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Dissipation and Distribution Behavior of Azoxystrobin, Carbendazim, and Difenoconazole in Pomegranate Fruits

Sagar C. Utture,[†,‡,§] Kaushik Banerjee,[*,†,§] Soma Dasgupta,[†,§] Sangram H. Patil,[†,‡,§] Manjusha R. Jadhav,[†,§] Sameer S. Wagh,[†,§] Sanjay S. Kolekar,[‡,§] Mansing A. Anuse,[‡] and Pandurang G. Adsule[†]

[†]National Referral Laboratory, National Research Centre for Grapes, P.O. Manjri Farm, Pune-412 307, India

[‡]Department of Agrochemicals and Pest Management, Shivaji University, Kolhapur-416 004, India

Supporting Information

ABSTRACT: The dissipation behavior and degradation kinetics of azoxystrobin, carbendazim, and difenoconazole in pomegranate are reported. Twenty fruits/hectare (5 kg) were collected at random, ensuring sample-to-sample relative standard deviation (RSD) within 20−25%. Each fruit was cut into eight equal portions, and two diagonal pieces per fruit were drawn and combined to constitute the laboratory sample, resulting in RSDs <6% ($n = 6$). Crushed sample (15 g) was extracted with 10 mL of ethyl acetate (+ 10 g Na_2SO_4), cleaned by dispersive solid phase extraction on primary secondary amine (25 mg) and C_{18} (25 mg), and measured by liquid chromatography tandem mass spectrometry. The limit of quantification was ≤0.0025 $\mu g\ g^{-1}$ for all the three fungicides, with calibration linearity in the concentration range of 0.001−0.025 $\mu g\ mL^{-1}$ ($r^2 \geq 0.999$). The recoveries of each chemical were 75−110% at 0.0025, 0.005, and 0.010 $\mu g\ g^{-1}$ with intralaboratory Horwitz ratio <0.32 at 0.0025 $\mu g\ g^{-1}$. Variable matrix effects were recorded in different fruit parts viz rind, albedo, membrane, and arils, which could be correlated to their biochemical constituents as evidenced from accurate mass measurements on a Q-ToF LC-MS. The residues of carbendazim and difenoconazole were confined within the outer rind of pomegranate; however, azoxystrobin penetrated into the inner fruit parts. The dissipation of azoxystrobin, carbendazim, and difenoconazole followed first + first order kinetics at both standard and double doses, with preharvest intervals being 9, 60, and 26 days at standard dose. At double dose, the preharvest intervals extended to 20.5, 100, and 60 days, respectively.

KEYWORDS: fungicides, azoxystrobin, carbendazim, difenoconazole, pomegranate, residues, method validation, dissipation, preharvest interval (PHI)

INTRODUCTION

Pomegranate is an important tropical fruit crop extensively cultivated in India in an area of around 0.1 million ha with an export value of INR 92 million.[1] The incidence of fungal diseases, e.g. wilt caused by *Fusarium oxysporum*, *Rhizoctonia solani*, *Ceratocystis fimbriata*, leaf and fruit spots caused by *Cercospora punicae*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, and fruit rot caused by *Rhizopus* sp. and *Colletotrichum* spp.,[2] is one of the major causes of economic loss in pomegranate production, which necessitates regular application of fungicides [3] to secure desired yield and fruit quality for domestic sales as well as to promote export. From a preliminary survey of pomegranate farms, carbendazim (methyl benzimidazole-2-ylcarbamate), difenoconazole (3-chloro-4-[(2*RS*,4*RS*;2*RS*,4*SR*)-4-methyl-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl] phenyl 4-chlorophenyl ether), and azoxystrobin (methyl (2*E*)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) were identified to have strong potential for the management of various fungal diseases of pomegranate. Carbendazim is a traditionally used fungicide, whereas the other two are relatively new entries in Indian horticulture. Literature survey reflects nonavailability of residue dissipation data for either of these fungicides in pomegranate as per the good agricultural practices, which creates apprehension of accumulation of their residues at levels above the maximum residue limits (MRLs) at the stage of harvest,[4] resulting in food safety restriction issues.

Pomegranate fruit is characterized by a thick outer rind that encloses the soft and edible aril. Because of this typical nature of pomegranate fruits, the residue dynamics information generated for other fruits may not be applicable to it. In our earlier studies in grapes, the residues of carbendazim were reported to dissipate following first-order rate kinetics,[5] while the dissipation of difenoconazole followed first + first order rate kinetics.[6] The residues of thiabendazole and imazalil were identified to get mostly localized in the peel of the orange fruits, with a small fraction of imazalil being translocated into the pulp.[7] Teixeira et al. investigated the levels of 13 fungicides and one insecticide in grapes and concluded that there were no significant differences between the pesticide levels in the whole grape berry (skin and pulp) and the berry skin for most of the test chemicals, with exception of Pyrimethanil, the residues of which was accumulated in pulp.[8] Such kind of information regarding fractionation of pesticide residues among different fruit parts of pomegranate is not available in literature, which could be the reason why the whole pomegranate fruits are considered for residue analysis despite the fact that the rind portion is nonedible to human beings.

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Table 1. Optimized LC-MS/MS Parameters with Retention Time for Three Fungicides

name of pesticide	RT (min)[a]	parent (m/z)	target (m/z)	CE (V)[b]	CXP (V)[c]	qualifier (m/z)	CE (V)[b]	CXP (V)[c]
azoxystrobin	5.2	404	372	19	6	344	27	4
carbendazim	3.2	192	160	27	4	132	41	4
difenoconazole	6.1	406	251	35	4	111	77	4

[a] RT = retention time. [b] CE = collision energy. [c] CXP = collision cell exit potential.

This research paper presents dissipation rate kinetics of the fungicides viz azoxystrobin, carbendazim, and difenoconazole residues in pomegranate fruits. The safety constants viz half-life and preharvest interval (PHI) of these chemicals have been estimated for recommendation to the growers to promote their safe usage when applied at standard and double doses as per the farmers' most critical usage pattern for disease management in pomegranate fields. Furthermore, the mobility and partitioning of the residues in different fruit parts after application was studied using a thoroughly validated analytical method.

MATERIALS AND METHODS

Chemicals. Certified reference standards of azoxystrobin, carbendazim, and difenoconazole (>98% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Residue analysis grade (dried) ethyl acetate and HPLC grade methanol were obtained from Thomas Baker (Mumbai, India). Primary secondary amine (PSA, 40 μm, Bondesil), graphitized carbon black (GCB), and C_{18} were procured from United Chemical Technology (Bristol, PA, USA). Anhydrous sodium sulfate (analytical reagent grade) was purchased from Merck (Mumbai, India) and activated by heating at 450 °C for 6 h and kept in desiccators.

Preparation of Standard Solution. The stock solutions of the individual pesticide standards were prepared by accurately weighing 10 (±0.1) mg of each analyte in volumetric flasks (certified A class) and dissolving in 10 (±0.1) mL of methanol. These were stored in dark vials at 4 °C. A working standard mixture of 1 mg L^{-1} was prepared by appropriate dilution of the stock solution, from which the calibration standards (0.001−0.025 μg mL^{-1}) were prepared by serial dilution with methanol: water (1:1, v/v).

Field Experiments. Field experiments were conducted on pomegranate crop (*Punica granatum* L., cultivar. Bhagwa) at a good agricultural practices (GAP) certified farm, located at Kalas village (latitude 18°9′58.74″N, longitude 74°48′22.78″E), Indapur district, Pune, India, as per the United States Environmental Protection Agency guidelines for crop field trials.[9] There were 440 plants in 1 ha area with row-to-row spacing of 8 ft and plant-to-plant spacing of 10 ft. The productivity was ≈4500 kg/ha, and each plant was bearing on an average of 40 fruits. The agricultural formulations of azoxystrobin (Amistar 23.5% SC; Syngenta India Ltd., Pune), carbendazim (Bavistin 50% WP; BASF India Ltd., Mumbai), and difenoconazole (Score 25% SC; Syngenta India Ltd., Pune) were applied by foliar spray at the standard rate of 1000 (mL ha^{-1}), 1000 (g ha^{-1}), and 500 mL ha^{-1}, respectively. Treatment at the double dose of 2000 (mL ha^{-1}), 2000 (g ha^{-1}), and 1000 mL ha^{-1} were given in separate plots. For each treatment, the applications were done two times at 15 days interval during June−July 2010. The meteorological conditions during the field experiments included average maximum and minimum temperatures of 32 and 20 °C, respectively, with relative humidity ranging between 55 and 90%. Each treatment, including the untreated control, was replicated thrice in separate plots each of 1 ha area. The crop was grown under drip irrigation on Vertic Ustropepts (USDA class) soil.

Samples comprising around 20 fruits (approximately 5 kg) were collected at random from each replicate of the treated and control plots separately at regular time interval on 0 (1 h after spraying), 1, 3, 5, 7, 10, 15, 30, 45, and 60 days after the final foliar spray. The fruits hidden inside the canopy or those showing signs of infestation of insect pests, diseases, or any physiological disorders were not included in sampling. All the samples were collected from field in morning hours (before 9 a.m.), transported to the laboratory within 1 h of sampling, and analyzed immediately to prevent any degradation losses of the residues in storage.

Strategy for Selection of Representative Subsample from Bulk. The processing of the laboratory samples was optimized with an objective to obtain a representative test sample. Among four trial strategies (described in Results and Discussion), the final method included cutting each of the fruits out of ≈5 kg sample directly into eight pieces without washing or any kind of pretreatments and from this, two diagonally ended pieces (picture provided in Supporting Information A) were collected to account for 1 kg test sample. This sample was then chopped into small pieces (around 1 cm^2) and crushed thoroughly in a blender after adding water (0.5 L).

Sample Preparation. Approximately 300 g of the crushed sample (as above) was further homogenized at high speed, and from this, a portion of a 15 g sample was drawn in a 50 mL polypropylene centrifuge tube. The sample was extracted with ethyl acetate (10 mL) and anhydrous sodium sulfate (10 g) by homogenization, followed by centrifugation at 3000 rpm for 5 min. A portion of a 3 mL aliquot was withdrawn and subjected to dispersive solid phase extraction (d-SPE) cleanup with 25 mg of PSA and 25 mg of C_{18}. After centrifugation, 2 mL of aliquot was separated, mixed with 200 μL of 10% diethylene glycol (in methanol), and evaporated to near dryness in a low-volume concentrator (TurboVap LV; Caliper Life Sciences, Russelsheim, Germany) at 35 °C under gentle stream of nitrogen. The residues were reconstituted in a mixture of 1 mL of methanol +1 mL of 0.1% acetic acid in water, and 10 μL of it was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

To study the fractionation of the residues in different parts of the fruit, the treated whole fruits were divided into four sections viz outer rind, albedo (white fleshy substance directly under the rind), membrane, and arils. Each portion was then crushed and analyzed separately using the method described above.

LC-MS/MS Analysis. The LC-MS/MS analysis was carried out on Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) hyphenated to an API 4000 Q trap (AB Sciex, Toronto, Canada) mass spectrometer equipped with an electrospray ionization (ESI) probe. The instrument was controlled using Analyst 1.5 software. The HPLC separation was carried out by injecting 10 μL onto an Atlantis dC_{18} column (100 mm × 2.0 mm ID, 5 μm). The mobile phase was composed of (A) methanol:water (20:80 v/v with 5 mM ammonium formate) and (B) methanol:water (90:10 v/v with 5 mM ammonium formate); gradient 0−1 min 85% A phase, 1−5 min 85−2% A phase, 5−8 min 2% A phase, 8−8.5 min 2−85% A phase, and 8.5−12 min 85% A phase. The column oven temperature and mobile phase flow rate were maintained at 30 °C and 0.4 mL min^{-1}, respectively. The estimation was performed in positive mode by multiple reaction monitoring (MRM). The MS/MS parameters with the corresponding retention times are presented in Table 1. The first mass transition was used for quantification, while the second mass transition was used for confirmation of the residues. The ratio of the peak area of quantifier to confirmatory MRM

transitions was 6.54, 4.16, and 1.55 for azoxystrobin, carbendazim, and difenoconazole, respectively, which were used for confirmation within ±10% of tolerance in accordance with the EC guidelines.[10]

LC-QToFMS Analysis. The LC-QToFMS analysis was carried out on Agilent 1200 series HPLC hyphenated to Agilent 6530 Accurate Mass QToF (Agilent Technologies, Palo Alto, CA). The HPLC parameters were as described in above section. The MS parameters were: capillary voltage 4000 V, gas temperature 325 °C, drying gas 8 L min^{-1}, nebulizer 45 psig, sheath gas temperature 325 °C, and sheath gas flow 9 L min^{-1}, fragmentor voltage 70 V, and skimmer voltage 65 V. The data was acquired at both positive and negative modes at resolution of 20000.

Method Validation. The analytical methodology was validated as per the single laboratory validation approach of Thompson et al.[11] The following validation parameters were considered to evaluate the performance of the method.

Calibration Range. The calibration curves for both the solvent and matrix matched standards were obtained by plotting the peak area against the concentration of the corresponding calibration standards at five concentration levels ranging between 0.001 and 0.025 $\mu g\ mL^{-1}$.

Sensitivity. The limit of detection (LOD) of the test compounds was determined by considering a signal-to-noise ratio of 3 with reference to the background noise obtained for the blank sample, whereas the limits of quantification (LOQ) were determined by considering a signal-to-noise ratio of 10.

Matrix Effect. The responses of the matrix-matched standards (peak area of postextraction spike) were compared with the corresponding peak areas of solvent standards in ten replicates. The matrix effect (ME) was quantified as average percent suppression or enhancement in peak area using the following equation:

$$\mathrm{ME}(\%) = \frac{(\text{peak area of matrix standard} - \text{peak area of solvent standard}) \times 100}{\text{peak area of solvent standard}}$$

The negative and positive values of the ME signify matrix induced signal suppressions or enhancements respectively. The ME for different parts of the fruit was separately evaluated in similar way using the above equation.

Precision. The precision in the conditions of repeatability (for six analyses in a single day) and the intermediate precision (for six analyses in six different days) were determined separately for a standard concentration of 0.0025 $\mu g\ g^{-1}$ of all the analytes. The Horwitz ratio (HorRat)[12,13] pertaining to intralaboratory precision, which indicates the acceptability of a method with respect to precision was calculated at a fortification level of 0.0025 $\mu g\ g^{-1}$ in the following way:

$$\mathrm{HorRat} = \mathrm{RSD}/\mathrm{Prsd}$$

where RSD is the relative standard deviation and Prsd is the predicted relative standard deviation = $2C^{-0.15}$, where C is the concentration expressed as mass fraction ($0.0025\ \mu g\ g^{-1} = 2.5 \times 10^{-9}$).

Accuracy-Recovery Experiments. The recovery experiments were carried out on fresh untreated pomegranate fruits by fortifying the crushed samples (15 g) in six replicates with the pesticides under study at three concentration levels viz, 0.0025, 0.005, and 0.010 $\mu g\ g^{-1}$. The recovery at above-mentioned concentrations was also evaluated from different parts of the fruit.

Measurement Uncertainty. The reproducibility of the method was assured by evaluating the measurement uncertainty. The global uncertainty in the determination of the pesticides was calculated at 0.0025 $\mu g\ g^{-1}$ as per the EURACHEM/CITAC Guide CG 4.[14] Five individual sources of uncertainty were considered for the assessment of global uncertainty as described earlier[15] and reported as expanded uncertainty, which is twice the value of the global uncertainty.

Application of Method to Real Samples. The validated method was applied for the assessment of residues of the selected pesticides from 25 fresh pomegranate samples collected randomly from the fruit retailers across the Pune district. The method was also evaluated on 10 each of fresh aril and pomegranate juice samples collected from the supermarkets and local retailers.

Data Analysis of Field Study. A number of publications have shown that simple first-order kinetics cannot adequately explain the degradation behavior of pesticides in natural systems, where the degradation pattern may follow a nonlinear path.[16,17] A nonlinear two compartment first + first order model can adequately fit to the degradation pattern of many pesticides, and could also predict the DT_{50} and PHI in a more realistic manner than a linear first-order model. In view of this, we attempted to analyze the time wise residue data of the pesticides in pomegranate by linear as well as nonlinear regression analysis with the following mathematical expressions:

$$\text{first-order model}: \quad [A]_t = [A]_1 \exp(-k_1 t)$$

$$\text{first + first order model}: \quad [A]_t = [A]_1 \exp(-k_1 t) + [A]_2 \exp(-k_2 t)$$

$$\text{square root first-order model}: \quad [A]_t = [A]_1 / k_1 t^{1/2}$$

where $[A]_t$ is the concentration (mg kg^{-1} pomegranate) of A at time t (days), $[A]_1$ and $[A]_2$ are the initial concentrations of A at time 0 degraded through first-order processes 1 and 2, and k_1 and k_2 are the degradation rate constants for 1 and 2. The units of k depend on the model used. The half-life (DT_{50}), which is the time at which the concentration of initial deposits reaches the 50% level, was determined by the following equation:

$$\text{first-order model}: \quad DT_{50} = \ln 2 {k_1}^{-1}$$

$$\text{square root first-order model}: \quad DT_{50} = (\ln 2 / \ln k_1)^2$$

DT_{50} is an important parameter that signifies the speed of degradation. The PHI, i.e. the time period (in days) required for dissipation of the initial residue deposits to below the maximum residue limit (MRL) for first-order kinetics, was determined by the equation

$$\mathrm{PHI} = [\log(\text{intercept}) - \log(\mathrm{MRL})]/\text{slope of first-order equation}$$

Because the first + first order model cannot be described in a differential form, DT_{50} and PHI could only be calculated by an iterative procedure. The equation parameters were calculated by use of a commercially available program TableCurve 2D (v 5.01). The EU-MRL values of 0.05, 0.1, and 0.1 mg kg^{-1} were used for azoxystrobin, carbendazim, and difenoconazole, respectively,[4] for estimation of PHI.

RESULTS AND DISCUSSION

Strategy for Selection of Representative Subsample from Bulk. The following strategies were devised to account for differences in sample representativeness during reduction of bulk sample to subsample for laboratory analysis.

Strategy 1. The whole bulk sample (5 kg) was cut into small pieces and crushed in a blender (a) without addition of water and (b) with addition of water in separate batches.

Strategy 2. The whole bulk sample (5 kg) was cut into small pieces, and around 20% of the sample was randomly separated and crushed in a blender (a) without addition of water and (b) with addition of water in separate batches.

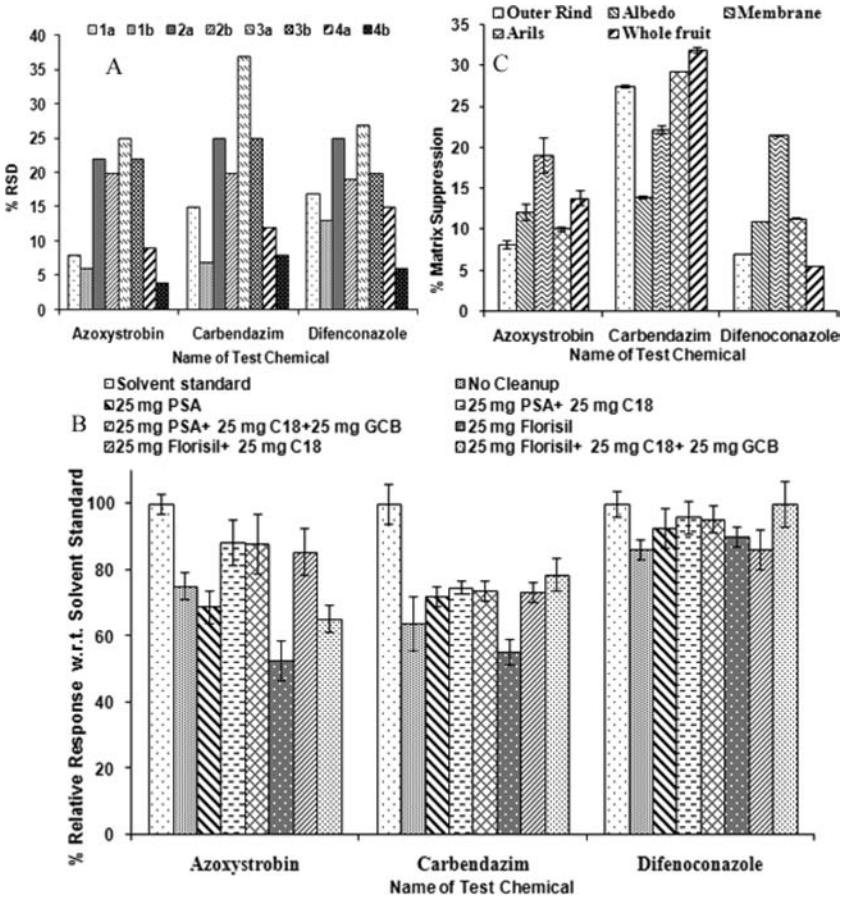

Figure 1. Optimization of the sampling strategies (A), cleanup (B), and matrix effects in different fruit parts (C).

Strategy 3. Fruits were selected at random from the bulk to account for nearly 40% and crushed (a) without addition of water and (b) with addition of water in separate batches.

Strategy 4. Each of the fruits of the bulk sample (5 kg) were cut into eight pieces, and from this, two diagonally ended pieces were collected and combined to account for around 1 kg of subsample (20% of the bulk). The sample was then chopped into small pieces of the size of around 1 cm^2 and crushed thoroughly in a blender (a) without addition of water and (b) with addition of water in separate batches.

The RSDs (n = 6) for the test results of the subsamples were lowest (Figure 1) when the samples were processed as per strategy 1 and 4 b. However, we chose 4 b over 1 b as it offers comparative ease of sample handling and operation. The addition of water facilitated in achieving satisfactory sample homogenization. For all the four strategies described above, it was observed that the RSDs were lower in (b) as compared to (a). The volume of water added was also optimized from a range of volumes (0.1−1 L kg^{-1} sample). For lesser volumes of water (up to 400 mL) added, the crushed material was only partially homogenized with RSD for the concentrations of six samples out of 1 kg lab sample to be >15%. However, when 500 mL of water was added while crushing the samples, they were uniformly homogenized, the corresponding RSD reduced to <8%. Beyond this volume, the sample attained a watery consistency with loss in recovery of azoxystrobin and thus, the water content was optimized to 500 mL per kg sample. Consequent compensation of the added water was taken into account during sample preparation to account for the dilution of residues.

Sample Preparation. A portion of 300 g of the sample obtained as per strategy 4b was further homogenized and 15 g of the homogenized material was withdrawn for analysis. Because the sample to water ratio was 2:1 (10 g sample + 5 mL water) for the homogenized material, no further water was added externally during extraction as reported earlier.[18] The extraction was carried out with 10 mL of ethyl acetate in presence of 10 g of Na_2SO_4 followed by centrifugation (3000 rpm, 5 min). A portion of a 3 mL aliquot was withdrawn and subjected to d-SPE cleanup. In the previously reported method,[18] matrix induced signal suppressions were observed for azoxystrobin, carbendazim, and difenoconazole (up to 30%). Consequently, the cleanup step employing different cleanup agents (PSA, C_{18}, and GCB), alone and/or in combination (seven combinations, including no cleanup) were attempted in order to minimize the ME (Figure 1).

Method Validation. *Accuracy and Precision.* The recovery of all the pesticides at three concentration levels, i.e. 0.0025, 0.005, and 0.010 $\mu g\ g^{-1}$ were within the range of 77−105% with RSDs (n = 6) below 9% (Table 2). The recovery of the pesticides from different fruit parts (i.e., outer rind, aldedo, membrane and arils) was also within 79−101% for all the compounds and the results are summarized in Table 2. The coefficient of regression for calibration curves were $r^2 > 0.999$ for all the compounds. The HorRat calculated at 0.0025 $\mu g\ g^{-1}$ for azoxystrobin, carbendazim and difenoconazole were 0.16, 0.32, and 0.21 respectively,

Table 2. Validation Parameters of Pesticides in Whole Fruit and Different Parts of Fruit

	whole fruit			outer rind			albedo			membrane			arils		
	recovery (% RSD) ($ng\,g^{-1}$)			recovery (% RSD) ($ng\,g^{-1}$)			recovery (% RSD) ($ng\,g^{-1}$)			recovery (% RSD) ($ng\,g^{-1}$)			recovery (% RSD) ($ng\,g^{-1}$)		
name of pesticide	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
azoxystrobin	77 (±8)	82 (±8)	101 (±5)	84 (±5)	78 (±2)	81 (±11)	81 (±11)	79 (±2)	72 (±7)	80 (±8)	88 (±10)	93 (±6)	88 (±4)	82 (±5)	82 (±5)
carbendazim	105 (±4)	87 (±9)	81 (±4)	95 (±6)	79 (±7)	97 (±3)	92 (±9)	87 (±6)	101 (±5)	99 (±11)	94 (±9)	86 (±9)	95 (±10)	94 (±6)	96 (±7)
difenoconazole	79 (±9)	85 (±12)	98 (±8)	78 (±11)	83 (±3)	76 (±13)	92 (±7)	76 (±1)	85 (±7)	82 (±8)	90 (±10)	93 (±7)	79 (±8)	93 (±3)	87 (±6)

indicating satisfactory intralaboratory precision. The limit of quantification for all the pesticides was 0.001 $\mu g\,g^{-1}$.

Matrix Effect (ME). The suppression in S/N for azoxystrobin was around 25% (±4) when no cleanup was used, which could be reduced to around 12% (±7%) when a combination of PSA (25 mg) and C_{18} (25 mg) were used. Similarly, the signal suppressions for carbendazim and difenoconazole were 36% (±8%) and 14% (±3%) for no cleanup and 25% (±2%) and 4% (±5%) for PSA and C_{18} cleanup, respectively. The addition of GCB to above mixture did not give any significant effect in minimizing the matrix induced signal suppressions. Thus, combination of 25 mg of PSA and 25 mg of C_{18} was selected as optimum cleanup strategy for minimizing the ME. For azoxystrobin and difenoconazole, ME was significantly minimized to 14% and 6%, respectively, when the extracts were cleaned by dispersive SPE with the combination of 25 mg of PSA + 25 mg of C_{18}. Because it was not possible to completely nullify the matrix effect, hence, it was essential to quantify the residues through matrix-matched calibration. Interestingly, the ME related to different components of pomegranate fruit was different. In case of carbendazim, the ME were around 28%, 14%, 22%, and 29% for the outer rind, albedo, membrane, and arils, respectively; whereas, for difenoconazole, there was matrix induced signal suppression (below 22%) in all parts, and in the outer rind, suppression was nominal (7%). Matrix suppressions for azoxystrobin were 8%, 12%, 19%, and 10%, respectively, for outer rind, albedo, membrane, and arils (Figure 1).

The variable degree of ME for different parts of pomegranate fruit could be due to their inherent difference in terms of biochemical composition. To investigate it further, the extract from different fruit parts were analyzed by HPLC-diode array detector ((Dionex Corp., Sunnyvale, CA, USA) with DAD (UVD340U) in the wavelength range of 200−600 nm (resolution 2 nm), which revealed prominent peaks at 520 nm during the first 1.0−3.5 min of the chromatographic run, which is characteristic of anthocyanins. Intense peaks were also observed at the wavelengths of 280 and 320 nm. The relative intensity of such matrix peaks varied in the order, outer rind > albedo ≈ membrane > arils, which could be attributed to different nature of distribution of polyphenolic compounds in the extracts of different components of pomegranate fruit. Owing to the presence of higher water content in the arils, the relative intensity of coextracted polyphenols was lower. Arils also showed intense peaks at 230 nm (retention time >5.5 min), tentatively corresponding to fatty acids.

For tentative identification of the coeluting biochemical compounds, the samples were also screened on a LC-QToFMS (Agilent 6530 Accurate Mass QToF, Bangalore, India) at resolution of 20000 for the plausible identification of the matrix components. The matrix components identified by accurate mass measurements (mass error <1 ppm) mostly belonged to the classes of polyphenols and fatty acids (Supporting Information Table S1), which are in agreement with the compounds reported by Borges et. al[19] in pomegranate juice. As per the tentative identifications, the polyphenols eluted in the retention time range of 1.0−3.5 min while the fatty acids eluted during 5−8 min of the chromatographic run.

The relatively higher ME of carbendazim in the rind and arils could thus be attributed to the presence of the coextracted anthocyanidins and their glucosides (e.g., pellargonidin, cyanidin, and delphinidin) at the retention time range of 1−3.5 min and that is why d-SPE clean up with GCB remained unsuccessful. The ME was relatively lower in albedo because the above matrix components were present in lower amounts. The observed ME for difenoconazole in albedo, membrane, and arils could be due to the presence of coextracted fatty acids (retention time 5−8 min; tentatively identified as palmitic, stearic, oleic, and punicic acids) in these fruit parts. The cleanup of the extract was performed with different quantities of PSA (no cleanup, 10, 25, 50, and 75 mg), and the response was compared with solvent standard at same concentration. The order of the matrix suppression for difenoconazole in samples without cleanup was membrane > aril > albedo > rind. After cleanup with PSA, the suppressions reduced in membrane, albedo, and rind. The matrix suppressions for arils were not minimized even after cleanup with higher amount of PSA (25, 50, and 75 mg). This implies that even at higher amount of PSA the suppressions could not be reduced, which could be due to the high amount of fatty acids.

During the retention time range of 4.0−5.5 min, we did not observe any intense peaks in the matrix blank extract of outer rind, albedo, and arils, which could be the reason why the ME was nominal for azoxystrobin that eluted at retention time of 5.2 min. However, in the case of membrane, the relatively higher presence of punicic and stearic acid resulted in comparatively higher ME for azoxystrobin. Thus, it was understood why the matrix induced signal suppressions could not be satisfactorily minimized when PSA or C_{18} was used individually or in separate combinations with GCB.

Measurement Uncertainty. The uncertainty in the determination was evaluated in order to validate the authenticity and accuracy of the method. The total uncertainty was evaluated assuming all the contributions independent of each other. A coverage factor of 2 was decided to evaluate the expanded uncertainty at a confidence level of 95%. The expanded

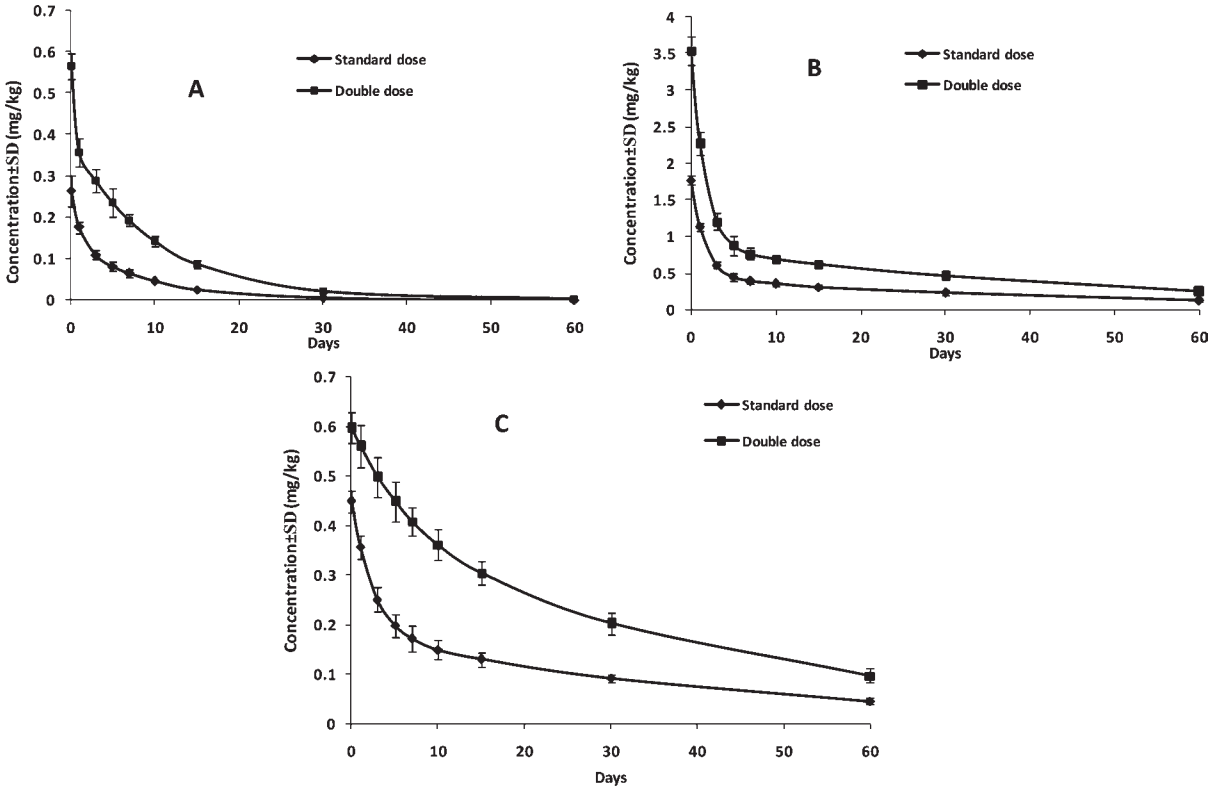

Figure 2. Dissipation of fungicides viz azoxystrobin (A), carbendazim (B), and difenoconazole (C) in pomegranate.

uncertainties for azoxystrobin, carbendazim, and difenoconazole at 0.0025 μg g^{-1} were 18.7%, 18.5%, and 19.7%, respectively, establishing ruggedness of the method. The uncertainties associated with calibration were below 5% for all the compounds at levels of 0.01 μg g^{-1}. However, the uncertainty increased with further decrease in analyte concentration. Up to 10% uncertainty was observed at analyte concentration of 0.0025 μg g^{-1}. This effect was primarily owing to the increase in the RSDs resulting from peak tailing at lower concentrations, which could however be resolved by incorporating appropriate bunching and smoothing factors during integration. The uncertainties associated with calibration were more for difenoconazole (9.57) followed by carbendazim (9.18) and azoxystrobin. The low uncertainties were associated to satisfactory precisions (below 1.18%) both on a day-to-day as well as analyst-to-analyst basis for all the three compounds with the corresponding uncertainty in accuracies below 1.64%, which could be due to stable nature of the compounds and their lower detection limits.

Field Study and Dissipation of Residues. The dissipation of all the three fungicides was faster initially and slowed down with the passage of time (Figure 2), indicating a nonlinear pattern of degradation. This indicated that simple first-order kinetics (where the initial dissipation rate is not as rapid) might not be appropriate to explain the dissipation behavior of their residues. The PHIs estimated through first-order kinetics were inadequate in achieving the dissipation of the residue load of these chemicals to the MRL, indicating the inappropriateness of this model to explain the residue dynamics. Hence, the kinetics of the residue data were also evaluated by fitting the data set into a nonlinear first + first order model and square root first-order model for estimation of the parameters like half-life (DT_{50}) and PHI (Table 3). The fit of the data set to a two-compartment first + first order kinetics model was comparatively greater, indicating the partitioning of the residues into two phases; where one part of the added pesticide, which was immediately available in one of the phases, degraded rapidly, leaving the other part possibly remaining in dynamic equilibrium as an adsorbed fraction on cellular components that becomes available for degradation slowly with passage of time.

The RSDs for the samples collected were within 20−25% for both the standard and double dose of application. The initial residues (1 h after application) of azoxystrobin, carbendazim, and difenoconazole were 0.263 (±14%), 1.767 (±9%), and 0.448 (±12%) mg kg^{-1} respectively at the standard dose, with the corresponding levels of 0.565 (±9%), 3.536 (±11%) and 0.597 (±8%) mg kg^{-1} at the double dose. Almost 58% of the initial deposits of azoxystrobin got dissipated within the third day of foliar spray, with the corresponding rate of 49% dissipation at double dose. For carbendazim, around 66% of the initial deposits got dissipated within 3 day of application, with only 10% residues remaining after 30 days at both the doses. The dissipation of difenoconazole residues was slower as compared to other two fungicides where around 44% of the initial deposits dissipated by third day at standard dose. At double dose, the dissipation of difenoconazole was further slower as evidenced by around only 17% residue decline after 3 days of application. The DT_{50} of azoxystrobin and carbendazim in standard dose was 2 days, while at the double dose, DT_{50} was 3.25 and 2.25 days, respectively. In case of difenoconazole, the dissipation at standard dose was much faster (DT_{50} = 4 days) than the double dose (DT_{50} = 16 days). The dissipation of azoxystrobin was significantly faster than the other two chemicals for which the PHI value was found to be 9 days for standard dose and 20.5 days at the double dose. Because the dissipation rate of carbendazim in later stages was

Table 3. Dissipation Data of Three Fungicides in Pomegranate

		azoxystrobin		difenoconazole		carbendazim	
dissipation kinetic model		SD[a]	DD[b]	SD[a]	DD[b]	SD[a]	DD[b]
first + first order	r^2	0.998	0.995	0.999	0.998	0.998	0.999
	$[A]_1$	118.41	177.14	266.40	185.57	1343.53	2682.64
	k_1	0.91	3.54	0.40	0.16	0.63	0.45
	$[A]_2$	144.60	387.85	181.77	411.03	424.67	761.23
	k_2	0.12	0.10	0.02	0.02	0.02	0.02
	$t_{1/2}$[c]	2.00	3.25	4.00	16.00	2.00	2.25
	PHI[c]	9.00	20.50	26.0	60.00	60.00	100.00
first order	r^2	0.974	0.964	0.851	0.971	0.893	0.912
	$[A]_1$	254.74	535.24	400.81	556.08	1700.45	3419.01
	k_1	0.22	0.16	0.11	0.04	0.25	0.23
	$t_{1/2}$[c]	3.00	4.25	5.50	17.25	3.00	3.00
	PHI[c]	7.50	15.50	13.50	47.00	11. 50	15.50
square root first order	r^2	0.928	0.921	0.768	0.921	0.819	0.854
	$[A]_1$	242.07	500.67	381.37	524.28	1683.60	3305.63
	k_1	2.38	2.39	1.55	0.58	8.22	9.67
	$t_{1/2}$[c]	3.50	4.75	6.00	19.75	3.00	3.25
	PHI[c]	7.50	13.00	12.50	45.00	7.50	10.00

[a] SD = standard dose. [b] DD = double dose. [c] $t_{1/2}$ and PHI are in days.

quite slower, the PHI appeared much longer (60 and 100 days at standard and double dose, respectively) despite the faster dissipation at initial days. The PHI for difenoconazole was found to be 26 and 60 days at standard and double dose, respectively. Considering the first + first order equation, partitioning of the residues into two compartments was observed with greater distribution of residues in the compartment where the dissipation rate constant (k_2) was comparatively lower than k_1. No significant changes in k_2 values were observed between the doses of any chemical, although changes in k_1 were noted. When the residue contents at PHI were calculated mathematically on the basis of the parameters of the kinetic equation obtained, it was observed that the residues in the first compartment, i.e. $[A]_1$, dissipated to below detection levels for all three chemicals. The residues distributed in the second compartment, i.e. $[A]_2$ were detected and were equivalent to the corresponding MRL values of the chemicals. The lower dissipation rate (k_2) of the residues in this compartment contributed to the relative increase in PHI as compared to first and square root first-order equations. Moreover, realistic PHIs were obtained from the first + first order equation when the experimental residue dissipation data set was compared to the calculated data set.

Residues in Different Parts of Fruit. From the data obtained by analysis of different parts of fruit, it was observed that the residues of all the three fungicides were mostly accumulated in outer rind of the fruit. Small amount of azoxystrobin was found in the arils initially, which could occur as a result of the translaminar diffusion of this relatively polar chemical through rind (thickness ≈3−4.4 mm). The day-wise data set of azoxystrobin residues in arils shows gradual decline, which could be attributed to the dissipation with passage of time and concurrent dilution of the residues owing to juice filling and growth of the arils. In the case of carbendazim and difenoconazole, the residues were not translocated into the albedo or any other parts of the fruit and the residues were confined to the rind of the fruit only. Thus, carbendazim and difenoconazole had very limited movement or penetration through the cuticle and their residues remained in the outer rind only.

Monitoring the Residues in Farm Samples. Out of the 25 fresh fruit samples analyzed (whole fruits), the residues of carbendazim were detected in eight samples with concentrations below the EU-MRL of 0.1 mg kg^{-1}. Analysis of such carbendazim incurred samples in six replicates resulted in RSDs less than 10%. The residues of the other two fungicides viz, azoxystrobin and difenoconazole were not detectable in any of the samples analyzed. All the aril and juice samples were free from the residues of these three fungicides.

The optimized method was effective in trace level determination of azoxystrobin, carbendazim, and difenoconazole residues from pomegranate with high precision, accuracy, and low measurement uncertainties. Residues mostly accumulated in the rind portion with no penetration of carbendazim and difenoconazole residues into arils. It could be concluded that due to slower dissipation of carbendazim, this chemical needs to be applied with caution ensuring adequate time gap before harvest to avoid detection of its residues. Although azoxystrobin residues translocated into the inner parts of fruit, it degraded fast and the residues could not be detected in the later stages of fruit growth. The PHIs for these fungicides will be useful to the farmers to ensure safe usage to minimize residues at harvest.

■ ASSOCIATED CONTENT

Supporting Information. Additional information on subsampling of the bulk sample; matrix components tentatively identified by Q-ToF mass spectrometry This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +91 26914245. Fax: +91 26914246. E-mail: kbgrape@yahoo.com.

Author Contributions

§These authors contributed equally in this project.

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