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Independent laboratory method validation for the determination of myclobutanil residues in cucumber

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ABSTRACT

A simple and inexpensive method was developed using solid-phase extraction extraction, together with high performance liquid chromatographic method with UV detection for determination of Myclobutanil residues in cucumber. The evaluated parameters include the extracts by silica gel sorbent using Ethyl acetate and acetonitrile solvents. The method was validated using cucumber samples spiked with Myclobutanil at different fortification levels (0.01 and 0.1 mg/kg). Average recoveries (using each concentration six replicates) ranged 86-94%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.01-5.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.003 mg/kg and 0.01 mg/kg respectively. Finally the cucumber samples were re analyzed by HPLC.

KEYWORDS

Myclobutanil;
HPLC-UV;
Cucumber;
LOD;
LOQ;
Residues.

INTRODUCTION

Myclobutanil is a board spectrum systemic fungicide of the substituted triazole chemical class of compounds[1,2]. The mode of action of myclobutanil is by inhibition of sterol biosynthesis in fungi. It is used heavily to control fungi effecting wine and table grapes, especially in California. It also has a number of other food crop and commercial or residential landscaping applications. Although it has a low acute toxicity, myclobutanil has been found to affect the reproductive abilities of test animals[9]. Myclobutanil is registered for use on a wide range of food and feed crops[7,8]. It may also be used in greenhouses, public rights of way, turf, and in landscaping applications. California accounts for roughly 50% of all myclobutanil use in the US, using 70,000 to 90,000 lbs. annually. Grapes are the most heavily treated crop, using 60% of all myclobutanil in California[10]. Almonds and strawberries are also account for a notable percentage of myclobutanil use in California.

Various methods have been described for the determination of these residues, using solid-phase micro extraction (SPME)[11] Supercritical fluid extraction (SFE) and liquid – liquid extraction[12]. However, none of the published researches to date have reported the residue analysis of Myclobutanil in cucumber.

EXPERIMENTAL

Materials and methods

Reference analytical standards of myclobutanil (purity 99.2%) were obtained from Sigma Aldrich. Ethyl acetate AR Grade, Acetonitrile and Water, HPLC grade, silica gel were obtained from the Merck India limited.

Preparation of standard stock solution

Accurately 5.78 mg of Myclobutanil reference standard, purity (99.2 %) was weighed into 10 mL volumetric flask. The content was dissolved in 10 mL of acetonitrile, sonicated and made up to the mark with the same solvent. The concentration was 573.38 mg/L solution, 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 50.0 gram portions of cucumber fruit fortified with 0.1 mL of working standard stock solution. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

Extraction procedure

The representative homogenised samples (Cucumber fruit) were taken in different 250 mL round bottom flasks. To the flasks 20 mL of HPLCwater was added and soaked for twenty minutes prior to extraction. A 50 mL volume of acetonitrile was added to each bottle and extracted the residues. The extracts were decanted into 250 mL graduated measuring cylinders. The samples were re extracted using 50 mL of acetonitrile. The collected acetonitrile solvent extracts were reduced to 10 mL volume on a rotary evaporator and diluted with 40 mL of water. The extracts were filtered through whatmann filter paper. Residue was eluted from the SPE cartridge into a 50 mL centrifuge tube by addition of 4 mL of 50% hexane: 50% ethyl acetate. A glass pipette was used to remove any water present in the bottom of the centrifuge tubes. The extracts were evaporated to approximately 2mL using rotary evaporator (50°C). The extracts were quantitatively transferred to a derivatization tube and reduced to dryness using rotary evaporator. The extracts were reconstituted in acetonitrile and diluted with a 0.15% ammonium hydroxide solution. The extracts were placed in a rotary evaporator and held at 70°C for 2 hours. After the extracts are cooled, ethyl acetate was added, the samples were horizontal mixed, and a partition was allowed to form. The ethyl acetate layer was transferred to a centrifuge tube and evaporated to dryness at 50°Cusing rotary evaporator. The extract was reconstituted in ethyl acetate.

Clean-up

Glass column of length 2 meter and 4 cm diameter was packed with 10 g of silicagel in 20 mL of ethyl acetate and then covered with 1 cm layer of anhydrous sodium sulfate. Allowed ethyl acetate to drain down to sodium sulfate layer. The residual material in 5 ml of ethyl acetate was poured over the column. The column was prewashed with 20 mL of ethyl acetate. Residue was eluted with 50 mL of acetonitrile. The eluate was concentrated to dryness and taken in 10 mL of acetonitrile.

Instrumentation

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 oven temperature was maintained at 30°C. The injected sample volume was 20µL. Mobile Phases A and B was Acetonitrile and 0.1% ortho phosphoric acid (30:70 (v/v)). The flow- rate used was kept at 1.0 mL/min with a detector wavelength at 230 nm. 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, lin-

earity and Limits of Detection (LOD) and Quantification (LOQ) were considered[3,4]. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.01 and 0.1 mg/kg. Linearity was determined by different known concentrations (0.01, 0.1, 0.5, 1.0 and 5.0, 10.0 µg/mL) which were prepared by diluting the stock solution. The Limit of Detection (LOD, mg/L) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control sample. The Limit of Quantification (LOQ, mg/L) was determined as the lowest concentration of a given fungicide giving a response of 10 times the baseline noise[5,6].

RESULTS AND DISCUSSION

Specificity

Specificity was confirmed by injecting the Mobile phase solvents i.e., Acetonitrile and 0.1% Orthophosphoric acid, HPLC water, sample solution standard solution and control. There were no matrix peaks in the chromatograms to interfere with the analysis of fungicide residues shown in Figure 1. Furthermore, the retention time of Myclobutanil was constant at 6.3 ± 0.2 min.

Linearity

Different known concentrations of fungicides (0.01, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/mL) were prepared into a different 10 mL volumetric flasks by diluting the stock solution. The serial dilution details were presented in TABLE 1. These standard solutions were directly injected into a HPLC. A calibration curve has been plotted for concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six standard concentration solutions. The peak areas obtained from different concentrations of standards were used to calculate linear regression equation. This was Y=24535.96X + 36.28 with correlation coefficient of 0.9998 respectively. A calibration curve is showed in (Figure 2).

Accuracy and precision

The analytical method was validated for the recovery of the test item at two concentration levels with cucumber fruit.

Preparation of 0.01 mg/L fortification level

0.1 mL aliquot of 5.0 mg/L linearity solution was fortified into a 50g of cucumber and extract the sample followed by above extraction procedure This was followed for 6 replications.

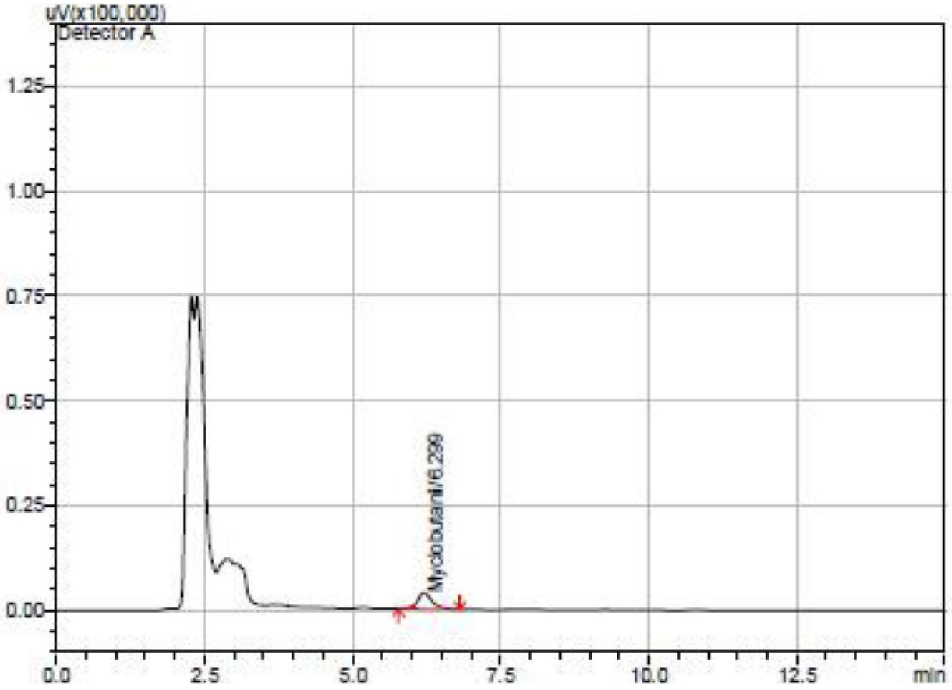

Figure 1 : Representative chromatogram at fortification level of 0.01 mg/kg

TABLE 1 : Serial dilutions for linearity standard solutions

Stock solution concentration (µg/mL)	Volume taken from stock solution (mL)	Final make up volume (mL)	Obtained concentration (µg/mL)
573.38	1.744	10	10
573.38	0.872	10	5
10	2.000	10	2
10	1.000	10	1
10	0.500	10	0.5
10	0.100	10	0.1
1	0.100	10	0.01

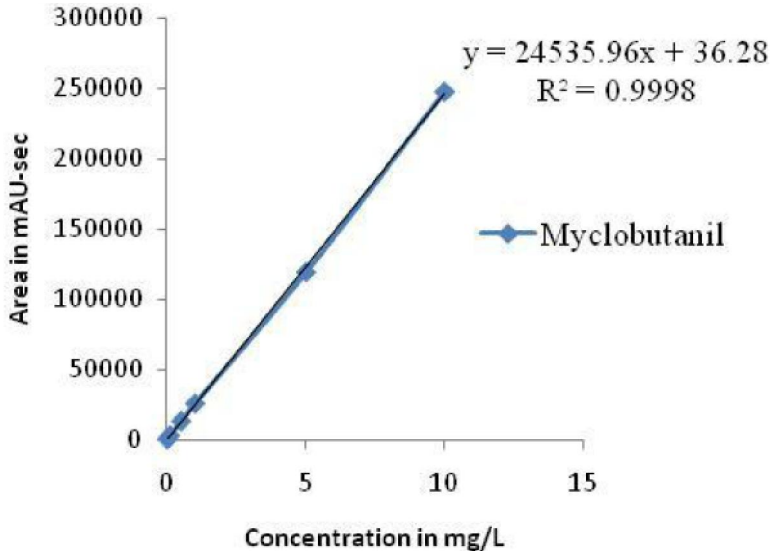

Figure 2 : Representative calibration curve of myclobutanil standard

Preparation of 0.1 mg/L fortification level

1.0 mL aliquot of 5.0 mg/L linearity solution was fortified into a 50g of cucumber and extract the sample followed by above extraction procedure. This was followed for 6 replications.

The samples were assayed for accuracy and repeatability in HPLC. Accuracy was calculated as %recovery and precision as %RSD and the results are mentioned in TABLE 2.

Detection and quantification limits

The limit of quantification was determined to be 0.01 µg/mL. The quantification limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (86-94%, RSD < 2%) were achieved. This quantification 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 0.003 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 refrigerator condition (5 ± 3°C) and Ambient temperature (25 ± 5°C) of 0.1 mg/kg level fortified fruit samples were stored for a period of 30 days at this temperature[13,14]. Analysed for the content of Myclobutanil before storing and at the end of storage period. The percentage dissipation observed for the above storage period was only less than 3 to 4% for Myclobutanil showing no significant loss of residues on storage. The results are presented in TABLE 3 and 4.

Calculations

The concentration of Myclobutanil in the samples analyzed by HPLC was determined directly from the standard curve.

TABLE 2 : Recoveries of the myclobutanil from fruit samples samples (n=6)

Fortification Concentration in mg/kg	Replication	Recovery (%)
		Cucumber
0.01	R1	86
	R2	85
	R3	86
	R4	87
	R5	88
	R6	87
	Mean	86.50
	RSD	1.21
0.1	R1	94
	R2	93
	R3	95
	R4	96
	R5	94
	R6	93
	Mean	94.17
	RSD	1.24

TABLE 3 : Storage stability details at refrigerator condition (5 ± 3°C)

Fortification Concentration in mg/kg	Storage Period in Days	Recovery in %
0.1	0	95
		94
		93
		96
		95
		95
	Average	94.67
	STDEV	1.03
	RSD in %	1.09
	30	90
		91
		91
		92
		90
		92
	Average	91.00
	STDEV	0.89
	RSD in %	0.98

Y = mx + c

Where, Y = peak area of standard (mAU*sec), m = the slope of the line from the calibration curve, x = concentration of injected sample (mg/L), c = 'y' intercept of the calibration curve

The recovered concentration or Dose concentration was calculated by using the formula:

$$\text{Recovered concentration or Dose concentration} = \frac{(x - c)\ X\ D\ X\ 100}{m\ X\ P}$$

Where, m = the slope of the line from the calibration curve, x = sample area of injected sample

TABLE 4 : Storage stability details at ambient temperature (25 ± 2°C)

Fortification Concentration in mg/kg	Storage Period in Days	Recovery in %
		93
		92
		94
		93
	0	94
		93
	Average	93.17
	STDEV	0.75
	RSD in %	0.81
0.1		89
		90
		90
	30	92
		91
		91
	Average	90.50
	STDEV	1.05
	RSD in %	1.16

(mAU*sec), c = 'y' intercept of the calibration curve, D = Dilution Factor, P = Purity of Test item

$$\% \text{ Recovery} = \frac{\text{Recovered Concentration}}{\text{Fortified Concentration}} \times 100$$

CONCLUSION

This paper describes a fast, simple sensitive analytical method based on HPLC-UV to determine the Myclobutanil residues in cucumber samples. The mobile phase Acetonitrile and 0.1% ortho phosphoric acid showed good separation and resolution and the analysis time required for the chromatographic determination of cucumber samples is very short (around 15 min for a chromatographic run).

Satisfactory validation parameters such as linearity, recovery, precision and LOQ values were established by following South African National Civic Organization (SANCO) guidelines[15]. Therefore, the proposed analytical procedure could be useful for regular monitoring, residue labs and research scholars to determine the Myclobutanil residues in different commodities (crop, water and soil samples).

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