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Toxicity of Insecticides in the Adult and Larva Olive Fruit Fly, after Estimation of the Dislodgeable Foliar and Fruit Residues in Olive Trees by LC-MS/MS and GC-MS/MS

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Abstract: "Can systemic insecticides be used in bait spots in order to kill the adult olive fly?" Effort was directed toward providing an answer to that question. Both field and laboratory tests were implemented to detect the dislodgeable residues of dimethoate, phosmet and b-cyfluthrin in olive leaves and fruit using the LC-MS/MS and GC-MS/MS chromatographic techniques. Residues of dimethoate declined more over time than those of phosmet, while levels of beta-cyfluthrin remained almost stable, both in leaves and fruit. Additionally, significantly higher and faster toxicity of dimethoate and beta-cyfluthrin (>92%) compared to phosmet (80%) to fly adults was shown, which was reduced significantly after a two-week period. Conversely, 100% mortality of the larval stages within olive flesh was observed at the 2nd day for dimethoate and at the 7th day for phosmet. Although phosmet was not expected to contribute to preventing larval development, its application in bait sprays presented similar toxicity to that of dimethoate. However, no larval toxicity was recorded in beta-cyfluthrin. As a primary conclusion, we recommend the avoidance of the use of systemic insecticides in bait sprays.

Keywords: dislodgeable residues; bait spays; olive leaves; olive fruit; olive fly; toxicity

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1. Introduction

Bactrocera oleae Rossi (*Tephritidae*, Diptera) is considered one of the most serious olive crop pests worldwide [1]. Olive growers all over the world struggle to cope with the reduction in infestation rates in olive fruit, the avoidance of crop yield and the deterioration of olive oil production losses. In Greece, the population levels of the pest usually increase between September and October, a period that coincides with the harvesting period of olives, and therefore, there is a risk of impairment of olive oil quality.

Generally, the most common procedure followed during the control of olive fruit fly populations is insecticide applications with cover sprays, especially in intensively cultivated olive orchards. In Greece, the Ministry of Rural Development and Food funds bait spray applications for the management of this dipteran pest since 1937, which can be considered a milder approach compared to cover sprays [2]. The main differences between cover and bait sprays are in the plant part in which the insecticide is applied, the actually applied dose, and the number of trees sprayed. In the case of cover sprays, the treatment is conducted in the total of the trees in the orchards and the plant protection product is applied to the whole canopy of the olive trees. Conversely, in bait applications, only a segment of the

foliage (each location receiving 300 mL of spraying solution) is treated and only half of the trees per hectare in the orchards are sprayed [3]. Additionally, in cover sprays the applied systemic compounds should penetrate plant tissues and kill the developing larvae in the mesocarp, while in bait sprays the insecticide should remain as a dry residue above the plant tissue, attracting the adults to this bait spot (due to the presence of hydrolyzed protein as a food attractant of the fly) and then kill them by contact or ingestion [4]. The actual dose in the spray solution of bait applications is ten times that of the corresponding cover sprays (e.g., organophosphate 0.03%). Initially, bait spays were focused on the total eradication of invasive flies and the non-survival of any individual in the environment [5] while nowadays they are mainly applied against female adults of *B. oleae* on olives and *Ceratitis capitata* Wiedenmann on citrus crops.

To date, during olive fly management, the applied insecticides for both applications, have mainly been organophosphosphates (dimethoate and phosmet), pyrethroids (a-cypermethin, lambda-cyhalothrin, beta-cyfluthrin), spinosad, etc. It is, however, often observed, based on registration procedures, that their registered labels may change from bait-use to cover-use or vice versa independently of their systemicity. The organophosphate compounds used include dimethoate, which can act by contact and through ingestion. It is promptly absorbed and distributed throughout plant tissues, and it shows rapid degradation [6]. Dimethoate was usually used by olive growers during the autumn period, by cover applications due to its effective and systemic action, in combination with its water-soluble nature (pKow = 0.704) [7,8]. Phosmet is a non-systemic, organophosphate insecticide, used in both plants and animals [9]. In plants, it breaks down quickly, primarily through oxidation in air and hydrolysis [10]. It was applied in olive orchards until 2022 and it could be characterized as fat-soluble [7,8]. Beta-cyfluthrin is a non-systemic, broad-spectrum, insecticidal pyrethroid, acting as a contact and stomach poison, characterized by a direct knock-down effect with durable efficacy. Beta-cyfluthrin is a mixture, mainly of two diastereoisomers, II and IV, and lipophilic (pKow = 6.18 at 22 °C) with a tendency for bioaccumulation [7,11].

The increased application density associated with cover sprays in olive trees may have various negative effects, such as the development of insecticide-resistant pest populations [12] and a negative impact on farmers' (worker exposure) and consumers' health (pesticide residues) [13] since olive fruit and olive oil are often contaminated with pesticide residues. The monitoring of dislodgeable foliar residue (DFR) in olive leaves may contribute to the estimation of actual residues (otherwise the pesticide concentration that can be washed from the surface of the leaf with the use of a water/surfactant solution) to which the olive fruit fly is exposed [14–17].

As already stated by Varikou et al. (2018) [18], after investigating the toxicity of insecticides against olive fruit flies through the monitoring of their residual degradation in homogenized olive leaves, a more or less accurate estimation of their DFR at the surface of the leaves would be of great importance. This information would elucidate and clarify the actual efficacy of the applied insecticides in the mechanistic action of bait sprays, meaning "the attract and kill" procedure described above. To achieve this goal the active ingredients dimethoate, phosmet and beta-cyfluthrin were selected as representatives of a systemic, a non-systemic and a contact insecticide, respectively, and their residual behavior in the surface of olive leaves and fruit (after bait applications) over time was investigated. Although it is acknowledged that dimethoate and beta-cyfluthrin have been withdrawn [19,20], they were chosen due to their intensive use in previous years for the management of *B. oleae* and the frequent positive detections in olive fruit and olive oil [13,21], to extrapolate the obtained results to similar compounds. The determination of dislodgeable residues on the surface of leaves and fruit was achieved after the development and validation of an appropriate analytical method combined with LC-MS/MS and GC-MS/MS. This knowledge of DFR combined with entomological trials of toxicity records of adult flies upon contact with sprayed olive stems or larvae within olive mesocarp, lead us to an answer concerning the efficacy of the bait sprays and further improvements of the

method. To this end, semi-field and laboratory tests were conducted in an effort to obtain primary information on whether a given applied and registered plant protection product remains outside olive leaves or fruit and consequently, remains active to 'attract and kill' olive fruit fly, or if it penetrates plant tissues (residual toxicity over time) following the inactivation of the purpose of the bait spray.

2. Materials and Methods

2.1. Field Application

2.1.1. Experimental Area-Olive Orchard

Semifield trials were conducted in the South of Greece (island of Crete) in two olive groves (cv. Koroneiki with small fruit, the common Cretan oil-producing olive variety) in Nerokourou (1.1 ha, 35°28′36.76″ N–24°02′36.44″ E-51 m) and Marathokefala (0.7 ha, 35°31′45.10″ N–23°46′38.12″ E-185 m).

Olive trees at the Nerokourou site were 30–50 years old, 3–6 m tall, 6–7 m apart and not irrigated. The tree density at the time of the study was approximately 200 trees/ha, and the orchard consisted of 140 olive trees. The mean olive fruit production per tree in 2018 was estimated at approximately 80% of the normal yield (approximately 60–70 kg/tree). This orchard, which belongs to the Institute of Olive Tree, Subtropical Plants & Viticulture of Chania, is mainly cultivated for experimental purposes.

Similarly, the olive trees of the Marathokefala site were 50–70 years old, 4–7 m tall, 6–7 m apart and not irrigated. The mean olive fruit production per tree was estimated to be approximately 80% of the normal yield (approximately 80–100 kg/tree) during the tested year. The orchard consisted of 200 olive trees.

The phenological growth stages of olive trees during experimentation were those of the 75th to 89th on the BBCH scale according to Sanz-Cortés et al. (2002) [22].

2.1.2. Application of the Insecticides in Olive Trees

The bait spraying solution consisted of mixing a hydrolyzed protein (2%) with an insecticide in a knapsack (15 L). The liquid hydrolyzed protein used was Protein 75% (Entomela 75 SL, 25% *w*/*w* urea and percentage of protein equal to 75% *w*/*w*; Stavrakis, Viotia, Greece). The tested insecticides were dimethoate (class: organophosphate; Efdakon 40 EC; 625 mL/hL; BASF Hellas), phosmet (class: organophosphate; Phosmetar 50 WP; 600 g/hL; Arista Hellas) and beta-cyfluthrin (class: pyrethroid; Bulldock 2.5 SC; 350 mL/hL; Alfa Georgika Efodia AEBE, Athens, Greece). Each pesticide was applied separately.

Two trials took place in the orchards on Crete, the first under summer, and the second under autumn, conditions. Moreover, the experimental design used for the study was completed randomized blocks with two (dimethoate and control-unsprayed) or four treatments (dimethoate, phosmet, beta-cyfluthrin and control—unsprayed) (as the number of the tested formulated insecticides including unsprayed treatment) for Nerokourou (during summer) and Marathokefala (autumn) orchards respectively, with five replicates per treatment, meaning a total of 10–20 plots. Each plot consisted of one tree—although each plot had a buffer zone of at least 10 m to avoid contamination from pesticide drift. The bait spraying solutions were applied to the whole tree canopy (instead of a part of the tree canopy) to collect as many uninfested olive fruit and leaves for the samplings as necessary. The second spray application was conducted in a different orchard (Marathokefala) to avoid contamination from pesticide residues from the first trial.

The application of the spraying solution of dimethoate took place on 23 July 2018 at Nerokourou and of dimethoate, phosmet and beta-cyfluthrin on 10 September of the same year at Marathokefala orchard, early in the morning (~8 am).

Mean daily temperature and relative humidity were recorded at the official weather station of the Institute of Olive Trees and Subtropical Plants of Chania (for Nerokourou) and the Regional Center of Plant Protection and Quality Control of Crete (for Marathokefala trial) about 2 km away from the experimental area.

2.2. Sampling

2.2.1. Sampling of Olive Leaves and Fruit to Assess Insecticide Residues

Olive leaves: During the selection of leaves, consistency should be taken into consideration in an effort to avoid variability matters from inconsistent sample collection [23]. Following guidelines adopted by Iwata et al. (1977) [14], when the size of the leaves does not allow the use of a leaf puncher, whole leaves are usually sampled. When collecting whole leaves, it is of merit importance that samplers should avoid contact with the leaf surface with their hands or sampling tools. Therefore, samples of olive leaves were collected from all the treated trees of each treatment with sampling intervals of 2 days for the first three time points and of 7 days for the next, meaning 0, 2, 4, 7 and 14 days after each bait spray application. Not more than 50 g of olive leaves/tree were collected randomly all around the tree canopy. Each sample comprised a total of 200–300 g (corresponding to approximately 400 cm^2) olive leaves per treatment, to achieve a representative sample from the whole tree canopy.

Olive fruit: Additionally, samples of uninfested olive fruit were collected from all the treated trees of each treatment at 2, 4, 7 and 14 days after the bait spray application. A total of 300 g of olive fruit were harvested in a similar fashion to the collection of leaves per sample in order to achieve a representative sample from the whole tree canopy.

The staff involved wore gloves and all samples were packed in sample containers, sealed tightly, placed on ice, and sent on the same day to the laboratory for the analysis and determination of dislodgeable residues of the insecticides.

2.2.2. Dislodgeable Foliar Residue (DFR) Sampling

When the samples arrived at the laboratory, subsampling was conducted. The measurement of DFR is based on the weight of residue/foliar surface area, with the preestablished correlation of leaf weight to the surface area of olive leaves, since leaf weight is easier to measure than area [24]. Therefore, the subsampling of whole leaves occurred at a customized surface of 100 cm^2 (10 × 10 cm), corresponding to (4.75 ± 0.54) g, so that all residue calculations could be based on two-sided leaf surface area.

2.3. Analytical Procedure

2.3.1. Chemicals, Reagents, and Standard Solutions

Pesticide reference standards (purity > 99%) of all analytes were purchased as well as a docusate sodium salt (>96%) from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC gradient grade) and water (LC-MS grade) were bought from Fischer chemicals. Ethyl acetate (plus for residual analysis) and formic acid (99%, for analysis) were bought from Carlo-Erba reagents (Sabadell, Spain).

2.3.2. Preparation of Standard Solutions

Stock solutions of dimethoate, phosmet and beta-cyfluthrin were prepared in acetone and stored at −20 °C. Working solutions of all analytes were then prepared by appropriate dilutions of the stock solution in acetonitrile.

2.3.3. Washing Residue DFR Technique and Sample Preparation

Washing and extraction of DFRs were accomplished within 24 h of sample collection. A total of 150 mL of 0.01% (w/v) dioctylsulfosuccinate solution was used for the washing of dislodgeable foliar residues. This solution, having detergent properties, was prepared on the first day of washing and was fully consumed within 2 days. More specifically, olive leaves and fruit were triple washed with 3 × 50 mL of dioctylsulfosuccinate solution (0.01% w/v) after shaking on a platform shaker for 5 min per wash. A total of 10 mL of each of the combined extracts (the extracts of three washings) were transferred to a 50 mL polytetrafluoroethylene (PTFE) tube and 10 mL of ethyl acetate was added. After vigorous shaking of the tube for one minute, centrifugation (4000 rpm for 5 min) was followed and then 1 mL of the supernatant was evaporated under a gentle nitrogen stream,

reconstituted in acetonitrile and injected into the LC-MS/MS system for the determination of dimethoate and phosmet or in the GC-MS/MS system for the determination of beta-cyfluthrin. A similar approach was also followed for the blank samples (unsprayed trees) and the field-fortified (sprayed) samples. All samples were analyzed in triplicate.

2.3.4. Instrumentation

LC-MS/MS analysis: Analysis of dimethoate and phosmet was carried out using a Varian liquid chromatography system equipped with two pumps (Prostar 210) and an automatic sampler (Prostar 420). An Atlantis dC18 Column (100 mm × 2.1 mm × 3 µm, 100 Å) was used at 25 ± 4 °C and the injection volume was 10 µL. The elution gradient was carried out with a binary solvent system consisting of MeCN:H_2O (10:90), 1 mM $HCOONH_4$, 0.5% HCOOH (solvent A) and MeCN:H_2O (90:10), 1 mM $HCOONH_4$, 0.5% HCOOH (solvent B) at a constant flow rate of 270 µL/min. Having 5 min of re-equilibration, the mobile phase was applied in a linear gradient profile with the proportions (*v*/*v*) of solvent A as followed (t (min)): (0.80% A), (8.5% A) (15.5% A).

A Varian 1200 L triple quadrupole mass spectrometer (Varian) was used with an electrospray ionization (ESI) interface, operating in the positive mode with the following setting: source temperature 50 °C, drying gas (N_2) was heated to 320 °C and pressure 18 psi, nebulizing gas (air) 45 psi. Infusion experiments with individual standard solutions of dimethoate and phosmet at 1 µg/mL were tested to optimize capillary voltage (CV) and collision energy (CE). The standards were diluted in mobile phase A. The above solutions were infused into the mass spectrometer, at a flow rate of 10 µL/min, using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). For dimethoate the retention time was 5.24 min; the transition 230-> 199 *m*/*z* (40 CV and 6.5 CE) was used for quantification and 230-> 125 *m*/*z* (40 CV and 6.5 CE) for qualification purposes. A typical SRM chromatogram is presented in Figure 1.

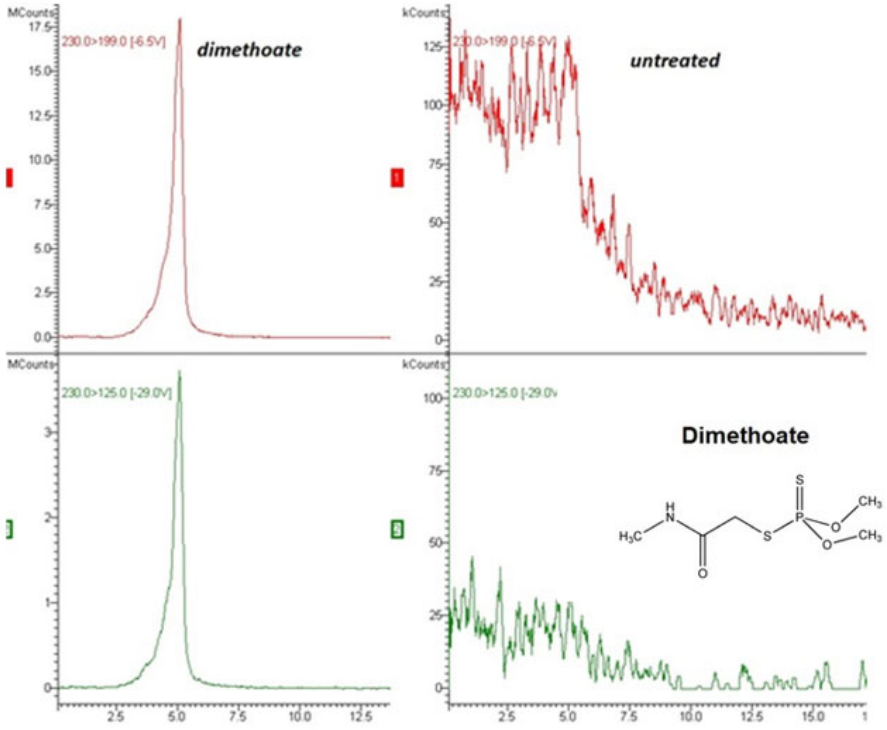

Figure 1. Determination of dimethoate residues in the surface of olive leaves using LC-MS/MS system.

For phosmet the retention time was 7.65 min; the transition 317.9-> 133.1 *m/z* (48 CV and 27.5 CE) was used for quantification and 317.9-> 160.1 *m/z* (31 CV and 19 CE) for qualification. A dwell time of 50 msec was set for the scanning of each transition. A typical SRM chromatogram is presented in Figure 2.

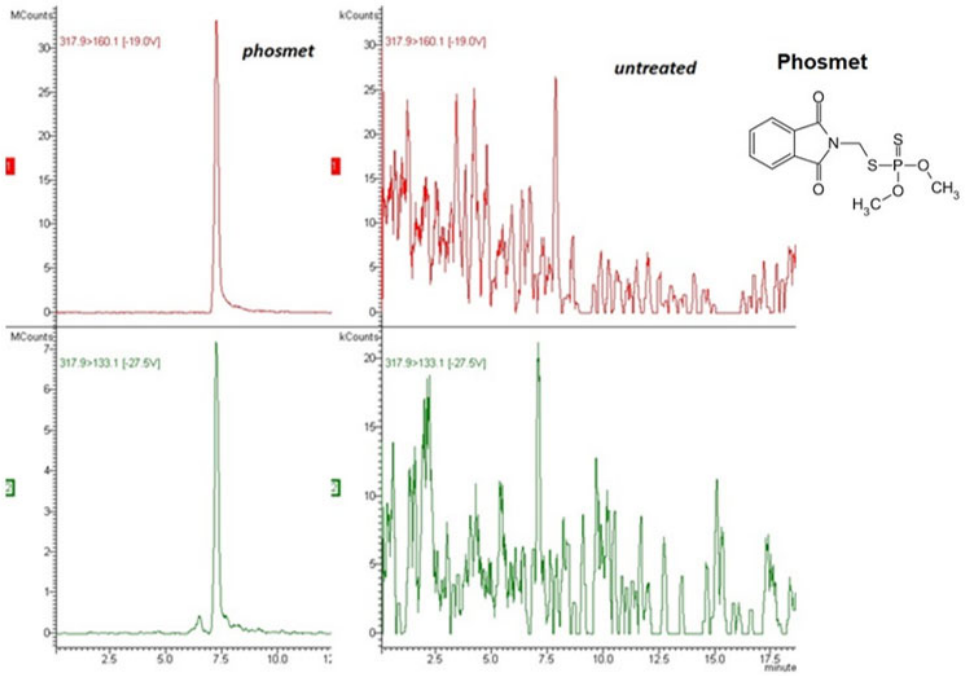

Figure 2. Determination of phosmet residues in the surface of olive leaves using LC-MS/MS system.

GC–(EI)-MS/MS: A gas chromatography system interfaced with a triple quadrupole mass spectrometer (Varian 1200 L, Lake Forest, CA, USA) was involved for residue identification and quantification. The autosampler of the GC was the CP-8400 model and the relative injector was a split/splitless operating in the splitless mode. The analytical capillary column used was the VF-5 ms, Varian 25200, (30 m × 0.25 mm I.D., coated with 0.25 μm film thickness). The injector temperature was initially set to 90 °C, held for 0.75 min, increased at 200 °C/min to 280 °C, and held for 5 min again and then decreased to 90 °C at 200 °C/min and held for 10 min. As regards the oven temperature program, it comprised of 2 min hold at 70 °C, ramp at 30 °C/min to 160 °C, then increased to 300 °C at 10 °C/min and held for 15 min. The operation temperature of the injector was set at 300 °C, the pulse pressure 69.95 kPa and the pulse duration 0.25 min. The carrier gas was He at 1.2 mL/min, while the injection volume was 5 μL. The source temperature was 200 °C and the corresponding interface temperature was 290 °C and the solvent delay was 5 min. The total GC run time was 35 min. Electron Impact EI-MS/MS mode and selection reaction monitoring data acquisition mode were applied in the operation of the mass spectrometer. The transfer line, manifold and ionization source temperatures were 280, 40 and 250 °C, respectively. The EI energy used was 70 eV as in that region the maximum abundance was observed. The retention time of beta-cyfluthrin was 19 min; the transition 165 > 127 m/z (40 CV and 5 CE) was used for quantification and 206 > 151 m/z (40 CV and 16 CE) for qualification purposes. The collision gas used for the operation in MS/MS mode, was Argon 99.999% at a pressure of 0.17 Pa, while the electron multiplier voltage was 1300 V and the dwell time was 50 ms. A typical SRM chromatogram for beta-cyfluthrin is presented in Figure 3.

2.3.5. Validation Design

Regarding the DFR washing step, initial tests showed that triple wash was adequate since no detectable residues were determined after a fourth stage of wash. The extraction efficacy of the second stage DFR analysis, which is liquid-liquid extraction, was validated at 4 levels (0.05, 0.1, 0.5 and 1 mg/L).

Validation of the method was accomplished in accordance with EU guidelines [25] regarding "Analytical Quality Control and Method Validation Procedures for Pesticides Residues Analysis in Food and Feed". The analytical parameters assessed were accuracy and precision (based on the results of recovery experiments), linearity and sensitivity.

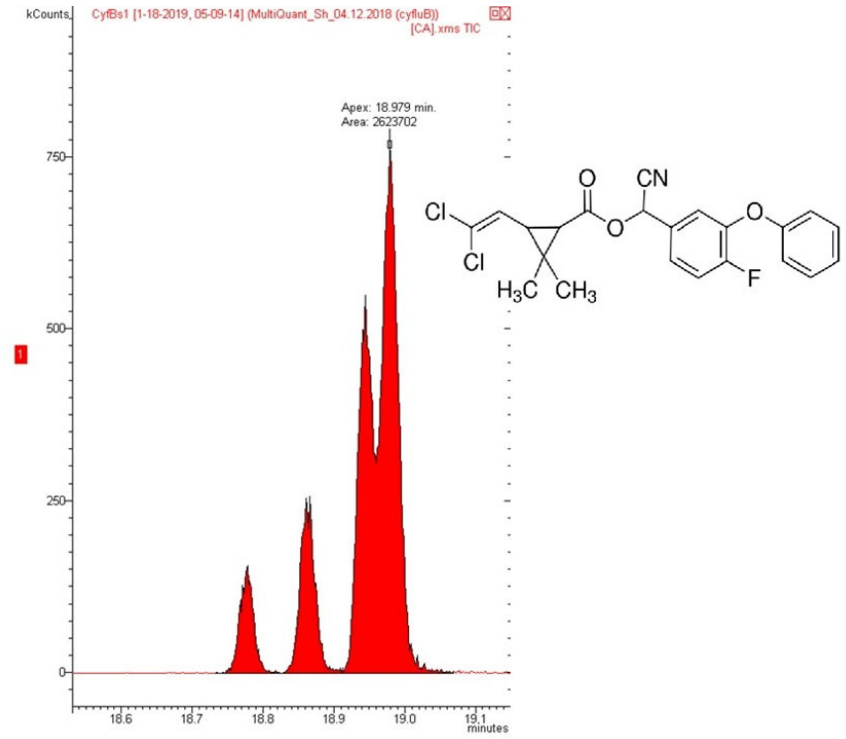

Figure 3. Determination of beta-cyfluthrin residues in the surface of olive leaves using GC-MS/MS system.

Linearity was examined at seven calibration levels (0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 mg/L) using solvent calibration standards.

Accuracy was estimated by measuring recoveries after spiking blank solutions (derived by the washing of untreated olive leaves with dioctylsulfosuccinate solution) with the appropriate quantity of working standard solution at the four concentration levels of 0.05, 0.1, 0.5 and 1 mg/L. The spiked samples followed the sample preparation as described above.

Precision was expressed as the relative standard deviation (RSD, %) and was confirmed by accessing the repeatability of the recovery experiments. The RSD was calculated for each spiking level.

The LOQ was set at the lowest fortification level tested, for which acceptable accuracy and precision were confirmed.

2.3.6. Confirmation Criteria

The identification of the compounds was initially based on the criterion of retention time (R.T.). Applying a tolerance of ±0.1 min, the R.T.s of dimethoate, phosmet and beta-cyfluthrin were matched based on calibration standards. The final confirmation of a target compound was performed in accordance with the criteria laid down in SANTE/11312/2021 [25]. According to this guideline, the permitted tolerances for the relative ion intensities (% of base peak) in MS/MS techniques are ±30% of the average of the calibration standards of the same sequence.

2.4. Toxicity Assessment

2.4.1. Toxicity Assessment in Olive Fruit Fly Adults-Leaf Trial

Five small olive seedlings were sprayed with each tested insecticide plus bait solution until runoff in order to estimate their efficacy to adult flies (the spraying date was 9 September 2019). After the sprayed trees were dried (2 h after the spray application) as well as 2, 4, 7 and 14 days (11/9, 13/9, 16/9 and 23/10, respectively), olive stems of uniform size (length of approximately 10 cm with about eight leaves) were cut off the

sprayed trees and transferred to small cylindrical cages (9 cm in diameter and 11 cm high, one stem/cage) of PVC (0.4 mm thick). The tops of the cages were covered with fine muslin. Adults of olive fruit flies (three pairs of individuals, not more than 7–10 days old) were released into each plastic cage and were supplied with sugar and water droplets. Unsprayed olive stems were used as a control. Ten replications were tested per treatment. The cages were kept in a growth room at 25 ± 0.5 °C, 65 ± 5% R.H. and a 16L:8D photoperiod. The efficacy (toxicity as was indicated by the percentage of mortality of the adult flies) of the bait-sprayed stems with various insecticides was evaluated by counting the number of *B. oleae* adults that died after 48 h. Dead flies were considered those that were totally lifeless without movement (flying or walking) observed.

2.4.2. Toxicity Assessment in Olive Fruit Fly Larval Stages-Olive Fruit Trial

Ninety-five groups of about forty-five green and uninfested olive fruit (*cv* Koroneiki) of uniform size were randomly collected from olive trees of an orchard of Nerokourou. Each group of fruit was placed in a petri dish with wet cotton while twenty-five (25) Petri-groups were placed in a large plastic and well-ventilated cage (100 × 100 × 50 cm). An olive fruit fly population of about 50 adults of each sex, was introduced to the cage and left to oviposit for two days (on Monday 14 October). On the 2nd (Thursday) and 5th day (Friday), these groups were replaced with 25 new ones while the old samples were isolated in a separate cage without the presence of olive flies. All olive group-samples were mixed the following Monday in a way to obtain infested fruit of all immature stages. On the 14th day (Monday), 30 randomly selected groups of infested fruit were dipped separately to dimethoate, phosmet and beta-cyfluthrin bait spraying solution the same day. A similar number of groups of fruit collected and infested by the olive fly in a similar way were left untreated and used as a control. The trial took place in a growth room at 25 ± 0.5 °C, 65 ± 5% R.H. and a 16L:8D photoperiod. Olive samples of each tested insecticide were subsequently examined every 2, 4 and 7 days after dipping each insecticide, for recording the mortality of olive fly larval stages (L1, L2, L3) under a binocular stereomicroscope and determine their systemic toxicity or efficacy; pupal stages escape from the toxic impact of the insecticide and excluded from the trial.

2.5. Statistical Analysis

Two-way repeated measures were employed, with the studied active ingredients (dimethoate, phosmet and beta-cyfluthrin) and observation time (0, 2, 4, 7 and 14 days after application) being the two factors examined. Both the recorded percentage of dead flies, after having contact with the sprayed olive stems, as well as dead larval stages after systemic penetration of each active ingredient were analyzed separately for each day, using two-way Repeated Measures ANOVA. Control was excluded from both the toxicity analysis of adult and larval stages due to no recorded mortality. The number of dead larval stages was presented as percentages of the means were separated using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

As regards the DFR values calculated in olive leaves and fruit for the three compounds, data were fitted to the first-order (Equation (1)) and second-order (Equation (2)) dissipation kinetic model.

$$C(t) = C_0 \times \exp(-K_f \times t) \quad (1)$$

$$C(t) = C_0/(1 + C_0 \times K_s \times t) \quad (2)$$

where C_0 is the initial pesticide concentration and K_f, K_s is the first and second-order dissipation rate constants respectively, while C(t) is the concentration at time t. The model parameters (C_0, K_f, K_s) were estimated using nonlinear regression, while DT_{50} values using inverse prediction, after the calculation of the model parameters.

3. Results

The results of the validation procedure and the adequacy of the developed analytical method are presented in Figures 1–3 and Table 1. The DFR values calculated for olive leaves and olive fruit expressed as μg/cm^2 and mg/kg, respectively, are presented in Figures 4 and 5, while the toxicity assessments of the calculated residues in the adult and larval stages of the olive fly are presented in Figures 6–8.

Table 1. Accuracy (Recoveries, %), precision (RSDs, %) values and linearity results calculated after the fortification of blank samples.

Compound	Fortification Level (mg/kg)	Mean Recovery (%) (n = 6)	RSD (%) (n = 6)	Equation of the Calibration Curve	Correlation Coefficient Squared (R^2)
Dimethoate	**0.05**	114	5.67	$y = 5 \times 10^8 x + 6 \times 10^6$	0.999
	0.1	72	7.42		
	0.5	112	2.23		
	1	100	8.65		
Phosmet	**0.05**	99	6.7	$y = 4 \times 10^7 x + 534{,}035$	0.996
	0.1	113	11.22		
	0.5	97	17.4		
	1	119	6.21		
Beta-cyfluthrin	**0.05**	74	6.44	$y = 4 \times 10^8 x + 643$	0.998
	0.1	87	3.76		
	0.5	94	7.01		
	1	79	8.44		

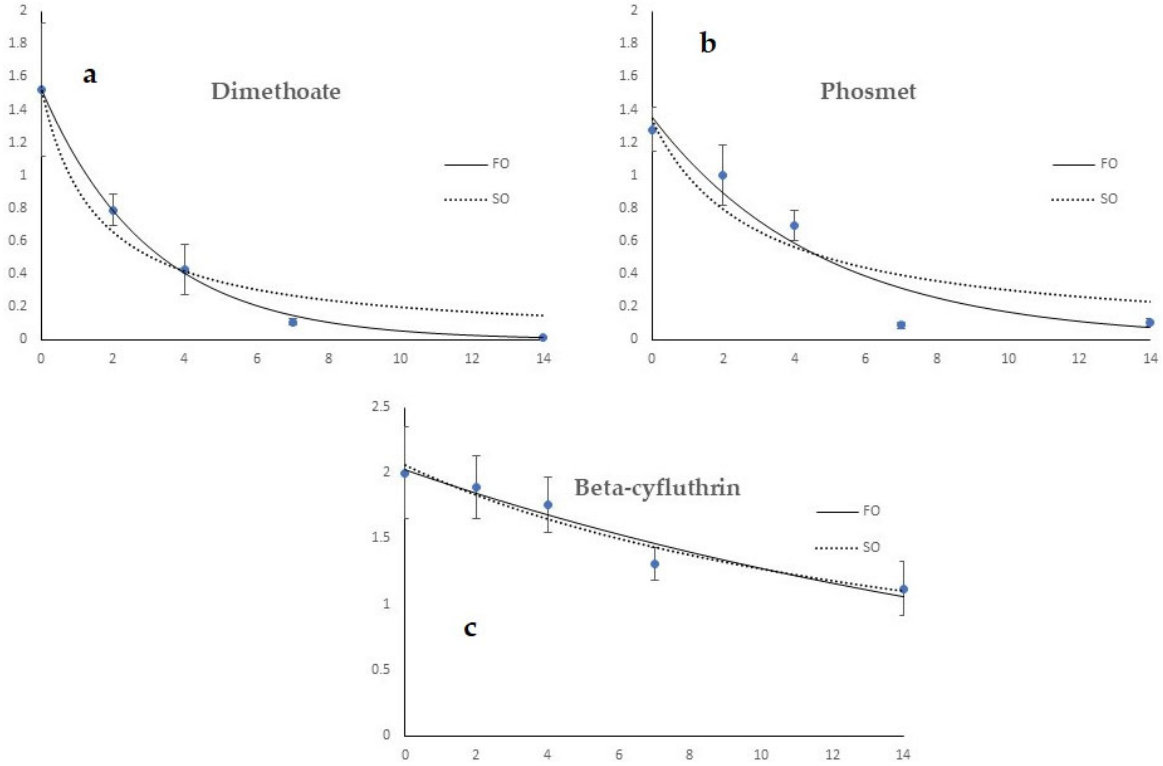

Figure 4. Changes in residual concentrations of dimethoate (**a**), phosmet (**b**) and beta-cyfluthrin (**c**), in olive leaves after determination as dislodgeable foliar residues. Solid and dashed lines indicate the results predicted using first-order (FO) and second-order kinetic models, respectively.

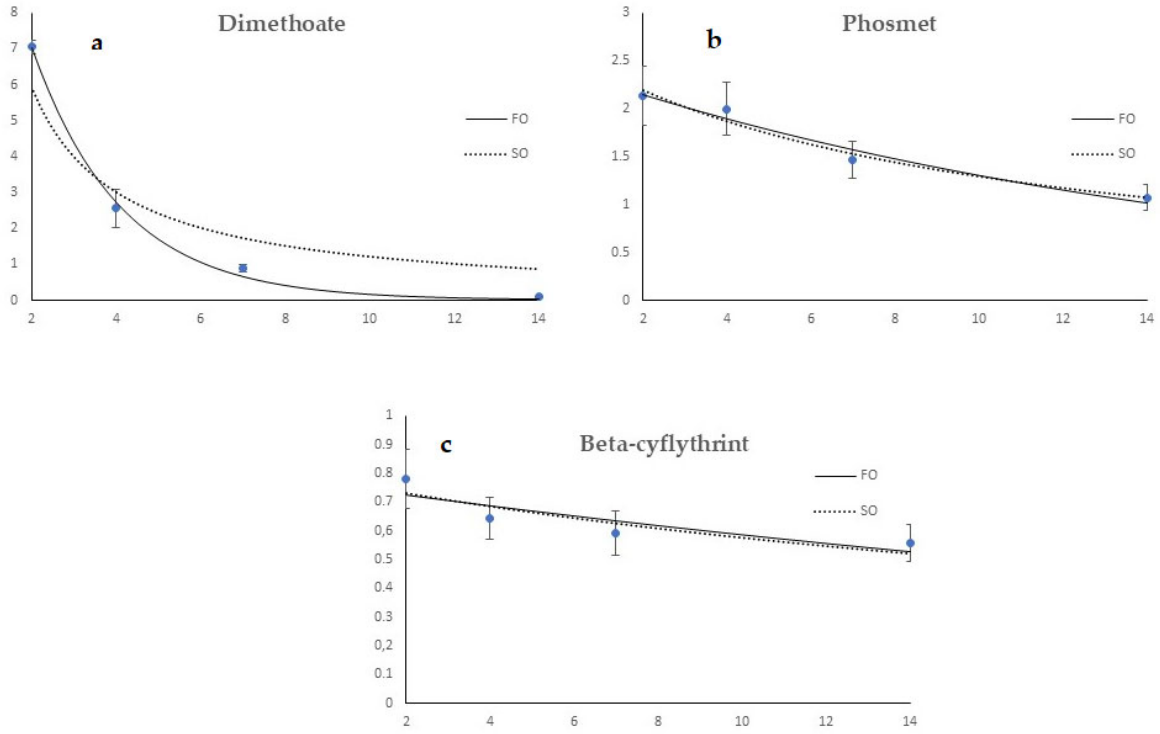

Figure 5. Changes in residual concentrations of dimethoate (**a**), phosmet (**b**) and beta-cyfluthrin (**c**), in olive fruit after determination as dislodgeable foliar residues. Solid and dashed lines indicate the results predicted using first-order (FO) and second-order kinetic models, respectively.

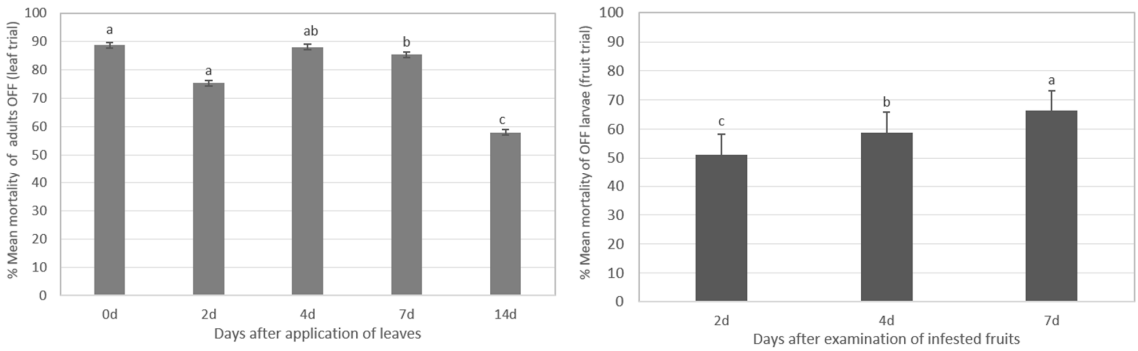

Figure 6. Mean ± SE recorded mortality of olive fruit fly adults (**left**) and larval stages (**right**) versus time. Records of mortality with the same letter are not significantly different at $p < 0.05$.

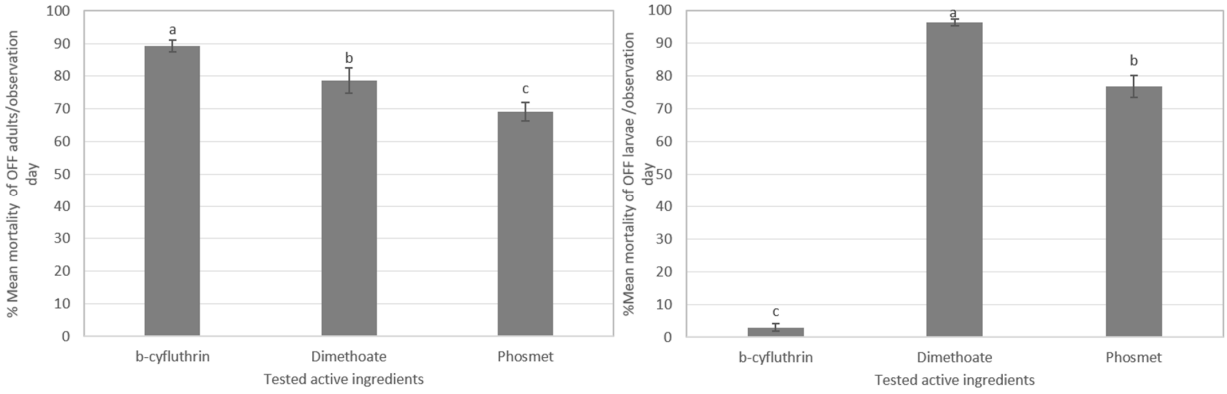

Figure 7. Mean ± SE recorded mortality of olive fruit fly adults (**left**) and larval stages (**right**) for each tested insecticide. Records of mortality with the same letter are not significantly different at $p < 0.05$.

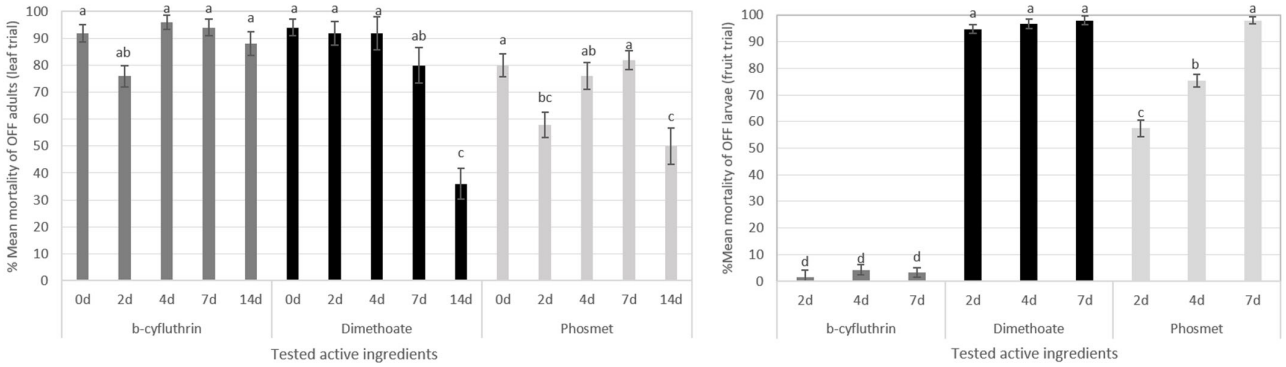

Figure 8. Mean ± SE recorded mortality of olive fruit fly adults (**left**) and larval stages (**right**) for each tested insecticide and observation day. Records of mortality with the same letter are not significantly different at $p < 0.05$.

3.1. Analytical Method Performance

Linearity was estimated through calibration curves after injecting calibration standards for the three insecticides at seven concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 mg/L). As observed, acceptable linear parameters were found with correlation coefficients (r) being higher than 0.996.

Recoveries were calculated from fortified blank samples (solutions derived by the washing of untreated olive leaves with dioctylsulfosuccinate solution). The obtained values ranged from 72% to 119% (Table 1) and were considered satisfactory, based on the criterion set by SANTE/11312/2021, the average recovery for spiked levels tested should be between 70% and 120%. Moreover, all the calculated relative standard deviation (RSD, %) values met the requirement of <20%, as shown in Table 1.

The Limit of Quantification (LOQ) was set at 50 μg/kg, as the lowest fortification level with unequivocal identification of the target compounds and acceptable accuracy and precision [26,27]. The Limit of Detection (LOD) was set at 16.7 μg/kg, taking into account that LOD is related to LOQ by the equation 10 LOD = 3 × LOQ.

The analytical method developed was proved to be fit for purpose for the extraction of dimethoate, phosmet and beta-cyfluthrin and the obtained validation results indicate its capacity for their determination from the surfaces of olive leaves and fruit and ensure the accuracy of the dislodgeable foliar residue results.

3.2. Dislodgeable Foliar Residue Results

Dislodgeable foliar residue results are the residue concentrations calculated after applying the standard leaf-washing technique. The results found in the control samples (untreated olive leaves) didn't exceed the limit of quantification set in the analytical method (<0.05 mg/kg).

As shown in Figure 4, the DFR levels on olive leaves declined over time after insecticide application. For dimethoate, this decrease was evident in the second sampling (2 days after application), where 51.9% of the initial concentration (t = 0) was recovered, while by the last sampling (t = 14 days) only 0.72% was present. The corresponding results for phosmet were 78.2% at 2 days of sampling and 8.44% by the end of the experiments. A different situation was observed for beta-cyfluthrin, showing a recovery of almost 90% of the initial concentration up to 4 days and a decrease to 56% in the last sampling (14 days). As observed, the dissipation rate of dimethoate was higher than those of phosmet and beta-cyfluthrin in all sampling points.

As can be seen, the first-order model seems to achieve better fitting better to dimethoate and phosmet dissipation rates, while for beta-cyfluthrin both models show a similar fit.

The half-lives (DT_{50}) of the tested pesticides in the leaves were calculated based on the parameter values of both kinetic models. For dimethoate, a DT_{50} of 2.1 days was calculated with the first-order model and a DT_{50} of 1.39 days with the second-order model. The

respective values of phosmet were 3.31 and 2.91 days, while for beta-cyfluthin they were 17.69 and 16.2 days, respectively.

Similar reduction levels were also observed in olive fruit (Figure 5). By the last sampling of 14 days, only 1.27% of the initially determined concentration was recovered for dimethoate, while the corresponding levels of phosmet and beta-cyfluthrin were 48.86 and 71.31%, respectively.

As can be seen, the first-order model seems to achieve better fitting to dimethoate dissipation rates, while for phosmet and beta-cyfluthrin, both models show a similar fit. The half-lives (DT_{50}) of the tested pesticides in the fruit were calculated based on the parameter values of both kinetic models. For dimethoate, a DT_{50} of 1.391 days was calculated with the first-order model and a DT_{50} of 0.59 days with the second-order model. The respective values of phosmet were 11.15 and 9.53 days, while for beta-cyfluthin they were 25.98 and 27.71 days, respectively.

3.3. Assessment of Toxicity of Olive Fly Adults and Immature Stages

3.3.1. Assessment of Toxicity of Olive Fly Adults

According to the statistics, both factors of the tested active ingredient and the time after application (days after spraying application) significantly influenced the recorded fly toxicity. The efficacies of the applied insecticides against olive fruit flies on the epidermis of the leaf tissue are significantly reduced 7 days after the application (ranging from 75–88%) to 58% ($F = 25.06$; d.f. = 4, 108; $p < 0.0001$) (Figure 6 leaf trial). The nature of the active ingredient can also strongly affect the fly's mortality ($F = 16.50$; d.f. = 2, 27; $p < 0.0001$).

Specifically, significantly higher mortality was recorded for the olive stems sprayed with beta-cyfluthrin (89.20%) than those sprayed with dimethoate (78.80%) or phosmet (69.20%) (Figure 7 leaf trial). Their interaction was also significant, just after the application, when the mortality levels were significantly reduced ($F = 9.23$; d.f. = 8, 108; $p < 0.0001$).

The highest mortality (94–96%) was recorded immediately after the application of beta-cyfluthrin and dimethoate, which slightly reduced for the former ingredient and significantly reduced for dimethoate to 36%, during the observation period of two weeks. The efficacy of phosmet ranged from 50–82% (Figure 8 leaf trial).

In the control cages with the unsprayed stems, no mortality of olive fruit fly adults was observed.

3.3.2. Toxicity Assessment in Olive Fruit Fly Larval Stages

As it was recorded, the percentage of dead larval stages of the *B. oleae* was significantly influenced by the insecticide ($F = 2127.57$; d.f. = 2.30; $p < 0.0001$) and the time (days) after the dipping process ($F = 38.53$; d.f. = 2.54; $p < 0.0001$). Specifically, only the dimethoate spraying solutions significantly increased the mortality of larvae (as was indicated by the dead larval stages during dissection of treated olive mesocarp) from 0% (recorded larval mortality at control fruit) to 96.46% of dimethoate-, 76.95% of phosmet- and 2.99% of the pyrethroid beta-cyfluthrin-treated fruit; this reduction was also significant at the 2nd day (Figures 6 and 7, fruit trial). Their interaction was also significant as the levels were significantly reduced just after the application ($F = 27.51$; d.f. = 4.53; $p < 0.0001$). Higher and faster penetration was recorded at dimethoate-treated fruit as was indicated by low survival of the larval stages from the 2nd day while it took 7 days for phosmet to have similar results (Figure 8, right fruit trial). At the control fruit, no mortality of larval stages was recorded. It is also important to note that almost all larval stages of the olive fly tested with the two organophosphates were recorded as dead from the 2nd day of application except the older third larval stage (as was indicated by the larger size compared to the younger ones) and the lateral immature stage of the fly, the pupal stage. Conversely, phosmet had a lower penetration with the olive mesocarp and reached similar toxicity with dimethoate on the 7th day of spraying while beta-cyfluthrin showed light epidermal toxicity to young larval stages of the fly.

4. Discussion

As reported above, the dislodgeable residue levels of both dimethoate and phosmet on olive leaves decreased rapidly after the insecticide application, having for dimethoate a decrease to half of the initially determined concentration at 2 days, and an almost complete depletion after 14 days, while the corresponding values for phosmet were slightly slower. Conversely, the pyrethroid beta-cyfluthrin residues remained almost stable at the beginning of the trial and were decreased to half of the initial concentration by the end of the experiment, a residual behavior that can be anticipated by its physicochemical properties [7]. The entomological trials showed that each active ingredient, according to its chemical group, is mainly focused at a specific stage of the olive fly; there was high toxicity for both larval and adult stages of the fly especially for the two organophosphates versus beta-cyfluthrin, due to their penetration process; significantly highest mortality was recorded for adults treated with the pyrethroid while there was almost no mortality to larvae. A lower toxic effect was recorded from the organophosphates toward individuals of the mature third larval stage while no toxic effect was recorded at the pupal stage.

The degradation process of a pesticide can be affected by its structure, its physicochemical properties and by the prevailing environmental conditions. Additionally, there are several studies reporting that higher pesticide concentrations exposed to crop plants and the environment result in their greater dissipation [26,27]. These processes mainly consist of photolysis (photocomposition), hydrolysis and volatilization [28]. The rapid decrease in the dimethoate and phosmet on olive leaves at 2 days cannot be attributed to their vapor pressure, as they are both considered slightly volatile having vapor pressure (v.p.) values of 0.025 mPa (dimethoate) and 0.065 mPa (phosmet) at 25 °C [7]. In addition, the application dose of the tested pesticides in the field was relatively constant for all experimental orchards/insecticides and therefore, DFR variability cannot be attributed to the parameter of variety, since all the olive trees sprayed were of the same variety (Koroneiki). As regards the prevailing climatic conditions, high temperatures were recorded during the implementation of applications (Supplementary Figure S1) and therefore, it cannot be excluded that the degradation of dimethoate and phosmet, having an aerobic DT_{50} of 2–4.1 days and of 3.6 days may have been also influenced by those high-temperature conditions, while no influence may have occurred in the case of beta-cyfluthrin which presents an aerobic DT_{50} of 20 days [29,30]. Finally, no rainfalls were recorded during the experimental period of spraying and sampling dates, a fact that excludes the possibility of wash-off.

In general, there is limited information regarding the extraction of DFR from whole leaves [9,31]. DFR determination of methamidophos in staked tomatoes using leaf disks has been reported [32], while Kasiotis et al. (2017) [16] assessed the field re-entry exposure to tebufenozide and bupirimate sprayed in greenhouses through a dislodgeable study on pepper and tomato leaves, while Goh et al. (1986) [24] studied the dissipation of DFR of chlorpyrifos and dichlorvos on turf. Additionally, Krieger et al. (2010) [33] used DFR to clarify the significance of pesticide use practices to growers, regulators and consumers. In all the above studies, DFR results were used for the estimation of worker exposure, and to the best of our knowledge, no study has used those values to estimate other parameters, such as the toxicity toward pests.

The implemented entomological trials followed this decrease in the dimethoate and phosmet residues at the upper plant tissue of leaves and fruit and showed that all tested compounds can be considered harmful to the adult olive fruit fly until the 7th day and then only the organophosphates are slightly harmful. The International Organization for Biological Control (IOBC) has classified the adverse effects of pesticides as harmful, which can also be applied to crop pests. Regarding their toxicity in larvae as penetration of the insecticides was proceeding from day to day in the olive fruit mesocarp, dimethoate's classification was altered from moderately harmful to harmful at day 4 of the fruit dissection, while phosmet moved from slightly harmful to harmful at day 7. Although phosmet is considered to be not systemic, it has, however, been reported that it is absorbed into the

wax layer of the leaf in the plant, and this provides better residual activity and reduces wash-off [34]. Conversely, beta-cyfluthrin was totally harmless to the larval stages of the olive fruit fly as expected.

According to the obtained results from the analytical and applied research bioassays of the present study, it might have been assumed that, as regards dimethoate, the purpose (described above) of the method of bait spray application with the systemic/cytotropic dimethoate was not accomplished after the 7th day of the experimental, since the detected dislodgeable foliar residues of the active ingredient decreased rapidly to not much more than half of the initially determined residues. Although, at that specific sample point, high toxicities of dimethoate to both adult and larval stages of the olive fruit fly were recorded (close to 100%) in the bioassay lab trials. One week later, dimethoate was recorded as harmless to the adult fly. Therefore, it is important to note that even a very low DFR of dimethoate (0.108 μg/cm^2) can perform high toxicity to the adult fly, as was indicated by the small cage trials. It is also worth mentioning that almost all immature stages of the olive fly within the olive mesocarp were recorded as dead from the 2nd day of application (except for the mature third larval stage and the pupal stage, which is resistant to the insecticides); a fact that indicates quick systemicity and excellent toxicity to the larval stages of the fly. It seems that the larval stage is very sensitive even in low insecticide quantities. No other toxicity or resistance bioassay has been carried out for the larvae of the olive fruit fly.

As far as phosmet is concerned, it was also observed that very low DFR values can cause high toxicity to the adult fly until the 7th day, while its toxicity to the larval stages was significantly lower compared with that of dimethoate. Phosmet was not expected to contribute to preventing larval development, though its application in bait sprays presented similar performance toxicity to dimethoate. Seven days after application, the larval toxicity of phosmet didn't differ significantly from dimethoate. Regarding phosmet, comparable with the present study, penetration in olive tissues has been also reported on the surfaces of apples [35].

The pyrethroid indicator compound remained on the surface of the leaf tissue and its toxicity to the adult fly was high (similar to dimethoate) and almost zero to the larval stages within the olive mesocarp, indicating no systemicity.

Varikou et al. (2018) [18] reported the high toxicity and low degradation of pyrethroids against organophosphorus in the bait dose; high residuality and toxicity of dimethoate to the adult flies of *B. oleae* only just after application and similarly for phosmet while l-cyhalothrin and a-cypermethrin were toxic for longer periods. Residues were determined in homogenized olive leaves. Additionally, no studies concerning the tissue penetration combined with toxicity of insecticides in larval stages of the olive fruit fly have been published up to now, because larval stages are hidden within olive mesocarp and are difficult to manipulate in toxicity or resistance bioassays.

According to our results, and after dealing with several different approaches, a primary conclusion can be reached that the inclusion of an (e.g., organophosphorus) insecticide with a systemic/cytotropic mode of action should be avoided in bait sprays and additional parameters (e.g., a trophical attractant) have to be examined in order to activate successfully a bait spot to the adult olive fruit fly. Therefore, these recent scientific results should be adapted in their broad application by olive growers; bait spray can be further improved with the proper choice of a suitable, not systemic, insecticide according to its mode of action.

5. Conclusions

Considering the strong correlation between mortality and pesticide quantity on the sprayed fruit or leaf, this paper aspires to add data and throw more light on this important field. Results obtained from comparative trials through the monitoring of DFR of the applied insecticide dimethoate on olive leaves by bait sprays, in parallel with entomological trials, showed that the use of such insecticides should be restricted in methods that are focused on killing insects by feeding and contact exposure of dry residues. As observed,

the development and validation of a robust and sensitive analytical method coupled with GC-MS/MS and LC-MS/MS ensured the calculated residue results for further assessment. The residues of dimethoate 4 days after application were significantly reduced either through degradation or penetration within plant tissues and therefore the use of systemic insecticides in bait spray applications against olive fruit fly adults may be an issue to which further consideration has to be given. Moreover, dimethoate and phosmet insecticides differed significantly in their effectiveness against adults and larvae of the olive fly and this may be due to their differentiation in physicochemical properties. However, both ingredients were observed to have similar action toward both larval and adult stages 7 days after their application. The question for further investigation that arises from this study, is whether phosmet demonstrates a systemic action or if its observed action is based only on the mobility that all the active ingredients show, especially in olives fruit, the flesh of which is not more than 5 mm thick. This is an issue that must be reconsidered for all contact insecticides applied for the control of *B. oleae*, such as phosmet. Therefore, a potential extrapolation of the derived results to compounds of similar action is proposed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13030543/s1, Figure S1: Mean temperature, humidity and rain on the days of the bait spray applications of the tested products in each experimental orchard.

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