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Research Article

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Development and validation of a matrix solid-phase dispersion method coupled to high-performance liquid chromatography with ultraviolet detection for the determination of acylamino acid fungicide residues in green chilli

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ABSTRACT

A simple, sensitive and inexpensive method was developed using matrix solid-phase dispersion (MSPD), together with high performance liquid chromatographic method for determination of acyclamino acid fungicide residues (Metalaxyl and Benalaxyl) in green chilli. The evaluated parameters included the type and amount of sorbent (silica gel, C18 and neutral alumina) and the nature of eluent (ethyl acetate, dichloromethane and acetonitrile).The best results were obtained using 1.0 g of green chilli sample, 1.0 g of C18 as sorbent and 20ml of ethyl acetate-dichloromethane (1:1, (v/v)).The method was validated using in green chilli samples spiked with fungicides at different concentration levels (0.03 and 0.3 µg/mL). Average recoveries (using each concentration six replicates) ranged 90-98%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.01-2.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.01 µg/mL and 0.03 µg/mL respectively.

Key words: matrix solid-phase dispersion, acyclamino acid fungicides, HPLC-UV

INTRODUCTION

Fungicides are a group of chemicals which are used primarily to control spoilage of crops through fungal attack. Fungicides can be divided into protectant and specific types.Protectants are the older type and includes copper and sulfur based products. They form a protective film on the plant surface and inhibit the germination of fungal spores. Specific type fungicides are so called because they act on one specific chemical reaction in the fungus. Acyclamino acid fungicides are one of the Specific type fungicides. Their invention was inspired by a group of fungicidally active natural products. The outstanding benefits they deliver are currently being utilized in a wide range of crops throughout the world.

Various methods have been described for the determination of these Fungicides, using solid-phase extraction(SPE) [3], solid-phase micro extraction (SPME) [4], supercritical fluid extraction (SFE) and matrix solid-phase dispersion (MSPD) [1], However, none of the published researches to date have reported the simultaneous analysis of chemical classes such as Metalaxyl and Benalaxyl in green chilli.

The matrix solid-phase dispersion (MSPD) technique was developed by Barker in 1989 [2]. It has advantages over conventional techniques because it employs small amounts of sample and solvent, and the extraction procedure consists of only a few experimental steps. MSPD evolved from the solid-phase extraction (SPE) technique, modified

for application to solid and semi-solid matrices. The MSPD procedure is based on the use of a sorbent, which acts as an abrasive in order to produce a modified "opening" of the solid matrix, facilitating the extraction process when using a suitable solvent for eluting the analytes. The use of MSPD for fungicide recovery depends on the solubility of the fungicide in the eluting solvent, as well as the interactions between the matrix components, sorbent and eluent.

Due to the lack of literature reports concerning the use of MSPD as an extraction technique for fungicides belonging to different chemical classes from plants, this paper presents an MSPD method for determination of residue of fungicides in green chilli. So, the present research considered two different chemical classes, namely Metalaxyl and Benalaxyl which analysis by high-performance liquid chromatography with ultraviolet detector (HPLC-UV).

EXPERIMENTAL SECTION

Standards, Reagents and samples

Certificated analytical standards of Metalaxyl (99.5%), and Benalaxyl (99.2%) were obtained from international institute of biotechnology and toxicology (IIBAT). Common names and structures of the acyclamino acid fungicides evaluated here are shown in **Fig. 1**. Acetonitrile was purchased from Rankem, New Delhi, Analytical grade solvents, dichloromethane and ethyl acetate, were supplied from Merck Limited, Mumbai, C18-bonded silica (50 µm) from phenomenex (Torrance, CA, USA), neutral alumina from Merck Limited, Mumbai, AR grade sodium sulphate from Merck Limited, Mumbai and green chilli were purchased from local market. They were brought to the laboratory and stored in plastic bag at refrigerator condition until they were processed in the laboratory.

Standard stock solutions

The fungicide standard stock solutions were individually prepared in acetonitrile at a concentration level 100 µg/mL and stored in a freezer at -18°C. The stock standard solutions were used for up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

Sample preparation

Representative 1.0 g portions of green chilli fortified with 100 µL of working standard solution. The mixture was then gently blended in the mortar for 30 min, to assess the homogeneity of the sample. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

Extraction procedure

1.0 g of green chilli sample was weighed out and homogenized with 1.0 g of C18 –bonded silica for 5 min. The homogenized sample was transferred to an MSPD column consisting of a 20mL capacity polyethylene syringe containing 1.0 g neutral alumina and 1.0 g of anhydrous sodium sulfate. The elution was performed under vacuum with 20 mL of ethyl acetate-dichloromethane (1:1).The eluent was collected into a round bottom flask and evaporated to near dryness. Finally make up with 5mL of acetonitrile and analysed by HPLC-UV system.

Chromatographic separation parameters

The HPLC-UV system used, consisted shimadzu high performance liquid chromatography with LC- 20AT pump and SPD-20A interfaced with LC solution software, equipped with a reversed Phase C18 analytical column of 250 mm x 4.6 mm and particle size 5 µm (PhenomenexLuna-C18) Column temperature was maintained at 40°C. The injected sample volume was 20µL. Mobile Phases A and B was Acetonitrile and 0.1% formic acid (70:30(v/v)). The flow- rate used was kept at 1.0 ml/min. A detector wavelength was 220nm. The external standard method of Calibration was used for this analysis.

Method validation

Method validation ensures analysis credibility. In this study, the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ) were considered. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.03 and 0.3 mg/kg. Linearity was determined by different known concentrations (0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 µg/ml) were prepared by diluting the stock solution. The limit of detection (LOD, µg/mL) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ, µg/mL) was determined as the lowest concentration of a given fungicide giving a response of 10 times the baseline noise.

RESULTS AND DISCUSSION

Specificity

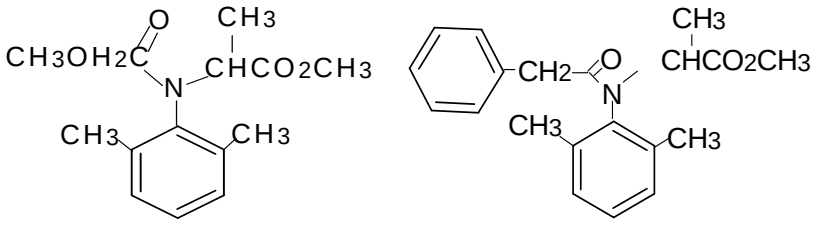
Specificity was confirmed by injecting the green chilli control. There were no matrix peaks in the chromatograms to interfere with the analysis of fungicide residues shown in **Fig. 2.** Furthermore, the retention times of Metalaxyl and Benalaxyl were constant at 4.2±0.2, 8.4±0.2 min.

Linearity

Different known concentrations of fungicides (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 µg/mL) were prepared in acetonitrile by diluting the stock solution. Each solution was prepared in triplicate. Injected the standard solutions and measured the peak area. A calibration curve has been plotted of concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six solutions. The peak areas obtained from different concentrations of fungicides were used to calculate linear regression equations. These were Y= 78768.54X+43.35 and Y=60533+42.86, with correlation coefficients of 0.9999and 0.9999 for Metalaxyl and Benalaxyl respectively. A calibration curve showed in **Fig. 3.**

Accuracy and Precision

Recovery studies were carried out at 0.03 and 0.3 µg/mL fortification levels for Metalaxyl and Benalaxyl in green chilli. The recovery data and relative standard deviation values obtained by this method are summarized in **Table 1.** These numbers were calculated from two (6) replicate analyses of given sample (Metalaxyl and Benalaxyl) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<2 %).



Metalaxyl Benalaxyl

Fig.1. Names and structures of two acyclamino acid fungicides evaluated

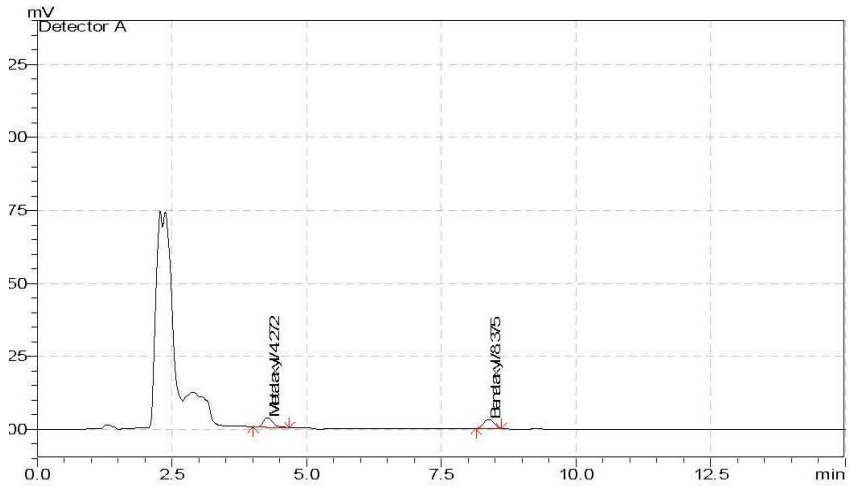


Fig.2. Representative Chromatogram at fortification level of 0.03µg/mL

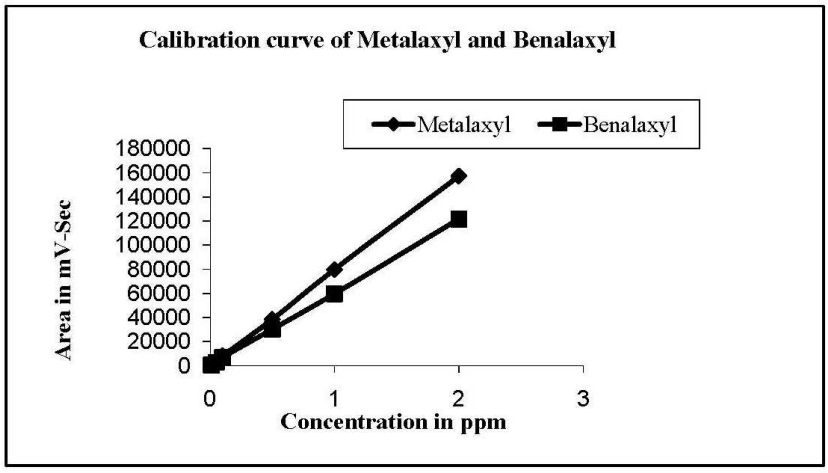


Fig.3. Representative Calibration curve of acyclamino acid fungicides.

Table 1. Recoveries of the acyclamino acid fungicides from fortified Green chilli control sample (n=6)

Fortification Concentration in µg/mL	Replication	Recovery (%)	
		Metalaxyl	Benalaxyl
0.03	R1	88	90
	R2	90	91
	R3	91	89
	R4	89	93
	R5	89	90
	R6	90	90
	Mean	**90**	**91**
	RSD	**1.17**	**1.52**
0.3	R1	95	95
	R2	98	93
	R3	96	96
	R4	95	92
	R5	95	94
	R6	93	92
	Mean	**95**	**94**
	RSD	**1.71**	**1.74**

Table 2. Storage stability Details (n=6)

Fortified concentration in µg/mL	Storage Period in Days	Replication	Recovery in %	
			Metalaxyl	Benalaxyl
0.1	0	R1	92	92
		R2	94	93
		R3	95	95
		R4	92	94
		R5	93	93
		R6	92	94
		Mean	**93**	**94**
		RSD	**1.36**	**1.12**
	30	R1	90	91
		R2	89	90
		R3	90	91
		R4	91	93
		R5	91	92
		R6	92	93
		Mean	**91**	**92**
		RSD	**1.08**	**1.32**

Detection and Quantification Limits

The limit of quantification was determined to be 0.03 µg/mL. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (90-98%, RSD<3%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of detection was determined to be 0.01 µg/mL at a level of approximately three times the back ground of control injection around the retention time of the

peak of interest.

Storage Stability

A storage stability study was conducted at -20±1°C with green chilli samples spiked with 0.1 µg/mL of Metalaxyl and Benalaxyl Samples were stored for a period of 30 days at this temperature. Analysed for the content of Metalaxyl and Benalaxyl before storing and at the end of storage period. The percentage dissipation observed for the above storage period was only less than 3% for Metalaxyl and Benalaxyl showing no significant loss of residues on storage. The results are presented in T**able 2.**

CONCLUSION

This paper describes for the first time a fast, simple sensitive analytical method based on MSPD-HPLC-UV was developed and validated for the simultaneous determination of two acyclamino acid fungicide residues in green chilli. The MSPD extraction procedure of the described method is very simple and requires no sample preparation or pre-treatment, providing adequate clean-up of the matrix. Whole green chilli extracts are very clean, with no interfering peaks at the retention time of the target compounds, indicating good selectivity of the proposed method. The mobile phase Acetonitrile and 0.1% formic acid yields good separation and resolution and the analysis time required for the chromatographic determination of the two acyclamino acid fungicides is very short (around 15 min for a chromatographic run).

Satisfactory validation parameters such as linearity, recovery, precision and very low limits were obtained and according to the SANCO guidelines [7]. For all of the acyclamino acid fungicides the sensitivity of the method was good enough to ensure reliable determination levels lower than the respective MRLs. Therefore, the proposed analytical procedure could satisfactorily be useful for regular monitoring of Strobilurin fungicide residues on a large number of fruit samples.

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