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Adjuvant Effects on Pyraclostrobin and Boscalid Residues, Systemic Movement, and Dietary Risk in Garlic under Field Conditions

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Abstract: Adjuvants are supplemental substances added to pesticide tank mixtures to enhance their efficacy by altering the dispersing, emulsifying, spreading, sticking, and wetting properties of the spray mixture. The goals of this study were an assessment of the effectiveness of pyraclostrobin and boscalid against garlic rust; the fungicide's dissipation and translocation within plant tissue in the presence of commercial adjuvants Dash® HC EC; and the validation of an analytical method for concurrently determining boscalid and pyraclostrobin in green garlic and garlic bulbs. Pyraclostrobin and boscalid retention in green garlic and the dietary exposure risks were also evaluated. The adjuvant Dash® HC EC is an emulsifiable concentrate containing fatty acids, methyl esters, and alkoxylated alcohol–phosphate esters. The test plots were sprayed with the maximum recommended dosage of 400.5 g a.i. ha^{-1} boscalid and 100.5 g a.i. ha^{-1} pyraclostrobin. The residues of pyraclostrobin and boscalid in garlic bulb and green garlic samples were determined using a mini-Luke multi-residue solvent extraction method paired with a high-performance liquid chromatography diode array detector (HPLC–DAD). This method is based on the extraction of a homogeneous sample with acetone, petroleum ether, and dichloromethane. Boscalid and pyraclostrobin recoveries in green garlic and garlic bulbs ranged from 71% to 107% on average, with a relative standard deviation (RSD) of 6.8% to 18.1%, and limits of quantification were 0.02 mg/kg. In green garlic and garlic bulbs, boscalid residues ranged from 0.14 to 3.51 mg/kg and pyraclostrobin from lower than LOQ to 1.43 mg/kg, during the experiment. The adjuvant increased the fungicidal efficacy of the fungicides and their retention on green garlic. Although boscalid is considered a nonsystemic fungicide, it showed higher translocation from garlic leaves to garlic bulbs than pyraclostrobin. Regarding the dietary risk assessment, the use of the adjuvant in a tank mixture with boscalid and pyraclostrobin is considered safe 14 days after application, which agrees with the recommended PHI.

Keywords: garlic; adjuvant; fungicides; dietary risk assessment; HPLC–DAD

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1. Introduction

Garlic (*Allium sativum* L.), a member of the genus Allium, is widely cultivated around the world and is the greatest bulb crop after onion. Greece is an essential garlic producer in Europe, and the garlic harvest area in Greece was 960 ha, with a production rate of approximately 5613 tons in 2021 [1]. The garlic plant consists of two consumable parts: green garlic and the garlic bulb. In Greece, consumers usually eat both when they are fresh. There are many health claims related to frequent garlic consumption. Many disorders, including hyperglycemia, cancer, and cardiovascular disease can be prevented by consuming garlic, which contains high amounts of proteins, lipids, minerals, and carbohydrates [2].

Garlic crops are susceptible to various diseases. Garlic rust is one of the most-frequent fungal diseases, significantly reducing the yield and product quality. The rust disease can severely damage garlic crops. The first symptoms are small (2 mm), circular-to-elongated white flecks that appear on both leaf surfaces. These little flecks transform into rectangular lesions as the disease advances. The final symptoms are the lesions becoming pustules [3]. Applying fungicides to protect garlic is a common and efficient way to manage garlic rust [4]. However, the increased or inappropriate use of fungicides could lead to crops containing a significant amount of pesticide residues, which could expose consumers to health risks [5,6]. Pesticide residue levels are typically regulated by rules to reduce consumer exposure to hazardous or unnecessary pesticide ingestion. The European Union Council Directive No. 396/2005 (Regulation 2005) has set maximum residue limits (MRLs) for pesticides in foodstuffs and animal feed [7].

Human health may be negatively impacted by acute or chronic pesticide exposure, especially in youngsters and pregnant women, who are more susceptible to toxic effects. There is increasing evidence from epidemiological and molecular research that pesticides can have harmful impacts on human health, including cancer, birth defects, reproductive abnormalities, toxicity, and even death. Considering the potential hazards of pesticide residues, the European Union (EU) and numerous countries have established maximum residue limits (MRLs) for pesticides in agricultural products to reduce pesticide residue levels, help ensure that pesticides are not overused, and help ensure that residues found in food are acceptable for humans, especially since vegetables are typically consumed fresh [8].

A broad-spectrum commercial formulation of 33.4% wettable granules (Signum® WG: 26.7% boscalid + 6.7% pyraclostrobin) is currently used to manage garlic rust in Greece. The application of this formulation is recommended to be mixed with a commercial emulsifiable concentrate adjuvant (Dash® HC EC) containing 37.5% oil and 22.5% alkoxylated alcohol–phosphate esters. This combination is commonly used by farmers during garlic disease management in northern Greece, which is considered a more effective approach than the individual application of Signum® 26.7/6.7 WG to control rust; consequently, it could be useful for public health safety to conduct controlled field studies to evaluate the dietary risks and pesticide residues of these fungicides with the adjuvant addition when they are applied to garlic in accordance with Integrated Pest Management Strategies (IPMs), following the proposed Good Agricultural Practices (GAPs). Boscalid (CAS name: 2-chloro-N-(4′-chloro(1,1′-biphenyl)-2-yl)-3-pyridine carboxamide) shows local systemic and translaminar activity, which belongs to the carboxamide chemical class, acting as a succinate dehydrogenase inhibitor (SDHI). Boscalid is effective against a variety of pathogen fungi, such as *Sclerotinia* spp., *Alternaria* spp., and *Botrytis* spp. Many crops, such as ornamentals, fruits, and vegetables, are treated with boscalid [9]. However, many studies have reported it in various matrices, such as cucumber [10], strawberries [11], and agro-food industrial sludge [12].

Pyraclostrobin (CAS name: methyl-N-[2-[[1-(4-chlorophenyl)pyrazol-3-yl]oxymethyl] phenyl]-N-methoxycarbamate) is also a fungicide belonging to the strobilurin chemical class. It has been approved for use to control plant diseases caused by ascomycetes, basidiomycetes, deuteromycetes, and oomycetes, which are hosted by carrots, grapes, peppers, tomatoes, wheat, potatoes, soybeans, and sugar beets [13]. Pyraclostrobin is among the most frequently detected fungicides in food (including garlic) and environmental matrices as a result of its continuous use [14].

Pesticide solutions can be mixed with adjuvants to improve the distribution, adhesion, and rainfastness of pesticides; decrease weathering; and pierce the waxy layer of the leaf surface to stimulate the absorption of active substances and thus enhance pesticides' effectiveness [15]. However, there is a lack of studies about how the addition of adjuvants to spraying solutions could affect the behavior of pesticides within plants (retention, translocation, and systemic action) and the dietary risk of these pesticides.

Recently, HPLC with a diode array detector (DAD) was employed to examine the dissipation and residue dynamics of pyraclostrobin in banana and soil under field conditions [16]. Also, in 2023, Dost et al. [17] described a method for the determination of boscalid and pyraclostrobin in dried grape and apricot using an HPLC–UV chromatographic system. For the determination of various pesticide classes, numerous methods have been proposed that involve sample blending with acetone during the extraction stage, followed by repeated separating stages with petroleum ether and dichloromethane. For the purpose of analyzing pesticide residues in an effective way, the initial acetone-based extraction method has been regularly improved or changed, and consequently, many of the resulting methods have been validated, such as mini-Luke multi-residue solvent extraction [18]. However, only a few studies have been published on the mini-Luke method in recent years. To the best of our knowledge, an HPLC–DAD method combined with mini-Luke multi-residue solvent extraction has not been performed thus far to determine pesticide residues in garlic samples. Also, the development of an analytical method, such as mini-Luke multi-residue solvent extraction, that reduces the extraction time and the volume of the used organic solvents could be an eco-friendly and low-cost approach.

Method validation includes the definition and assessment of the standard performance parameters (or features) of accuracy/recovery, precision (repeatability, intermediate precision, and reproducibility), linearity and application range, the limit of detection/quantification (LOD/LOQ), selectivity/specificity, robustness, ruggedness, uncertainty, trueness, stability, and system appropriateness studies, as well as a comprehensive and in-depth protocol on how to use and describe analytical methods and the associated procedures [19].

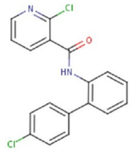
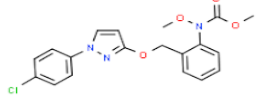
Therefore, the aim of this study was to (1) validate a simple, sensitive, and effective method for determining boscalid and pyraclostrobin in green garlic and garlic bulb based on the mini-Luke multi-residue solvent extraction method using a high-performance liquid chromatograph equipped with a diode array detector (HPLC–DAD); (2) determine the residues of pyraclostrobin and boscalid in green garlic and garlic bulb treated with Signum® WG 26.7, 6.7 and Signum® WG 26.7, 6.7 mixed with adjuvant Dash® HC EC under field conditions; (3) explore the translocation potential of boscalid and pyraclostrobin from green garlic to garlic bulb; (4) investigate how the adjuvant mixed with boscalid and pyraclostrobin could affect the retention of green garlic; (5) estimate the dietary intake risk of fungicides in garlic crop under field conditions; and (6) compare the effectiveness of the two fungicides against garlic rust in the presence or absence of the adjuvant in spay solution.

2. Materials and Methods

2.1. Reagents and Materials

Pyraclostrobin and boscalid analytical standards (purity 99.5%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Table 1 presents their physicochemical parameters and characteristics. Methanol and water of HPLC-grade purity were obtained from Riedel de Haen (Seelze, Germany) and utilized for HPLC analysis. To perform the extraction procedure, acetone, dichloromethane, petroleum ether, and HPLC-grade acetonitrile were also purchased from Riedel de Haen (Seelze, Germany). Na_2SO_4 was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The commercial formulations Signum® 26.7/6.7 WG and the adjuvant DASH HC EC were provided by BASF Hellas A.G. (Thessaloniki, Greece) and used for spray solution preparation.

Table 1. Physicochemical properties and characteristics of boscalid and pyraclostrobin [20].

Parameter	Boscalid	Pyraclostrobin
Molecular formula	$C_{18}H_{12}Cl_2N_2O$	$C_{19}H_{18}ClN_3O_4$
Substance group	Carboxamide	Strobilurin
Mode of action	Succinate DeHydrogenase Inhibitor	Respiration inhibitor
Structural formula		
Molecular weight (g/mol)	343.21	387.82
Water solubility at 20 °C (mg/L)	4.6	1.9
Octanol–water partition coefficient (25 °C) LogK_{ow}	2.96	3.99
Adsorption coefficient K_{foc} (mL/g)	772	9304
Soil degradation $DT_{50field}$ [1]	118	33.3

[1] Half-life for field studies.

2.2. Field Trial and Sampling

The field experiment was conducted on a farm in northern Evros (41°33′58.9″ N 26°32′48.2″ E, 0.03 Ha). The commercial formulations Signum® 26.7/6.7 WG and DASH HC EC, mixed or individually, were applied on garlic crops to protect against garlic rust (Figure 1) using an experimental Azo field plot sprayer (length 2.4 m) equipped with 6 fan flat nozzles. The fungicide application was conducted on 22 April, during the entry stage of the bulb formation of garlic. During fungicide spray, the wind speed was 1.37 mph, and no spay drift was recorded. In April and May, the temperatures range from 9.3 to 27.9 °C, and the average rainfall values are 35.6 and 48.1 mm, respectively. During the experiment, all cultivation practices were upheld following the GAP. The experimental setup included three different treatments with four replications corresponding to an experimental randomized complete block design consisting of 12 plots. The different treatments encoded "A", "B", and "C". Treatment A was a blank control that was sprayed with water; treatment B was sprayed with Signum® WG 26.7, 6.7; and treatment C was sprayed with Signum® WG 26.7, 6.7 and adjuvant Dash® HC EC. In all cases, agrochemicals were applied at the maximum recommended doses. The test plots were sprayed with the maximum recommended dosage of 400.5 g a.i. ha^{-1} (boscalid) and 100.5 g a.i. ha^{-1} (pyraclostrobin). The maximum recommended dose of Dash® HC EC is 1 L/ha. The tank mixtures were prepared according to the manufacturer's instructions. The application of agrochemicals was performed as a foliage spray. Table 2 provides the treatments' variations, and Figure 2 shows the design of the field trial.

Figure 1. Garlic rust symptoms (lesions) during the field trial.

Table 2. Signum® and Dash® treatments.

Treatment	Signum® Dose	Dash® Dose	Spray Solution Volume (Water)
A	-	-	1000 L/ha
B	400.5 g a.i. boscalid + 100.5 g a.i. ha^{-1}	-	1000 L/ha
C	400.5 g a.i. boscalid + 100.5 g a.i. ha^{-1}	500–1000 mL/ha	1000 L/ha

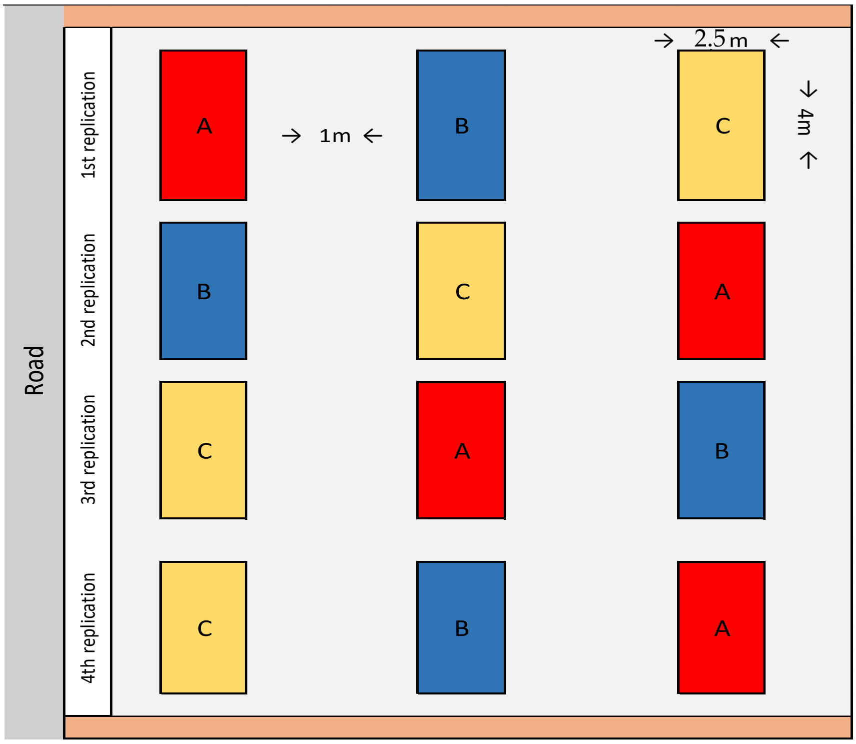


Figure 2. Design of the field trials.

After the application of agrochemicals, approximately 2 kg of garlic plants presenting normal growth were collected randomly from each experimental plot, with four replications and at three time intervals of 1, 7, and 14 days. Each plant was separated into two subsamples, the aboveground plant (green garlic) and the underground plant (garlic bulb), and the subsamples were stored in plastic bags at −20 °C until their preparation for HPLC analysis. The adjuvant effect on fungicide residue levels in the two edible garlic parts, the retention of fungicides on green garlic, their translocation from green garlic to garlic bulb, and the dietary risk of fungicides in garlic tissues were assessed. The suggested preharvest interval (PHI) for garlic is 14 days. Setting the harvest (sampling) intervals at 1, 7, and 14 days allowed us to determine the optimal PHI, considering the human health risk.

2.3. Disease Severity and Fungicide Effectiveness Assessment

Disease assessment was performed with 8 random plant samples in each experimental plot. Five of the youngest leaves per plant were scaled for the percentage of the garlic leaf surface covered with lesions to estimate the effectiveness of the treatments [3].

2.4. Sample Preparation and Instrumental Analysis

An amount of 200 g was obtained from garlic samples (green garlic or garlic bulb) following the quartering method, homogenized using a blender, and a representative portion of 10 g was extracted. Sample preparation was conducted following a slightly modified approach of the mini-Luke multi-residue solvent extraction procedure, reducing the extraction time and the volume of the used organic solvents [18]. Initially, samples were transferred in 250 mL PTFE centrifuge tubes. Following the addition of 15 mL of acetone and 10 g of Na_2SO_4, the samples were mixed using a probe blender for 1 min. The sample extracts were then segregated using a probe blender for an additional 20 s after the addition of dichloromethane (15 mL) and petroleum ether (15 mL). The samples were then centrifuged at 4000 rpm for 2 min. The organic phase volume was measured using an Eppendorf pipette. The organic phase of each sample was evaporated in a rotary evaporator at 60 °C until total dryness. The methanol solubility of boscalid and pyraclostrobin is 45.000 and 100.800 mg/L, respectively [20]. Therefore, a volume of 5 mL of methanol is considered appropriate to reconstitute the residues by shaking the flask thoroughly. The solvent was evaporated under a nitrogen stream, and the residue was taken with 1 mL of methanol. The samples were filtered through a 0.45 μm filter (Millipore, Eschborn, Germany) and then placed in HPLC vials and kept under refrigeration until HPLC analysis.

Samples were analyzed using an HPLC–DAD chromatographic system equipped with an autosampler (Finnigan Surveyor, Thermo Scientific, Waltham, MA, USA). The analytical column Hypersil GOLD 100 × 4.6 mm (Fortis Technologies Ltd., Cheshire, UK) was adjusted at 25 °C and guarded with a precolumn Hypersil GOLD 10 × 4 mm. Quantitative data analysis was conducted using the ChromQuest 5.0 software (Finnigan Surveyor, Thermo Scientific). The gradient mobile phase consisted of acetonitrile (M) and water (W), and the flow rate was 1.0 mL/min, including the following stages: 20%-M/80%/-W (0–10 min), 80%-M/20%/-W (10–11 min), 95%-M/5%-W (11–25 min), and 20%-M/80%-W (26–30 min). The overall run time analysis was 30 min, and the injection volume was set at 25 μL. Data were extracted at 235, 240, and 263 nm of ultraviolet (UV) spectra.

2.5. Dietary Risk Assessment

To guarantee that the acceptable daily intake (ADI) of the pesticides is not exceeded, the risk assessment method includes the potential exposure to pesticide residues. Consumers are thought to be sufficiently protected as long as the pesticide residues do not exceed the ADI. This is helpful for understanding the scope of health concerns and estimating human exposure to pesticides through the food supply [7]. Human health risk assessment was conducted at intervals of 1, 7, and 14 days, following the method of Sivaperumal et al. [21].

The toxicological profile of pyraclostrobin was assessed considering the framework of peer review under Directive 91/414/EEC, and the data were sufficient to derive an ADI of 0.03 mg/kg bw per day and an acute reference dose (ARfD) of 0.03 mg/kg bw [22]. Similarly, according to Directive 91/414/EEC, the toxicological profile of boscalid was evaluated, and an ADI of 0.04 mg/kg bw per day was determined. Also, the use of an ARfD was unnecessary. Therefore, an acute risk assessment was not conducted [23]. Furthermore, the risk assessment was performed with the assumption that consumers consume fresh garlic. Consequently, the consideration of food processing was unnecessary.

2.5.1. Daily Intake

The daily intake of boscalid and pyraclostrobin residues in green garlic and garlic bulb was calculated using the following equation (Equation (2)):

$$\mathrm{EDI} = \frac{\mathrm{C}}{\mathrm{CR}} \times \mathrm{BW} \tag{1}$$

where EDI is the estimated daily intake, C is the mean detected concentration of green garlic or garlic bulb, CR is the average daily consumption rate, and BW is body weight (bw), which was set at 60 kg [24]. Several researchers have provided evidence indicating that the intake of garlic at a dose of 0.1 g/d could be beneficial for human health [2,25]. Therefore, the average daily intake concentration (CR) was set at 0.1 g/day.

2.5.2. Long-Term Risk Assessment

Hazard quotient (HQ) and hazard index (HI) ratios were determined for the evaluation of long-term risk assessment in green garlic and garlic bulb samples, individually. The consumer was deemed to be sufficiently protected if the ratio was found less than unity (1). Equation (2) was used to estimate the HQ by dividing the EDI by the ADI. The ADIs were obtained from the Pesticide Properties DataBase [20].

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{ADI}} \times 100 \tag{2}$$

By summing the obtained hazard quotients (HQs) for each fungicide in garlic bulb and green garlic individually, the HI was determined (Equation (3)).

$$HI = \sum_{i}^{n} HQ_i \quad (3)$$

Pesticide exposure can pose a health risk if HI exceeds the value of 1. This approach has been shown to provide information about vegetables with increased health risks.

2.6. Method Validation

Methanol was used to generate stock solutions of the aforementioned fungicides at 100 mg/mL. To estimate the matrix effect, calibration standards in methanol and matrix-matched calibration standards were prepared. The linearity was assessed using the correlation coefficient of matrix-matched calibration curves considering five different concentrations (0.01, 0.05, 0.1, 0.5, and 1 mg/L). The linearity of the calibration curve in methanol was also tested using these 5 concentrations (0.1, 0.5, 1, 2, and 5 mg/L). Prior to use, all of the standard solutions were stored in a refrigerator at −20 °C.

To determine the accuracy of the analytical method and the recovery of the extraction method, pesticide-free garlic samples (green garlic and garlic bulb) that had been spiked with boscalid and pyraclostrobin at concentrations of 0.02, 0.5, 1, and 2 mg/kg (five replicates) were analyzed. For recovery assessment, blank samples were used, which were obtained from treatment A and sprayed with water. To evaluate reproducibility, three recovery assessments were conducted on three different days. The limit of detection (LOD) was determined as three times the signal–background noise ratio on each chromatogram, and the limit of quantification (LOQ) was the concentration of the lowest spiked level in samples that would provide reliable and reproducible results [26].

The matrix effect (ME) was estimated as follows:

$$ME = \left(\frac{\text{slope of the calibration curve in matrix}}{\text{slope of the calibration curve in solvent}}\right) - 1 \times 100\% \quad (4)$$

Positive and negative MEs showed that the matrix had either enhanced or suppressed the signal, respectively. There is a mild matrix effect when the ME is between −20% and 20%. There is a medium matrix effect when the ME is between 20% and 50% or −50% and −20%, and a strong matrix effect when the ME is 50% or higher [14].

2.7. Statistical Analysis

The least significant differences (LSDs) ($p < 0.05$) in the disease severity scaled as percentage were determined via ANOVA and Fisher's LSD test to compare the effectiveness of the treatments against rust disease. Similarly, LSDs were found between treatments to compare the pesticide amounts accumulated in garlic bulbs and green garlic. Therefore, the effect of adjuvants on pesticide residues in green garlic and the differences between pesticide translocation can be determined. Statistical analysis of data was performed using MSTAT-C (Michigan State University, East Lansing, MI, USA, MSTAT-C 1989).

3. Results and Discussion

3.1. Method Validation

The preparation of calibration curves involved both matrix-matched standards and standards in methanol, and they were analyzed in triplicate. Calibration curves in methanol were linear in the range of 0.1–5 mg/mL and had an $R^2 > 0.99$ (correlation coefficient) for each compound, providing satisfactory results. The results were obtained from UV spectra at 263 nm. The retention time of boscalid and pyraclostrobin was 11.81 min and 14.85 min, respectively. Similarly, the linearity of the matrix-matched calibration curves was confirmed with the correlation coefficients using the five concentrations (0.01, 0.05, 0.1, 0.5, and 1 mg/L). Fungicide chromatographs are presented in Supplementary Figure S1. The regression equations of methanol and matrix-matched calibration curves with their respective R^2 values are presented in Table 3. Calibration curves prepared with standards in methanol were compared with the calibration curves with standards in green garlic and garlic bulb matrix to determine a potential matrix effect. In all cases, the matrix effects were mild, ranging from −7.62 to −4.86 (Table 4). A positive matrix effect suggests that the detector signal was elevated, whereas a negative matrix effect signifies that the detector signal for the compound diminished [27]. The outcomes demonstrated that the signal suppression of the two pesticides varied in different matrices. Consequently, the matrix calibration curves were employed for the quantification of fungicides in this study to confirm the accuracy of the analytical method. Yu and Xu [14] determined the matrix effect of pyraclostrobin in garlic parts ranging from −32.83 to 19.32, using a QuEChERs method with LC–MS/MS analysis. To the best of our knowledge, the effects of boscalid matrix on garlic tissue have not been reported before. The mini-Luke extraction method applied in our case exhibited a lower matrix effect than the previously reported QuEChERs method.

Table 3. Calibration curves, matrix effects, and R^2 values of boscalid and pyraclostrobin in methanol, garlic bulb, and green garlic.

Matrix	Calibration Curve		Matrix Effect (%)		R^2	
	Boscalid	**Pyraclostrobin**	**Boscalid**	**Pyraclostrobin**	**Boscalid**	**Pyraclostrobin**
Methanol	y = 1.035x + 0.0461	y = 1.049x + 0.005	/	/	0.9987	0.9994
Garlic bulb	y = 0.978x − 0.136	y = 0.998 + 0.028	−5.5	−4.86	0.9901	0.995
Green garlic	y = 0.9561x − 0.174	y = 0.982 + 0.01	−7.62	−6.38	0.9906	0.9912

Table 4. Recovery rate and RSD of boscalid and pyraclostrobin in garlic bulb and green garlic.

Matrix	Spiked Level (mg/kg)		Average Recovery (%)		RSD (%) (n = 5)	
	Boscalid	**Pyraclostrobin**	**Boscalid**	**Pyraclostrobin**	**Boscalid**	**Pyraclostrobin**
Green garlic	0.020		81	75	9.3	6.8
	0.5		93	98	7.5	10.1
	1		107	71	10.1	9.5
	2		90	102	13.1	14.6
Garlic bulb	0.020		83	84	6.6	18.1
	0.5		82	86	16.3	11.5
	1		101	78	10.1	8.2
	2		99	105	12.8	16.4

LOD and LOQ were calculated. For boscalid, the outcomes were, respectively, 0.01 and 0.02 mg/kg. The LOD and LOQ of pyraclostrobin were 0.015 and 0.02 mg/kg, respectively. LOQs were below the maximum residue limit (MRL) of boscalid and pyraclostrobin in garlic, which are 5 and 0.3 mg/kg in the EU, respectively. Boscalid and pyraclostrobin recovery rates in green garlic and garlic bulb at four different concentrations (0.02, 0.5, 1, and 2 mg/kg) (n = 5) ranged from 71% to 107% on average, with RSD % values ranging from 6.8% to 18.1% (Table 4). The LOQ method was defined as the lowest spike level of the validation that can be quantified with acceptable trueness (70–120%) and precision (RSD < 20%), as described in Document N° SANCO/12571/2013 [28].

Previously, Gikas et al. [13] and Papaevangelou et al. [29] quantified pyraclostrobin and boscalid in aquatic and plant samples. However, they followed different extraction procedures, including solid-phase extraction, liquid–liquid extraction, and QuEChERs. In general, liquid chromatography is considered a popular approach to detect simultaneously boscalid and pyraclostrobin. Lv et al. [30] reported eight different matrices (e.g., watermelon and grape) for which boscalid and pyraclostrobin were detected via LC/MS-MS, presenting LOQs ranging from 0.0001 to 0.01 mg/kg.

3.2. Disease Severity and Efficacy of Fungicides against Rust Disease

The effectiveness of treatments A, B, and C against garlic rust was analyzed at 1, 7, and 14 days after spraying, and the results are presented in Table 5. The fungicides' efficacy was determined by considering the leaf coverage percentage with lesions. The mean leaf coverage with lesions of the untreated control was 2%, 3.7%, and 4.5% after 1, 7, and 14 days, respectively. Treatment C showed a slightly higher performance than treatment B against garlic rust. For treatment B, the mean leaf coverage of the three time intervals of 1, 7, and 14 days were 1.9%, 2.6%, and 2.1%, respectively, while for treatment C, these values were 1.9%, 2%, and 1.8%, respectively. Therefore, the addition of the adjuvant could improve the performance of the fungicides against rust in garlic.

Table 5. Leaf coverage with lesions (%) after applications of fungicides and the adjuvant. The percentages are the means of eight plants. The means denoted with a different letter indicate significant differences between treatments ($p < 0.05$).

Treatment	1d	7d	14d
A (control)	2.0 A	3.7 B	4.5 C
B (fungicides)	1.9 A	2.6 B	2.1 A
C (fungicides + adjuvant)	1.9 A	2.0 A	1.8 A

To the best of our knowledge, this is the first study in which the boscalid and pyraclostrobin efficacy against rust disease is investigated in the presence or absence of an adjuvant. Previously, Koike et al. [3] examined the efficacy of metalaxyl + mancozeb, chlorothalonil, sulfur, copper hydroxide, and maneb, following the same disease assessment protocol. The results showed that the most effective fungicide was maneb (1.9% leaf coverage), and the less effective was the preventative fungicide copper hydroxide (3.9% leaf coverage).

3.3. Detected Pesticide Residues in Green Garlic and Garlic Bulb

Following the experimental setup, the commercial fungicide Signum and the commercial adjuvant Dash® were applied to garlic samples in accordance with GAP, in northern Evros, Greece. The concentrations of fungicide residues are summarized in Table 6. In treatment B, Signum® WG 26.7, 6.7 was applied to garlic. Boscalid and pyraclostrobin mean residues were 0.175 mg/kg and below the LOQ in garlic bulb and 0.175 and 0.022 mg/kg in green garlic, respectively, on day 14 (PHI). At sampling day 7, boscalid and pyraclostrobin mean residues were 0.167 and 0.031 mg/kg in garlic bulb and 0.863 and 0.362 mg/kg in green garlic, respectively. The initial mean concentrations (day 1) of boscalid and pyraclostrobin were 3.280 and 1.251 mg/kg in green garlic, and in garlic bulbs, they were 0.139 and 0.036, respectively.

Table 6. The residues of fungicides in garlic samples (mg/kg) after Signum® and Dash® application during the experiment. ± SD values are standard deviations (n = 4). EU (mg/kg).

Treatment	Green Garlic			Garlic Bulb			
	1d	7d	14d	1d	7d	14d	MRL [27]
Boscalid	3.280 (±0.47)	0.863 (±0.22)	0.149 (±0.016)	0.139 (±0.013)	0.167 (±0.022)	0.175 (±0.021)	5
Boscalid + adjuvant	3.510 (±0.51)	0.994 (±0.26)	0.162 (±0.02)	0.147 (±0.028)	0.168 (±0.019)	0.177 (±0.026)	
Pyraclostrobin	1.251 (±0.23)	0.362 (±0.081)	0.022 (±0.009)	0.036 (±0.004)	0.031 (±0.003)	<LOQ	0.3
Pyraclostrobin + adjuvant	1.426 (±0.3)	0.401 (±0.075)	0.023 (±0.005)	0.042 (±0.008)	0.044 (±0.005)	<LOQ	

Treatment C included the application of Signum® WG 26.7, 6.7 mixed with adjuvant Dash® HC EC. At sampling day 14, boscalid and pyraclostrobin mean residues were 0.175 mg/kg and below the LOQ in garlic bulb and 0.149 and 0.023 mg/kg in green garlic, respectively. Boscalid and pyraclostrobin mean residues were 0.168 and 0.044 mg/kg in garlic bulbs and 0.994 and 0.401 mg/kg in green garlic, respectively, after 7 days. On day 1, in green garlic and garlic bulbs, the mean concentrations of boscalid and pyraclostrobin were 3.510 and 1.426 mg/kg and 0.147 and 0.042, respectively.

The EU has established MRLs for pesticides in foodstuffs and animal feed in order to ensure public health safety. The analysis of pesticide residual levels in food to determine safe PHIs is essential for effective pest management approaches and guarantees consumer health. Both MRLs and PHIs are crucial components of GAP compliance [31]. The PHI is the amount of time following the final application of the targeted pesticide that is needed for the concentration to drop below the MRL. The results of treatments B and C showed that boscalid concentration levels in garlic bulb and green garlic were below MRL (5 mg/kg) on days 1 and 7, but pyraclostrobin residual levels exceeded the MRL (0.3 mg/kg). However, the detected concentrations of both pyraclostrobin and boscalid were below MRLs on day 14. Therefore, the current findings demonstrated that both application conditions (Signum® and Signum® + Dash®) after a PHI of 14 days were safe, with residue levels below the respective MRLs.

Concentrations of fungicide residues detected in green garlic were considerably higher than those detected in garlic bulbs. These findings are closely related to the morphological traits of garlic plants and spray conditions. Garlic bulb grows in the soil, but when pesticides are applied, they are applied straight to the plant (green garlic), not to the soil or bulbs [14].

The aforementioned data showed that the two fungicides were dissipated rapidly in garlic. This conclusion has also been corroborated by earlier studies. According to Malhat et al. [32], the half-life of pyraclostrobin in strawberries was 7 days, and on day 14 after treatment, its concentration decreased by more than 82%.

Regarding boscalid, its half-life ranged from 4.9 to 6.4 days in strawberries and 9.8 days in ripe apples [10]. The physical and chemical characteristics of fungicides, as well as environmental factors including light, temperature, and humidity, may contribute to their rapid dissipation within plant tissue. Moreover, crop variety potentially affects the dissipation rate [33].

Due to increased volatilization and rapid degradation, which are adversely affected by high moisture content, high temperatures, and direct sunshine, climate change may lead to a decrease in pesticide concentrations in plant tissues. These factors also have an impact on the chemical alterations in pesticides. Higher precipitation levels, along with temperature, degradation, and sorption, are favorable for pesticide dissipation in general. Higher temperatures enhance the uptake and cause equilibrium of semi-volatile pesticides within leaves. Furthermore, local environmental conditions have a major influence on pesticides' dispersion through the atmosphere. Therefore, the persistence and effectiveness of pesticides are influenced by the timing and severity of rainfall. Furthermore, heat and light modify the chemical composition of pesticides, affecting their durability.

3.4. Effect of the Adjuvant on Pesticide Residues in Green Garlic

Even though tank mixtures with adjuvants are frequently employed to boost the retention, coverage, uptake, and biological activity of foliage-applied sprays of various agrochemicals, there is little evidence of their long-term impact on pesticide residues in treated crops. The results of this study showed that the green garlic samples treated with a tank mixture of the fungicides and adjuvant (treatment C) presented significantly higher concentrations of pyraclostrobin and boscalid than treatment B. The use of the adjuvant increased the retention of fungicides on garlic foliage and decreased their dissipation rate (Figure 3). It is evident that the higher absorption of green garlic induced a better fungicidal performance (Table 5). The adjuvant Dash® HC EC contains 349 g/L oil (fatty acids and methyl esters) and 209 g/L alkoxylated alcohol–phosphate esters. Oil adjuvants may enhance the cuticular uptake of pesticides applied on plants by 10–20% [34] and reduce the decomposition of pesticides [35]. Also, Dash® HC EC is a nonionic and hydrophilic adjuvant that can reduce the acidity of the tank mixture. Therefore, pesticide molecules can be transformed to nondissociated, and leaves could absorb higher amounts of pesticides [36,37]. Holloway et al. [38] investigated the effect of an adjuvant named Toil, containing fatty acid esters on propiconazole residues in wheat foliage. All the samples sprayed with fungicides containing the adjuvant exhibited a similar notable increase in residues (0.10–0.16 mg/kg) compared with the no-adjuvant treatment (0.05 mg kg) in wheat.

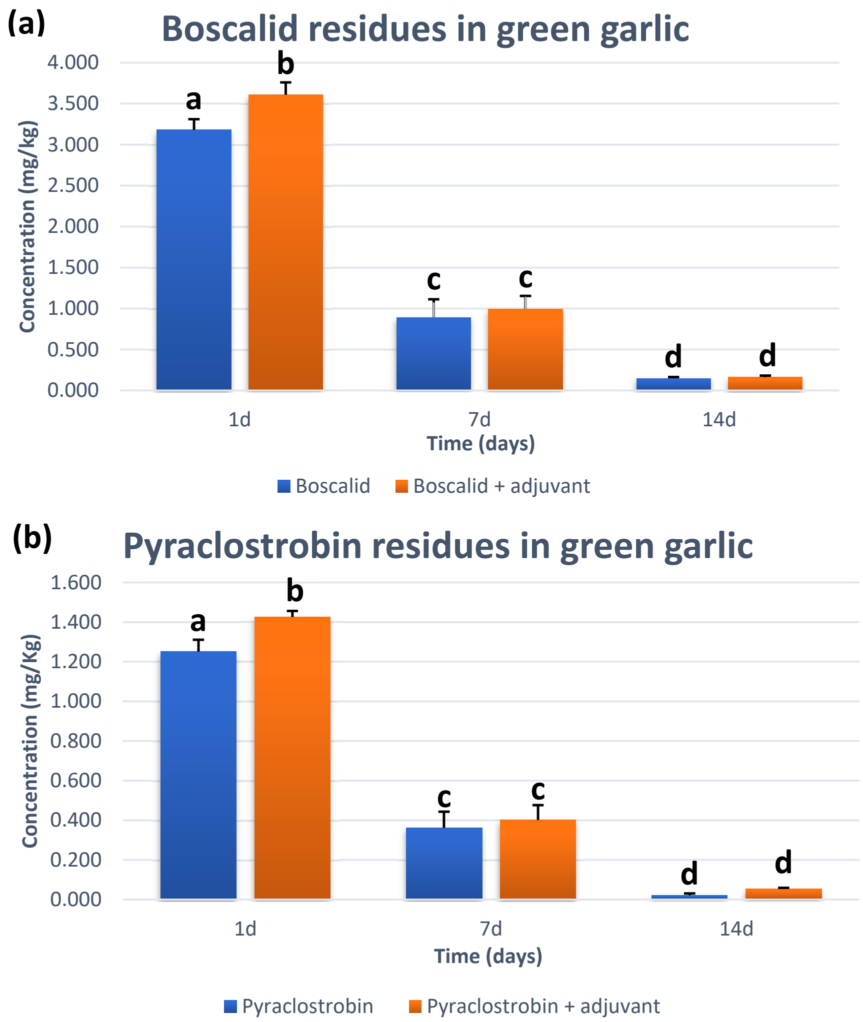


Figure 3. Boscalid (**a**) and pyraclostrobin (**b**) residues in green garlic (mg/kg). The plotted values are the means ± SD. Means denoted with a different letter indicate significant differences between treatments ($p < 0.05$).

3.5. Translocation of Boscalid and Pyraclostrobin from Green Garlic to Garlic Bulb

The results of this study showed that boscalid had higher translocation from green garlic to garlic bulbs than pyraclostrobin. The translocation to garlic bulb was not significant for boscalid on days 1, 7, and 14. Pyraclostrobin was not significantly translocated to garlic bulbs on days 1 and 7, but on day 14, pyraclostrobin was highly dissipated in bulbs ($p < 0.05$), presenting residues below the LOQ (Figure 4). The phytoaccumulation and translocation in plants treated with pesticides are strongly related to plant species and pesticide characteristics, such as octanol–water partition coefficient ($LogK_{ow}$) and water solubility. Pesticides with $LogK_{ow}$ values ranging from 0.5 to 3.0 are less hydrophobic and demonstrate high translocation in plants [39]. Pyraclostrobin with a $LogK_{ow}$ value of 3.99

is highly hydrophobic, and thus, low translocation in garlic tissue is expected, whereas boscalid with a $LogK_{ow}$ value of 2.9 exhibits local systemic and translaminar movement. Water solubility is another crucial parameter that influences the outcome of pesticide application in plant tissue. The water solubility of boscalid and pyraclostrobin is 4.6 and 1.9 mg/L, respectively. In accordance with Wang et al. [40], pesticides with greater $LogK_{ow}$ values and lower water solubility are absorbed more readily by plants, whereas pesticides with lower $LogK_{ow}$ and higher water solubility are favorably translocated within plants. Therefore, boscalid is more conducive than pyraclostrobin to translocation in garlic. Malhat et al. [32] and Fraser et al. [41] suggested that pyraclostrobin is a translaminar fungicide, while Papaevangelou et al. [29] and Simon-Delso et al. [42] reported that boscalid could present systemicity in some cases, due to its moderate hydrophobicity. However, extensive research on the translocation of boscalid and pyraclostrobin in plant tissues is limited.

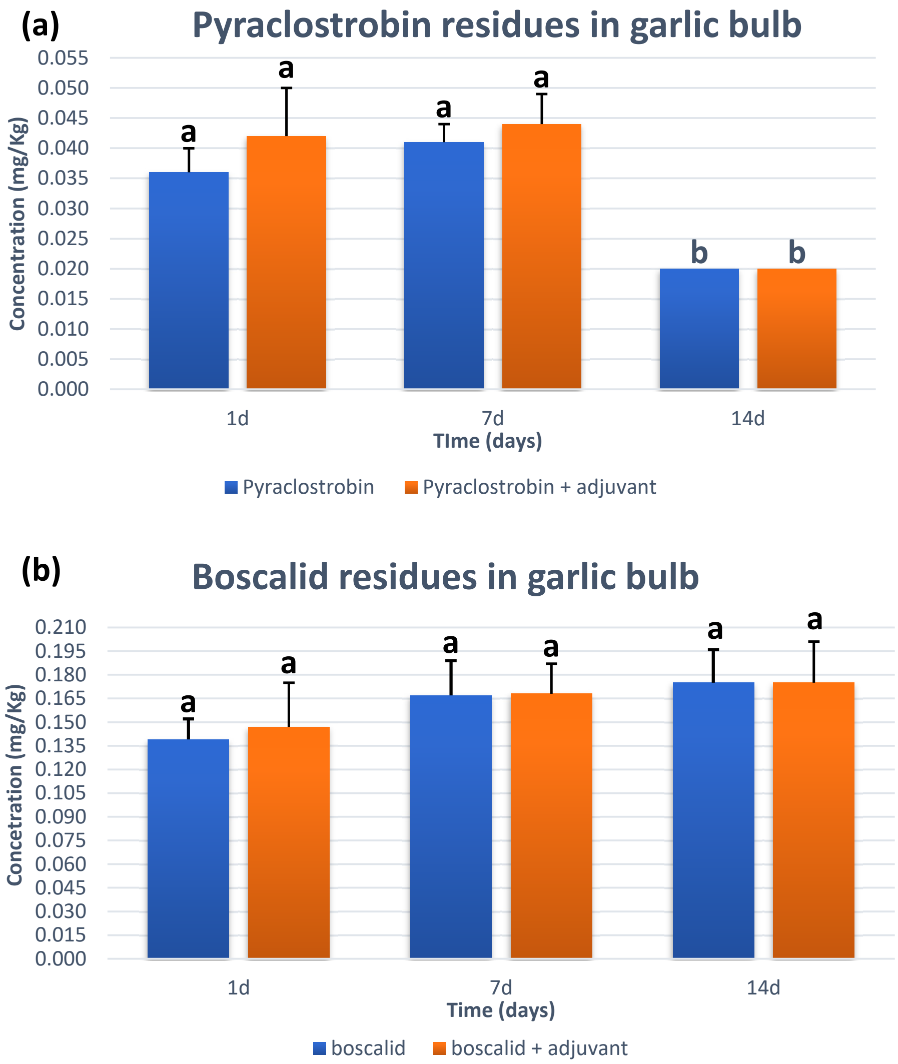


Figure 4. Boscalid (**a**) and pyraclostrobin (**b**) residues in garlic bulb (mg/kg). The plotted values are means ± SD. The means denoted with a different letter indicate significant differences between treatments ($p < 0.05$).

3.6. Long-Term Human Risk Assessment

The EDI, HQ, and HI values of boscalid and pyraclostrobin for treatments B and C were determined in green garlic and garlic bulb samples during the experiment (Table 7).

Table 7. EDI (estimated daily intake), HQ (hazard quotient), and HI (hazard index) for long-term risk assessment of boscalid and pyraclostrobin.

Treatment	Green Garlic			Garlic Bulb		
	1d	7d	14d	1d	7d	15d
Boscalid EDI	0.0055	0.0014	0.0002	0.0002	0.0003	0.0003
Boscalid + adjuvant EDI	0.0059	0.0017	0.0001	0.0002	0.0001	0.0003
Pyraclostrobin EDI	0.0021	0.0006	0.0003	0.0001	0.0003	<1 [1]
Pyraclostrobin + adjuvant EDI	0.0024	0.0007	0.00004	0.0001	0.0001	<1
Boscalid HQ	13.6667	3.5958	0.6208	0.5792	0.6958	0.72917
Boscalid + adjuvant HQ	14.6250	4.1417	0.6750	0.6125	0.7000	0.73750
Pyraclostrobin HQ	6.9500	2.0111	0.2889	0.2000	0.2278	<1
Pyraclostrobin + adjuvant HQ	7.9222	2.2278	0.1278	0.2333	0.2444	<1
HI (fungicides)	20.6167	5.6069	0.9097	0.7792	0.9236	<1
HI (fungicides + adjuvant)	22.547	6.3694	0.8028	0.8458	0.9444	<1

[1] Below the LOQ.

Even though boscalid was detected in higher concentrations in garlic bulbs and presented higher translocation than pyraclostrobin, the HQ values did not exceed the unity value for garlic bulbs on days 1, 7, and 14 (PHI). Regarding pyraclostrobin, the garlic bulb consumption was also considered safe during the experiment (HQs < 1), due to its limited movement. However, the consumption of garlic bulbs on days 1 and 7 is not recommended because the entry stage of bulb formation does not provide edible bulbs.

The addition of the Dash® HC in tank mixtures has been proposed as a new promising fungicidal strategy, which provides several advantages, including higher retention of fungicides on leaves. In order to reach conclusions about agrochemicals' safety, risk assessment is essential. To ensure that new disease management approaches continue to meet the necessary safety standards, they must be routinely re-evaluated. In spite of the ability of the adjuvant to enhance the retention of both fungicides on green garlic, the adjuvant did not negatively affect the safety of green garlic consumption on day 14 (HQs < 1). Green garlic consumption on days 1 and 7 was found to be unsafe because the HQs and HIs values of boscalid and pyraclostrobin exceeded the unity value. Consequently, the use of the adjuvant in the tank mixture was considered safe on day 14, providing good rust management, which agrees with the recommended PHI. According to our results, the consumption of garlic bulbs 1 or 7 days after the application of agrochemicals is safe if the growth stage of the bulb is appropriate.

4. Conclusions

The concentrations of boscalid and pyraclostrobin were determined in samples of green garlic and garlic bulb. In this study, a mini-Luke multi-residue solvent extraction method paired with HPLC–DAD was validated. This approach showed acceptable levels of linearity, accuracy, sensitivity, precision, and reproducibility. The addition of the adjuvant can increase the effectiveness of fungicides without increasing the dietary risk of garlic. Also, the dietary risk was not negatively affected by the addition of the adjuvant in the tank mixture. Green garlic consumption is safe only fourteen days after agrochemical application, but consumers do not need to avoid garlic bulb consumption one day after application, provided that bulbs are consumable. The results of this study could provide new insights into the use of boscalid and pyraclostrobin in garlic ecosystems and fill a research gap regarding the effects of adjuvants on fungicides' efficacy and dietary risk. In our opinion, authorities need to consider the information gaps and uncertainties in the risk assessment of agrochemical combinations, particularly with adjuvants. Thus, an additional safety factor for adjuvants should be included.

In the United States and the European Union, the active substance is the main parameter of the current health risk evaluation of pesticides. However, adjuvants can also be harmful; multiple adverse impacts on both human health and the environment have been documented. Adjuvants are considered harmful compounds, but the procedures for their regulation differ from those of active ingredients, and their toxic effects are frequently disregarded. Adjuvants are excluded from the dietary risk assessment and are not taken into account in acceptable daily intake [43]. Therefore, future studies should focus on adjuvant residues in food.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081636/s1. Figure S1: HPLC-DAD chromatographs of (a) garlic bulb blank, (b) spiked garlic bulb matrix with boscalid and pyraclostrobin (0.020 mg/Kg), (c) green garlic blank, and (d) spiked green garlic.

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