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Research Article

Analysis of Flubendiamide and Des-Iodo Flubendiamide in Capsicum and Grape by LC-MS/MS

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Abstract

Flubendiamide is an insecticide belonging to the chemical class of pthalic acid diamide. The major metabolite of flubendiamide is des-iodo flubendiamide. An efficient analytical method was validated for analysis flubendiamide and des-iodo flubendiamide by liquid chromatography coupled with mass spectrometry (LC-MS/MS). Extraction and purification of capsicum and grapes samples were carried by the quick easy cheap effective rugged safe (QuEChERS) method. Flubendiamide and des-iodo flubendiamide was analyzed by operating the LC-MS/MS in the multiple reactions monitoring (MRM) mode. The recoveries were in the range of 81.50-106.33% with relative standard deviation (RSD), 1.8-12.0% and measurement uncertainty (MU) within 9.1-19.7%. The limit of detection (LOD) and limit of quantification (LOQ) of the method was 0.0015 µg mL^{-1} and 0.005 mg kg^{-1}, respectively. The method was linear in the range of 0.005-0.1 µg mL^{-1}. The method developed was suitable to analyze flubendiamide below its maximum residue limits in capsicum and grapes.

Keywords: Liquid chromatography-tandem mass spectrometry (LC-MS/MS)**,** Method validation, QuEChERS, Flubendiamide, Des-iodo flubendiamide.

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Introduction

Flubendiamide N2-[1,1-dimethyl-2-(methylsulfonyl) ethyl]- 3-iodo-N1-[2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl] phenyl]-1,2-benzenedicarboxamide) is the first commercial insecticide which belongs to the class of chemicals pthalic acid diamide. The major metabolite of flubendiamide is des-iodo flubendiamide, which is produced when foliar application of flubendiamide is given to plants. Flubendiamide attacks the lepidopteron group of insects by acting on their ryanodine receptors, a unique property that lies on its chemical structure [1]. In contrast to most other commercially successful insecticides which act on the nervous system, flubendiamide disrupts proper muscle function in insects. It has a novel biochemical action as it affects calcium ion balance which causes contraction of insect skeletal muscle. It is environmental friendly and has low toxicity to mammals [1]. Flubendiamide is being co-developed by Nihyaka & Bayer crop science globally and is used for foliar application in many crops of vegetables and fruits.

Capsicum (*Capsicum annum* L.) is one of the most economically important vegetable crops belonging to the family Solanaceae. Capsicum is very rich in vitamins like A, C and E and also has antioxidant properties [2]. Grape (*vitis vinifera*) is an important fruit crop commercially in the world. Grape is consumed as fresh fruits, juice, raisins (dried form) and application in wine production. India is considered as a major producer of grapes cultivated in 40,000 ha area (1.3% of the total crop area) [3]. Flubendiamide could control lepidopteron pests of capsicum and grapes which affect the crops severely. It gave effective control of fruit borer (*Helicoverpa armigera* and *Spodoptera litura*) of capsicum [4, 5] and berry plume moth and sylepta tunalis on grapes [6].

QuEChERS is a commonly used simplified method for the analysis of pesticide residues in vegetables and fruits. It requires low volume of solvent compared to other methods [7]. It is a very flexible method that can be modified depending on the analyte, matrix and analytical instrument. Analysis of flubendiamide and des-iodo flubendiamide has been carried out in several matrices at the limit of quantification of 0.01 mg kg^{-1} [8, 9]. Liquid chromatography coupled with mass spectrometry detection is a rapid and powerful tool to analyze pesticides at trace levels. It also

provides identification of the analytes and confirms presence of analytes in various matrices. The present study was carried out to validate an analytical method (QuEChERS) for analyzing flubendiamide and des-iodo flubendiamide in grapes and capsicum by LC-MS/MS. The analytical method was validated by studying the parameters as per [10] to provide evidence that the method is fit for the purpose.

Materials and methods

Chemicals and reagents

Certified reference materials of flubendiamide (purity 99.5%), and its metabolite des-iodo flubendiamide (purity 99.2%) were procured from Sigma Aldrich Pvt. Ltd. (Bangalore, India). The chemical structure of flubendiamide (a) and des-iodo flubendiamide (b) is given in **Figure 1**. The commercial formulation, Fame 480 SC, was purchased from the local market. Primary secondary amine (PSA), particle size 40 µm, was procured from Agilent technologies (Bangalore, India). Anhydrous magnesium sulphate ($MgSO_4$), sodium sulphate (Na_2SO_4), sodium acetate ($C_2H_3NaO_2$) and sodium chloride (NaCl) were procured from Rankem Avantor Performance Materials India Ltd (Bangalore, India). $MgSO_4$ was activated by heating in a muffle furnace at 600°C for 5 hr and kept in desiccators before use. Na_2SO_4 was activated in an oven at 110^0C for 5 hr and kept in desiccators before use. Acetonitrile (LC-MS/MS-grade), ammonium formate (NH_4HCO_2) and formic acid (CH_2O_2) were procured from Sigma Aldrich Pvt. Ltd. (Bangalore, India).The deionized water for the mobile phase was obtained from Millipore water purification system (ELIX, Merck Millipore, India Pvt Ltd) and filtered using Millipore GV filter paper of pore size 0.22 µm.

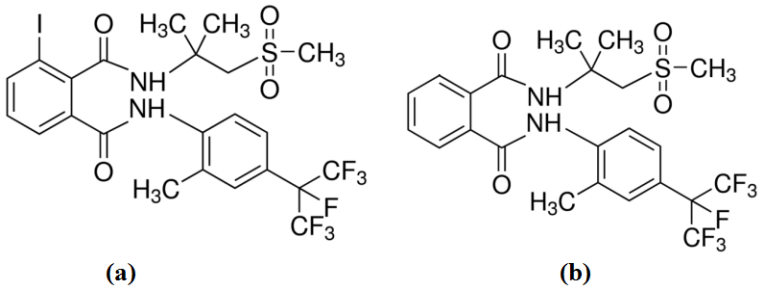

Figure 1 (a) Flubendiamide, (b) Des-iodo flubendiamide.

Preparation of standard solutions

The stock solutions of of flubendiamide and des-iodo flubendiamide pesticide standards (200 µg mL^{-1}) were prepared by dissolving 20±0.1 mg in 100 mL LC-MS/MS-grade acetonitrile. The dilutions were carried out to prepare further working standards. The standards were prepared at the concentrations of 0.0025 - 1.0 µg mL^{-1} for calibration curve. Matrix-matched standards were prepared by adding the insecticides to the blank samples of grapes and capsicum. Blank sample (1 mL) extract was evaporated to dryness by using nitrogen gas in a TurboVap LV Concentration Workstation (Zymark Corporation, Hopkinton, MA, USA) and reconstituted with 1 mL of working standard solution at the appropriate concentrations. The prepared stock and working standards were stored at -20^0C before use.

Sample preparation and clean up

About 2 kg of capsicum and grapes samples was collected from the crops grown at the experimental farm of Indian Institute of Horticultural Research (IIHR), Bangalore, India after the application of target pesticides. The samples were cut into small pieces, homogenized in a waring blender and 15 g representative samples in 3 replicates were taken for analysis of flubendiamide and its metabolite. The capsicum and grapes samples were spiked with flubendiamide and des-iodo flubendiamide at 0.005, 0.01, 0.025, 0.05 and 0.1 mg kg^{-1}. Fifteen grams of spiked samples were weighed in 50 mL Restek polypropylene centrifuge tubes to which 15 mL of 1% acetic acid in LC-MS/MS grade acetonitrile was added. Further, 6 g of anhydrous magnesium sulphate and 1.5 g of sodium acetate were added to the tubes and mixed for 2 min. The tubes were centrifuged at 4100 rpm for 10 min using a Restek Centrifuge (Q-Sep 3000, Bellefonte, PA, USA). An aliquot (3 mL) of the upper acetonitrile extract was placed in 15-mL centrifuge tubes containing 150 mg PSA and 450 mg anhydrous magnesium sulphate. The tubes were mixed for 2

min and centrifuged for 10 min at 4100 rpm. From the supernatant acetonitrile phase, 2 mL was taken, passed through 0.2 µm membrane filters (Phenomenex Pvt. Ltd, India) and analyzed by LC-MS/MS.

Analysis by LC-MS/MS

The quantification and conformation of flubendiamide and its metabolite, des-iodo flubendiamide in capsicum and grapes samples was analysed by LC-MS/MS. An Agilent Infinity 1290 HPLC connected to Agilent 6460 Triple Quad mass spectrometer was used for the analysis. Agilent Zorbax Eclipse Plus C18 column (2 X 100 mm id, 1.8 µm particle size) was used for HPLC separation. The column temperature was maintained constantly at 30^0C. The mobile phases were (A) water with 0.1% ammonium formate (5 mM) and 0.01% formic acid, v v^{-1} and (B) methanol with 0.1% ammonium formate (5 mM) and 0.01% formic acid, v v^{-1}. A flow rate of 0.4 mL min^{-1} was maintained. A gradient programme started with 85% A and 15% B phase (0-1 min). A linear gradient was then established in order to reach a 50% A and 50% B composition at 6 min, 5% A and 95% B at 12 min; return to the initial conditions at 18 min. The samples were transferred to 1.5 mL vials and 2 µL was injected using an auto-sampler. The instrument parameters were optimised and two most abundant MS/MS (precursor–product) ion transitions were monitored; one for quantification and another for confirmation (quantifier and qualifier). For confirmation, the ion ratio (calculated as percent ratio of peak areas of the qualifier and quantifier MRMs) was used [11]. The chromatograms of LC-MS/MS matrix-matched calibration standard and spiked capsicum samples are presented in **Figure 2.** The electrospray ionization (ESI) probe was operated in negative mode by multiple reactions monitoring (MRM) for determination of the insecticides **Figure 3**.

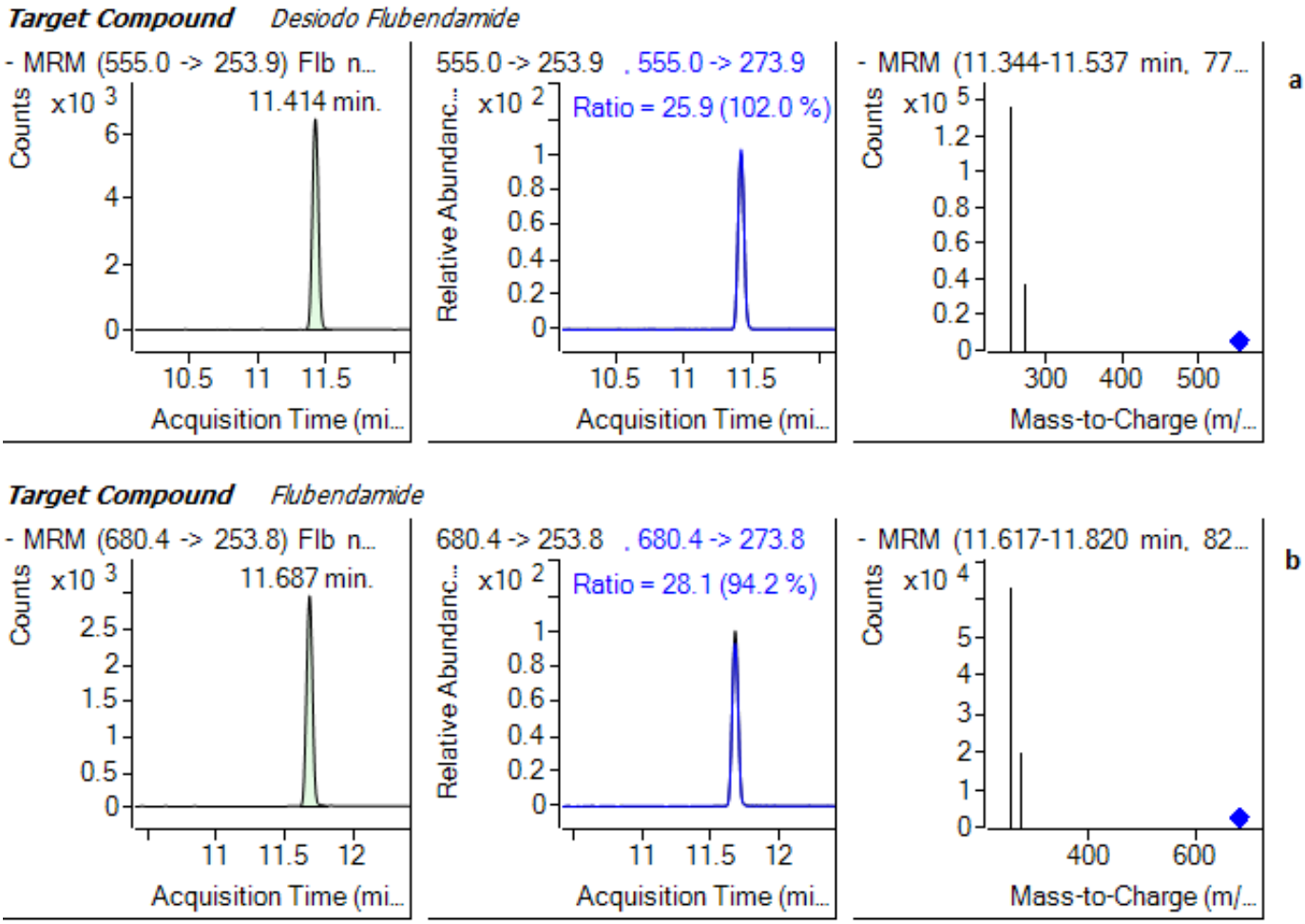

Figure 2 Extracted ion chromatograms of (a) Des-iodo flubendiamide MRM transitions, Flubendaimide MRM transitions (ESI negative ion mode).

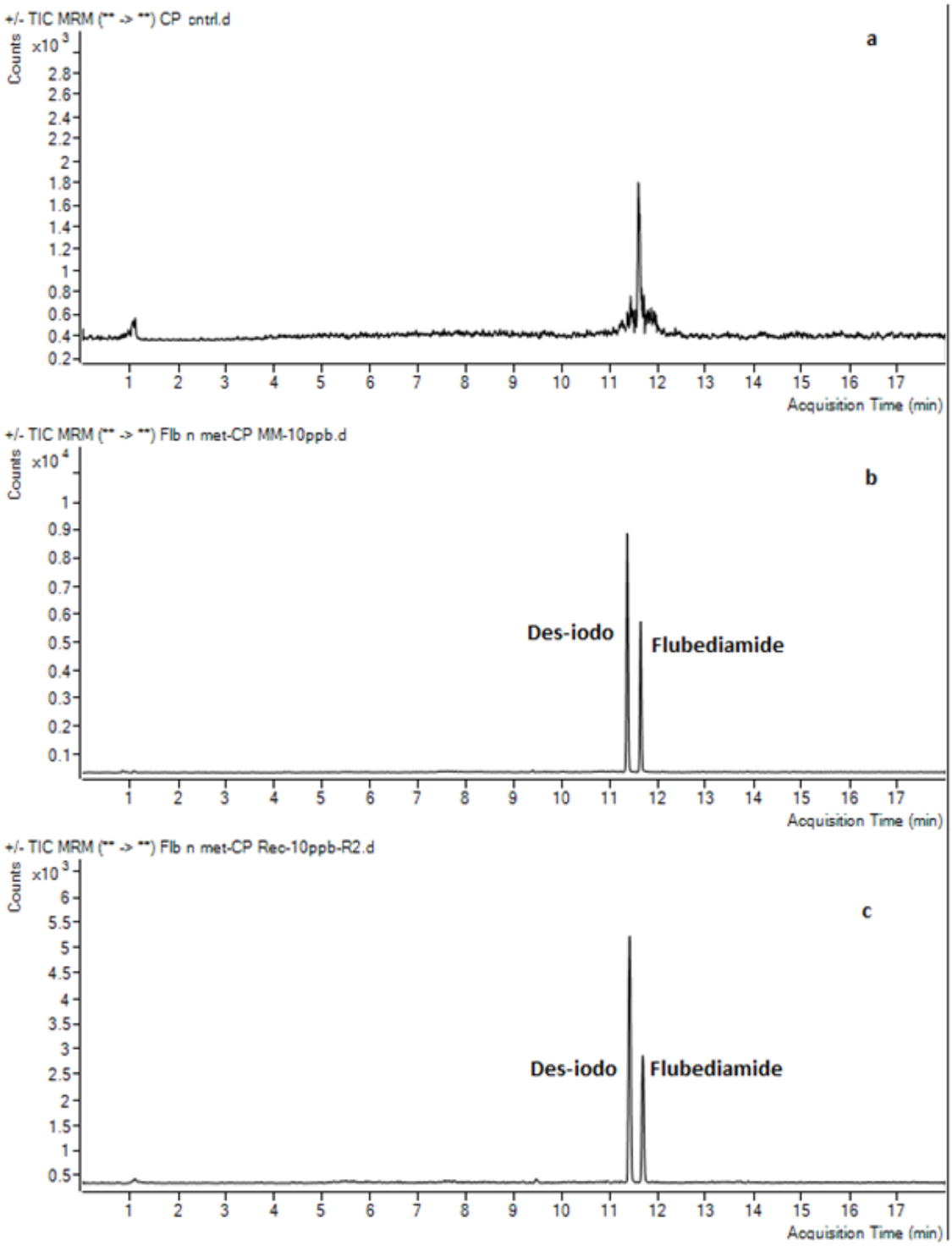

Figure 3 LC-MS/MS extracted ion chromatogram of capsicum (a) matrix-matched standard of flubendiamdie at 0.01 mg kg^{-1}, (b) matrix-matched standard of des-iodo flubendiamide at 0.01 mg kg^{-1} (c) capsicum sample spiked at 0.01 mg kg^{-1}

Method validation

The analytical method was validated by studying various parameters such as recovery, linearity, accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), and measurement uncertainty (MU) [10].

Accuracy, selectivity and precision

Selectivity is the ability of the detector to detect a specific analyte without any interference with other compounds in a complex matrix. The selectivity of the method for flubendiamide was studied by analyzing the blank and spiked samples from the lower levels to higher levels concentrations in all the matrixes. The precision of the method was studied by analyzing the spiked samples of grapes and capsicum by flubendiamide and des-iodo flubendiamide at five different levels (0.005, 0.01, 0.025, 0.05 and 0.1 mg kg^{-1}). Six replicates of each spiked sample were analyzed by LC-MS/MS. The six replicates were analyzed on the same day and again after six days to evaluate the intra (within lab repeatability) and inter-day precision (within lab reproducibility). The precision of the method is expressed as the percent relative standard deviation (% RSD).

Limit of detection and limit of quantification

LOD is where the level at which the target analyte can be detected accurately. It is determined by analyzing known concentration of standards from 0.1-0.005 µg mL^{-1} in LC-MS/MS. The lowest concentration at which flubendiamide and its metabolite is detected at a signal to noise ratio of 3:1 is considered as LOD. LOQ is the lowest concentration at which the compound can be determined without any interference with other compounds in spiked matrix samples at a signal to noise ratio of 10:1.

Linearity

Linearity curve was determined by analyzing pesticides at five different levels in the concentration ranges of 0.005-0.1 µg mL^{-1} (LC-MS/MS). Each level was analyzed six times. Analyte concentrations in solvent and matrix match samples against the peak areas were plotted to obtain the calibration curves.

Measurement uncertainty

The MU of the method was calculated by considering various components of uncertainties to obtain the measured results. It is a parameter which indicates the dispersion of the measured results with that of the true value. The individual uncertainties were measured considering all contributions independent of each other. Major sources of uncertainty are listed as type A and B uncertainties. Type A uncertainty is determined by calculating the standard deviation of six replicates of recovery sample. Type B uncertainty is determined by considering factors such as reference standard purity, reference standard preparation, sample weight, volume of solvent used, calibration of balance, volumetric glasswares used and final volume of the sample. The individual uncertainties were measured. The combined uncertainties were evaluated using the formula given below. The expanded uncertainties were determined using the coverage factor K=2, to give a confidence level of 95 % (EURACHEM/CITAC Guide CG 4, 2012).

Matrix effect

Matrix effect of an analytical method is due to all other components in the matix sample along with target analyte to be quantified [12]. Matrix components which are present are non-ignorable even though the sample is subjected to clean-up step by primary secondary amine (PSA). These components might effect by suppressing or enhancing the specific analyte concentration during chromatographic analysis, the possibility of other impurity components may elute from the sample with the same retention time as that of known analyte which might report false negative results. To avoid such results, the matrix match standards are analyzed along with solvent standards. The matrix effect is calculated and expressed in the form of percentage by using the equation given below. Quantification of flubendiamide and its metabolite were done by calibration curve of matrix matched standards in LC-MS/MS.

Results and discussion

The analytical method used for analysis of flubendiamide and des-iodo flubendiamide in capsicum and grapes gave satisfactory results. Capsicum and grapes samples were spiked with flubendiamide and des-iodo flubendiamide at 0.005, 0.01, 0.025, 0.05, 0.1 mg kg^{-1}. Six replicates were analyzed at all concentration levels. The recoveries of flubendiamide and des-iodo flubendaimide were within the acceptable range of 70-120% **Table 1**. Recoveries of

flubendiamide from capsicum were 84.06-101.45 % and in grapes were 86.33-102.97%. Recoveries of des-iodo flubendiamide from capsicum were 81.50-98.67 and in grapes were 88.17-106.33, respectively. The precision of the analytical method was calculated and expressed as %RSD (n=5) was between (2.5-10.1) in capsicum and (1.8-12.0) in grapes. The % RSD values were withing the acceptable range of ≤20 [10].

Table 1 Recovery of flubendiamide and its metabolite des-iodo flubendiamide from capsicum and grapes at various spiked levels.

Spiked concentration (mg kg^{-1})	Average recovery (%) ± SD*							
	Capsicum				Grapes			
	Flubendiamide	% RSD	Des-iodo flubendiamide	% RSD	Flubendiamide	% RSD	Des-iodo flubendiamide	% RSD
0.005	84.06 ± 7.8	9.2	81.50 ± 8.3	10.1	86.33 ± 10.4	12.0	88.17 ± 8.1	9.2
0.010	87.33 ± 5.7	6.5	82.62 ± 6.4	7.7	87.67 ± 8.7	9.9	91.32 ± 7.4	8.1
0.025	90.54 ± 5.1	5.6	88.5 ± 5.2	5.8	92.74 ± 6.0	6.4	93.82 ± 6.1	6.5
0.050	93.71 ± 3.8	4.0	92.83 ± 4.6	4.9	100.0 ± 5.9	5.9	103.31 ± 5.5	5.3
0.100	101.45 ± 2.6	2.5	98.67 ± 3.5	3.5	102.97 ± 4.2	4.0	106.33 ± 2.0	1.8

*Average of six replicate analysis ± standard deviation

The calibration curve was linear in the range of standard concentrations 0.005 to 0.1 µg mL^{-1}. The correlation coefficient for flubendiamide and des-iodo flubendiamide (r^2) was > 0.99. The LOD of the method was found to be 0.0015 µg mL^{-1}. This was the lowest level at which the peak was detected at signal to noise ratio of 3:1. The LOQ of the method for flubendiamide and des-iodo flubendiamide was found to be 0.005 mg kg^{-1}. At this concentration level the peak was detected at signal to noise ratio of 10:1.The uncertainties of type A and type B were combined to obtain the measured value. It was determined by using coverage factor of K=2, to give a confidence level of 95 %. MU of the analytical method was in the range of 9.3-15.7 in capsicum and 9.1-17.7 in grapes **Table 2**. Higher the spiked concentration levels lower the uncertainty values obtained and lower the spiked concentration levels higher the uncertainty values obtained.

Table 2 Uncertainty of measurement of the analytical method.

Spiked concentration (mg kg^{-1})	Expanded uncertainty (%)			
	Capsicum		Grapes	
	Flubendiamide	Des-iodo Flubendiamide	Flubendiamide	Des-iodo Flubendiamide
0.005	14.8	15.7	19.7	14.7
0.01	12.2	13.3	15.4	13.6
0.025	11.5	11.6	12.1	12.2
0.05	10.3	10.9	11.7	10.2
0.1	9.3	10.0	9.3	9.1

Conclusions

A method for the quantification of flubendiamide and des-iodo flubendiamide using LC-MS/MS was developed and validated. Satisfactory results were obtained and the analytical method was found to be fit for the purpose. The LOD and LOQ of the analytical method for the analysis of flubendiamide and des-iodo flubendiamide were 0.0015 µg mL^{-1} and 0.005 mg kg^{-1}. The calibration curve was linear in the range of 0.005 to 0.1 µg mL^{-1}. The correlation coefficient (r^2) was > 0.99 for flubendiamide and des-iodo flubendiamide. The recoveries obtained were within the acceptable range [10]. The analysis of flubendiamide and des-iodo flubendiamide was performed using high performance liquid chromatography (HPLC) which could be detected at 0.01 µg mL^{-1}[8, 9] but could not be detected at 0.0025µg mL^{-1}. It is required to analyze both the compounds to its maximum residue levels (MRL). The present study has been carried out to validate an analytical method (QuEChERS) for analyzing flubendiamide and des-iodo flubendiamide in grapes and capsicum by LC-MS/MS at below their MRLs.The method developed was suitable to analyze flubendiamide and des-iodo flubendiamide below the Codex MRL of 0.02 mg kg^{-1} in capsicum and 2.0 mg kg^{-1} in grapes.

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