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HPLC METHOD VALIDATION AND UNCERTAINTY DETERMINATION FOR QUANTIFICATION OF KRESOXIM METHYL FUNGICIDE IN CHILI

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Abstract

A simple and efficient multi-residue analytical method was developed and validated for the determination of Kresoxim methyl (KM) fungicide applied on chili crop for controlling of several fungal diseases. The fungicide residues from chili samples were extracted using modified QuEChERS method, followed by analysis through HPLC–UV. Method validation parameters viz. specificity, linearity, matrix effects, LOD, LOQ, recovery, accuracy and precision, robustness and estimation of measurement uncertainty were evaluated. Specificity of the method based on the chromatographic peak purity was observed in the chromatograms of KM, and calibration curve was found to be linear with R^2> 0.99. Matrix effect for KM in chili was < ±20 %. The recovery studies were conducted by spiking the samples at three (LOQ, 5LOQ and 10LOQ) concentration levels. The average recovery of KM at all the three concentrations ranged between 84–86 % with percent RSD < 3 %. Measurement uncertainty (MU) can be helpful while deciding the compliance of chili samples against the established MRL. Hence both type A and type B uncertainties were considered for calculation of combined uncertainty (Uc) in the method validation process. The combined uncertainty (Uc) was below 25 % default value which is normally considered satisfactory for nonfatty matrixes (fruit, vegetables, and grain) by many regulatory authorities for enforcement decisions. The proposed method validation for KM in chili was simple, rapid, and cost effective with high accuracy and sensitivity requiring minimum use of organic solvents.

Key words: kresoxim methyl; method validation; HPLC; measurement uncertainty.

ВАЛІДАЦІЯ МЕТОДУ ВЕРХ ТА ВИЗНАЧЕННЯ НЕВИЗНАЧЕНОСТІ ДЛЯ КІЛЬКІСНОГО АНАЛІЗУ ФУНГІЦИДУ КРЕЗОКСИМ-МЕТИЛ У ПЕРЦІ ЧИЛІ

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Анотація

Був розроблений та валідований простий та ефективний метод аналізу багатозалишкових речовин для визначення фунгіциду крезоксим-метил (КМ), який застосовується на культурах чилі для боротьби з декількома грибковими захворюваннями. Залишки фунгіциду зі зразків чилі були екстраговані за допомогою модифікованого методу QuEChERS, після чого проведений аналіз ВЕРХ-УФ. Були оцінені параметри валідації методу, а саме: специфічність, лінійність, матричні ефекти, LOD, LOQ, відновлення, точність і прецизійність, надійність та оцінка невизначеності вимірювання. Специфічність методу, заснованого на чистоті хроматографічного піку, була виявлена в хроматограмах КМ, а калібрувальна крива виявилася лінійною з R^2> 0.99. Матричний ефект для КМ в перці чилі становив < ±20 %. Дослідження відновлення проводили шляхом додавання зразків у трьох концентраціях (LOQ, 5LOQ і 10LOQ). Середнє відновлення КМ у всіх трьох концентраціях становило 84–86 % з відсотком RSD < 3 %. Невизначеність вимірювання (MU) може бути корисною щодо прийняття рішення про відповідність зразків перцю чилі встановленому MRL. Тому для розрахунку комбінованої невизначеності (Uc) в процесі валідації методу були враховані невизначеності типу А і типу В. Комбінована невизначеність (Uc) була нижчою за 25 % за замовчуванням, що зазвичай вважається задовільним для нежирних матриць (фрукти, овочі та зерно) багатьма регуляторними органами для прийняття рішень щодо застосування. Запропонована валідація методу для КМ у перці чилі була простою, швидкою та економічно ефективною, з високою точністю та чутливістю, й вимагала мінімального використання органічних розчинників.

Ключові слова: крезоксим-метил; валідація методу; ВЕРХ; невизначеність вимірювання.

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Introduction

Kresoxim methyl, [methyl(2E)-2-methoxyimino-2-[2-[(2methyl phenoxy) methyl] phenyl] acetate], (KM) is a broad spectrum Strobilurin fungicide with a protective, curative and eradicative mode of action (Figure.1). It is effective against a wide spectrum of fungi like ascomycetes, basidiomycetes, imperfecti, and oomycetes. The mode of action of strobilurins is inhibition of mitochondrial respiration by binding to the ubihydroquinone oxidation center of the mitochondrial bcl complex (complex), thereby blocking electron transfer [1; 2]. In a short span of time, strobilurins have represented 10 % of the global fungicide market [3] and have led to major changes in disease control programs by being registered for cereals, turf-grass, grapevines, potatoes, fruit, nut, and vegetable crops.

Exposure to pesticides can occur either directly, via inhalation (sprays), ingestion or dermal contact, mostly to persons using them daily, like farmers, etc., while to consumers they affect via pesticide residues found in food and water [4]. The contamination of water, soil, air and the accumulation in crops, e.g., fruit and vegetables, pose a potential risk to human health as it leads to several types of health problems [5]. Hence, the determination of pesticides residues in food matrices has now become essential in view of the toxicity and also due to the risk involved in export potential.

Chili (*Capsicum annum L.*), a vegetable crop of family Solanaceae is important commercially due to its two major attributes, i. e. color and pungency. The "capsaicin" present in its pericarp contains the alkaloid which is responsible for the pungency. However, pathogenic fungi severely affect the chili crop and cause heavy losses [6]. Due to considerable pesticide exposure to humans and consequent toxic effects, highly, sensitive, reliable, and easy-to-use analytical methods are needed for monitoring pesticide residues. The development of a method that enables the concurrent assessment of Kresoxim methyl (KM) fungicide in chili specifically becomes essential from the perspective of toxicology. Method validation includes determination of suitability, linearity, specificity, precision, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ) [7]. Uncertainty determination is also compulsory for any measurement as it ensures the reliability of the measured results [8]. Studies pertaining to method validation of KM in soil using HPLC and in Korean plum using GC have been reported earlier, too [9; 10]. Some other studies pertaining to the analysis of KM in grapes and water by HPLC [11] and evaluation of KM in green chilli by GC- μECD have been reported [12].

The present study was, however, aimed to develop and optimize a simple, quick, robust, and reproducible method that was both cost effective, appropriate and reproducible for quantification and uncertainty determination of KM fungicide in chili by using commonly available equipment like HPLC which is usually found in pesticide residue analysis laboratories.

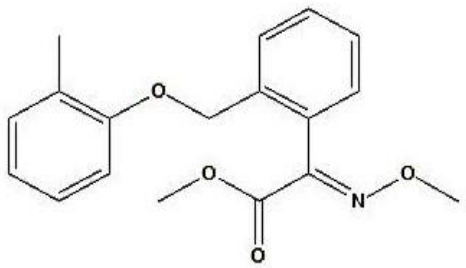

Fig. 1. Structure of Kresoxim methyl

Materials and methods

Chemicals and reagents

Kresoxim methyl was procured from Dr. Ehrenstorfer, GmbH, Germany. Other reagents like HPLC grade acetonitrile, distilled water (HPLC grade) were procured from M/s Merck, India, and analytical grade anhydrous magnesium sulfate and primary secondary amine (PSA) were purchased from M/s Merck / Thermofisher, India.

Preparation of analytical solution and calibration curve

A stock solution of Kresoxim methyl was prepared at a concentration of 100 mg/kg by dissolving in HPLC grade acetonitrile (CH_3CN). This solution was further diluted to 10 mg/kg and thereafter serial dilutions in a concentration range of 0.1 to 5 mg/kg were done using CH_3CN as solvent for the purpose of linearity check. To prepare the calibration curve, 20 μL of each concentration in triplicate were injected into the HPLC system using the complete loop injection technique.

Collection of chili and KM extraction

The chili samples without any previous history of pesticide usage were collected from Vegetable Research Centre (VRC), GB Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand, India for method validation purpose.

Extraction of the fungicide from chili was done using the modified QuEChERS extraction method

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[13]. The QuEChERS method presents numerous advantages over the conventional methods earlier used for solid – phase and liquid – liquid extraction processes in terms of recovery, precision and consumption of solvent etc. [14; 15].

Instrumentation

HPLC analysis was done using Dionex Ultimate 3000 HPLC system having RP-C18 column (25×0.46 cm i.d.) (particle size-5 µm), with injector loop of 20 µl, a dual pump and UV-VIS detector within a temperature range of 22 ± 3 °C. The RP-C18 chromatographic column was of Thermofisher make and the software used for HPLC chromatograms processing was that of Chromeleon. Ultrasonic Bath (make Spectra lab), Centrifuge (make Remi) and the analytical balance (Citizen make) utilized for weighing the chemicals and reagents were all calibrated by the NABL 17034 certified firm.

Chromatographic conditions

The chromatographic parameters optimized for quantification of KM were RP-C18 column (250×4.6 mm i.d. packed with 5 µm particle size silica adsorbed octadecyl silane) and UV detector at 260 nm. The flow rate was 0.5 mL/min, injection volume was 20 µL, and column oven temperature was 25 °C. The mobile phase consisted of CH_3CN and H_2O in a ratio of 80: 20 in an isocratic mode.

Statistical analysis

Statistical analysis was conducted using Excel software to calculate the average, regression equation, standard deviation (SD), and relative standard deviation (RSD).

Results and discussion

Validation experiments

The method validation of KM was done as per Europeon Commission document (SANTE guidelines 2021) [16] with special interest to specificity, linearity, matrix effects, limits of detection (LOD), limits of quantification (LOQ), recovery, accuracy and precision, robustness and estimation of measurement uncertainty.

Specificity / selectivity

The ability of a method to measure a target analyte without interference from other components in a sample is termed as specificity / selectivity. It was observed that after all specific injections KM gave the same response in sample as well as standard with a Rt (retention time) value of 5.92 min. Specificity of the method based on the chromatographic peak purity was observed in the chromatogram, as no interference of any other matrix peak that would impede the analysis of the KM was present in the chromatograms (Figure. 2a & 2b)

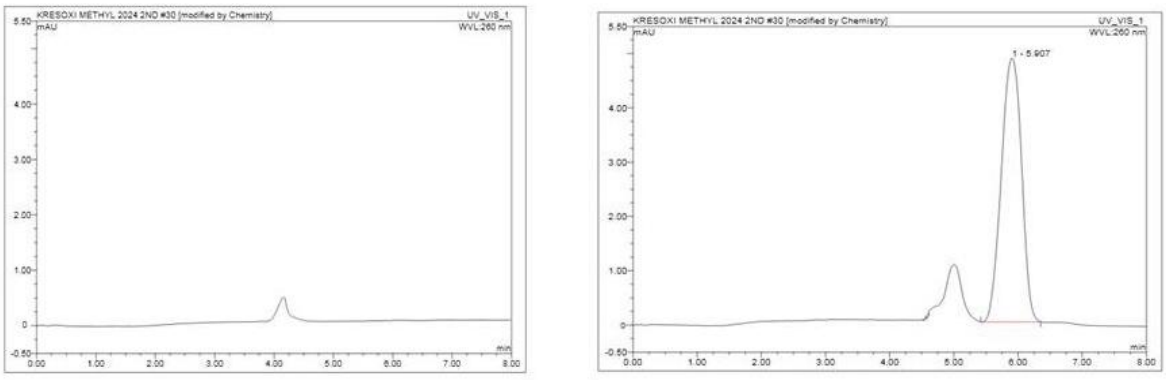

Fig. 2.Chromatogramsof (a) Control chili matrix (b) Kresoxim methyl standard in chili matrix

Linearity and matrix effect

Linearity was assessed using the matrix – matched calibration curve with concentrations ranging from 0.1 to 3.0 mg/kg. The linearity of the method was established by plotting a graph between mean peak area and concentration. The results obtained through regression data analysis and matrix effect determination using the formula given below are presented in Table 1.

$$\%\ \text{Matrix interference} = \frac{\text{Peak area of pesticide in solvent} - \text{Peak area of pesticide in matrix}}{\text{Peak area of pesticide in solvent}} \cdot 100\ \%$$

LOD and LOQ

The linearity graph plotted was used for assessing the LOD and LOQ values with the help of regression equation by using the mathematical equations

$$\text{LOD} = 3.3 \times \sigma/S \text{ and } \text{LOQ} = 10 \times \sigma/S,$$

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where σ = Standard deviation of the intercept and S = Slope of calibration curve

LOD and LOQ were found to be 0.044 and 0.134 mg/kg respectively (Table 1).

Table 1

Regression and statistical parameters for the determination of KM in chili matrix

Parameters	Chili (matrix)
Linearity range	0.1 – 3.0 mg/ kg
Regression equation	y = 0.937x + 0.006
R^2 (Correlation coefficient)	0.999
Slope	0.937
Intercept	0.006
LOD (mg/Kg)	0.044 mg/ kg
LOQ (mg/Kg)	0.134 mg/ kg
SEm	0.0124
Matrix effect (ME) %	2.070
Significance F	3.77944E-10

Recovery, accuracy and precision

Recovery studies were conducted at three different concentrations (LOQ, 5LOQ and 10 LOQ) in five replicates of each. Extraction and clean-up was done as per the modified QuEChERS method described above. The recovery percent was obtained using the given formula.

$$\% \text{ Recovery} = \frac{\text{Recovered concentration}}{\text{Spiked concentration}} \cdot 100\ \% \quad (1)$$

All the recovery values were in the range of 70–120 % with % RSD < 20 (Table 2), and thereby acceptable as per SANTE (2021) guidelines [16]. Accuracy of the developed method was established using the data of recovery studies, SD and % RSD values. The accuracy in terms of % recovery at three different concentrations were within the acceptance limit (70–120 %). The % RSD was also below 20 %. Hence, the developed method meets the requirement of accuracy.

Table

Recovery data of Kresoxim methyl at three concentrations from chili matrix

Parameter	LOQ	5 LOQ	10 LOQ
% Recovery (Mean of five replicates)	84.85	88.22	88.5
SD	1.810	2.117	2.631
% RSD	2.133	2.399	2.974

The precision of a method which refers to the closeness of agreement between a series of measurements of a homogeneous sample is also considered as repeatability (intra-day precision) and intermediate precision (inter-day precision [17]. For precision studies repeatability tests were performed by injecting ten replicate samples of KM in chili at one test concentration and calculating SD and % RSD of both the Rt and concentration.

The values obtained for SD and % RSD are presented in Table 3. The % RSD values were < 20 % which confirmed that the method met the requirement of method precision as per SANTE guidelines 2021.

Table 3

Precision / Repeatability results of KM in chili matrix

Conc. (mg/Kg)	Replicate	Rt (min.)	Peak area(mAU*min)	Obtained conc.
0.5	R1	5.920	0.468	0.493
0.5	R2	5.910	0.481	0.507
0.5	R3	5.920	0.486	0.512
0.5	R4	5.910	0.481	0.507
0.5	R5	5.920	0.476	0.502
0.5	R6	5.930	0.484	0.510
0.5	R7	5.910	0.481	0.507
0.5	R8	5.930	0.478	0.504
0.5	R9	5.910	0.476	0.502
0.5	R10	5.920	0.477	0.503
	Mean	5.918	0.479	0.505
	SD	0.008	0.005	0.005
	% RSD	0.133	1.055	1.068

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Robustness

The robustness / ruggedness of an analytical procedure isa measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [18].

In HPLC method validation for pesticide analysis, robustness is assessed by deliberately changing parameters like absorption wavelength, mobile phase concentration and flow rate etc. Acceptable limits are typically defined by a relative standard deviation (RSD) of less than 2–5 %. In the present study robustness of the test method was demonstrated by change in mobile phase composition and absorption wavelength in detection of KM by HPLC-UV (Table 4). The robustness results of the proposed method when altering the mobile concentration ratios and detector wavelength were within the permissible accepted values in terms of Relative Standard Deviation (RSD). So, the proposed method can be used in quality control laboratories of the pesticide industry at even the commercial scale for the determination of KM in chili samples. In their HPLC method validation studies with nitenpyram insecticide Yar et al., 2023 [19] have also performed the robustness studies by altering the flow rate and the mobile phase concentration ratios and found that they were within the permissible accepted values in terms of Relative Standard Deviation.

Table 4

Robustness data of KM in chili

Replicate (R) no.	Change in mobile phase		Change in absorbance wavelength	
	Peak area at in (mAU) ACN : Water (80:20)	Peak area at in (mAU) ACN : Water (80:20)	Peak area at in (mAU) at 260 nm	Peak area at in (mAU) at 265nm
1	1.875	1.782	1.875	1.632
2	1.806	1.782	1.806	1.639
3	1.851	1.635	1.851	1.747
4	1.890	1.630	1.890	1.671
5	1.866	1.730	1.866	1.642
6	1.877	1.752	1.877	1.636
Mean	1.861	1.772	1.861	1.660
SD	0.03	0.065	0.03	0.046
% RSD	1.602	3.756	1.602	2.748

Measurement Uncertainty (MU) in Pesticide Residue Analysis

Measurement uncertainty (MU)is critical during compliance statements. The standard MU values in the present study were estimated using atop-down approachwhich is based on the trueness and precision data generated in the method validation experiment. The range of MU values indicates where thetrue valueof a measurement is likely to be, reflecting thevariabilityin the measurement process. Repeatability of determination of analytes in spiked samples and uncertainty associated with the preparation of the calibration standards solutions (weighing, diluting) are significant sources of combined uncertainty [20].

Calculation of MU Values

MU values were calculated usingintralaboratory validation data. Combined uncertainty in estimation was determined for KM at 5 LOQ level as per the statistical procedure of the EURACHEM/CITAC Guide CG 4, 2000 [21]. Sources of uncertainty that were taken into account were of two types: (i) type A, representing uncertainty related to repeatability at one concentration, and (ii) type B, related to uncertainty of purity of analytical standards, uncertainty of weighing and measuring, uncertainty of linearity and uncertainty associated with recovery and precision.

The combined uncertainty (Uc) was calculated as

$$Uc = [(U_1^2+U_2^2+U_3^2+U_4^2 \text{---})^{1/2}]$$

and reported as expanded uncertainty (2U) which is twice the value of the combined uncertainty at 95 % confidence level. Sanyal et al. (2011) in their studies based on development, validation, and uncertainty measurement of multi-residue analysis of organochlorine and organophosphorus pesticides have also used similar sources to account for uncertainty measurement. The MU values obtained for KM from different sources are presented in Table 5. The expanded uncertainty calculated as per uncertainty formula was ± 0.031 at 0.505 mg/kg concentration which was around six percent and hence can be considered satisfactory. The Uc of less than 25 % default value has been employed for nonfatty matrixes (fruit, vegetables, and grain) by many regulatory authorities for enforcement decisions [22].

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Table 5

Uncertainty Measurement of Kresoxim-methyl fungicide in chili matrix

Uncertainty Budget								
Source of Uncertainty	**Relative Uncertainty**	**Estimate value**	**Type**	**Distribution**	**F**	**Std. Uncertainty**	**Uncertainty Contribution**	**DOF**
Repeatability	**u1**	0.005	A	Normal	√10	0.001672845	0.00331	9
CRM-Kresoxim-methyl	**u2**	1.14	B	Normal	2	0.00574	0.00574	∞
Analytical Balance	**u3**	0.5	B	Normal	2	0.025	0.025	∞
Digital Balance	**u4**	0.3	B	Normal	2	0.015	0.015	∞
Volumetric Flask (10ml)	**u5**	0.01	B	Normal	2	0.0005	0.0005	∞
Micropipette (100 µl)	**u6**	0.1	B	Normal	2	0.0005	0.0005	∞
Micropipette (1000 µl)	**u7**	0.1	B	Normal	2	0.00005	0.00005	∞
Recovery	**u8**	0.946	B	Rectangular	√3	0.0062	0.0062	∞
Linearity	**u9**	0.001	B	Rectangular	√3	0.0006	0.0006	∞

Conclusion

No matter how impeccable the pedigree of the method is, the laboratory must ensure that the degree of validation of a particular method is adequate for the required purpose, and that the laboratory itself is capable of verifyingany claimed performance criteria. The present study used a small number of inexpensivechemicals that could be used to extract components for method validation. Analysis was done using HPLC equipment which is commonly available in pesticide residue analysis labs, thus making the method economical. All method validation performance characteristics tested in the present study were found to be satisfactory within the recommended limits, indicating the reliability of the method. Realistic uncertainty estimates, which are important for ensuring the reliability of results, were also significantly lower than the recommended limits. Hence, the proposed method is cost effective, brief and reliable to be adopted for commercial testing purposes even by the labs where sophisticated expensive equipments like GC and LC-MS/MS are not readily available. Even more pesticides can be included in the validated method to cover a broader range of pesticides.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that can influence the work reported in this paper.

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