

Analytical method validation and comparison of two extraction techniques for screening of azoxystrobin from widely used crops using LC–MS/MS

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Abstract A simple analytical method was developed and validated in chilli, tomato, grape and mango fruits using liquid chromatography tandem mass spectrometry. The method comprised of extraction with ethyl acetate and cyclohexane mixture followed by d-SPE cleanup employing modified quick, easy, cheap, effective, rugged and safe extraction method and quantified in LC–MS/MS using gradient elution. The method was validated in concentration ranging from 0.01 to 0.1 $\mu\text{g g}^{-1}$. The recovery of azoxystrobin in different crops was ranging from 84.36 to 95.64 % at three different concentration levels of analytes with relative standard deviation (HorRat < 20 %) of 4–14 %. The global uncertainty was calculated at limit of quantification level i.e. 0.01 $\mu\text{g g}^{-1}$. In order to evaluate in safety use in India, a field study was conducted with the following extraction method. The calculated half life periods of azoxystrobin were ranging from 3.10–3.46, 3.64–3.46, 1.65–1.96 and 1.32–1.36 days respectively for experimental substrates. The PHI values of azoxystrobin in chilli, tomato, grape and mango fruits were determined as 4.76, 3.90, 4.06 and 10.74 days respectively.

Keywords Dissipation · Azoxystrobin · QuEChERS · LC–MS/MS · Validation · Risk assessment

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Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable fruit in India, it occupy about 8.23 % of our total vegetable production. As chilli (*Capsicum annum* L.) is the most important and highest producing commercial spice crop named as “wonder spice” in India [1]. Grapes (*Vitis vinifera* L.) belong to the world’s largest fruit crops with a global production of around 69 million tons in 2006 [2]. Mango (*Mangifera indica* L.) is the major fruit of India and is known as “King of fruits”. These all crops contain a large medicinal aspect which offers health benefits.

Azoxystrobin [methyl(E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]Phenyl} 3methoxy-acrylate)] is a broad-spectrum fungicide with protectant, erradicant, translaminar, and systemic properties and is used on a wide range of crops. It belongs to the subclass Strobilurins may extract from the fungus *Strobilurus tenacellus*. They have a suppressive effect on other fungi, reducing competition for nutrients. They inhibit electron transfer in mitochondria, disrupting metabolism and preventing growth of the target (ambiguous) fungi. It can provide protection against downy mildew and powdery mildew [3]. The established acceptable daily intake (ADI) is 0.2 mg kg^{-1} body weight per day [4] and maximum residue limits (MRLs) in Indian subtropical climate are 1.0, 1.0, 0.5 and 0.01 mg kg^{-1} for chilli, tomato, grape and mango respectively [5]. So, persistence and degradation of azoxystrobin in/on these widely used crops is essential to understand its behavior in tropical climatic condition in India and to validate an efficient analytical method.

In general, pesticide residue analysis is carried out in a sequence of steps, viz. extraction of target compounds from sample matrix, cleanup and pre-concentration, followed by chromatographic analysis [6–10]. A large number of research

work were carried out with azoxystrobin in different substrate viz. orange (LOD and LOQ was 0.001, 0.004 $\mu\text{g g}^{-1}$ using QuEChERS in LC-MS/MS) [6], tomato (detected LOD and LOQ was 0.001, 0.0029 $\mu\text{g g}^{-1}$) [7], white wine and rose wine (experimental LOD and LOQ was 0.00025, 0.00396 $\mu\text{g mL}^{-1}$ using single-drop microextraction in GC-MS) [8], melon (experimental LOD and LOQ was 0.001, 0.01 $\mu\text{g g}^{-1}$ using QuEChERS in LC-MS/MS) [9], apple (found LOD and LOQ was 0.001, 0.01 $\mu\text{g g}^{-1}$ in GC-ECD & NPD using QuEChERS method of extraction) [10], solid-state Raman spectroscopic method was introduced for the quantitative determination of multiresidual treatments of fungicides viz. trifloxystrobin, tebuconazole, and propiconazole (LOD, LOQ was ranging from 0.003–0.014 to 0.01–0.046 $\mu\text{g g}^{-1}$ respectively for three fungicides) [11]. To understand the fate and degradation behaviour of target fungicide under field condition, Wilkinson et al. [12] gather the informations on azoxystrobin metabolism studied in wheat, grapes, peanuts, rice, and cotton. 2-((6-(2-(3-oxoprop-1-en-2-yl)phenoxy)pyrimidin-4-yl)oxy)benzotrile ($m/z = 343.10$) and methyl 2-(2-((6-(2-cyanophenoxy)pyrimidin-4-yl)oxy)phenyl)acrylate ($m/z = 373.36$) was found as daughter ions for confirmative analysis in mass spectrometer. Boudina et al. [13] studies the photochemical degradation of azoxystrobin under aqueous medium of different pH equipped with high-pressure mercury lamp. Degradation of azoxystrobin involves photoisomerization, photo-hydrolysis of the methyl ester and of the nitrile group, cleavage of the acrylate double bond, photohydrolytic ether cleavage between the aromatic ring giving phenol and oxidative cleavage of the acrylate double bond. Banerjee et al. [14–16] were explained the chemical and photochemical transformations of other strobilurin fungicides viz. trifloxystrobin.

The object of the present study was to optimize and validate an analytical method based on matrix solid phase dispersion (MSPD; modified QuEChERS approach) followed by dispersive solid phase extraction (d-SPE) clean up. After validation of proposed method, four field trials were conducted to determine the degradation kinetics of azoxystrobin on chilli, tomato, grape and mango matrices by LC-MS/MS. The study also estimated the significant advantages of MSPD over liquid-liquid extraction (LLE) method of analysis and focused on the risk assessment of azoxystrobin on human health under the tropical humid climatic condition of West Bengal.

Materials and method

Reagents and chemicals

Azoxystrobin reference standards (purity > 99.9 % m/m) and azoxystrobin formulation (23 % SC) was supplied by

M/S Indofil Industries Ltd., Mumbai. HPLC grade organic solvents as methanol, acetonitrile, ethyl acetate (JT Baker, Phillipsburg, USA), purified water (Millipore, Bedford, MA) were used. Analytical reagent grade formic acid, anhydrous sodium sulfate and sodium chloride, ammonium acetate crystal pure (Merck India Ltd., Mumbai, India), Primary secondary amine (PSA; 40 μm particle size), graphitized carbon black (GCB; United Chemical Technology, Bellefonte, PA), 0.2 μm ultipor N₆₆ nylon 6, 6 membrane filter from Pall Corporation, were used for sample analysis. Activated Na₂SO₄ was prepared by heated in a muffle furnace at 400–450 °C for 5 h before use and kept in desiccator.

Field trial details

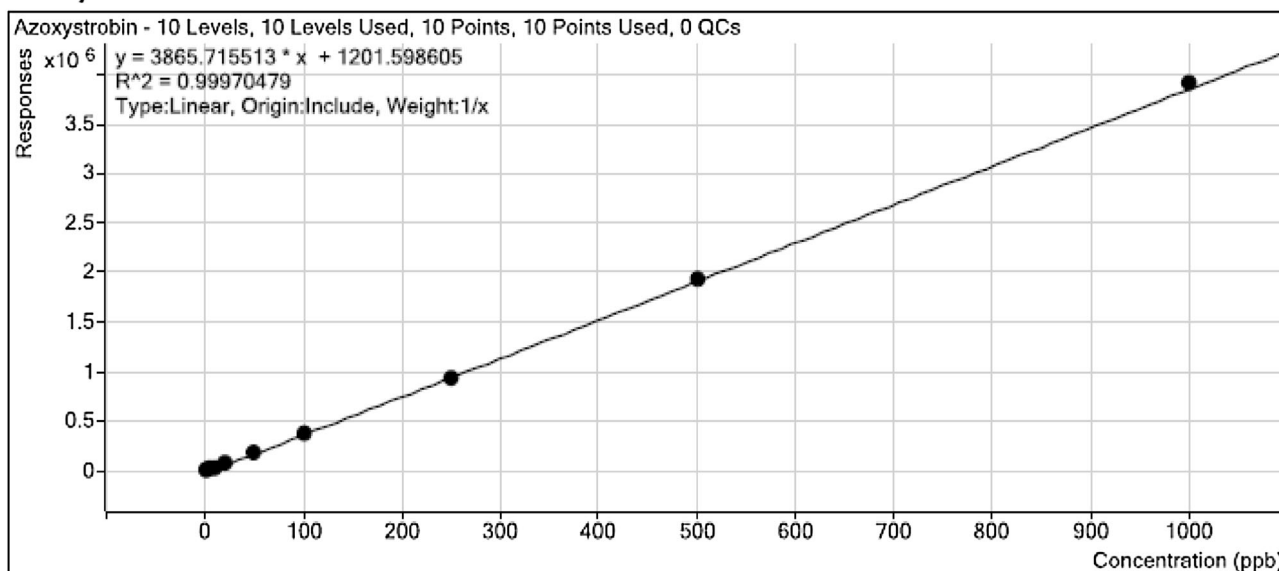
To validate the method and sample analysis for quantification of azoxystrobin, a field experiment was conducted at Experimental Research Farm, B.C.K.V., Mohanpur, Nadia, West Bengal for chilli, tomato, mango and at Malancha Farm, Birbhum, West Bengal for Grape (Variety: Suryamukhi, Patharkuchi, Amrapali and Pusa Navarang respectively). The fungicide, azoxystrobin 23 % SC was applied twice (15 days interval) in the field at the doses of 125 g a.i. h⁻¹ and 250 g a.i. h⁻¹ during Mar.'11 to May'11, Dec.'11 to Apr.'12, Apr.'12 to May'12 and Mar.'12 to May'12 respectively. The average temperature range was 9.70–34.50, 12.38–34.50, 24.20–36.70 and 18.09–38.60 °C with average relative humidity 40.57–94.77, 37.00–95.58, 49.00–90.40 and 29.90–83.65 %. Total rainfall was 9.77, 2.23, 4.20 and 56.40 mm respectively.

Preparation of standard solution

Stock solution of azoxystrobin standard was prepared by accurately weighing 10 ± 0.001 mg of the fungicide in volumetric flasks (certified “A” class) with a digital balance (Sartorius, CP 225D, Germany) and dissolving it in 10 mL methanol. This stock solution was stored in a refrigerator at 4 °C. A working standard of 1.0 mg L⁻¹ was prepared by diluting the intermediate stock standard solution. From freshly prepared working standards, the calibration standards within the range 0.001–1.0 $\mu\text{g mL}^{-1}$ (ten levels) were prepared by serial dilution [17, 18] with CH₃OH:H₂O (9:1, v/v) + 0.1 % formic acid (Fig. 1).

A simple dilution is one in which a unit volume of a liquid material of interest is combined with an appropriate volume of a solvent liquid to achieve the desired concentration. The dilution factor is the total number of unit volumes in which solute material will be dissolved. The dilute material must then be thoroughly mixed to achieve the true dilution.

On the other hand serial dilution is simply a series of simple dilutions which amplifies the dilution factor quickly beginning with a small initial quantity of material. The

Azoxystrobin

* Expected and experimental concentrations are described in ppb (ng g^{-1})

Target Compound	Azoxystrobin					
Data File	Compound	RT	Resp	Final Conc	Exp Conc	Accuracy
Azoxy-Cal-1.d	Azoxystrobin	7.373	5425	1.0924	1.0000	109.24
Azoxy-Cal-2.d	Azoxystrobin	7.379	9249	2.0817	2.0000	104.09
Azoxy-Cal-3.d	Azoxystrobin	7.379	21011	5.1243	5.0000	102.49
Azoxy-Cal-4.d	Azoxystrobin	7.379	37774	9.4606	10.0000	94.61
Azoxy-Cal-5.d	Azoxystrobin	7.373	74607	18.9887	20.0000	94.94
Azoxy-Cal-6.d	Azoxystrobin	7.379	189769	48.7795	50.0000	97.56
Azoxy-Cal-7.d	Azoxystrobin	7.379	385044	99.2941	100.0000	99.29
Azoxy-Cal-8.d	Azoxystrobin	7.379	934241	241.3628	250.0000	96.55
Azoxy-Cal-9.d	Azoxystrobin	7.379	1936048	500.5144	500.0000	100.10
Azoxy-Cal-10.d	Azoxystrobin	7.373	3910605	1011.3014	1000.0000	101.13

Fig. 1 Calibration curve of analytical standard ($0.001\text{--}1 \mu\text{g g}^{-1}$; 8 levels) of azoxystrobin (solvent only)

source of dilution material for each step comes from the diluted of the previous. In a serial dilution the total dilution factor at any point is the product of the individual dilution factor in each step up to it.

$$\text{Dilution} = \frac{\text{sample volume}}{\text{total volume}}$$

$$= \frac{\text{sample volume}}{\text{sample volume} + \text{diluent volume}}$$

For the preparation of calibration curve, standard solutions are prepared by serial dilution method (In calibration curves, correlation coefficient (R^2) values are 0.8973 and 0.9997 for simple dilution and serial dilution respectively).

Fortifications and recovery study

Chilli, tomato, grape and mango samples were collected from the control plots of the trial field. The collected samples were used in fortification experiments and as matrix blanks for

matrix-matched calibration standards. By using matrix solid phase dispersion (MSPD) method of extraction, the recovery study of azoxystrobin was carried out by using the control samples (all four matrices) spiked at desired concentration level, viz. 0.01 , 0.05 and $0.10 \mu\text{g g}^{-1}$ (LOQ, fivefold of LOQ and tenfold of LOQ). As for comparison, conventional LLE method was studied, where the fortified concentration levels are 0.02 , 0.05 and $0.1 \mu\text{g g}^{-1}$. The fortified samples were mixed well and left standing for 2 h to distribute pesticides evenly and given time to interact with the matrix before extraction.

Extraction and cleanup

Extraction procedure by conventional LLE method

Fifty grams of tomato, chilli, grape and mango samples was blended separately using Polytron homogenizer (PT-

MR-3100, Kinemetica AG, Switzerland) → The homogenized sample (5 g) was taken in a 500 mL separatory funnel + 100 mL of 5 % of aqueous NaCl solution was added $\xrightarrow{\text{Shake for 15 min.}}$ Subsequently extracted with 200 mL (100 + 50 + 50) of ethyl acetate → The organic layer was passed through activated Na₂SO₄ + concentrated using rotary vacuum evaporator (EYELA) at 40 °C → The extract was evaporated and reconstituted in 6 mL ethyl acetate.

Extraction procedure by MSPD method

Fifty grams tomato, chilli, grape and mango samples homogenized $\xrightarrow{\text{homogenized}}$ The homogenized sample (5 g) was taken in a 50 mL centrifuge tube 10 mL Ethyl Acetate + cyclohexane (9 + 1; v/v) mixture was added $\xrightarrow{\text{Vortex for 2 min.}}$ Adding 5 g of activated Na₂SO₄ $\xrightarrow{\text{Vortex for 1 min.}}$ Aliquot was centrifuged by refrigerated centrifuge; Model Avanti J-30I (Beckman coulter, USA) with rotor heads JA-30.50 for 5 min at 5000 rpm → 6 mL supernatant extract was taken in 10 mL centrifuge tube.

Dispersive solid phase extraction for clean up

6 mL supernatant extract + 75 mg PSA + 75 mg GCB + 450 mg of activated Na₂SO₄ was added to it $\xrightarrow{\text{Vortex for 2 min.}}$ 2 mL supernatant liquid was collected for 5 min@5000 rpm centrifuged → evaporated to dryness under a gentle stream of N₂ in Turbo Vap LV instrument from Caliper Life Science (Hopkinton, MA, USA).

All the residue was then reconstituted with [MeOH:H₂O (9:1, v/v) +0.1 % Formic acid]. The sample was then filtered through 0.2 μ membrane filter. Now the sample is ready for the final analysis in LC–MS/MS.

LC–MS/MS analysis

The HPLC separation was performed on a Agilent 1200 Series separation module liquid chromatography (Agilent Technologies) equipped with a quaternary solvent delivery system by 10 μL via autosampler on a reversed phase Zorbax Eclipse XDB-C18 (5 μm; 4.6 × 150 mm) column (Agilent Technologies, USA) and a Agilent 6410 triple-quadruple spectrometer equipped with an electrospray source (ESI) was used for detection and quantification. Injection volume was 10 μL, column temperature was 25 ± 2.0 °C and the analysis performed with a flow rate of 0.5 mL/min. The mobile phase was composed of (A) water, 5 mM ammonium acetate and 0.1 % acetic acid and (B) methanol, 5 mM ammonium acetate and 0.1 % acetic

acid. Gradient: 0.0–2.0 min—5.0 % B to 95 % B, 2.0–10.0 min—back to the initial condition of 5 % B, at 15.0 min, it ends with 5 % B.

Estimation of the fungicide was performed in positive mode by a single multireaction monitoring (MRM) with mass transition from parent ion 403.90 to daughter ion 371.90. A second mass transition was used 403.90 > 343.90 for confirmation. The collision energy was 5 V, gas flow was 8 mL min⁻¹ with temperature 300 °C and the fragmenter, nebulizer was 60 V and 20 psi respectively. The ratio of the peak area of these two daughter ions for azoxystrobin was 28.1. The corresponding ratio in the positive samples was determined and confirmed in accordance with European Union guidelines [21].

Preparation of matrix-matched calibration standards

For calibration in LC–MS/MS, ten concentration levels (0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.10, 0.25, 0.50 and 1.00 μg L⁻¹) were prepared. The standard calibration curve is reported in Fig. 1. Chromatograms of qualifier, quantifier ion ratio as well as mass transition of azoxystrobin analytical standard in limit of detection (LOD) level (0.001 μg g⁻¹) are reported in Fig. 2. To evaluate the matrix effect, matrix-matched calibration curve was prepared by using the extracts of blank matrix.

Method validation

The analytical method was validated as per the single laboratory validation (SLV) approach [20, 21]. A brief comparison was done between conventional LLE and MSPD method of extraction. The performance of the method was evaluated considering different validation parameters that include the precision, accuracy-recovery experiment, matrix effect (%ME) and uncertainty measurement.

The calibration curve for azoxystrobin in pure solvent and matrix were obtained by plotting the peak area against the concentration of the corresponding calibration standards at ten calibration levels ranging between 0.001 and 1.0 μg g⁻¹ (Fig. 1).

$$\text{HorRat}(r) = \frac{\text{RSD}(r)}{\text{PRSD}(R)} \quad (1)$$

$$U_C = (U_1^2 + U_2^2 + U_3^2 + U_4^2 + U_5^2 + U_6^2 + U_7^2)^{\frac{1}{2}} \quad (2)$$

In the single-laboratory validation (SLV) studies precision were determined from the reproducibility (R) and repeatability (r). The Horwitz ratio (HorRat) pertaining to within-laboratory precision, which indicates the acceptability of a method with respect to precision [19], was calculated for all of the pesticides by Eq. (1). The

Azoxystrobin

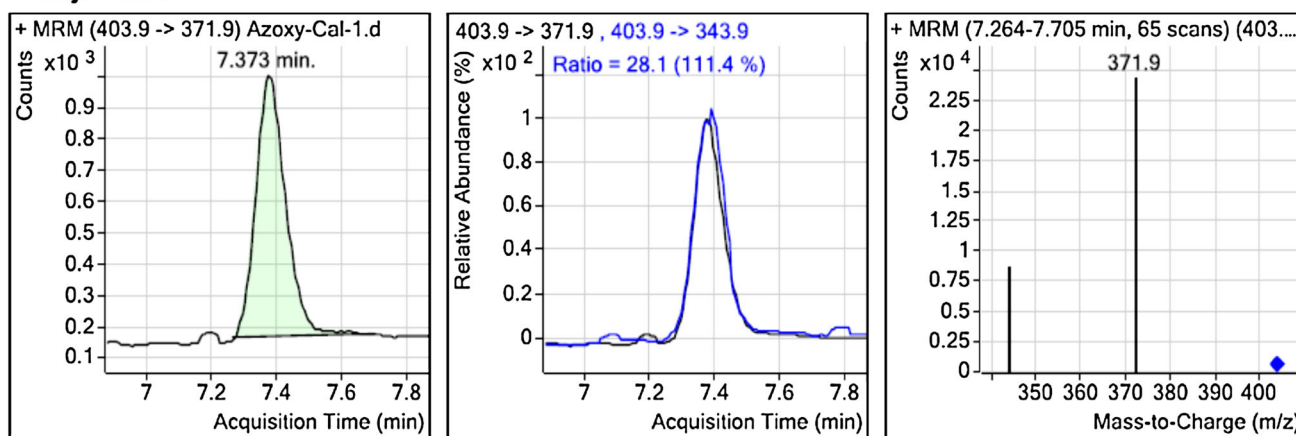


Fig. 2 Chromatograms of azoxystrobin standard (conc. $0.001 \mu\text{g g}^{-1}$) with qualifier, quantifier ion transition and ion ratio

percentage of matrix effect (ME %) was assessed by employing matrix matched standards. Global uncertainty was determined for azoxystrobin at the level of $0.01 \mu\text{g g}^{-1}$ (for MSPD) and $0.02 \mu\text{g g}^{-1}$ (for LLE) as per the statistical procedure of the EURACHEM/CITAC Guide CG 4 [22]. Seven individual sources of uncertainty were taken into account. The combined uncertainty (U_C) was calculated as in Eq. (2). The uncertainty values, %ME and HorRat values for azoxystrobin are reported as relative uncertainties in Table 1.

The limit of detection ($\text{LOD} = 0.001 \mu\text{g g}^{-1}$) was determined by considering a signal-to-noise ratio of 3 with reference to the background noise obtained from blank sample, whereas the limits of quantification ($\text{LOQ} = 0.02 \mu\text{g g}^{-1}$ for LLE and $0.01 \mu\text{g g}^{-1}$ for MSPD) were determined by considering a signal-to-noise ratio of 10 irrespective of the chilli, tomato, grape and mango matrices by using matrix-matched standards.

Results and discussion

Two extraction techniques were MSPD using QuEChERS approach and conventional LLE method of residual fungicide from widely used crops were compared and analyzed by liquid chromatography coupled with triply quadrupole mass spectrometry for conformation.

Conventional liquid-liquid extraction method

With pure ethyl acetate, the recovery was (considering three levels as $0.02, 0.05, 0.1 \mu\text{g g}^{-1}$) 52–81, 85–96, 90–93 and 86–92 % for chilli, tomato, grape and mango respectively in neutral (at $\text{pH} \approx 7.0$) medium with the HorRat values of 35–59 % ($>20\%$) and percentage of relative

standard deviation (%RSD) were in the range of 9–35 % as considered 20 ng g^{-1} level for all matrices with low matrix interferences (Table 1).

Matrix solid phase dispersion (QuEChERS approach) method

For ethyl acetate + cyclohexane (9:1, v/v) solvent mixture in neutral medium (at $\text{pH} \approx 7.0$) the recoveries were in between (considering three levels as $0.01, 0.05, 0.1 \mu\text{g g}^{-1}$) 89–96, 87–95, 84–88, 88–95 % respectively for all matrices with good precision when quantified with matrix-matched standards. Precision in terms of HorRat (SLV) at 10 ng g^{-1} level was 13–19 % ($\leq 20\%$) for azoxystrobin (Table 1) and percentage of RSD in the range of 5–13 %, indicating satisfactory repeatability and ruggedness of the methodology. This method was linear over the range assayed, $0.001\text{--}1.0$ (ten levels) $\mu\text{g g}^{-1}$ in matrix match calibration curve.

From the entire study it clearly revealed that at neutral medium the mixture of ethyl acetate and cyclohexane gave higher recovery percentage (84–96 % for all matrices), low matrix effect (suppression or enhancement), good sensitivity ($\text{LOD} = 0.001 \mu\text{g g}^{-1}$, $\text{LOQ} = 0.01 \mu\text{g g}^{-1}$) with satisfactory uncertainty parameters used in matrix solid phase extraction (MSPD) with d-SPE clean up for regular analysis of azoxystrobin residue in chilli, tomato, grape and mango matrices. Although, pure ethyl acetate produce good (52–96 % for all substrates) recoveries using LLE with d-SPE clean up but LLE method requires long time, large quantity of solvent, less sensitive ($\text{LOQ} = 0.02 \mu\text{g g}^{-1}$) which is not sufficient and useful for regular analysis. From the entire experiment it can be conclude that the very much appropriate solvent was ethyl acetate + cyclohexane mixture for extraction and PSA, GCB and Na_2SO_4 for d-SPE clean up.

Table 1 HorRat, % of matrix effect, Individual, combined, standard and expanded uncertainties for azoxystrobin in different matrices using MSPD method

Method	Substrate	HorRat ^a	ME ^b	Uncertainties									
				U ₁	U ₂	U ₃	U ₄	U ₅	U ₆	U ₇	U _C	U _S	U
MSPD	Chilli	0.167	-4.360	0.000210	0.0264	1.64 e ⁻⁰⁶	0.000577	0.0131	0.0013	0.0132	0.0323	0.31	0.62
	Tomato	0.199	-15.087	0.000128	0.0315	2.45 e ⁻⁰⁶	0.000577	0.0134	0.0013	0.0132	0.0367	0.35	0.69
	Grape	0.133	-15.640	0.000148	0.0211	1.48 e ⁻⁰⁶	0.000577	0.0054	0.0013	0.0132	0.0255	0.22	0.43
	Mango	0.164	5.138	0.000121	0.0260	1.32 e ⁻⁰⁶	0.000577	0.0027	0.0013	0.0132	0.0294	0.28	0.56
LLE	Chilli	0.556	-18.750	0.000105	0.0795	2.90 e ⁻⁰⁶	0.000577	0.0131	0.0013	0.0132	0.0816	0.66	1.33
	Tomato	0.346	-15.087	0.000064	0.0495	1.63 e ⁻⁰⁶	0.000577	0.0134	0.0013	0.0132	0.0529	0.47	0.95
	Grape	0.592	-7.197	0.000074	0.0845	2.70 e ⁻⁰⁶	0.000577	0.0054	0.0013	0.0132	0.0857	0.80	1.59
	Mango	0.484	5.138	0.000060	0.0691	2.22 e ⁻⁰⁶	0.000577	0.0027	0.0013	0.0132	0.0704	0.65	1.30

^a HorRat at 0.01 µg g⁻¹ (for MSPD) 0.02 µg g⁻¹ (for LLE) for all four matrices

^b ME (%) (Matrix Effect) pertains to matrix-induced signal suppression (“-” sign) or enhancement at 0.01 µg g⁻¹ (for MSPD) 0.02 µg g⁻¹ (for LLE). Uncertainty associated with U₁: Calibration curve; U₂: Recovery; U₃: Precision; U₄: Standard; U₅: Weighing; U₆: Volumetric flask; U₇: Pipette; Each contributing uncertainties (U₁-U₇) were reported as relative uncertainty U_C: combined uncertainty; U_S: standard uncertainty was calculated multiplying the combined uncertainty with the result of recovery experiment; U: Expanded or global uncertainty was twice the value of the standard uncertainty. A coverage factor of 2 was decided at a confidence level of 95 % to evaluate the expanded uncertainty at 0.01 µg g⁻¹ (LOQ for MSPD), 0.02 µg g⁻¹ (LOQ for LLE) of fortification. That results account a brief comparison of uncertainty measurement between two different methods of extraction

Analysis of real sample

After validation of the proposed method, we perform a real field trial of azoxystrobin on chilli, tomato, grape and mango as mentioned in field trial details. The T_{1/2} of azoxystrobin was calculated using Hoskins formula [23]. The half-life period (T_{1/2}) was presented in Table 2 and Fig. 3. Quantity of azoxystrobin in all the experimental samples was below detection limit (BDL) at 5–7 days from the date of application. In the present study no residues of azoxystrobin were detected at the time of harvest in chilli, tomato, grapes, mango and respective field soil samples irrespective of any doses, which refers that azoxystrobin does not possess any background contamination property in chilli, tomato, grapes and mango when applied at the recommended dose. There was no residue of azoxystrobin was found in the untreated field sample.

Risk assessment study of azoxystrobin

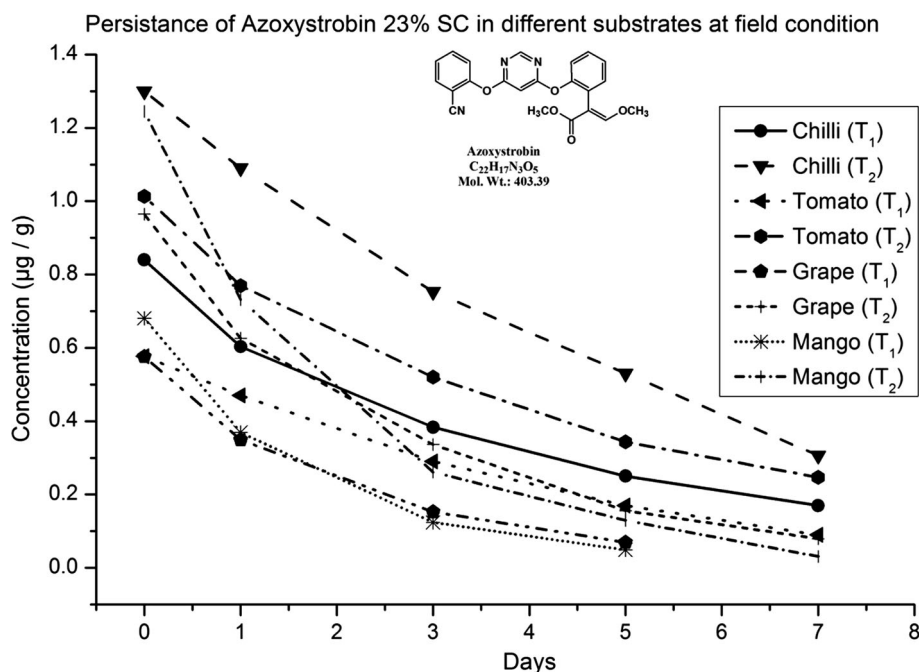
Risk assessment is crucial to the process of making decisions about pesticides, both new and existing. New pesticides must be evaluated before they can enter the market. Existing pesticides must be re-evaluated periodically to ensure that they continue to meet the appropriate safety standard. Generally a pesticide remains in soil, water and plant for some time after its application and before it breaks down. So, proper risk assessment study is required accordingly so that a minimum amount of pesticide is left in environment as well as the food chain. Residues of pesticides may remain in treated products and get into human food chain. These residues should not exceed a limit above which they may pose risks to human health. The concepts of persistence, maximum residue limit (MRLs), acceptable daily intake (ADI) for pesticides has

Table 2 Statistical data on the degradation of azoxystrobin in chilli, tomato, grape and mango from the days versus log (residue × 1000) plot

Substrate	MRL (µg g ⁻¹)	PHI (days)	T ₁ (125 g a.i. h ⁻¹)			T ₂ (250 g a.i. h ⁻¹)		
			T _{1/2} (days)	Trendline equation	R ²	T _{1/2} (days)	Trendline equation	R ²
Chilli	1.0	4.76	3.103	Y = -0.097x + 2.895	0.993	3.460	Y = 0.087x + 3.127	0.990
Tomato	1.0	3.90	2.641	Y = -0.114x + 2.783	0.995	3.460	Y = -0.087x + 2.986	0.995
Grape	0.5	4.06	1.654	Y = -0.182x + 2.742	0.998	1.968	Y = -0.153x + 2.972	0.998
Mango	0.01	10.47	1.320	Y = -0.228x + 2.809	0.997	1.368	Y = -0.220x + 3.106	0.989

Gram active ingredient per hectare (g a.i. h⁻¹) is used to indicate the amount of target chemical present in azoxystrobin 23 % SC formulation, used in field

Fig. 3 Degradation kinetic study of azoxystrobin in field samples



been devised to keep a check on the pesticide residues in food chain and keep them within safe limits.

The persistence of any chemical is generally expressed in terms of half-life ($T_{1/2}$) or DT_{50} i.e. time for disappearance of pesticide to 50 per cent of its initial concentration. $T_{1/2}$ values are often obtained by fitting first-order kinetics to observed degradation patterns as: $C_t = C_0 e^{-kt}$, where C_t is chemical concentration ($\mu\text{g g}^{-1}$) at time t (h), C_0 is initial concentration ($\mu\text{g g}^{-1}$), and k is the first order rate constant (h^{-1}) independent of C_t and C_0 . The calculated $T_{1/2}$ of azoxystrobin was (using Hoskins' formula) [23] varied between 3.10–3.46, 2.64–3.46, 1.32–1.37 and 1.65–1.97 days in chilli, tomato, grape and mango respectively. In the present study no residues of azoxystrobin were detected in harvest chilli, tomato, grapes and mango samples irrespective of any doses, which refers that azoxystrobin does not possess any background contamination property in those matrices when applied at the recommended dose. The initial deposits (2 h after spraying) of azoxystrobin in those trials of each matrices were 0.84 & 1.30, 0.58 & 1.01, 0.58 & 0.96 and 0.68 & 1.24 $\mu\text{g g}^{-1}$ respectively for T_1 & T_2 doses which was found to be less than its Indian and European Union maximum residue limit (1.0, 1.0, 0.5 and 0.01 $\mu\text{g g}^{-1}$ for four matrices) [24]. The residues dissipated gradually to 0.17 & 0.31 (7 days), 0.09 & 0.25 (7 days), 0.069 (5 days) & 0.08 (7 days) and 0.048 (5 days) & 0.03 (7 days) $\mu\text{g g}^{-1}$ and 0.06–0.14 $\mu\text{g g}^{-1}$ (for four matrices respectively) and were below the quantifiable limit of 0.01 $\mu\text{g g}^{-1}$ at 10 days after the application. Based on these observations, it may be concluded that residual azoxystrobin concentrations at the time of harvest will be

below those associated with toxicological risk when applied at the recommended dose. Acceptable daily intake (ADI) for azoxystrobin has been observed to be 0.20 $\mu\text{g g}^{-1}$ body weight per day. Accordingly, an adult of 60 kg can tolerate an intake of 12,000 $\mu\text{g day}^{-1}$ without any appreciable exposure risks. A consumption of 200 g of test substrates (viz. chilli, tomato, grape and mango) by an adult with initial deposit as observed at double dose (1.30, 1.01, 0.96 and 1.24 $\mu\text{g g}^{-1}$ for chilli, tomato, grape and mango respectively) will lead to intake of only 260, 202, 192 and 248 $\mu\text{g day}^{-1}$ which is toxicologically acceptable and quite safe from health point of view of the consumer.

Conclusion

The proposed simple MSPD method of extraction with ethyl acetate + cyclohexane (9 + 1; v/v) mixture, cleanup by d-SPE and quantified by LC-MS/MS using MRM provided high cleanup efficiency and low matrix effects thus enabling adaptation of this sensitive and selective method for routine residue analysis of azoxystrobin on Chilli, tomato, grape and mango matrices with satisfactory recovery (84.36–95.64 %). The method offers low cost of analysis, short time as well as satisfactory uncertainty parameters. From the field data of residue analysis we can calculate the pre-harvest interval (PHI) and half life period of the target molecule, as from the established MRLs and ADI (0.2 $\mu\text{g g}^{-1}$) data of azoxystrobin (Table 1) we can demand that azoxystrobin is toxicologically acceptable and quite safe from health point of view of the consumer. The

proposed method is sensitive enough (i.e. LOD = 0.001 $\mu\text{g g}^{-1}$ and LOQ = 0.01 $\mu\text{g g}^{-1}$ for all matrices) for the analysis of azoxystrobin.

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References

- R.R. Karpate, R. Saxena, Post harvest profile of chilli. (Dept. of Ag. and Cooperation, Ministry of Agriculture, Nagpur, 2009), MRIN-2
- FAO Statistical Database, FAOSTAT, (2007), <http://www.fao.org>. Accessed 29 June 2013
- P.C. Adsule, Good Agricultural Practices for Production of Quality Table Grapes. (N.R.C for Grapes, Pune, 2013), pp. 38–57. www.drs.nio.org/drs/bitstream/2264/4073/1/Mar_Geol_307-310_88a.pdf. Accessed 18 Feb 2013
- FAO database, Azoxystrobin: 229, (FAO/WHO Plant Production and protection 215, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan, 2012), pp. 55–95. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report12/JMPR_2012_Report.pdf. Accessed 18 June 2014
- The gazette of India: Extraordinary (2009) Part II-Sec. 3(i): 8. (Dept. of Health and Public welfare, New Delhi), [www.drugscontrol.org/GSR%20157%20\(E\)%20td%204.3.09.pdf](http://www.drugscontrol.org/GSR%20157%20(E)%20td%204.3.09.pdf). Accessed 30 July 2013
- O. Golge, B. Kabak, J. Food Compos. Anal. **41**, 86–97 (2015). doi:10.1016/j.jfca.2015.02.007
- O. Golge, B. Kabak, Food Chem. **176**, 319–332 (2015). doi:10.1016/j.jfca.2015.02.007
- J.P. dos Anjos, J.B. de Andrade, Microchem. J. **120**, 69–76 (2015). doi:10.1016/j.microc.2015.01.009
- M.D.H. Prodhan, E.-N. Papadakis, E. Papadopoulou-Mourkidou, Int. J Environ. Anal. Chem. 1–11 (2015). doi:10.1080/03067319.2015.1025227
- E. Szpyrka, A. Kurdziel, A. Matyaszek, M. Podbielska, J. Rupa, M. Słowik-Borowiec, Food Control **48**, 137–142 (2015). doi:10.1016/j.foodcont.2014.05.039
- B. Ivanova, M. Spiteller, Analyst **137**(14), 3355–3364 (2012). doi:10.1039/C2AN35174A
- M. Wilkinson, (1994) ICIA5504: Metabolism in winter wheat, Jealott's Hill Research Station, Zeneca Agrochemicals, UK. FAO Report No. RJ1682B. Syngenta File No. ICI5504/0286
- A. Boudina, C. Emmelin, A. Baaliouamer, O. Païssé, J.M. Chovelon, Chemosphere **68**(7), 1280–1288 (2007). doi:10.1016/j.chemosphere.2007.01.051
- K. Banerjee, A.P. Ligon, M. Spiteller, Anal. Bioanal. Chem. **382**(7), 1527–1533 (2005). doi:10.1007/s00216-005-3336-8
- K. Banerjee, A.P. Ligon, M. Spiteller, Agric. Food Chem. **54**(25), 9479–9487 (2006). doi:10.1021/jf0620214
- K. Banerjee, A.P. Ligon, M. Spiteller, Anal. Bioanal. Chem. **388**(8), 1831–1838 (2007). doi:10.1007/s00216-007-1382-0
- G. Anderson, M.E. Lewiston, Preparing solutions and making dilutions. (Mississippi Genome Exploration Laboratory, Mississippi State, MS) <http://www.mgel.msstate.edu/pdf/solutions.pdf>. Accessed 10 July 2014
- A. Ben-David, C.E. Davidson, J. Microbiol. Methods **107**, 214–221 (2014). doi:10.1016/j.mimet.2014.08.023
- W. Horwitz, L.R. Kamps, K.W. Boyer, J. Assoc. Off. Anal. Chem. **63**(6), 1344–1354 (1980)
- Method validation and quality control procedures for pesticide residues analysis in food and feed (2009) Document No. SANCO/10684/2009. 6th EU AQC
- European Union Commission Directorate of General Health and Consumer Protection (2000) Guidance document on residue analytical methods. Document No. SANCO/825/00 rev. 6
- Quantifying uncertainty in analytical measurement, (2000) Document no. EURACHEM/CITAC Guide CG 4, 2nd ed., <http://www.measurementuncertainty.org.html>. Accessed 15 July 2013
- W.M. Hoskins, FAO Plant Prot. Bull. **9**, 163–168 (1961)
- EFSA, Reasoned opinion on the review of the existing maximum residue levels (MRLs) for azoxystrobin according to Article 12 of Regulation (EC) No 396/20051, European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal, **11**(12): 3497–3594 (2013). <http://www.efsa.europa.eu/en/efsajournal/doc/3497.pdf>. Accessed 25 Sep 2014