)	0.04	x	14 [b]		14		14
Lambda-Cyhalothrin (L)	0.7	0.0638	14 [b]	0	14	57.1	
Metalaxyl-M (R)	0.01 [a]	x	14 [b]		14		14
Metalaxyl-M (L)	3	0.0007	14 [b]	0	14	11.3	14
Pirimicarb (R)	0.05	x	7		7		7
Pirimicarb (L)	3	0.4201	7	2.0	7	56.5	
Spinosad (R)	0.02 [a]	0.0084 [n.s.]	14 [b]		14		14
Spinosad (L)	60	0.0861	14 [b]	0	14	37.2	
Tebuconazole (R)	0.4	0.0490	7 [c]	0	7	42.6	
Tebuconazole (L)	2	2.2325 [n.s.]	7 [c]		7		7
Thiacloprid (R)	0.05	0.0086	7	4.5	7	7.4	
Thiacloprid (L)	5	0.3081 [n.s.]	7		7		7

× the model was not established due to the rapid dissipation of the active substance in the crop; [n.s.]: non-significant model ($R^2 < 0.5$); [a] Limit corresponds to the practical limit of quantification (LOQ) of the analytical method; [b] PHI listed for pesticide application to another vegetable; for the APHI25 and APHI0.01 calculation, a longer PHI was used; [c] Currently only allowed on fruit trees (methoxyfenozide, tebuconazole) or cereals (fluoxastrobin, prothioconazole); [e] In cases when the calculated APHI was shorter than the PHI, the recommended PHI should be followed by farmers; Root (R); Leaves (L) (only in carrot and parsley).

3.2. Dissipation Course of Active Substances from Application to Harvest

Eight of the thirty-two active substances were sprayed on all experimental vegetable species. (Table S7). One fungicide (azoxystrobin) and three insecticides (cypermethrin, spinosad and thiacloprid) were selected to demonstrate differences in pesticide dissipation behavior (Figure 2). Higher levels of residues of four active substances in the first term after application were found on lettuce, leek, and carrot and parsley leaves. The highest levels were observed for azoxystrobin (20 mg kg^{-1}) and for tested insecticides (from 1.1 to 1.6 mg kg^{-1}). Low levels of residues of azoxystrobin (0.3 mg kg^{-1}) and tested insecticides (ranging from 0.02 to 0.014 mg kg^{-1}) were observed in the first term after application in onion. Less residues of active substances were found in the underground part of crops than in the above parts. The highest number of residues were found in carrot leaves, in descending order followed by parsley leaves, lettuce, leek, carrot root, parsley root, and onion (Figure 2). The highest levels of residues were found for azoxystrobin, followed by cypermethrin, and the lowest values of residues were observed for thiacloprid and spinosad.

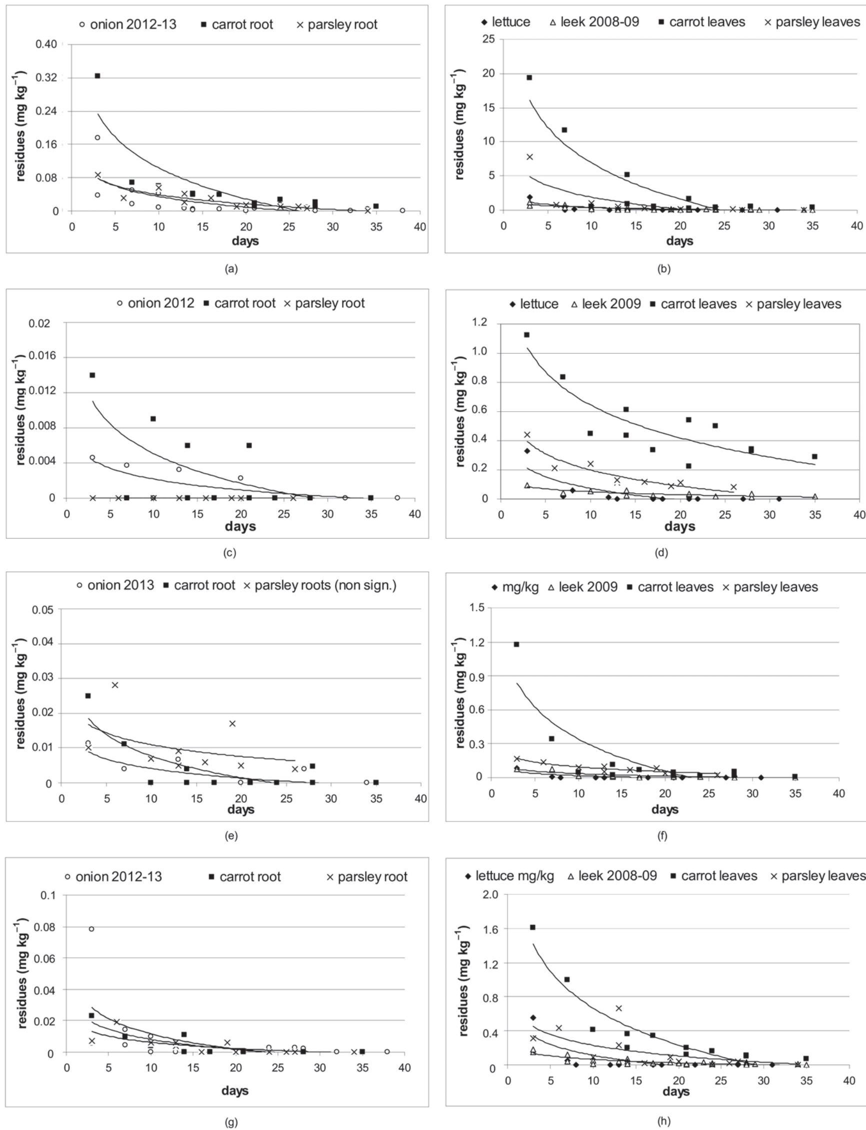

Figure 2. Dissipation of four active substances in vegetable species. (**a**) azoxystrobin on bulb and roots; (**b**) azoxystrobin on aboveground parts of crops; (**c**) cypermethrin on bulb and roots; (**d**) cypermethrin on aboveground parts of crops; (**e**) spinosad on bulb and roots; (**f**) spinosad on aboveground parts of crops; (**g**) thiacloprid on bulb and roots (**h**) thiacloprid on aboveground parts of crops.

4. Discussion

In the tested vegetables, the pesticide dissipation rate k^{diss} and the pesticide dissipation half-life $t_{1/2}$ varied depending on the active substances and vegetable species. The fastest pesticide dissipation occurred overall in lettuce (Table S5A; Figure 1a). Our results corresponded with the findings of Song et al. [27], who compared dissipation curves and half-lives of six pesticides on six leafy vegetables planted in field trails. The dissipation of dimethoate, chlorpyrifos, beta-cypermethrin and

deltamethrin in leaf lettuce expressed in k^{diss} and $t_{1/2}$ was in the range of −0.793 to −0.176 and 0.87 to 3.94 days, respectively.

The dissipation half-lives of the active substances were compared with the predicted geometric mean of the dissipation half-lives at 20 °C ($t_{1/2\ \mathrm{ref,i}}$) and the corrected $t_{1/2\ \mathrm{ref,i}}$ of pesticides applied to a specific vegetable under a given average temperature ($t_{1/2\ \mathrm{plant,\ active\ subst.}}$) calculated according to model II [14]. The results are given in Table S7. In many cases, the corrected $t_{1/2\ \mathrm{plant,\ active\ subst}}$ of the active substances correspond more to our results; e.g., the corrected $t_{1/2}$ in lettuce and onion were shorter than $t_{1/2\ \mathrm{ref,i}}$, whereas in leek, the corrected half-lives were prolonged for several days (Table S7). Two active substances, fluoxastrobin and prothioconazole, sprayed on onion did not appear in the pesticide list needed for $t_{1/2}$ calculation according to model II. We compared our results with $t_{1/2\ \mathrm{ref,i}}$ and $t_{1/2\ \mathrm{plant,\ active\ subst.}}$ computed according to model III [14]. Although the calculation according to model III has its limitations, such as higher uncertainty compared to half-lives based on model II [14], the results correlated with our values (Table S7). Computed predicted half-lives ($t_{1/2\ \mathrm{ref,i}}$; $t_{1/2\ \mathrm{plant,\ active\ subst.}}$) of fluoxastrobin 2012–13 (6.14; 6.73 days) and prothioconazole 2013 (7.19; 8.14 days) corresponded to our results of 7.20 and 6.78 days, respectively.

Eight of the thirty-two active substances were sprayed on all vegetable species. The results showed differences in concentrations of active substances in underground and aboveground parts of the plants after foliar application of the pesticides. The initial deposition of the active substance in aboveground part could be influenced by the foliar surface area, which can be defined as vegetation cover (VC), e.g., the proportion of soil area covered by leaves [8]. Leaves of carrot and parsley are overlapping, while the compact shape of iceberg lettuce head and leek could allow less deposition of the active substance. The role of foliar area in the initial deposition of active substances has been mentioned, e.g., by Lu et al. [28] and Song et al. [27]. Two fungicides (azoxystrobin, tebuconazole) and three insecticides (cypermethrin, spinosad, thiacloprid) were selected to demonstrate differences in pesticide dissipation.

The systemic fungicide azoxystrobin had robust models for all vegetables and its parts. The dissipation rate of azoxystrobin was as follows (ranked from the fastest dissipating): lettuce, parsley leaves, carrot root, onion, carrot leaves, leek, and parsley root. The coefficient k^{diss} ranged from −0.725 to −0.093, and $t_{1/2}$ ranged from 0.96 to 7.42 days. Azoxystrobin belonged to eight active substances most often detected in vegetables and mushrooms obtained during official inspection of pesticide residues in Czech supermarkets in 2018 [29]. Similarly, azoxystrobin was detected in eight vegetable species from southeastern Poland but never exceeded the MRL [30]. According to our results, azoxystrobin never exceeded the MRL and in all tested vegetables produced $APHI_{25}$ values that were equal to or lower than the PHI, but it was not suitable for zero-residue production, except for lettuce.

The second fungicide, tebuconazole, had significant effects on all vegetables except carrot root and parsley leaves. The dissipation rate of this systemic triazole fungicide was as follows (ranked from the fastest): lettuce, leek, onion, carrot leaves, and parsley root. The dissipation constant k^{diss} and $t_{1/2}$ ranged from −0.523 to −0.064 and 1.32 to 10.88 days, respectively. Tebuconazole is not allowed on any of the tested vegetables in the Czech Republic, and our results confirm that it is not suitable for low-residue or zero-residue production.

Spinosad, a fermentation product of the actinomycete bacterium *Saccharopolyspora spinosa*, dissipated in descending order: lettuce, carrot leaves, carrot roots, onion, parsley leaves, and leek. In parsley roots, the dissipation model was not significant. Based on the k^{diss} (−0.614 to −0.132) and $t_{1/2}$ (1.13 to 5.23 days) values, spinosad is a rapidly dissipating substance. In the Czech Republic, spinosad is permitted in lettuce (PHI 14 days), onion, and leek (PHI 7 days). In carrot and parsley, we used a longer PHI of 14 days. In all tested vegetables, spinosad was suitable for low-residue production with $APHI_{25}$. In lettuce, carrot root and onion, production with less than 0.01 mg kg^{-1} spinosad was feasible. Sikorska-Zimny et al. [31] calculated the half-life of spinosad in onion, carrot and cabbage and found the values to be 5.2, 3.6 and 2.9 days, respectively. Our calculated half-lives of spinosad in

onion (3.58 days), carrot root (2.55 days), and cabbage (3.85 days; data shown in Kocourek et al. [15]) confirm the fast dissipation process of this substance.

Similarly, thiacloprid, a neonicotinoid insecticide, dissipated fast in almost all tested vegetables (ranked from the fastest): lettuce, onion, carrot root, leek, carrot leaves, and parsley roots, with k^{diss} values of −0.660 to −0.102 and $t_{1/2}$ values of 1.05 to 6.81. The parsley leaf data did not enable the generation of any significant model. In the Czech Republic, thiacloprid is permitted in lettuce, carrot, and parsley with a PHI of seven days. In our study, we used the longest PHI of 21 days for leek and onion, as recommended for other vegetable species. In onion, the 21-day PHI was sufficient for 25% MRL as well as for zero-residue production. In the case of leek, a 21-day PHI was suitable for 25% MRL as well as for zero-residue production in one year only. In 2008, the calculated $APHI_{25}$ and $APHI_{0.01}$ prolonged the PHI for 6 and 18 days, respectively.

Cypermethrin, a synthetic pyrethroid, dissipated in descending order: lettuce, carrot root, onion, parsley leaves, leek, and carrot leaves, with k^{diss} values of −0.507 to −0.051 and $t_{1/2}$ values of 1.37 to 11.53. In parsley roots, cypermethrin was below the detection limit. Its dissipation strongly depended on the vegetable species. For example, no cypermethrin residues were detected in carrot roots after foliar application or later [32]. On the other hand, Yuan et al. [33] reported a high concentration of cypermethrin in some vegetable samples from supermarkets, where residue levels varied in different vegetables, high levels being found in radish and cauliflower. Cypermethrin was the insecticide observed frequently at concentrations above the MRL in leafy vegetables [34]. Cypermethrin is the most used pesticide in vegetables, but the residue levels were always below the European MRL [35].

Two others tested pyrethroids (deltamethrin and lambda-cyhalothrin) dissipated according to vegetable species and their parts. Both were decomposed very fast in onion, carrot, and parsley roots without the ability to create a dissipation curve. Similarly, Riplay et al. [32] did not detect deltamethrin residues in onion from the first day of foliar application. In lettuce, the k^{diss} coefficients −0.539 and −0.404 were obtained for deltamethrin and lambda-cyhalothrin, respectively. On the other hand, in leek, carrot, and parsley leaves, these two active substances dissipated very slowly and are thus not suitable for low-residue and zero-residue production.

Pesticides sprayed at the highest recommended dosages for vegetables in the Czech Republic dissipated under the MRL except for iprodione and methoxyfenozide (onion), acetamiprid, chlorpyrifos and thiamethoxam (leek) and tebuconazole (parsley leaves).

The MRLs of active substances in carrot and parsley roots can even be one thousand times lower than in leaves, e.g., the MRL of spinosad in parsley roots is 0.02 mg kg^{-1} and in parsley leaves is 60 mg kg^{-1} [24]. Our model for parsley roots was not robust enough, but the highest detected amount of spinosad was 0.03 mg kg^{-1} and in parsley leaves was 0.45 mg kg^{-1} three days after spraying. Similarly, the maximum level of spinosad detected in carrot roots and carrot leaves was 0.03 mg kg^{-1} and 1.18 mg kg^{-1} three days after spraying, respectively. The limit of 60 mg kg^{-1} appears to be very mild compared to the lettuce (10 mg kg^{-1}) and leek (0.2 mg kg^{-1}) MRLs. Leaves of parsley are often used raw in many dishes. High initial deposits of active substances in combination with mild MRLs allow PHI compliance.

Gonzáles-Rodríguez et al. [34] showed this problem in lettuce compared to other leafy vegetables. The MRLs for Swiss chards and spinaches can even be one hundred times lower than for lettuce because lettuce is highly sensitive to pests and needs successive applications of pesticides, consequently leaving a higher level of residues that are tolerated. Recently, there was a high decrease in the MRL of dimethoate in lettuce from 25 mg kg^{-1} (Reg. (EU) 2015/400) to 0.01 mg kg^{-1} (applicable from 31.7.2019; Reg. (EU) 2019/38) [24]. However, the MRLs of many active ingredients evaluated in lettuce were still much higher than those in leek (Tables 2 and 4).

Models of pesticide dissipation in vegetables are suitable for the regulation of pesticide residues in harvested crops. In this paper, significant models were established for 114 pesticide/crop combinations. According to these models, it is possible to predict the APHI for the requested "action threshold" of residues in crops at the time of harvest. For pesticides with a very low incidence of residues in the short

term after treatment, it is not necessary to prolong the PHI. Fast dissipation of residues was found for deltamethrin and lambda-cyhalothrin in onion, carrot roots, and parsley roots and for cypermethrin, metalaxyl-M and pirimicarb in parsley roots. Similarly, a very fast drop in residue levels was found for abamectin in onion and leek. For these pesticides, it is not necessary to establish $APHI_{25}$ or $APHI_{0.01}$ because the pre-harvest interval is achieved in any case. A strict limit for zero-residue production (0.01 mg kg^{-1}) is achieved when pyrethroids are applied in root vegetables. In contrast, high deposits of pyrethroids in the leaves of carrot and parsley after treatment do not enable us to achieve this limit. Very low incidence of residues of several pesticides in the short term after treatment was also detected in cauliflower (beta-cyfluthrin) and head cabbage (acetamiprid, beta-cyfluthrin, cypermethrin, deltamethrin, lambda-cyhalothrin, pymetrozine, thiamethoxam) [15].

The difficulty of vegetable growing for low-residue or zero-residue production depends on the crop and pesticides used. The order of crops for which the established $APHI_{25}$ or $APHI_{0.01}$ are longer than the PHI corresponds to the order of crops descending from the lowest to the fastest dissipation of pesticides. For low-residue production, the order of crops is based on the number of pesticides with $APHI_{25}$ values exceeding the PHIs from the total number of evaluated pesticides for which a significant model was established: leek (7/13), carrot leaves (2/9), iceberg lettuce (3/21), onion (4/29), parsley leaves (0/7), carrot roots (0/7), and parsley roots (0/8). In comparison, the number of pesticides with $APHI_{25}$ values exceeding the PHIs was 7 of 20, 8 of 17, and 1 of 18 evaluated pesticides in Chinese cabbage, cauliflower, and head cabbage, respectively [15]. The growth of Chinese cabbage, leek and cauliflower for low-residue production is difficult, while the growth of iceberg lettuce, onion, head cabbage, parsley and carrot for roots seems to be easier. Growing of parsley and carrot for leaves in low-residue production is also achievable owing to the very mild MRLs discussed above. For zero-residue production, the order of crops is based on the number of pesticides with $APHI_{0.01}$ values exceeding the PHIs from the total number of evaluated pesticides for which a significant model was established: leek (13/13), carrot leaves (9/9), parsley leaves (6/7), iceberg lettuce (9/21), onion (11/29), parsley roots (2/8), and carrot roots (1/8). In comparison, the number of pesticides with $APHI_{0.01}$ values exceeding the PHIs were 19 of 20, 13 of 17 and 1 of 18 evaluated pesticides in Chinese cabbage, cauliflower, and head cabbage [15]. The growth of Chinese cabbage, leek, cauliflower, parsley, and carrot for leaves for zero-residue production is difficult, while the growth of head cabbage, iceberg lettuce, onion, parsley, and carrot roots is feasible.

According to the exceeding of the $APHI_{25}$, it is possible to select the pesticides tebuconazole (onion, iceberg lettuce, carrot leaves, leek), chlorpyrifos (leek, iceberg lettuce), iprodione (onion, iceberg lettuce), cypermethrin (carrot leaves), methoxyfenozide (onion), azoxystrobin, pyridaben, thiacloprid, and thiamethoxam (leek) as unsuitable for low-residue production.

According to the exceeding of the $APHI_{0.01}$, the number of "risky" pesticides is higher. Chlorpyrifos, acetamiprid, thiacloprid, and thiamethoxam were the insecticides that most often exceeded the $APHI_{0.01}$ in vegetables. Except for acetamiprid, these pesticides were recently restricted by EU regulation [36–38]. Azoxystrobin, boscalid, iprodione, spinosad, pyridaben, propamocarb-hydrochloride, and tebuconazole were the fungicides that most often exceeded the $APHI_{0.01}$ in vegetables. However, the limited number of pesticides available for the control of vegetables such as leek, Chinese cabbage or cauliflower did not enable the exclusion of these unsuitable pesticides from pest management.

The half-lives of pesticides set employed in this study for treatment of lettuce were lower than half-lives of those when used in onion, leek, and parsley and carrot roots, including the carrot leaves (Figure 1a). This means that dissipation rates of pesticides in lettuce were higher compared to other vegetable species (Supplementary Table S5 online). In this context it is rather surprising that relatively high levels of some fungicides (e.g., azoxystrobin) were found in samples of lettuce collected in a Spanish market, and in some of them the MRL was exceeded [34]. It is worthwhile to point out that MRLs for the same pesticides in other crops are in some cases lower than in lettuce [24]. For instance, the MRL for azoxystrobin in lettuce is 1.5-times higher than in onion and 15-times higher than in

carrot root. The MRL for difenoconazole in lettuce is 10-times higher than in carrot and MRL for mandipropamid in lettuce is even 250-times higher than in onion [24]. MRLs for leafy vegetable as Swiss chard or spinach can be one hundred times lower than for lettuce [34]. The reason of such apparent discrepancies in MRLs setting is a high sensitivity of lettuce to pests and thus the need to apply more treatments, which, consequently, leave higher residues. However, in an earlier Spanish study, the residues of azoxystrobin in greenhouse lettuce harvested at PHI day were in all cases below the officially set MRL mentioned in the study. The results indicate that the time between application and harvest is at least as important as the application dose [8].

It is necessary to mention that high half-life values of some pesticides do not necessarily lead to occurrence of residues at harvest. For instance, thiamethoxam had in lettuce a relatively high level of half-live in comparison to onion (Table S5A,B). However, in lettuce the thiamethoxam level lowered at harvest below a limit of detection when PHI was kept [39].

The occurrence of pesticide residues in lettuce in a Spanish market was reported more often than in other vegetables [34] and may potentially pose a threat to consumer health. Interestingly, fungicides were most detected, especially in lettuce. Although concentrations of insecticides used to be lower, residues of cypermethrin, chlorpyrifos, difenoconazole, or lambda-cyhalothrin were found in 50% of the lettuce samples, while no detectable pesticide residues were present in potatoes and onions. In 20% of the lettuce samples, residues exceeded MRL; moreover, in some samples, this was the case for two or three pesticides [40].

Pesticide dissipation half-lives in plants are largely dependent not only on their characteristics (e.g., surface morphology) but also on environmental conditions [13]. Moreover, dissipation rate and half-lives of pesticides are variable for various vegetable species. Therefore, the mathematic models are suitable only for the vegetable species for which they were developed. One of the key factors affecting pesticide half-lives is temperature in the period between application and harvest, with higher temperatures resulting in higher dissipation rates and thus shorter half-lives. Generally, the PHI forecast according to developed models will be more reliable under conditions with higher temperatures. The application of the predictive models on vegetables grown in glasshouses is rather complicated as the temperature conditions are much higher than those for which they were developed; half-lives are fairly lower, what can be also documented by our earlier data comparing the fate of azoxystrobin under field and glasshouse conditions [8].

It is noteworthy that the developed models of pesticide dissipation for a forecast of PHI and incidence of residues in plant products before harvest are applicable in regions with similar climatic conditions for the growing of that vegetable. For expanded use of dissipation models developed in this study, parameters such as plant characteristics, substance properties and environmental conditions including temperature are summarized in regression models developed by Fantke et al. [14]. As an example, corrected values of half-live $t_{1/2\ \mathrm{ref,i}}$(day) and $t_{1/2\ \mathrm{plant,\ active\ subst.}}$ (day) according to these regression models of Fantke et al. [14] are presented in Table S7.

Degradation is for many pesticides the most relevant dissipation process, followed by growth dilution [2]. Growth of plants, depending on water accessibility, is another limiting factor of this model's reliability. A better forecast power can be expected when water access for vegetable is sufficient, regardless of whether it is due to irrigation or precipitation in the growing locality. On the other hand, limited plant growth increases the share of growth dilution on the overall dissipation.

The major outcome of this study is 114 regression model equations of tested pesticide dissipation of in five vegetable species. These models can be used in practice for monitoring of pesticide residues in products at harvest in dependence on term of application; the knowledge of dissipation rate enables establishing APHI for targeted limits of pesticide residues in product at harvest, e.g. for a limit of 25% MRL or 0.01 mg·kg^{-1}. In addition, based on the models, it is possible to differentiate between the pesticides for which prolonging of the PHI is not necessary and those for which some adjustment of PHI is needed. Models of pesticide dissipation and the procedure of APHI calculation can also serve as a basis for expert systems aimed at the regulation of pesticide residues in vegetables. Under these

conditions, the key users might be vegetable growers, extension services or retail chains that require vegetable products with pesticide residues lower than official MRLs. Overall, protection of consumer health will occur due to a reduction of pesticide vegetable contamination by residues. The other challenging application of the developed models is their use for registration purposes and updating of respective MRLs and PHIs. However, it remains unclear if reduced pesticide application amounts will remain effective against the relevant target pests. Hence, understanding the efficacy of reduced pesticide application for controlling relevant pest organisms requires further research.

The outcomes of this study might nevertheless help to identify a selection of candidate pesticides for stricter pesticide legislation, specifically in the case of pesticides with a higher incidence of residues in food crops, longer half-lives and longer $APHI_{0.01}$. Considering the focus of future research in this field, the monitoring of levels of rapid dissipation of active substances from the first day after application would be useful as it provides important data for comparison of initial deposits in particular crops with the literature data (18). Evaluation of relationships between MRL, PHI, and acute reference doses (ARfDs) is a challenge for further research of pesticide residues in products. Finally, the models characterizing pesticide dissipation can also be used for screening residue-related consumer exposure, thus informing related risk assessments.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/5/680/s1, Table S1: Overview of active substances, pesticides, and application rates in crops in semi-field experiments for pesticide residue analyses, Table S2: Optimized MS/MS transition parameters of the LC-based method, Table S3: Ions (*m*/*z*) monitored by the GC-MS analytical method, Table S4: Performance characteristics of the validated analytical method (broccoli spiked at 0.1 mg kg^{-1} and 0.01 mg kg^{-1}, six replicates), Table S5: Parameters of pesticide residue dissipation models including dissipation half-lives of pesticides in A) iceberg lettuce; B) onion; C) leek; D) carrot; E) parsley, Table S6: The list of active substances of pesticides presented in Figure 1, Table S7: Dissipation half-lives ($t_{1/2}$) of pesticide residues in plants compared with predicted geometric means of the dissipation half-lives ($t1_{1/2\ ref,i}$) at 20 °C and the corrected $t_{1/2\ plant,\ active\ subst.}$ according to the Model II, resp. Model III [14], Table S8: The highest detected concentrations of pesticide residues in tested vegetables three to seven days after final treatment.

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Determination of Three Typical Metabolites of Pyrethroid Pesticides in Tea Using a Modified QuEChERS Sample Preparation by Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry

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Abstract: Pyrethroid pesticides are widely used on tea plants, and their residues of high frequency and concentration have received great attention. Until recently, the residues of typical metabolites of pyrethroid pesticides in tea were unknown. Herein, a modified "quick, easy, cheap, effective, rugged and safe" (QuEChERS) method for the determination of three typical metabolites of pyrethroid pesticides in tea, using ultra performance liquid chromatography tandem mass spectrometry, was developed. The mixture of florisil, octadecylsilane, and graphite carbon black was employed as modified QuEChERS adsorbents. A Kinetex C18 column achieved good separation and chromatographic peaks of all analytes. The calibration curves of 3-phenoxybenzoic acid (3-PBA) and 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA) were linear in the range of 0.1–50 ng mL^{-1} (determination coefficient R^2 higher than 0.999), and that of *cis*-3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid (TFA) was in the range of 1–100 ng mL^{-1} (R^2 higher than 0.998). The method was validated and recoveries ranged from 83.0% to 117.3%. Intra- and inter-day precisions were lower than or equal to 13.2%. The limits of quantification of 3-PBA, 4-F-3-PBA, and TFA were 5, 2, and 10 μg kg^{-1}, respectively. A total of 22 tea samples were monitored using this method, and 3-PBA and TFA were found in two green tea samples.

Keywords: tea; pyrethroid pesticide metabolite; ultra-high performance liquid chromatography tandem mass spectrometry; modified QuEChERS

1. Introduction

It is estimated that 2.4 million tons of active pesticide ingredients are used annually, mainly in agriculture [1]. A more significant estimate indicates that pesticides are generally persistent; about half of the detected substances have already been eliminated, and another 10% to 20% are stable transformation products [2]. The issue of pesticide transformation products is important because they leave the active moiety intact, generate toxicologically more potent structures, and have smaller molecular mass and more polarity than their parent compounds, which increases their solubility and perdurability. The transformation products and/or metabolites of pesticides in crops may be due to impurities in formula products applied in the field, or the results of abiotic transformation and plant metabolism. Pesticides with their transformation products and metabolites enter the food chain and then are exposed to human beings, resulting in chronic and acute poisoning.

Pyrethroid pesticides with low mammalian toxicity have been used worldwide since the 1980s as a replacement for the high toxic class of pesticides, such as organophosphorus and organochlorine compounds [3]. Pyrethroid pesticides have become one of the most important classes of insecticides in crops, and they have the largest market share, accounting for 38% in 2015 [4]. In China, pyrethroid pesticides were the only increased pesticide class in terms of use, with annual demands in 2016 approaching approximately 3800 tons [5]. Pyrethroids such as bifenthrin, λ-cyhalothrin, cypermethrin, deltamethrin, fenpropathrin, fenvalerate, permethrin, and cyfluthrin provide effective insecticide control for crop protection. The mass application of pyrethroid pesticides in agricultural crops inevitably involves the issue of their residues in crops and related foods. Li et al. found that 378 of 1450 fruit samples contained pyrethroid residues in the range of 0.005 to 1.2080 mg kg^{-1} [6]. High frequencies for pyrethroids, such as 63.4% for bifenthrin (ND-3.848 mg kg^{-1}), 55.4% for λ-cyhalothrin (ND-3.244 mg kg^{-1}), 46.5% for cypermethrin (ND-0.499 mg kg^{-1}), and 24.8% for fenvalerate (ND-0.217 mg kg^{-1}), were found in 101 tea samples [7]. Morgan et al. investigated 8 pyrethroid residues in repeated duplicated-diet solid food samples of 50 adults during the period of 2009–2011, and found that at least one pyrethroid or pyrethroid degradate was found in 49% and 2% of the monitored samples, respectively [8]. Due to the neurotoxicity, hepatotoxicity, development toxicity, and digestive system toxicity of pyrethroid pesticides for animals and human beings, such high residues at the mg kg^{-1} level in food present a healthy risk [9]. European Food Safety Authority (EFSA) formulated a maximum residue level of cypermethrin in the range of from 0.05 mg kg^{-1} to 2 mg kg^{-1}, based on the risk assessment, and cypermethrin residues in some monitored food samples reported by previous studies exceeded the maximum residue levels (MRLs) [10].

Pyrethroids are readily transformed into intermediate products and metabolites by hydrolysis and photolysis. As shown in Figure 1, 3-phenoxybenzoic acid (3-PBA) can be formed from several pyrethroid pesticides, such as esfenvalerate, cypermethrin, deltathrin, permethrin, and cyhalothrin, which are widely used in tea gardens. *Cis*-3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid (TFA) can be transformed from bifenthrin and cyhalothrin, while 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA) can be formed from cyfluthrin [3]. Hydrolysis is an important degradation pathway for pyrethroid pesticides, highlighting that hydrolysis intermediates could be formed in the field or surfaces of agricultural crops. Following the disappearance of pyrethroid pesticides, 3-PBA was concurrently formed [11]. Although pyrethroid pesticides are used in tea gardens and the issue of their residues is concerning, their transformation products and metabolites have not been monitored and assessed, except in a paper reported by Mortimer et al. [12].

The potential adverse effects of 3-PBA on human beings and environments should be further considered, and knowledge regarding the dissipation pattern of 3-PBA in agricultural crops and environments needs to be addressed. Wang et al. found that 3-PBA inhibited the phagocytic ability of macrophages, increasing the reactive oxygen species level. These results illustrated that 3-PBA has an immunotoxic effect on macrophages [13]. 3-PBA showed antiestrogenic, antiandrogenic, and thyroid hormone receptor antagonistic activities [14]. Considering the toxicity of pyrethroids transformation products and metabolites, an analytical technique for these compounds in foods should be developed for the determination of their residues in foods and further risk assessment.

Several techniques have been developed for the determination of pyrethroid transformation products and metabolites using gas chromatography tandem mass (GC-MS/MS) [15], ultra-high performance liquid chromatography (UHPLC) [16], and UHPLC coupled with tandem mass spectrometry (UHPLC-MS/MS) [17] or high-resolution orbitrap mass spectrometry (UHPLC-Orbitrap MS) [18]. Due to the high polarity and low volatility of pyrethroids transformation products and metabolites, these compounds were derived with bis(trimethylsilyl) trifluoroacetamide (BSTFA) prior to GC-MS/MS analysis [19]. The UHPLC-MS/MS technique provided clear advantages for the analysis of transformation products and metabolites attributing to high selectivity, sensitivity, and accuracy. Al-

though several approaches for the analysis of pyrethroid metabolites have been developed using UHPLC-MS/MS, these analytical protocols focused on environment water [17], soil [20], human urine, and plasma [21,22]. There are few methods for the determination of pyrethroid metabolites in agricultural crops and foods. For some simple matrices, such as human plasma and water [17], sample preparation did not involve clean-up [22]. However, for complex samples, such as soil [20], urine [21], wastewater [23], vegetables and fruits [24], and other substrates, it is necessary to carefully optimize the adsorbents and their dosage of dispersive solid-phase extraction (d-SPE) or SPE cartridges to obtain satisfactory purification effect. Therefore, the development of the UHPLC-MS/MS method for the complicated matrix is different from other current methods due to the optimization of sample preparation techniques.

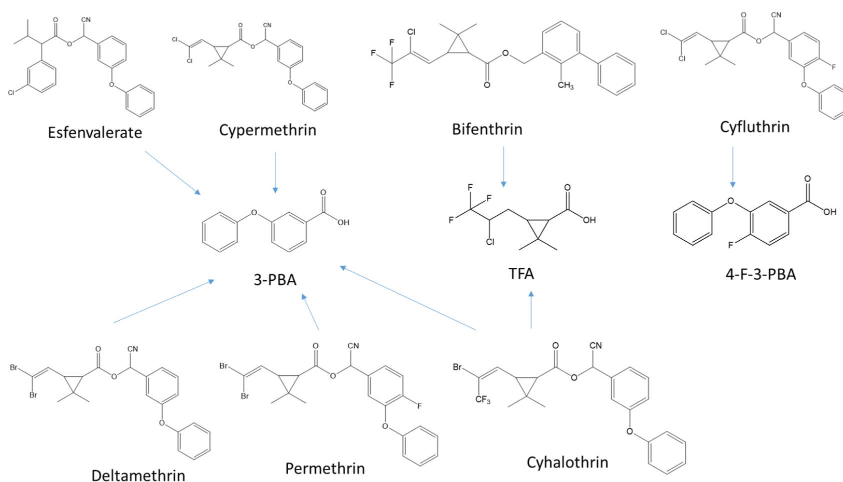


Figure 1. Transformation compounds from pyrethroids pesticides.

Tea is a complex matrix, rich in tea polyphenols, caffeine, polysaccharides, and other polar substances, which greatly interferes with the analysis of polar compounds, like pyrethroid pesticide metabolites. In this study, we developed a sensitive method based on UHPLC-MS/MS for the determination of typical pyrethroid metabolites in tea samples. UHPLC-MS/MS parameters, such as chromatographic column and mobile phase, were optimized, and three modified "quick, easy, cheap, effective, rugged and safe" (QuEChERS) techniques, i.e., original QuEChERS [25], Official CEN 15662 [26], AOAC 2007.1 [27], were evaluated, and the adsorbents were optimized step by step.

2. Experimental

2.1. Reagents and Materials

3-PBA (purity 99%), 2-phenoxybenzoic acid (2-PBA) (purity 99%), 4-F-3-PBA (purity 99%), and TFA were obtained from Dr.Ehrenstorfer (Augsburg, Germany). HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (Shanghai, China). A Milli-Q-Plus system (Millipore, Milford, DE, USA) was used to obtain de-ionized water. Anhydrous magnesium, sodium chloride, dehydrate citrate sodium, sodium acetate, and disodium monohydrogen citrate were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). LC-MS-grade acetic acid and formic acid were obtained from Sigma-Aldrich (Shanghai, China). Amino carbon nanotubes (NH_3-NTs), multi-walled carbon nanotubes (MWCNTs), graphite carbon black (GCB), primary secondary (PSA), octadecylsilane (C18), florisil, zirconia (ZrO), and polyvinylpyrrolidone (PVPP) were purchased from Bonna-Agela Technologies (Tianjin, China). Organic green tea

and black tea, which were free of pesticide residues and pyrethroid pesticide metabolites, were supplied by the Key Laboratory of Tea Quality and Safety, Ministry of Agriculture. Commercial tea samples, including green tea (12) and black tea (10), were purchased from a supermarket, a whole market, and a monopoly shop located in Zhejiang province.

2.2. Sample Preparation

A 2.0 g portion of previously grounded tea was weighed into a 50 mL centrifuged tube. An internal standard, named 2-PBA, was added, and its concentration was 50 μg kg^{-1} by adding 100 μL of 2-PBA standard solution at 1 μg mL^{-1}. The internal standard fortified in tea samples was used to calibrate the loss of target compound during sample preparation. For the matrix-matched standard solutions, the internal standard 2-PBA was added to the concentration at 10 μg L^{-1}. Then, 2 mL of water was added and the tea sample was mixed on a vortex for 1 min. After that, 10 mL acetonitrile containing 1% acetic acid was used to extract the target compounds using an oscillator for 10 min. Then, 1.0 g of dehydrate citrate sodium and 0.5 g of disodium monohydrogen citrate were added to the mixture. The mixture was homogenized on a vortex for 1 min and then centrifuged at 10,000 rpm for 10 min. Following this, 2 mL of supernatant was transferred into a 5 mL centrifuge tube containing 200 mg florisil, 200 mg C18, and 100 mg GCB. The mixture was mixed on a vortex for 1 min and then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a 0.22-μm membrane into the LC vial for UHPLC-MS/MS analysis.

2.3. UHPLC-MS/MS Analysis

Instrumental analysis was performed using an API 6500 triple quadrupole instrument mass spectrometry (AB SCIEX, Foster City, CA, USA) coupled to a Sciex 4000 UHPLC system (AB SCIEX, USA), both from Sciex Corporation (Foster City, CA, USA). Chromatographic separations employed a Kinetex C18 column (2.1 × 100 mm, particles 2.6 μm) (Phenomenex Co., Ltd, California, USA). A 10 μL sample loop was used for injection. The injection volume was 2 μL. The mobile phase consisted of water (A) and acetonitrile (B). Gradient elution was as follows: initial to 1 min, 5% B; 1–3 min, 95% B; 3–10 min, 95% B; 10–10.5 min, 5% B; 10.5–12 min, 5% B. UHPLC-MS/MS was performed in negative polarity (−4500 V). The source temperature was 550 °C. Gas 1, gas 2, curtain gas, and collision gas were set for nitrogen, and the flow rate was 60, 60, 30, and 10 psi, respectively. Scheduled multi reaction monitoring (sMRM) mode was employed for detecting the target compounds, and sMRM parameters are shown in Table 1.

Table 1. MS/MS parameters for the analysis of 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), *cis*-3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid (TFA), and internal standard 2-phenoxybenzoic acid (2-PBA).

Compound	Retention Time (min)	Precursor (m/z)	Declustering Potential DP (V)	Production (m/z)	Collision Energy CE (eV)
3-PBA	3.35	212.9	−27	92.9	−27
				169.1	−18
4-F-3-PBA	3.31	230.9	−25	92.9	−33
				167.0	−25
TFA	3.450	240.9	−30	35.0	−15
				121.0	−15
2-PBA	3.19	212.9	−27	92.9	−27
				169.1	−18

2.4. Validation Procedure

The method was validated in accordance with the SANTE/11813/2017 standard, in terms of linearity, matrix effect, sensitivity, accuracy, and precision [28]. Linearity was evaluated using a matrix-matched standard solution at seven levels in the range of

0.1–200 μg L^{-1} in triplicates. Matrix matched standard solutions were prepared using the same type of analyzed tea samples, and internal standard 2-PBA was added to the concentration at 10 μg mL^{-1}. Matrix effect (ME) was evaluated comparing the slope of the calibration curve obtained from the solvent and matrix-matched standard solution in the same levels, according to the following equation: ME (%) = (slope of matrix-matched calibration curve/slope of solvent calibration curve-1) × 100. The limits of detection (LOD) and quantification (LOQ) were estimated from the UHPLC-MS/MS signal through the lowest spiked level. LOD was defined as the lowest spiked concentration that provided a signal to noise ratio of 3, and LOQ was defined as the lowest spiked concentration with recovery in the range of 70 – 130% with relative standard deviations (RSDs) below 20%. Recoveries were calculated by comparing the measured values and spiked concentrations. Accuracy was expressed as recovery and assessed by the detecting concentrations in blank tea samples spiked with 10, 50, and 100 μg kg^{-1}. Precision was estimated as intraday and interday variability by recovery experiment of three replicates in three days.

3. Results and Discussion

3.1. UHPLC-MS/MS Optimization

The MRM parameters were optimized by direct injection of individual standard solution at 0.5 μg mL^{-1} through a syringe pump at the flow rate of 10 μL min^{-1}. Regarding analytes as acid compounds, ESI in negative mode was employed, and their deprotonated molecular ions $[M - H]^-$ were acquired by full scan in the mass range of m/z 50–300. Each deprotonated molecular ion of three target compounds and internal standard could achieve two transitions. The ion transition with a higher signal was used for quantification, and another transition was used for confirmation. The isomers of 3-PBA and 2-PBA had the same transitions. The declustering potential and collision energy were optimized to improve sensitivity. Table 1 shows MRM parameters for each compound.

Several LC columns, such as Luna C8 (4.6 × 150 mm, 5μm) [22], XSELECT™ CSH™ C18 (100 × 2.1 mm id, 2.5 μm particle size) [23], the Betasil C18 column (100 × 2.1 mm id, 3 μm particle size) [29], Inspire C18 (25 cm × 4.6 mm, id 5 μm particle size) [17], and the Zorbax Eclipse Plus C18 column (100 × 2.1 mm id, 1.8 μm particle size) [18], have been used for the separation of pyrethroid metabolites. However, there is no report for investigating the effect of LC columns and mobile phase on the separation of target compounds. In this study, seven octadecylselyl-based columns, e.g., Zorbax Elipse Plus C18 (150 × 3.0 mm id, 1.8 μm particle size), Zorbax SB-Aq (100 × 2.1 mm id, 2.7 μm particle size), Poroshell 120 EC-C18 (100 × 2.1 mm id, 2.7 μm particle size), Aquity UHPLC HSS T3 (100 × 2.1 mm id, 1.8 μm particle size), Zorbax Eclipse XDB-C18 (150 × 3.0 mm id, 1.8 μm particle size), Kinetex C18 (50 × 2.1 mm id, 2.6 μm particle size) and Kinetex C18 (100 × 2.1 mm id, 2.6 μm particle size), were employed for the separation of target compounds. The results are shown in Figure 2 and Figure S1. Chromatographic peak broadening for the front four columns was observed, and poor separation of isomers 3-PBA and 2-PBA on Zorbax Eclipse XDB-C18 occurred. A good chromatographic peak was obtained on the Kinetec C18 column due to its core–shell particles with high peak capacity and resolution ability [30]. However, the isomers of 3-PBA and 2-PBA could not be separated on the shorter column with a length of 50 mm.

The mobile phase was also investigated. Compared with methanol, acetonitrile had high elution ability, and better chromatographic peaks for all compounds were observed. Acid water with 0.1% formic acid resulted in no retaining on Kinetex column, poor separation of 3-PBA and 2-PBA, and mass spectrometric signal decreasing eight times. Therefore, a Kinetex C18 column with mobile phase using water and acetonitrile was employed for the separation of target compounds.

3.2. Modified QuEChERS Development

Three extraction methods, namely, original QuEChERS [25], Official CEN 15662 [26], and AOAC 2007.1 [27], were evaluated. As shown in Figure 3, the highest recoveries

of all analytes for CEN 15662 treatment were obtained among three extraction methods. Acidified acetonitrile was generally used for the optimal extraction of acid compounds to achieve high extraction efficiency [31,32]. In this study, the analytical compounds and internal standard 2-PBA were acid compounds with log P less than 4.0. Therefore, the citrate-buffering salts employed for the Official CEN 15662 method helped to improve the recoveries of target compounds when compared with the original QuEChERS and AOAC 2007.1 method, without using acidified acetonitrile.

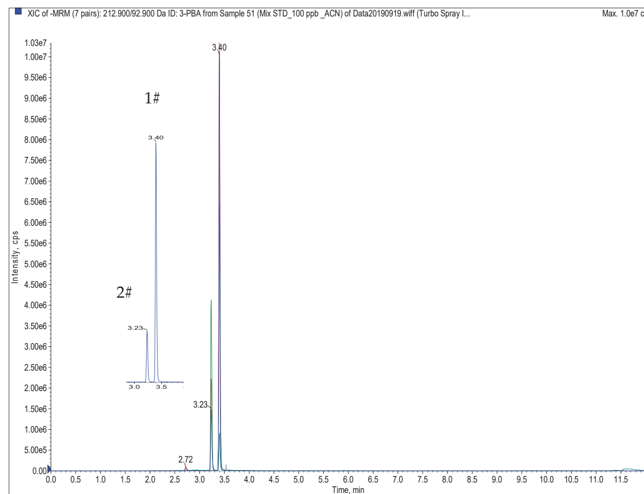
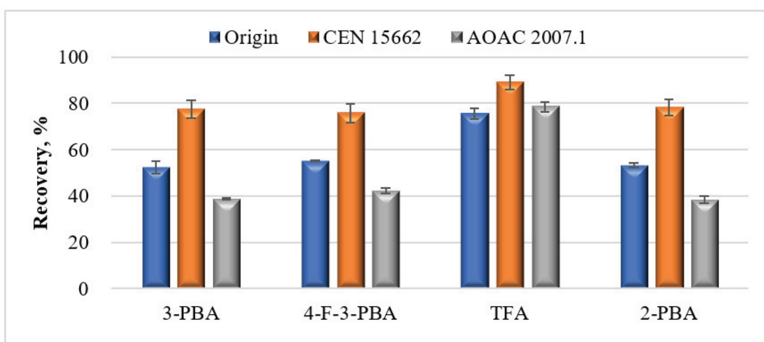


Figure 2. Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) chromatograms of three target compounds and internal standard 2-PBA (peak 1 # means 3-PBA and peak 2# means 2-PBA) obtained from Kinetex C18 (100 × 2.1 mm id, 2.6 µm particle size) under the mobile phase A water and phase B methanol. Note, gradient elution: 0–1 min 5% B; 1–3 min 95% B; 3–10 min 95% B, 10–10.5 min 5% B;10.5–12 min 5% B.



Extraction method	Extraction solvent	Salt
Original	Acetonitrile	4 g $MgSO_4$ and 1 g NaCl
CEN 15662	Acetonitrile with citrate buffer (1 g sodium citrate tribasic dehydrate and 0.5 g sodium citrate dibasic sesquihydrate)	None
AOAC 2007.1	Acetonitrile with acetate buffer (1.5 g NaAc)	6 g $MgSO_4$

Figure 3. Recoveries of three analytes and internal substances with the extraction methods of original "quick, easy, cheap, effective, rugged and safe" (QuEChERS), CEN 15662, and AOAC 2007.1.

The selection of QuEChERS adsorbents is crucial for the recovery of analytes and the removal of the tea matrix. Several adsorbents, such as PSA, C18, GCB, florisil, and MWCNTs, have been employed for cleaning up the tea matrix [33–35]. In this study, seven adsorbents, e.g., NH_3-NTs, MTCNTs, GCB, C18, PSA, florisil, and ZrO, were investigated. Two milliliters of tea extracts spiked with three target compounds and internal standard at 100 µg L^{-1} was added in a 5 mL centrifuged tube individually containing NH_3-NTs (100 mg), MTCNTs (100 mg), GCB (100 mg), C18 (200 mg), PSA (200 mg), florisil (200 mg), and ZrO (200 mg). The results are shown in Table 2. Satisfied recoveries of all target compounds were achieved in the range of 92.5–108.3% for five adsorbents, except PSA and PVPP, in which recoveries ranged from 55.7% to 111.9%, and from 86.4–97.9%, respectively. As shown in Figure 4, tea extracts became light yellow and transparent after they were cleaned up by three carbon materials, such as NH_3-NTs, MWCNTs, and GCB, because these adsorbents were particularly effective for tea co-extracted pigments [36]. Tea extracts were dark and opaque before and after clean up by ZrO, indicating that a few tea matrices were removed.

Table 2. Recoveries of target compounds after clean-up by amino carbon nanotubes (NH_3-NTs), multi-walled carbon nanotubes (MWCNTS), graphite carbon black (GCB), octadecylsilane (C18), primary secondary (PSA), polyvinylpyrrolidone (PVPP), florisil, and zirconia (ZrO).

Adsorbents	Recoveries (%)			
	3-PBA	2-PBA	4-F-3-PBA	TFA
NH_3-NTs	103.0 ± 2.4	101.6 ± 1.3	102.3 ± 4.2	103.8 ± 1.6
MWCNTs	103.2 ± 2.3	108.3 ± 1.8	103.1 ± 5.1	102.0 ± 7.8
GCB	99.8 ± 2.9	104.9 ± 1.4	99.7 ± 5.0	92.6 ± 1.9
C18	93.9 ± 3.0	103.6 ± 2.0	93.7 ± 6.8	93.8 ± 5.9
PSA	81.0 ± 3.7	55.7 ± 3.5	88.9 ± 2.8	111.9 ± 5.3
PVPP	86.4 ± 4.2	97.9 ± 0.9	88.4 ± 2.3	95.2 ± 2.5
Florisil	105.7 ± 3.9	98.4 ± 2.6	104.9 ± 2.7	104.9 ± 3.3
ZrO	94.0 ± 3.3	103.6 ± 0.7	94.1 ± 3.3	92.5 ± 4.4

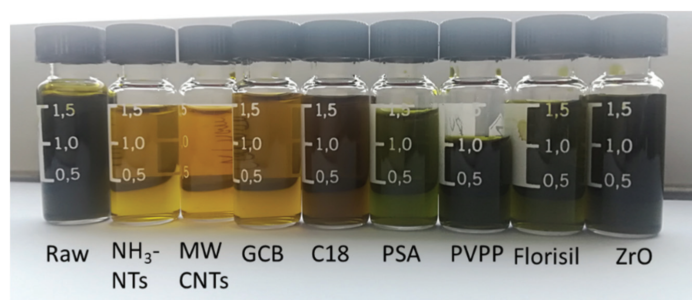


Figure 4. Tea extracts before (Raw) and after clean up by NH3-NTs, MWCNTS, GCB, C18, PSA, PVPP, florisil, and ZrO.

Although the decolorization effect of C18 and florisil was not obvious, considering the fact that C18 and florisil were useful and employed to remove some mid-polar and high lipid contents [37,38], the mixture of 200 mg C18 and 200 mg florisil combination with individual MWCNTs and GCB was further evaluated as modified QuEChERS. As shown in Table 3, the mixture of MWCNTs, C18, and florisil resulted in the loss of 3-PBA, 2-PBA, and 4-F-3-PBA, while the combination of GCB with C18 and florisil achieved good recoveries of all target compounds in the range of 84.2 – 98.8%. Therefore, the mixtures of 100 mg GCB, 200 mg C18, and 200 mg florisil were used as modified QuEChERS adsorbents.

Table 3. The effect of different amounts of GCB and MWCNTs combined with 200 mg C18 and 200 mg florisil on the recovery of analytes.

Adsorbents	Recoveries (%)			
	3-PBA	**2-PBA**	**4-F-3-PBA**	**TFA**
GCB (25 mg) + C18 + Florisil	86.0	87.1	87.2	91.6
GCB (50 mg) + C18 + Florisil	84.2	86.0	86.8	97.6
GCB (75 mg) + C18 + Florisil	84.5	87.7	87.2	97.1
GCB (100 mg) + C18 + Florisil	85.6	87.5	89.0	98.8
MWCNTs (25 mg) + C18 + Florisil	82.0	82.4	82.9	92.0
MWCNTs (50 mg) + C18 + Florisil	78.1	76.4	79.7	91.9
MWCNTs (75 mg) + C18 + Florisil	75.7	78.8	76.7	99.0
MWCNTs (100 mg) + C18 + Florisil	69.4	75.8	74.5	92.0

3.3. Method Performance

The linearity of the present method was evaluated using acetonitrile and matrix-matched calibration curves at seven different concentration levels in the range of 0.1 to 100 μg L^{-1} in triplicates at each level. 2-PBA at 10 μg L^{-1} was used as an internal standard for calibration of 3-PBA, 4-F-3-PBA, and TFA. The calibration curves of 3-PBA, 4-F-3-PBA, and TFA were obtained by plotting the peak area ratios against the concentrations. Satisfactory linearity of three analytes was obtained with determination coefficients (R^2) higher than 0.998 in both acetonitrile and tea matrix-matched calibration solutions (Table 4).

Table 4. Linear range and equation with correlation coefficient (R^2), matrix effect (ME, %), the limit of detection (LOD, μg kg^{-1}), and the limit of quantification (LOQ, μg kg^{-1}).

Compounds	Matrix	Linear Rang (ng mL^{-1})	Equation	R^2	MEs (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
3-PBA	Green tea	0.1–50	Y = 2.341x + 0.533	0.9994	−82.9	1.5	5
	Black tea	0.1–50	Y = 1.834x + 0.177	0.9999	−83.7	2.2	5
4-F-3-PBA	Green tea	0.1–50	Y = 5.426x + 0.628	0.9999	−60.3	0.5	2
	Black tea	0.1–50	Y = 3.810x + 0.330	1.0000	−63.6	0.5	2
TFA	Green tea	1–100	Y = 0.0263x + 0.0109	0.9993	−53.2	5.2	10
	Black tea	1–100	Y = 0.0147x + 0.0105	0.9984	−65.9	6.1	10

Matrix effects (MEs) were evaluated by comparison of the slope ratio between matrix-matched calibration curves and acetonitrile calibration curves. All target compounds showed a strong matrix suppression effect, ranging from −53.2% to −83.7%. The black tea matrix resulted in a more serious suppression effect than that of the green tea matrix. Strong Mes were also observed for the determination of pyrethroids degradation products in human urine samples [18]. Therefore, the matrix-matched calibrations were used for quantification purposes, employing blank green tea samples and black tea samples.

Blank tea samples spiked at 1, 2, 5, and 10 μg kg^{-1} were determined for the evaluation of LODs and LOQs. As shown in Table 4, LODs of 3-PBA, 4-F-3-PBA and TFA were 1.9–2.2, 0.5 and 5.2–6.1 μg kg^{-1}, respectively, while LOQs were 5, 2, and 10 μg kg^{-1} for 3-PBA, 4-F-3-PBA and TFA, respectively, in green tea and black tea samples. Mortimer et al. developed a sensitive gas chromatography electronic capture detector (GC-ECD)-based method for the determination of pyrethroid pesticides metabolites, where the LODs of 3-PBA and TFA were 5.0 and 1.4 μg L^{-1} [12]. Although the sensitivity of the previous study reported by Mortimer et al. was similar to this developed method, its complicated and time-consuming derivatization step was a shortage. Meanwhile, the LODs depended on the coextractive interferences because of the poor selectivity of GC-ECD.

Table 5 shows the intraday and interday accuracy and precision estimated through recovery trials, spiking blank samples at 10, 50, and 100 μg kg^{-1}. Satisfactory recoveries were obtained in the range of 83.0–108.6% for 3-PBA, 82.9–92.3% for 4-F-3-PBA, and

107.8–117.3% for TFA in green tea and black tea. The precisions expressed as RSDs of 3-PBA, 4-F-3-PBA and TFA ranged from 2.4% to 13.2%, 1.8% to 9.2%, and 1.2% to 9.5% for intraday precision, while the interday RSDs of 3-PBA, 4-F-3-PBA and TFA ranged from 3.1% to 11.0%, 4.0% to 11.1%, and 2.2% to 8.0%, respectively. Typical UHPLC-MS/MS chromatograms of three target compounds and the internal standard are shown in Figure 5.

Table 5. Recovery, relative standard deviations (RSDs), LOD, and LOQ were obtained for the target compounds in green tea (GT) and black tea (BT).

Compounds	Teas	Recoveries, %			Intra-Day Precision (RSD, %, n = 5)			Inter-Day Precision (RSD, %, n = 3)		
		Spiked Level (μg kg^{-1})			Spiked Level (μg kg^{-1})			Spiked Level (μg kg^{-1})		
		10	50	100	10	50	100	10	50	100
3-PBA	GT	108.6	83.0	95.4	4.6	3.1	2.4	11.0	6.0	10.3
	BT	101.0	100.1	99.7	13.2	3.1	5.4	7.9	3.5	3.1
4-F-3-PBA	GT	92.1	82.9	92.3	5.3	3.7	1.8	6.4	5.3	4.0
	BT	88.0	88.3	85.1	9.2	3.9	2.6	6.1	4.3	11.1
TFA	GT	110.4	113.4	109.7	3.6	1.2	4.9	8.0	4.0	6.1
	BT	117.3	112.0	107.8	9.5	1.6	3.2	5.1	2.6	2.2

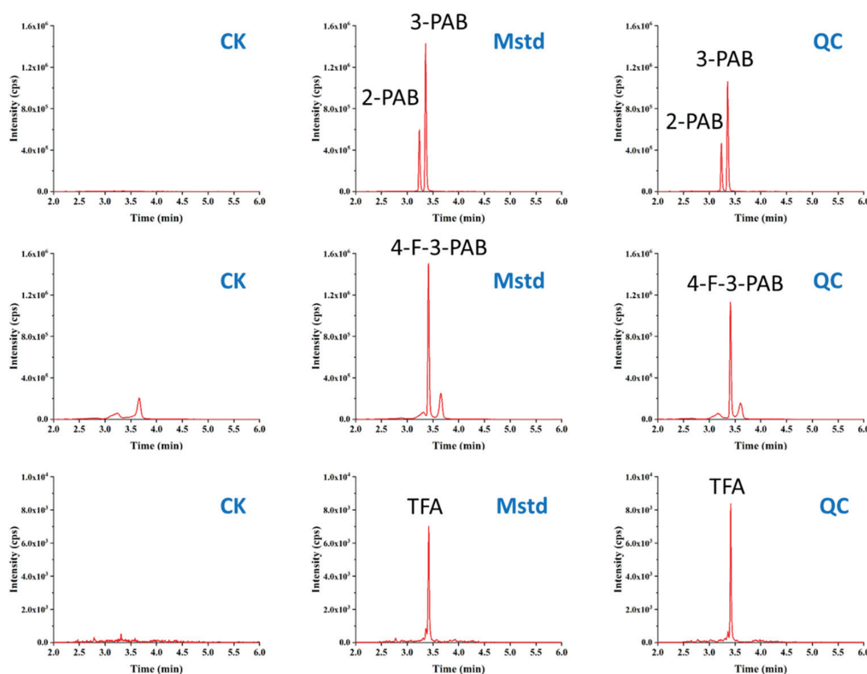


Figure 5. UHPLC-MS/MS chromatograms of 3-PBA, 4-F-3-PBA, TFA and internal standard 2-PBA in a blank green tea sample (CK), matched matrix standard calibration at 10 μg L^{-1} (Mstd), and spiked sample at 50 μg L^{-1} (QC).

3.4. Analysis of Real Sample

The proposed method was applied to analyze 12 green tea samples and 10 black tea samples. Only two green tea samples were found to be contaminated with 3-PBA at 28.7 μg kg^{-1} and TFA at 12.9 μg kg^{-1} for one sample, and 3-PBA at 9.5 μg kg^{-1} for another sample. All black tea samples did not contain 3-PBA, 4-F-3-PBA, and TFA. To

illustrate the formation of 3-PAB and TFA, we detected pyrethroid pesticides in both positive green tea samples referring to the previous method [39]. The results show that the positive sample with 3-PBA and TFA contained cypermethrin (649 μg kg^{-1}), bifenthrin (1021 μg kg^{-1}), and cyhalothrin (406 μg kg^{-1}), while another positive green tea sample with 3-PBA only contained cypermethrin (31 μg kg^{-1}). The results indicate that 3-PBA could be transformed from cypermethrin and cyhalothrin, while TFA was converted from bifenthrin and cyhalothrin.

Although pyrethroid pesticides were not found in the present black tea samples, 3-PBA and other metabolites could be produced during tea fermentation when pyrethroid pesticides were applied in the tea plant. Hu et al. found a novel golden flower fungus from fu brick tea, named *Eurotium cristatum* ET1 strain, which could efficiently degrade both cypermethrin and 3-PBA [40]. They also found that 3-PBA could be formed when ET1 degraded cypermethrin in brick tea, which was one of the heavy fermentation teas. Therefore, metabolites of pyrethroid pesticides could appear in both unfermented and fermented tea samples, and, thus, both pesticides and their metabolites should be monitored.

4. Conclusions

A modified QuEChERS method was developed for the determination of three typical pyrethroid metabolites, namely 3-PBA, 4-F-3-PBA, and TFA in tea using UHPLC-MS/MS. The UHPLC-MS/MS parameters were optimized and three versions of QuEChERS technique were compared. Various types of adsorbents were evaluated for obtaining high recoveries of target compounds and removal of tea matrices. Method validation was employed, and high recoveries and precisions were obtained, indicating the developed method fulfilled with the analysis of 3-PBA, 4-F-3-PBA, and TFA in tea.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/1/189/s1, Figure S1: UHPLC-MS/MS chromatograms of 3 target compounds and internal standard 2-PBA.

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Pesticides and Environmental Contaminants in Organic Honeys According to Their Different Productive Areas toward Food Safety Protection

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Abstract: Monitoring contaminant residues in honey helps to avoid risks to human health, as it is a natural product widely consumed in all population groups, including the most vulnerable, such as children and the elderly. This is important for organic honey production that may be negatively influenced by geographical area pollution. Considering the importance of collecting data on the occurrence of various xenobiotics in different geographical areas, this study aimed to investigate the presence of contaminant residues (persistent organic pollutants (POPs) and pesticides, including glyphosate and metabolites) in organic honey samples from different production areas using different analytical methods, in order to confirm their incidence and possible impact on the food safety traits of organic production. Regarding POPs, traces of benzofluoroanthene and chrysene were detected in honey from intensive orchards and arable lands. Traces of all polychlorobiphenyl (PCB) congeners were detected at different percentages in almost all of the samples, regardless of the origin area. Traces of polybromodiphenylethers (PBDE 28, 33, and 47) were found in different percentages of samples from all of the geographical areas examined. Traces of organochlorines (OCs) and organophosphates (OPs) were identified in honey samples belonging to all of the geographical areas. No glyphosate, glufosinate, and aminomethylphosphonic acid (AMPA) residues were detected.

Keywords: pesticides; persistent organic pollutants (POPs); glyphosate; AMPA; organic honey; GC-MS/MS; IC-HRMS; food safety

1. Introduction

Honey bees play a key role in the environmental ecosystem as pollinating species also contributing to the production of marketed honey, beeswax, and other bee products [1]. Food safety is essential for protecting consumer health and promoting food surveillance. It is fundamental to monitor contaminant residues present in foodstuff, such as honey, to prevent health risks in humans, as it is an aliment broadly consumed throughout the population, including the most vulnerable groups, such as children and the elderly [2]. Beebread, beeswax, and honey contamination by pesticides can also affect the colonies' vitality when contaminated matrices are present during larvae development, leading to serious ecotoxicological issues [3,4]. Moreover, honey is widely used to control oxidative deterioration

processes in fruit and vegetables and/or reactions of lipid oxidation in meat [5], avoiding pathogen and microorganism proliferation that leads to the decomposition of food [6].

In modern apiculture, honey contamination may occur directly (i.e., honey bee colonies treated for veterinary purposes) or indirectly, since honey bees, during foraging, are able to cover long distances, coming in contact with polluted pollen, nectar, and water [7–9]. Many researchers have conducted studies on honey bees and/or honey-bee products to assess the environmental pollution level of industrial areas [1]. In fact, other works have underlined that honey contamination is strongly related to the environmental scenario considering the different types of contamination sources [2,10,11]: Pesticides applied in agriculture may consequently contaminate honey and bee products, compromising food safety [12,13]. For these reasons, in the last decades, beekeeping practices have been implemented in order to ensure human health safety and to preserve the key role of honey bees in the environment, by reducing both direct and indirect beehive contamination [14]. Unfortunately, serious concerns regarding both organic and non-organic honey production are still present. In fact, many persistent environmental organic pollutants (POPs) may contaminate bee matrices [8]. Among the environmental contaminants, in the literature, the presence of polychlorobiphenyls (PCBs), polybromodiphenylethers (PBDEs), organochlorines (OCs), and organophosphates (OPs) in honey samples is well-recognised. In particular, OCs are extremely stable, slightly volatile, lipophilic, and persistent. For these reasons, OCs accumulate and bio-accumulate in foodstuff, representing a matter of concern for the consumer [2]. Organophosphates can induce acute poisoning via food consumption due to their acetylcholinesterase inhibition activity, representing a life-threatening concern [15,16]. This class of pesticides, widely used in agriculture to protect against crop-eating insects and to control *Varroa destructor*, represents a consistent contamination source [17,18]. Due to the common beekeeping practice of recycling old wax combs, OP residues accumulate over time, increasing the potential contamination of the following cycle [19,20]. Due to the high lipophilicity, OPs accumulate, particularly in beeswax, as reported in studies conducted in Belgium [21], France [22], Germany [23], Switzerland [12], Italy [24–26], and Spain [3,27,28]. Significant OP levels have also been found in apiaries outside of Europe, such as in North America [29–32] and South America [33]. Among pesticides, glyphosate (GLY) is the most widely used herbicide in the world [34] and represents a chemical model for estimation of the potential toxic effects on non-target organisms. Although this herbicide exhibits a low toxicity to adult honey bees [35], GLY has recently been associated with sub-lethal health issues in bees due to its chronic accumulation in the hive [36], representing a potential risk for food safety. Although other pesticides have been detected in honey bee products, such as royal jelly and wax combs, there is a lack of information on GLY incidence in these matrices [4]. Due to the analytical difficulties in detecting GLY and its metabolite—aminomethylphosphonic acid (AMPA)—by conventional methods due to their physical and chemical properties [37], it is important to develop reliable analytical methods to monitor their presence, fate, and levels in bee hive product samples, as reported in our previous work [7]. Honey is one of the matrices of animal origin monitored in the Italian National Residue Monitoring Plan (NRMP) concerning veterinary drugs, forbidden and unauthorized substances, and environmental contaminants, such as pesticides; its application aims to guarantee honey traceability and safety and preserve public health and apiaries' ecosystem [38]. In addition, honey could represent a useful indicator for addressing the environmental pollution of geographical areas.

According to the Council Regulation 1804/1999, the use of allopathic chemically-synthesized medicinal products for preventive treatments in organic beekeeping is prohibited and it is also established that plants that can be foraged by bees must be at least 3 km from any source of pollution and from any non-agricultural production sources [14]. At present, few data are available on the multiresidue screening of xenobiotics oriented to assess the relation between the production context and the consequent potential risk of honey contamination with POPs and non-persistent pesticides. In addition, scarce information is available with regards to the monitoring plans of glyphosate and their metabolites in honey. This is particularly important for organic honey production that may be negatively influenced by production area pollution. Considering this scenario and the constant need

to collect data regarding the presence of various xenobiotics in different geographical areas, the aim of the present study was to investigate the presence of contaminant residues using different analytical methods in organic honey samples, from different production areas, to confirm their incidence and possible impact on the food safety traits of organic production.

2. Materials and Methods

2.1. Honey Sample Collection

Ninety-eight honey samples were collected during 2019 and 2020 from different areas in southern Italy (Apulia region), as detailed in Table 1. The distribution of sampled areas according to their geographical location is illustrated in Figure 1.

Table 1. Characteristics of the different production areas of the 98 collected honey samples.

Sample No.	Area Characteristic in Relation to its Potential Pesticide Sources	Botanical Source
29	Intensive orchards	Acacia, Centaurea, Citrus, *Prunus avium* (Cherry), Eucalyptus, *Prunus dulcis* (Almond), Multifloral, Honeydew
25	Arable lands	Centaurea, Citrus, *Prunus avium* (Cherry), *Coriandrum sativum* (Coriander), Eucalyptus, *Prunus dulcis* (Almond), Multifloral, Honeydew
31	Areas close to the city without agriculture activities (anthropic sources, traffic)	Acacia, Centaurea, Multifloral, Honeydew
13	Intensive orchards and arable lands	Multifloral, *Prunus avium* (Cherry), *Coriandrum sativum* (Coriander), Acacia

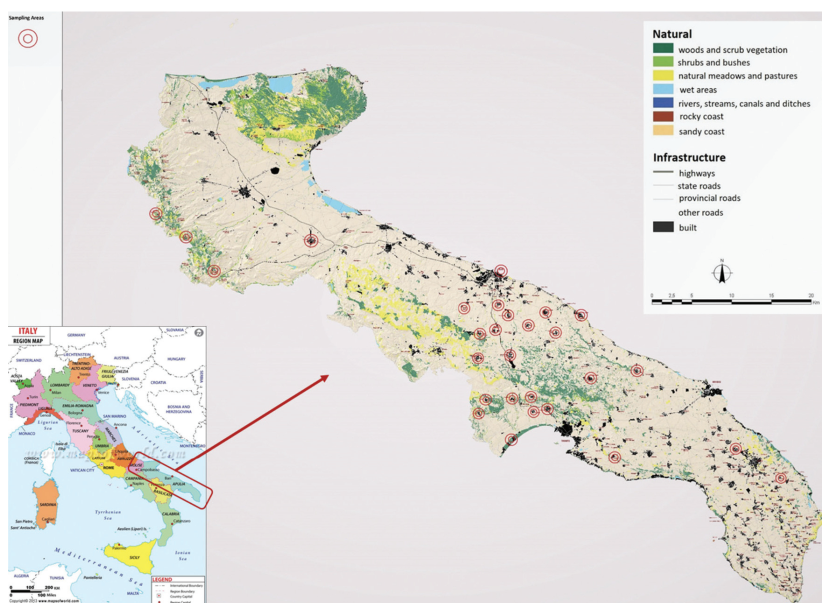

Figure 1. Geographical location distribution of the sampled areas [9].

2.2. Chemicals and Reagents

Glyphosate, glufosinate ammonium, AMPA, and the internal standard N-acetyl-d3-glufosinate were obtained from Merck (Darmstadt, Germany). The PCB congener mix containing PCB 28, PCB 52, PCB 101, PCB 138, PCB 153, PCB 180, and PCB 209 as an internal standard (IS), as well as the PBDE mixture made up of PBDE 28, PBDE 33, PBDE 47, PBDE 99, PBDE 100, PBDE 153, PBDE 154, and 3-fluoro-2,2,4,4,6-pentabromodiphenyl ether (FBDE) as IS, were bought from AccuStandard (New Haven, USA). The OC mix, composed of α-HCH, β-BHC, lindane, hexachlorobenzene, heptachlor, heptachlor epoxide, aldrin, dieldrin, endrin, endrin aldehyde, endosulphan I, endosulphan II, endosulphan sulphate, trans chlordane, 4,4′-DDE, 4,4′-DDT, 2,4′-DDT, 4,4′-DDD, and methoxychlor, was obtained from Restek (Bellefonte, PA, USA). The OP mix including anziphos methyl, boscalid, bupiramate, captan, chlorantraniliprol, chlorpyriphos, coumaphos, diazinon, disulphoton, ethoprophos, fenchlorphos, fenthion, fluazinam, iprodion, methyl paration, mevinphos, penconazol, phorate, protiofos, pyraclostrobin sulprofos, quinoxyfen, spirodiclofen, tetrachlorpirophos, tribuphos, trifloxystrobin, and 4-nonylphenol (IS for OCs and OPs) was sourced from Merck (Darmstadt, Germany). Florisil (100–200 96 mesh) was obtained from Promochem (Wesel, Germany). All of the solvents of special grade for pesticide residue analysis (Pestanal) were purchased from Merck. Formic acid (98–100%) was also sourced from Merck (Darmstadt, Germany).

2.3. Analysis of Pesticides and POPs

2.3.1. Extraction and Clean-Up

The extraction of POPs was performed according to Chiesa et al. [13], by pressurized liquid extraction with an ASE 350 Accelerated Solvent Extractor (Thermo-Fisher Scientific, Waltham, MA, USA). Briefly, 2 g of honey sample was homogenized with an equal weight of Diatomaceous earth and sodium sulphate and transferred into the extraction cell. Then, 1 mL of isooctane solution containing the three ISs was added and the remaining empty part of the cell was filled with Diatomaceous earth. The cells were packed with a cellulose filter at the bottom, followed by Florisil (5 g). The extraction solvent was a mixture of hexane/ethyl acetate (4:1, *v*/*v*). Organic extracts were then collected and treated with sodium sulphate to remove any possible humidity trace. Finally, the extract was dried in a centrifugal evaporator at 30 °C and dissolved in 200 μL of isooctane.

2.3.2. GC-MS/MS Detection

Pesticides and POPs in honey samples were analysed by triple quadrupole mass spectrometry in electronic impact (EI) mode. A GC Trace 1310 chromatograph through a Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane fused-silica capillary column (35 m × 0.25 mm, 0.25 μm film thickness, Restek, Bellefonte, PA, USA), coupled to a TSQ8000 detector (Thermo Fisher Scientific, Palo Alto, CA, USA), was used to confirm and quantify residues. The oven temperature program and all of the set mass parameters were described in Chiesa et al. [13]. The Xcalibur™ and Trace Finder 3.0 were the processing and instrument control software programs used.

2.3.3. Extraction of Glyphosate, Glufosinate, and AMPA

The determination of GLY, its metabolite, and glufosinate was conducted according to Chiesa et al. [7]. Briefly, 1g of honey was spiked with the internal standard (100 ng g^{-1}) and 3 mL of methanol, followed by the addition of 7 mL of acidified deionized water (1% formic acid). The sample was mixed and then sonicated for 15 min. After centrifugation, 1 mL of the supernatant was filtered by a mixed cellulose syringe filter (0.45 μm) directly into a plastic 2 mL vial.

2.3.4. IC-HRMS Orbitrap Analyses of Glyphosate, Glufosinate, and AMPA

The instrumental analyses were performed by a Dionex ICS-5000+ Ionic Chromatography (IC) system (Sunnyvale, CA, USA) coupled to a Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization (HESI) source. For the analyte separation, a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a Dionex IonPac AG19-4 µm guard column (2 × 50 mm) was used. The gradient, all IC, and HRMS parameters were described in Chiesa et al. [7].

Chromeleon™ (Thermo Fisher Scientific, San Jose, CA, USA) and Xcalibur™ 3.0 (Thermo Fisher Scientific, Waltham, MA) software was used to control the IC and HRMS system, respectively.

2.4. Validation Parameters and Quality Control

The methods were already validated according to SANTE/12682/2019 [39] and accurately described in the above-mentioned studies of Chiesa et al. [13] and Chiesa et al. [7]. For the limit of quantification (LOQ) of the methods, we used the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and a relative standard deviation RSD $\leq$ 20%, as defined by the European Commission [39]. Recovery of the studied analytes was carried out at a fortification level of 10 ng g^{-1}, while the method repeatability (expressed as the coefficient of variation, CV, %) was evaluated by analysing six replicates for each by adding known quantities of analyte standard solution (10 ng g^{-1}) to the honey samples.

3. Results and Discussion

This study represents the first survey on the presence of different classes of pesticides and POPs in honey from the Apulia region in Italy. This Italian area was selected for honey sample collection to evaluate the differences with previous research conducted in northern Italy, characterized by industrialized and intensive agricultural contexts [2,13,40]. The results regarding the 98 honey samples are presented in Table 2.

The analytical methods reported here were applied to the investigation of 98 honey samples collected from different Apulian areas to detect and link the occurrence of POPs and pesticides in relation to the contamination source, confirming honey as a suitable indicator of environmental pollution. Apulia region apiculture is strongly based on organic production. Therefore, an evaluation of contaminant residues is critical for sustaining and valorizing organic honey, as well as bee-derived products, such as royal jelly and propolis.

The percentage frequencies of detection in the different sampling areas of the different compounds divided into chemical classes are represented in Figure 2.

Regarding POPs, among the four PAHs investigated, traces of benzofluoroanthene were detected in all samples, with higher percentages in honey produced in intensive orchards (14%), arable lands (16%), and mixed intensive orchards/arable lands areas (15%) compared to that produced in anthropized areas (3%). Additionally, chrysene was detected in honey samples from arable lands (8%), anthropized areas (3%), and mixed intensive orchards/arable lands (15%). Another study [40] reported contamination by PAHs in samples of organic honey from various Italian regions, but with higher concentrations of benzofluoroanthene, anthracene, and benzopyrene in high and low anthropized areas. Furthermore, in this case, the presence of PAHs could be due to various sources, both natural (e.g., forest fires) and industrial (e.g., combustion processes at high temperatures), but without specific connections related to the geographical area considered.

Table 2. Pesticide residues in 98 honey samples from different geographical areas of the Apulia region (Italy).

Pesticides	Intensive Orchards (n = 29)		Arable Lands (n = 25)		Areas Close to the City without Agriculture Activities (n = 31)		Intensive Orchards and Arable Lands (n = 13)		MRLs
	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd
Polycyclic Aromatic Hydrocarbons (PAHs)									
Chrysene	n.d.	-	<LOQ	2 (8%)	<LOQ	1 (3%)	<LOQ	2 (15%)	-
Antracene	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Benzofluoranthene	<LOQ	4 (14%)	<LOQ	4 (16%)	<LOQ	1 (3%)	<LOQ	2 (15%)	-
Benzopyrene	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Polychlorobyphenils (PCBs)									
PCB 28	<LOQ	23 (79%)	<LOQ	25 (100%)	<LOQ	31 (100%)	<LOQ	10 (77%)	-
PCB 52	<LOQ	24 (83%)	<LOQ	25 (100%)	<LOQ	25 (81%)	<LOQ	9 (69%)	-
PCB 101	<LOQ	28 (97%)	<LOQ	25 (100%)	<LOQ	27 (87%)	<LOQ	11 (85%)	-
PCB 138	<LOQ	25 (86%)	<LOQ	23 (92%)	<LOQ	22 (71%)	<LOQ	10 (77%)	-
PCB 153	<LOQ	27 (93%)	<LOQ	25 (100%)	<LOQ	27 (87%)	<LOQ	12 (92%)	-
PCB 180	<LOQ	15 (52%)	<LOQ	18 (72%)	<LOQ	14 (45%)	<LOQ	5 (38%)	-
Polybrominated diphenyl ethers (PBDEs)									
PBDE 33	<LOQ	7 (24%)	<LOQ	5 (20%)	<LOQ	10 (32%)	<LOQ	4 (31%)	-
PBDE 28	<LOQ	6 (21%)	<LOQ	8 (32%)	<LOQ	10 (32%)	<LOQ	6 (46%)	-
PBDE 47	<LOQ	2 (7%)	<LOQ	5 (20%)	<LOQ	3 (10%)	<LOQ	1 (8%)	-

Table 2. *Cont.*

Pesticides	Intensive Orchards (n = 29)		Arable Lands (n = 25)		Areas Close to the City without Agriculture Activities (n = 31)		Intensive Orchards and Arable Lands (n = 13)		MRLs
	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd
PBDE 99	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
PBDE 100	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
PBDE 153	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
PBDE 154	n.d.	-	n.d.	-	n.d.	-	n.d.	-	
Organochlorines (OCs)									
α-BHC	<LOQ	1 (3%)	n.d.	-	<LOQ	1 (3%)	n.d.	-	10
Hexachlorobenzene	<LOQ	26 (90%)	<LOQ	24 (96%)	<LOQ	24 (77%)	<LOQ	9 (69%)	10
β-BHC	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
ϒ-BHC (Lindane)	n.d.	-	<LOQ	1 (4%)	n.d.	-	n.d.	-	10
Heptachlor	<LOQ	1 (3%)	n.d.	-	<LOQ	1 (3%)	n.d.	-	10
Aldrin	<LOQ	9 (31%)	<LOQ	6 (24%)	<LOQ	5 (16%)	<LOQ	1 (8%)	10
Heptachlor epoxide (isomer B)	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
trans-Chlordane	<LOQ	1 (3%)	<LOQ	1 (4%)	n.d.	-	n.d.	-	10
Endosulfan I	n.d.	-	<LOQ	1 (4%)	<LOQ	1 (3%)	n.d.	-	10
4,4′-DDE	<LOQ	17 (59%)	<LOQ	21 (84%)	<LOQ	22 (71%)	<LOQ	4 (31%)	50
Endrin	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
2,4′-DDT	<LOQ	1 (3%)	n.d.	-	<LOQ	1 (3%)	n.d.	-	50

Table 2. *Cont.*

Pesticides	Intensive Orchards (n = 29)		Arable Lands (n = 25)		Areas Close to the City without Agriculture Activities (n = 31)		Intensive Orchards and Arable Lands (n = 13)		MRLs
	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd	det. freq.	ng g^{-1} ± sd
Endosulfan II	<LOQ	2 (7%)	n.d.	-	<LOQ	1 (3%)	n.d.	-	10
4,4′-DDD	<LOQ	17 (59%)	<LOQ	15 (60%)	<LOQ	15 (48%)	<LOQ	7 (54%)	50
4,4′-DDT	<LOQ	5 (17%)	<LOQ	3 (12%)	<LOQ	4 (13%)	<LOQ	1 (8%)	50
Endosulfan sulfate	n.d.	-	n.d.	-	<LOQ	3 (10%)	n.d.	-	10
Organophosphorus (OPs)									
Dichlorvos (DDVP)	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Mevinphos	n.d.	-	n.d.	-	<LOQ	1 (3%)	n.d.	-	-
Demeton O & S	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
Ethoprophos	n.d.	-	<LOQ	1 (4%)	n.d.	-	n.d.	-	-
Phorate	n.d.	-	n.d.	-	<LOQ	1 (4%)	n.d.	-	10
Diazinon	<LOQ	2 (7%)	<LOQ	1 (4%)	n.d.	-	n.d.	-	10
Disulfoton	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
Methyl parathion	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
Fenchlorphos (Ronnel)	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Chlorpyrifos	n.d.	-	n.d.	-	n.d.	-	n.d.	-	50
Fenthion	n.d.	-	n.d.	-	n.d.	-	n.d.	-	10
Trichloronate	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-

Table 2. *Cont.*

Pesticides	**Intensive Orchards (n = 29)**		**Arable Lands (n = 25)**		**Areas Close to the City without Agriculture Activities (n = 31)**		**Intensive Orchards and Arable Lands (n = 13)**		**MRLs**
	ng g^{-1} ± sd	**det. freq.**	**ng g^{-1} ± sd**	**det. freq.**	**ng g^{-1} ± sd**	**det. freq.**	**ng g^{-1} ± sd**	**det. freq.**	**ng g^{-1} ± sd**
Merphos	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Tetrachlorvinphos	n.d.	-	<LOQ	1 (4%)	n.d.	-	n.d.	-	-
Prothiofos	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Fensulfothion	n.d.	-	n.d.	-	n.d.	-	n.d.	-	-
Sulprofos	<LOQ	1 (3%)	n.d.	-	<LOQ	1 (4%)	n.d.	-	-
Azinphos methyl	<LOQ	3 (10%)	<LOQ	1 (4%)	n.d.	-	n.d.	-	-
	1.32	1 (3%)							
Coumaphos	<LOQ	16 (55%)	<LOQ	18 (72%)	<LOQ	15 (48%)	<LOQ	7 (54%)	10
	0.66±0.2	2 (7%)	1.44±0.3	3 (12%)	0.87±0.2	3 (10%)	1.64±0.4	5 (38%)	

n.d. = not detected (<LOD, limit of detection); LOQ = limit of quantification; sd = standard deviation; det. freq = detection frequency; MRLs = maximum residue limits [38].

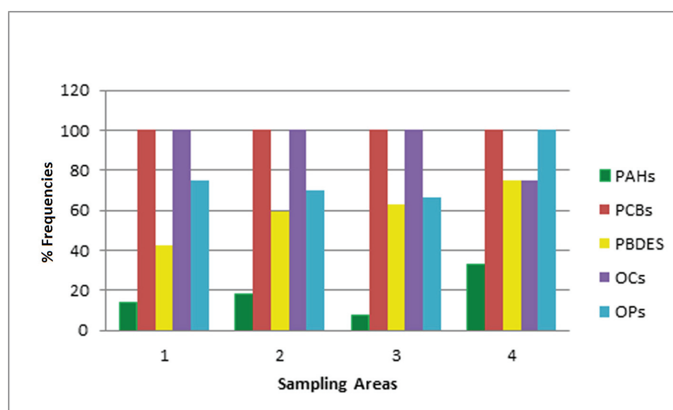

Figure 2. Detection frequencies in the different sampling areas: (1) Intensive orchards; (2) arable lands; (3) areas close to the city without agriculture activities (anthropic sources and traffic); and (4) intensive orchards and arable lands.

Among the six PCBs examined, traces of all congeners at different percentages were detected in almost all of the honey samples, regardless of the sample origin. Specifically, PCB 101 showed the highest (85–100% of samples) and PCB 180 the lowest (38–72% of samples) prevalence. Despite the low concentrations, it is crucial to consider that PCBs were found in all of the sampling areas, confirming that these pollutants are ubiquitous, as reported by other studies [11,13,41]. In fact, the results reported in this study confirm that the PCB concentration in honey, and therefore PCB contamination, is not influenced by the origin of the sample, as confirmed by other studies [2,40,42]. Regarding PBDEs, four congeners (PBDE 99, 100, 153, and 154) were not detected in our samples, according to data on honey collected from other Italian regions reported by Chiesa et al. [13]. Otherwise, traces of the other PBDEs (PBDE 28, 33, and 47) were found at different percentages in samples from all of the geographical areas examined (Table 2). In the literature, few studies concerning the detection of PBDEs in honey are reported (Table S1), with all imputing contamination to direct air transport or also through cross-contamination inside the hive. The results from this study confirmed the ubiquitous presence of many PBDE congeners, underlining the persistence of this class of contaminants in the environment and the consequent possible contamination of both organic and non-organic honey [11,40].

Among pesticides, all of the investigated OCs, with the only exception of β-BHC, which was not detected in this study, were identified in traces in honey samples belonging to all of the geographical areas, as reported in Table 2. The ability of these pesticides to accumulate in the environment and to enter the food chain not only via fatty products, but also via non-fatty products, such as honey, has been previously stated [2]. In particular, some OCs, such as DDT, tend to persist longer than other compounds in the environment, both in an unchanged or metabolized form (DDE and DDD), considering the past use of the parental DDT. The half-life of these pollutants in soil is reported to be over 25 years and strictly related to soil characteristics [43], remaining a threatening issue over time for public health and food chain safety. In this study, different percentages of 2,4′ DDT < 4,4′ DDT < 4,4′ DDD < 4,4′ DDE were detected, in terms of traces, in almost all honey samples tested. Regarding 4,4′ DDD and 4,4′ DDE, the detection percentages were constantly above 55% of samples from each area examined, with the highest percentages being found for DDE in honey from arable lands and anthropized areas (78% and 81%, respectively). These findings, although at higher concentrations, are similar to those reported in other Italian regions, where DDT metabolites were detected more frequently than the parental compound in organic honey samples [13].

Hexachlorobenzene (HCB) was the most frequently identified organochlorine in this study. In fact, traces of this compound were detected in honey samples from all of the geographical areas. As reported

in Table 2, intensive orchard areas showed the highest frequency of HCB detection (90%), followed by arable lands and anthropized areas (both 77%) and mixed intensive orchard/arable land areas (69%). This finding is in contrast to previous works regarding Italian honey. Chiesa and colleagues (2016) [13] did not detect this OC in organic honey from Lombardy, Piedmont, and Calabria regions, and Naccari et al. [44] did not report HCB in honey samples from the Sicily region. In another study, only one sample from Emilia Romagna was contaminated with HCB at the concentration of 69.7 ng/g [40]. Hexachlorobenzene can be released as a by-product of chlorination processes such as pesticide production, coal and fuel combustion, and waste incineration [45]. Due to its long atmospheric degradation lifetime and its relatively high vapor pressure and low water solubility, HCB is highly persistent in the environment [46]. In the Apulia region, the occurrence of these sources of contamination, together with the presence of highly polluting industrial sites, could have contributed to the ubiquitous contamination, even at low levels, of the apiaries examined.

The other class of pesticides investigated—OPs—was detected once again in all of the geographical areas examined. Traces of mevinphos, ethopropos, phorate, diazinon, tetrachlorpyrifos, and sulprofos were detected at different percentages (3–7%) in the various sampling sites. Azinphos methyl was detected in traces in 10% of samples from intensive orchard areas and in 4% of samples from arable land areas. Two honey samples from intensive orchard areas showed concentrations of 0.330 and 1.318 ng/g, similar to data reported by Chiesa et al. [13] on organic honey from the Calabria region. The most frequently detected OP in Apulia organic honey was coumaphos. This acaricide, which has been extensively used in recent decades against *Varroa destructor* outbreaks, showed a similar percentage of detection in all of the geographical areas examined. The coumaphos concentration in Apulia organic honey ranged between 0.322 and 2.132 ng/g, which is a result consistent with those reported for Calabria and Trentino organic honey [13]. Although this result could be considered surprising, since the use of allopathic chemically-synthetized medicinal products for preventive bee treatments is prohibited for organic system production, many studies have reported that coumaphos is persistent in wax and can migrate to other bee products, such as honey, in different proportions [17,18]. Moreover, coumaphos is a compound that can also resist the melting temperature of wax, so it is able to accumulate for years, as it is a common beekeeping practice to recycle wax almost continuously in the form of the foundations on which bees construct a complete comb [21]. Consequently, incorrect apiary management by means of *off-label* preventive treatments with acaricide formulations registered for other species and used fraudulently during organic beekeeping operations cannot be excluded either.

No traces of GLY, glufosinate, and AMPA were detected in the samples analysed, demonstrating the food safety of the analysed products and confirming the absence of contamination from agricultural and urban contexts close to the production areas. This result is in accordance with a previous study regarding Italian organic honey marketed in Italy [7]. Our evidence is also echoed in research by El Agraebi et al. [4], where no transfer of GLY from wax to honey was detected. Despite this finding, caution should be taken in the interpretation of the results since the literature confirmed GLY toxicity below regulatory limits [47] and the genotoxicity of AMPA [48].

Moreover, in the study by Berg et al. [49], agricultural lands demonstrated a strong correlation with GLY incidence, with high concentrations when extensive golf courses and/or highways were adjacent to them. In the same study, the authors suggest GLY migration from the site of use into other areas by bees, but in this case, the samples taken directly from 59 bee hives on the Hawaiian island of Kauaʻi were analysed using ELISA techniques.

From the few data reported in the literature, we can also find evidence of GLY's presence in honey samples, for example, in the study of Pareja et al. [50], where it was detected in 81% of the samples from different origins, with 41% above the MRL. However, none contained AMPA. In the study of Gasparini et al. [51], GLY was detected close to MRL in almost all of the 10 samples obtained from local beekeepers near an agriculture zone. In the study of Chamkasem and Vargo [52], 47% of the samples contained GLY higher than 16 ng g^{-1} (estimated LOQ), while glufosinate and AMPA were not detected in any of the samples.

In general, organic honey produced in the Apulia region displayed contamination by various compounds, although at much lower concentrations than those reported for other Italian regions [2,7,13,40]. Although there does not seem to be a relation in terms of the geographical area considered, generally, the different contaminations detected seem to be mainly linked to the critical points presented by the Apulia region (e.g., highly-polluting industrial sites), together with beekeeping practices that can be considered good, but could still be improved further, especially with regards to organic production. In this study, many POPs and pesticides were detected at a trace level (PCBs, BPDEs, and OCs) or at low concentrations (OPs). While this finding may seem irrelevant from a toxicological point of view, it could actually be a potentially threatening issue for consumer safety, especially for the more fragile categories, such as the elderly and children. In fact, the risk assessment and characterization in terms of the cumulative toxicological effect of low concentrations of multiple xenobiotics have already been addressed. On 29 April 2020, EFSA delivered the first pilot report assessing the cumulative risk from combined exposure to pesticide residues, based on the results of the EU annual monitoring programs for pesticide residues for the years 2014–2016 [53]. Consequently, the monitoring of the safety of honey as a foodstuff (especially from organic production) should be constantly focused on the development of increasingly sensitive analytical methods.

4. Conclusions

In this study, the presence of persistent organic pollutants and pesticides was investigated using different analytical methods in organic honey samples from different production areas in the Apulia region. The determination of contaminant residues in the environment and in foods is essential for assessing specific and cumulative human exposure, especially by dietary intake, guaranteeing that it does not exceed acceptable levels for health.

The results of this study show that honey contamination, even at low concentrations (from <LOQ to 2.13 ng g^{-1}), is strictly related to highly-polluting industrial site problems of the geographical area, confirming honey bees and beehive matrices as suitable tools for monitoring environmental contamination. With the aim of protecting and increasing the importance of honey production, especially organic production, it would seem mandatory to intensify the safety monitoring of this foodstuff and to keep improving good beekeeping practices, as suggested by the EU framework. Moreover, this approach is critical for developing an integrated strategy to select uncontaminated areas for organic production.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/12/1863/s1, Table S1: Literature data on contaminants and pesticides in honey.

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Conflicts of Interest: The authors declare no conflict of interest.

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Organochlorine Pesticides and PCBs in Traditionally and Industrially Smoked Pork Meat Products from Bosnia and Herzegovina

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Abstract: The aim of this study was to determine the concentration of 19 organochlorine pesticides (OCPs): (hexachlorocyclohexane (α-HCH, β-HCH, δ–HCH), lindane, aldrin, heptachlor, heptachlor epoxide, *trans*-chlordane, *cis*-chlordane, endosulfane I, endosulfane II, endosulfane sulfate, dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), dieldrin, endrin, dichlorodiphenyldichloroethane (DDD), methoxychlor and endrin ketone and 6 polychlorinated biphenyls (PCBs) (PCB 28, PCB 52, PCB 101, PCB 153, PCB 138 and PCB 180). The samples were taken from pancetta, dry pork neck (budiola), pork tenderloin and sausages produced in Rakitno (Bosnia and Herzegovina), smoked in both a traditional smokehouse and in an industrial chamber. Instrumental analysis was performed using gas chromatography–mass spectrometry (GC–MS). The reliability of the results, i.e., quality control is ensured by standard laboratory practice, which involves participation in proficiency test, the use of blank samples, reference materials and implementation of recommendations given by the relevant international organizations. The concentrations of α-HCH, lindane, PCB 28, PCB 52 and PCB 153 were detected and quantified. The concentrations of OCPs and PCBs did not significantly vary depending on product type and the conditions of production. All the examined samples were for human consumption.

Keywords: OCPs; PCBs; smoked pork meat products; pancetta; pork neck; pork tenderloin; sausage; traditional and industrial smoking

1. Introduction

In line with the current trend aimed at revitalizing and supporting traditional food manufacturing processes, autochthonous meat products made from locally available raw materials are becoming more and more significant. Smoking is a traditional method of food preservation and is a quite important in many countries worldwide. Smoked meat products in Bosnia and Herzegovina and the Balkans region alike are made in small smokehouses using open fire. Smoking is used not only to contribute the preservation and extension of shelf life of products, but also to affect sensorial properties of final products [1–4]. It should be pointed out, however, that the consumption of food produced in a traditional manner carries certain food safety risks. Consumption of foods containing chemical contaminants such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) can lead

to intoxication, mainly due to long-term exposure [5]. In case of smoked products, the greatest attention is paid to the content of polycyclic aromatic hydrocarbons (PAHs), which are discussed in numerous publications [1,2,6–9], while little attention is paid to the content of OCPs and PCBs, whose effects on human health are certainly not negligible. PCBs, DDT, HCH gamma isomer (lindane) are potentially carcinogenic, according to the International Agency for Research on Cancer (IARS). IARS listed PCBs in group I, which is made up of the most carcinogenic compound for humans [10]. In addition, very low concentrations of PCBs are known to cause adverse immunotoxicity and neurotoxicity effects in humans [11]. OCPs and PCBs were used in agricultural and industrial activities. OCPs have been extensively used for pest management in agriculture mainly due to their low cost but also high efficiency [5]. PCBs have been widely used as additives in industrial materials including plastics, paints and paper. Moreover, PCBs have been used in electronic industry like transformers and capacitors due to their low electrical conductivity and high thermo-resistance [10]. Because of their adverse effects on human health and environment the usage of most OCPs and PCBs were banned in many countries. However, these compounds are still widely detected both in environment and consequently in food.

It is known that the above-mentioned contaminants are very persistent, characterized by high lipophilic properties, and therefore accumulate in food chain, especially in fats. Consequently, food of animal origin is their main source [12,13]. Many people are most exposed to these compounds (>90%) through the food chain, while inhalation or skin exposure is significantly lower [14,15]. The control of OCPs and PCBs residue in food is important for consumer safety. Different organizations, such as regulatory, advisory and scientific bodies specified different maximum residue levels (MRLs) as well as acceptable maximum intake for these compounds. Numerous analytical methods for the analysis of OCPs and PCBs are widely available. Muir and Sverko [16] highlighted the importance of modern capillary gas chromatography (GC) equipment with either electron capture or low-resolution mass spectrometry (MS) detection for separation and quantification of OCPs and PCBs. There are also different screening methods such as commercially available enzyme-linked immuno-absorbent assays. Different methods for extracting OCPs and PCBs such as liquid:liquid (LLE), cavity-dispersed microwave-assisted (MAE), focused microwave-assisted (FME), solid-phase (SPE), and pressurized fluid (PFE) extraction techniques were previously described [17]. Limits of detection (LODs) and limits of quantification (LOQs) may vary between different methods and between different laboratories.

The aim of this study was the examination of chemical safety of pork meat products smoked in traditional and in industrial conditions. The parameters used to assess chemical safety were OCPs and PCBs. The analyzed PCBs (PCBs 28, 52, 101, 138, 153 and 180) belong to the so-called indicator PCBs that have been proposed to be monitored by several countries and international organizations [18]. The reliability and accuracy of analytical results were achieved by using a GC–MS method that was developed and validated as previously described [19].

2. Materials and Methods

2.1. Preparation of Samples and Smoking Procedures

All samples of pancetta, dry pork neck (budiola), pork tenderloin and sausages were produced in a local meat industry, which is located in the Municipality of Posušje, Rakitno, Bosnia and Herzegovina. The raw material used for the production of these meat products originated from local meat industry farms. Raw materials were processed using traditional technology. A part of a pig carcass was cut for the production of pancetta in such way that a part of the chest surrounded by the ribs with a big belly was separated from the back by a longitudinal incision and from the neck with cross-section between the third and fourth rib. The bones and cartilage ribs were left as a part of chest. Corresponding part of the belly was separated from the rest of the carcass by transverse section at the level of the lumbal part of spine. Rib bones and cartilage were separated from the chest muscles. The samples of pancetta had a rectangular shape. The samples of budiola were made from previously formed pieces

of pork necks. The samples of tenderloin were produced from long dorsal muscle *musculus longissimus dorsi*. After determining the weight of each individual sample, they were salted using a mixture of a mineral and a nitrite salt in a 50:50 ratio. The salting was done manually by using unspecified amount of salt put on the surface of meat in a cooling chamber at the temperature of +4 °C in duration of seven days. The samples were then rinsed with water and moved to a place for drying and smoking where they were dried and tempered for 12 to 20 h. The samples were smoked in both a traditional smokehouse (open combustion chamber) and in an industrial chamber produced by Maurer-Atmos Middleby GmbH., (Reichenau, Germany). In traditional smokehouse, the smoking process for all products lasted 20 days. The traditional products were smoked for 6–8 h per day for first 6 days and after that for 2–3 h every second or third day for 14 days. The industrial products were smoked for 4 h per day for 3 days in the industrial chamber. Experimental conditions during smoking procedures for both traditional and industrial conditions are shown in Table 1. The total time of sample preparation was for 45 days for both methods. Traditional sausages were made using the local recipe, from the mixture of the first-category meat with the addition of 30% of the second-category meat, mineral salt, sweet and hot peppers and minced garlic. The mixture was stuffed in natural casings. Smoking was performed in both a traditional smokehouse and in an industrial chamber. In traditional smokehouse, smoking lasted 20 days and in the industrial chamber for 3 days. The total length of production for industrial and traditional production was 30 days.

Table 1. Experimental conditions during smoking procedures of pork meat products.

Sample	Number of Samples	Production Conditions	Smoking Duration (days)	Duration of Production
Pancetta	12	Traditional	20 [1]	45
Pancetta	12	Industrial	3 [2]	45
Tenderloin	12	Traditional	20	45
Tenderloin	12	Industrial	3	45
Budiola	12	Traditional	20	45
Budiola	12	Industrial	3	45
Sausage	12	Traditional	20	30
Sausage	12	Industrial	3	30

[1] first 6 days for 6–8 h per day, 14 days every two or three days for 2–3 h; [2] 4 h per day.

2.2. Reagents and Materials

All chemicals and reagents used were of analytical grade with high purity. Calibration solutions were prepared using the pesticides mix of 20 pesticides—Organochlorine pesticides mixture produced by Ultra Scientific Inc., (Nort Kingstown, RI, USA), lot CL-1069; PCB Mix 1 that include PCB 28, PCB 52, PCB 101, PCB 153, PCB 138 and PCB 180, produced by Dr Ehrenstorfer, lot G126821IO (Bgm.Schlosser-StraBe 6A-Ausburg, Germany). In order to eliminate the influence of the matrix, calibration through matrix blank sample was performed according to European Commission Document No. SANCO /12571/2013 [20].

Working standard solution of Pesticides mix that contains 19 pesticides—Chlorinated Pesticides-herbicides lot: 213091108, was obtained from AccuStandard Inc. (New Haven, CT, USA), used for spike preparation for internal control. Spiked samples were used with the purpose of internal control of the following parameters: recovery, precision, limit of quantification (LOQ) and limit of detection (LOD). Chromatogram of the standard mixtures is shown in Figure 1.

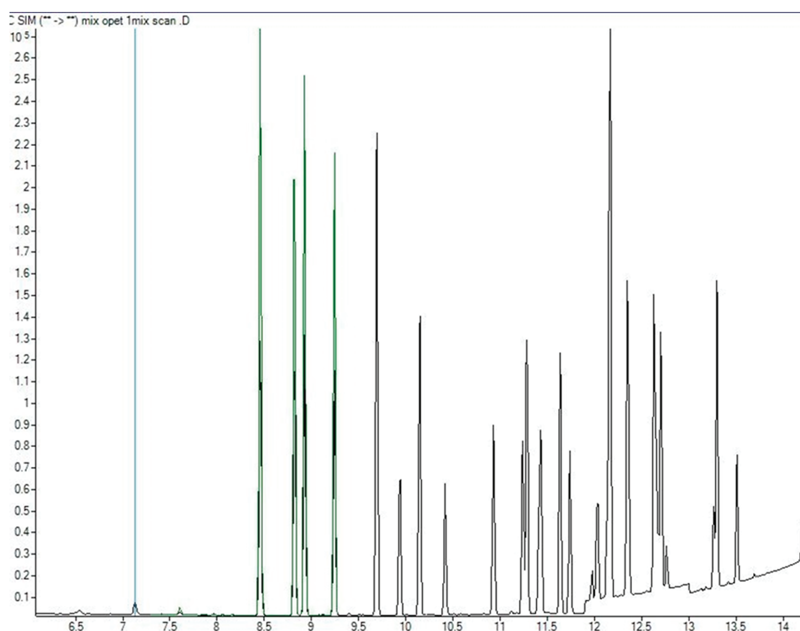

Figure 1. Chromatogram of the standard mixture.

2.3. Sample Preparation

The method of sample preparation was based on the extraction with acetonitrile (ACN) produced by Sigma-Aldrich (St. Louis, MI, USA) in the presence of anhydrous magnesium sulfate ($MgSO_4$) and anhydrous sodium acetate (CH_3COONa), produced by Merck (Darmstadt, Germany). Sample (3 g) was measured and transferred into centrifuge tube and 3 mL of water and 3 mL of acetonitrile were added. After intensive stirring on a vortex, 3 g of anhydrous magnesium sulfate and 1 g of anhydrous sodium acetate were added. Exothermic reaction occurred within 1 min after the intense stirring on vortex. The sample was then centrifuged for 5 min at 1110× *g* (approximately 3000 rpm). 1 mL of upper acetonitrile extract was transferred into a 5 mL tube, which contained 150 mg of anhydrous magnesium sulfate, 100 mg of primary and secondary amine (PSA) and 50 mg of C18 [21–23]. The tube content was centrifuged for 5 min at 1110× *g*. After centrifuging, purified and clear extract was obtained. After that, 0.5 mL of the extract was evaporated in nitrogen and reconstituted with hexane. The sample prepared in this way was ready for the analysis on GCMS-Agilent 7890B/5977A obtained from Agilent Technologies Inc., (Santa Clara, CA, USA).

2.4. GCMS Analysis and Instrumentation

The identification of OC pesticides was based on a comparison of retention times of the peaks and target ions with those obtained from a standard mixture of OC pesticides (standards supplied by instrument manufacturer). The quantification was based on external calibration curves prepared from the standard solution of each of the pesticides.

The gas-mass chromatography was Agilent 7890B/5977A MSD (Santa Clara, CA, USA). The GC operating conditions are shown in Table 2. The verification of the peaks was carried out, based on the retention times and target ions compared to those of external OC pesticides. Procedural blank and solvent blanks were analyzed and quantified, but no OC pesticides were found in these blanks. Determination was made in splitless mode, because of that we don't have split mode, carrier gas was Helium, velocity—32.098 cm/sec; pressure—7.0 psi. Determination was made at constant flow.

Table 2. The GC operating conditions.

Descriptions	Conditions
Instrument	Agilent 7890B/5977A MSD (Santa Clara, CA, USA)
Column	Fused silica column (30 m × 0.25 µm film of HP-5M-thickness) Agilent Technologies, Inc., (Santa Clara, CA, USA)
Temperature	Injection 280 °C
	MSD 280 °C
	Column 50 °C (0.4 min hold) to 195 °C at 25 °C/min; hold to 265 °C for 1.5 min at 8 °C/min; maintained at 315 °C for 1.25 min at 20 °C/min
Carrier gas	Helium
Injection volume	4 µL

2.5. Accuracy and Precision

The accuracy of the method was calculated as percent recovery of pesticides from spiked samples. 3 g of homogenized sample was spiked prior to the determination procedure by adding a mixed pesticide standard working solution to reach the final fortification levels of 5, 10, 50, 100 and 500 µg kg^{-1}. For each level, five replicates were analyzed. After the addition of each concentration in the matrix, the mixture was equilibrated by shaking, and the samples were allowed to settle for 30 min prior to extraction in order to ensure the sufficient contact of the analytes with the whole matrix. Then, the samples were prepared according to the method which was described earlier.

The precision in case of repeatability (RSDr) was determined at fortification levels of 0.05 mg kg^{-1} with five replicates on the same day. Precision in case of reproducibility (RSDR) was determined at fortification levels of 0.05 mg kg^{-1} with five replicates at three-week intervals. The limit of detection (LOD) was calculated according to Magnusson and Örnemark [24]. In order to determine the LOD of each analyte, ten independent sample blanks fortified at the lowest acceptable concentration of 0.005 mg kg^{-1} were injected, and the LOD was expressed as the analyte concentration corresponding to three times the standard deviation. Limit of quantification (LOQ) was determined according to European Commission Document SANCO/12571/2013 [20]. LOQ was set as the lowest fortification level for each pesticide that was achieved in the acceptable accuracy (mean recoveries for individual pesticides in the range of 70–130%) and precision (RSDr ≤ 20%).

2.6. Statistical Analysis

Data analysis was performed using Statistica version 12 from StatSoft® (Tulsa, OK, USA) and Excel (Microsoft Excel, 2007) to determine the descriptive statistic parameters (mean, standard deviation, range) and one-way analysis of variance (ANOVA). ANOVA was used for the assessment of variation in different meat products before smoking and after smoking. Post-hoc Tukey's test was used for statistical analysis of differences with a statistical significance defined at $p < 0.05$.

3. Results

3.1. Method Validation

Method validation and quality control were conducted following the European Commission SANTE /11813/2017 [25]. The method was validated in terms of the optimal linearity ($r^2 > 0.99$). Precision was evaluated by repeatability in triplicate (50.0 µg kg^{-1}, $n = 20$) and it ranged from 0.78–17.91%. Recovery ranged from 81.61% to 116.33%. The limits of quantification were lower than the maximum residual limits prescribed for examined products. The obtained results are shown in Tables 3 and 4.

Table 3. The average values of LOD, LOQ, precision, linearity, recovery and RSD in blank smoked meat samples, spiked with 50 µg/kg (n = 20).

OCPs	LOD (µg/kg)	LOQ (µg/kg)	Precision (%)	Linearity (r^2)	Recovery (%)	RSD (%)
α-HCH	1.38	4.66	4.26	0.9991	96.13	5.26
β–HCH	0.56	1.89	17.9	0.9992	99.12	8.89
δ–HCH	0.28	1.18	0.78	0.9991	100.3	18.2
Lindane	0.28	1.10	8.88	0.9993	99.50	8.28
Heptachlor	0.27	1.10	3.36	0.9991	88.10	14.6
Aldrin	1.44	4.64	3.62	0.9993	98.31	3.41
Heptachlor epoxide	0.55	1.59	3.49	0.9991	94.39	3.38
Trans-chlordane	0.42	1.22	4.42	0.9991	90.22	8.23
Cis-chlordane	1.18	3.89	4.28	0.9990	91.56	8.18
Endosulfane I	0.88	2.78	9.26	0.9991	87.27	8.68
DDE	1.38	4.76	3.29	0.9990	96.87	3.88
Dieldrin	1.49	5.20	3.48	0.9992	94.36	3.37
Endrin	0.86	3.12	8.49	0.9998	83.36	16.2
DDD	1.22	3.89	5.72	0.9997	81.61	14.3
Endosulfane II	1.51	4.92	7.81	0.9991	91.21	10.3
DDT	1.38	4.78	3.48	0.9991	94.36	3.37
Endosulfane sulphate	1.45	4.64	13.7	0.9994	116.3	15.3
Metoxichlor	0.59	2.07	7.66	0.9994	106.1	1.78
Endrin ketone	1.26	4.36	5.59	0.9991	85.58	10.5

LOD—Limit of detection; LOQ—Limit of quantification; r—Correlation coefficient; RSD—Precision in case of repeatability; DDE—dichlorodiphenyldichloroethylene; DDD—dichlorodiphenyldichloroethane; DDT—dichlorodiphenyltrichloroethane.

Table 4. The average values of LOD, LOQ, precision, linearity, recovery and RSD in blank smoked meat samples, spiked with 50 µg/kg (n = 20).

PCBs	LOD (µg/kg)	LOQ (µg/kg)	Precision (%)	Linearity (r^2)	Recovery (%)	RSD (%)
PCB 28	0.6	1.9	3.4	0.9994	106.3	3.7
PCB 52	1.0	4.6	7.8	0.9992	101.4	8.6
PCB 101	1.0	3.7	6.4	0.9991	101.1	7.7
PCB 138	1.0	3.2	6.4	0.9990	99.80	7.7
PCB 153	0.9	3.1	5.5	0.9991	110.9	8.3
PCB 180	1.2	4.0	7.3	0.9981	112.3	10

LOD—Limit of detection; LOQ—Limit of quantification; r—Correlation coefficient; RSD—Precision in case of repeatability.

In calculating measurement uncertainty, the contributions PT (FAPAS: Pesticides and PCB in Milk Powder, July–September 2019, Round 05136), the contribution of reproducibility and contribution of bias were taken into account.

3.2. Concentrations of OCPs and PCBs

The study results are presented in Table 5. The concentrations of OCPs and PCBs for each product were examined before smoking, after traditional and industrial smoking and at the end of the manufacturing process. For each product the concentrations of β–HCH, δ-HCH, heptachlor, aldrin, heptachlor epoxide, *trans*-chlordane, *cis*-hlordane, endosulfane I, DDE, dieldrin, endrin, DDD, endosulfan II, DDT, endosulfan sulphate, metoxichlor, endrin keton as well as PCB 101, PCB 138 and PCB 180 were under the limit of detection and limit of quantification in all examined samples. Concentrations of α-HCH, lindane, PCB 28, PCB 52 and PCB 153 were detected and quantified. All these values are below the prescribed maximum residual levels according to European Union legislation [26–29]. Statistical analysis revealed that there are no statistically significance differences between examined pork meat products before smoking and after smoking and manufacturing.

Table 5. Level of OCPs and PCBs (above the LOD and LOQ values) in different smoked pork meat products (μg/kg).

Sample		α-HCH	Lindane	PCB 28	PCB 52	PCB 153	Σ6PCB
Pancetta before smoking	X ± SD	5.1 ± 1.1	15.1 ± 1.08	3.3 ± 0.3	7.003 ± 4.42	3.8 ± 0.1	
	Range frequency	<loq–6.2 33.3%	12.03–28 100%	<loq–10.1 50%	<loq–10.005 33.3%	<loq–4.62 33.3%	<loq–20.1
Pancetta after traditional smoking	X ± SD	<loq	12.5 ± 6.6	<loq	7 ± 6.0005	<loq	
	Range frequency		2.2–26.3 100%		<loq–20 58.3%		<loq–20
Pancetta after industrial smoking	X ± SD	<loq	12.11 ± 1.08	<loq	4.003 ± 2.02	4	
	Range frequency		8.003–14.19 100%		<loq–5 66.7%	<loq–4 16.7%	<loq–8
Pancetta ripening after traditional smoking	X ± SD	<loq	15 ± 14.07	<loq	3.004 ± 4.002	4 ± 1	
	Range frequency		3–46 100%		<loq–9.907 41.7%	<loq–8 83.3%	<loq–15.8
Pancetta ripening after industrial smoking	X ± SD	7.015 ± 5	12.13 ± 5.03	1.2 ± 0.4	1.8 ± 0.2	8.003 ± 6.001	
	Range frequency	<loq–12 33.3%	4.11–22.17 100%	<loq–1.7 83.3%	<loq–6.001 83.33%	<loq–10.005 25%	<loq–10.2
Tenderloin before smoking	X ± SD	30 ± 1	18.15 ± 8.02	<loq	3.001 ± 2.00015	6.005 ± 4.001	
	Range frequency	<loq–34 33.3%	3.12–28.16 100%		<loq–5 66.7%	<loq–10.006 83.3%	<loq–13.5
Tenderloin after traditional smoking	X ± SD	15 ± 4	15.11 ± 2.08	<loq	1.003 ± 0.5	3 ± 1	
	Range frequency	10–20 66.7%	12.01–18.23 100%		<loq–2.004 66.7%	2–6 100%	<loq–6.2
Tenderloin after industrial smoking	X ± SD	<loq	11.16 ± 8.03	<loq	<loq	5.003 ± 6.001	
	Range frequency		3.1–19.19 100%			<loq–10.004 66.7%	<loq–10.004
Tenderloin ripening after traditional smoking	X ± SD	<loq	22.11 ± 7.12	<loq	4.003 ± 2.001	<loq	
	Range frequency		15.004–33.31 100%		1.001–7.005 100%		<loq–7.005
Tenderloin ripening after industrial smoking	X ± SD	<loq	11.17 ± 8.03	3 ± 1	20	<loq	
	Range frequency		10–23 100%	2–4 45.5%	<loq–20 9.1%		<loq–20.4

Table 5. *Cont.*

Sample		α-HCH	Lindane	PCB 28	PCB 52	PCB 153	Σ6PCB
Dry neck before smoking	X ± SD	9 ± 0.7	17 ± 3	<loq	<loq	3 ± 1	<loq–5.005
	Range frequency	<loq–20.02 33.3%	11.11–23.23 100%			<loq–5.005 75%	
Dry neck after traditional smoking	X ± SD	<loq	11.15 ± 1.14	<loq	3.003 ± 1.004	4.004	<loq–9.1
	Range frequency		4.003–31.46 100%		<loq–5.0099 75%	<loq–4.004 8.33	
Dry neck after industrial smoking	X ± SD	15.05	13.15 ± 3.066	<loq	1.1 ± 0.2	3.007 ± 1.006	2.002–6.2
	Range frequency	<loq–15.05 33.3%	11.002–17.26 100%		<loq–1.2 66.7%	2.002–5.02 100%	
Dry neck ripening after traditional smoking	X ± SD	8.05	15.15 ± 2.1	1 ± 0.4	1.003 ± 0.15	5.007 ± 1.004	4.002–8.01
	Range frequency	<loq–8.05 33.3%	12.02–16.28 100%	<loq–2 66.7%	<loq–1.05 66.7%	4.002–6.01 100%	
Dry neck ripening after industrial smoking	X ± SD	<loq	15.02 ± 2.01	<loq	1.004 ± 0.101	4.1 ± 0.004	<loq–5.2
	Range frequency		12.008–16.04 100%		<loq–1.005 66.7%	<loq–4.2 16.7%	
Sausages before smoking	X ± SD	7.007 ± 5.005	18.12 ± 4.05	<loq	1.18 ± 0.2	1.08 ± 0.6	<loq–4.1
	Range frequency	<loq–12.012 33.3%	14.04–22.22 100%		<loq–2.06 83.3%	<loq–2.01 25%	
Sausages after traditional smoking	X ± SD	<loq	16.16 ± 3.03	<loq	3.003 ± 1.001	2.02 ± 0.01	<loq–7.1
	Range frequency		10.1–20.2 100%		<loq–4.004 40%	1.01–3.03 50%	
Sausages after industrial smoking	X ± SD	<loq	15.25 ± 6.06	<loq	5 ± 2	3 ± 0.4	2–6.8
	Range frequency		8.20–24.31 100%		2–7 100%	<loq–3.3 25%	
Sausages ripening after traditional smoking	X ± SD	<loq	25.27 ± 6.05	<loq	<loq	<loq	<loq
	Range frequency		20.21–31.31 100%				
Sausages ripening after industrial smoking	X ± SD	<loq	27.15 ± 5.06	<loq	<loq	<loq	<loq
	Range frequency		21.08–31.24 100%				

X ± SD—mean ± standard deviation; loq—limit of quantification.

4. Discussion

European Commission (EC) Regulation 178/2006 [26] and European Commission (EC) Regulation 149/2008 [27] prescribed MRLs for DDT and metabolites in various meat products are set at the maximum level of 100 μg/kg. The measured value for DDD was several times below the limits specified in the EU.

The maximum levels for PCBs residues in the EU were set in 2006 [28], allowing maximum levels for sum of dioxins and dioxin-like PCBs expressed in World Health Organization (WHO) Toxic Equivalents (WHO-PCDD/F-PCB-TEQ) for pork and meat products at 1.5 pg/g fat. An amendment of European Commission (EC) 1125/2011 [29], with new limits based on WHO TEF (toxicity equivalency factors) from 2011 set the maximum permitted level of six PCBs at 40 ng/g fat for meat and pork products.

Food and Agriculture Organization (FAO) [30] set the maximum acceptable limits at 500 ng/g DDT and 300 ng/g for β-HCH and lindane. In addition, they determined the maximum total PCBs level at 200 ng/g lipid weight for PCBs congeners 28, 52, 101, 126, 138, 153 and 180. WHO [11] established TDI (tolerable daily intake) for total PCBs at 20 ng/PCBs/kg of body weight per day.

The production and use of PCBs is either banned or restricted in most countries, but these compounds are still very important chemical pollutants found in the environment. They can easily migrate to the food chain, which makes them a significant hazard to human health. The presence of chemical contaminants, including OCPs and PCBs in pork muscle tissue and generally in animal tissues is a direct consequence of environmental pollution [23]. It can also be a result of contaminated pig feed [1]. In the past, the cases of PCBs occurrence in food of animal origin at concentrations higher than regulatory levels were connected with industrially produced animal feed. In recent years, the cases of PCBs level higher than the EU limit most commonly came from either "free range" animals, with the unknown source of contamination [31], or animal feed containing ingredients contaminated with PCBs [32,33]. A case where the source of contamination was determined was an old farm tank stained with PCBs day that peeled off at the place where the pigs were kept [34]. Adequate management can significantly reduce the exposure to PCBs.

The major sources of contamination of food of animal origin with PCBs are the following: PCBs from previously contaminated land, PCBs emitted from buildings and PCBs present on farms [31].

The OCPs and PCBs detected in the examined products at the end of the smoking process were α-HCH, lindane, PCB 28, 52 and 153 while other contaminants were below the detection limit (Table 5). In this study raw material pollutant content was low, but higher than the concentration of the examined contaminants in the products. This is most likely due to free range pig production and smoking process which can affect the concentration of OCPs and PCBs. According to the results obtained by Zabik et al. [35], smoking resulted in significantly higher reductions (40–50%) in OCPs and PCBs levels than other thermal treatments used during culinary processing (baking, cooking). Also, higher concentrations of OCPs and PCBs detected in products after smoking than in the raw material can be explained by higher water content and lower fat content in the pre-smoked samples compared to the post-smoked samples. This was confirmed in the case of PAHs content [1], which at the end of the production process was higher than after the smoking process. Škaljac et al. [36], also found that the concentration of PAHs increases with the length of storage time in traditionally produced sausages.

Some studies have shown higher levels of PCBs than allowed. In the analyzed samples [37], five out of six PCBs indicators were above the recommended maximum limit of 15 μg/kg of fat.

Dioxin-like PCBs (DL-PCBs), including 6 indicator PCBs (PCBs 28, 52, 101, 138, 153, and 180) in meat products analyzed in Germany [38], WHO-PCB-TEQ ranged from 0.06 ng/kg fat for raw ham to 0.13 ng/kg fat for raw sausage. The most common were PCB 118, PCB 126, and PCB 156. In the study conducted in Albania [39], the PCBs profile was the following: PCB 153 > PCB 52 > PCB 138 > PCB 180. The average PCBs content was 6 ng/g. Costabeber et al. [40] examined the concentration of six PCBs in 55 samples of meat (pork and beef) and meat products (different types of sausages, salami, canned meat) from Brazil. They found the following concentrations: PCB 52 (5.18 ng/g fat) > PCB 180 (1.69 ng/g fat) > PCB 101 (1.35 ng/g fat) > PCB 28 (1.19 ng/g fat) > PCB153 (0.47 ng/g fat) > PCB 138

(0.43 ng/g of fat). The sum of 6 congeners was 10.3 ng/g. Meat products have a higher PCBs content than meat (the highest content was in the products made from several types of meat, followed by pork, and the lowest was in cattle). The results of a study conducted by Boada et al. [41] showed that the consumption of meat and sausages increases the risk of DL-PCBs detection in human serum.

OCPs and PCBs belong to the environmental pollutants of anthropogenic origin [42]. Production and use of PCBs is practically completely stopped today, but their remains can still be found primary in electrical installations and the environment, and therefore in food. PCBs have been detected in fish from the Mediterranean 30 years after their ban [43]. DL-PCBs can form during cooking meat. Dong et al. [44] found that the concentrations of DL-PCBs in cooked beef was lower than those in raw beef, but a relatively high concentration of PCBs was detected in the oil vapors generated during meal preparation.

In addition to the concentrations measured in meat and its products, it is also necessary to calculate TDI. TDI for 6PCBs at 10 ng/kg bw (0.01 µg/kg bw) [45–47]. The calculation for a 70 kg man is the following: daily intake of Σ6PCBs (ng/kgbw) = conc. Σ6PCBs (ng/g) × x(g)/70 (kg).

However, most OCPs have not been in use in the EU for many years, but they are still detected in food chain because of their persistence in the environment. Also, the risk can be unconscious and illegal use of OCPs which can lead to food poisoning.

In the present study, α-HCH and lindane were the only OCPs detected under the LOQ values. Lindane (γ-HCH) is the most toxic and the least stable of the HCH compounds. It is transformed into more stable α-HCH over the time [48]. Similar results to these were obtained in a study conducted by Pine and Nuro [39]. They found that average pesticide concentration in meat samples is 13.5 ng/g, while the lowest concentration was measured in pork samples. They also found that the presence of HCHs pesticides was a result previous use of lindane in agriculture.

The report on official controls conducted by Iceland and Norway in 2016 and the results obtained indicate that the potential of EU citizens being exposed to pesticide residues at a concentration that would have a negative impact on health is very low. Products of animal origin (pig fat), were analyzed for the presence of 22 pesticides. 11 of them were below the LOQ, while the remaining 11 were detected and qualified. The most commonly detected and quantified were DDT, hexachlorobenzene and chlordane. The MRL exceeded by a small amount in pig fat samples. For the pesticides for which MRLs are not prescribed, a default MRL of 0.01 mg/kg applies. 11 pesticides were sporadically detected, including DDT, hexachlorobenzene and chlordane in 2.8%, 1.8% and 1%, while the others were present in 0.4% of samples (mainly pesticides whose use is not allowed due to their persistence: dieldrin, α-HCH, lindane). Endosulfane, heptachlor, β-HCH and methoxychlor were not detected in any samples. During 2016, 919 pig fat samples were analyzed, with 97.2% of samples having a pesticide concentration lower than the quantification limit. 2.8% of samples contained one or more pesticides. Up to three different residues were detected and quantified in four samples (0.4%) [49].

As the methodology in analytical chemistry is evolving [50], the number of incidents related to food contamination is expected to increase.

Studies regarding the effect of culinary food processing on OCP concentration showed a significant decrease in OCP levels during thermal processing of food of animal origin [51,52]. Frying, roasting, grilling, cooking and other forms heat treatment of raw meat are effective in reducing pesticide levels in food [53,54]. Concentrations of OCPs and PCBs are associated with the fat content of food. During heat treatment of food, fat is reduced or released from the product, so there is less fat in the final product. Muresan et al. [52] noted that OCPs content decreased only by 1% during cold smoking. After the combined treatment of warm smoke and pasteurization, a reduction of 15% to 16% occurred. Roasting decreased OCPs by 46–56%, respectively. In a review paper [55], it was also concluded that culinary procedures can modify the concentration of contaminants in food. However, this depends on the initial OCPs content in the treated food. Thermal treatments that release or remove fat can reduce the overall concentration of OCPs and PCBs in treated meat and products.

In conclusion, concentrations of α-HCH, lindane, PCB 28, PCB 52 and PCB 153 were detected and quantified. All these values are under the prescribed maximum residual levels. Low levels of contaminants tested could be attributed to increased awareness of pesticides usage and their more proper use, i.e., compliance with the ban on its use, which results in reduction of their presence in the environment. The concentrations of OCPs and PCBs were not significantly affected by product type and by conditions of production. However, further study is required in order to monitor the presence and concentrations of OCPs and PCBs in these products.

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