RESEARCH ARTICLE

METHOD VALIDATION AND HARVEST TIME RESIDUES OF CLOTHIANIDIN 50 WDG IN SUGARCANE USING UHPLC

B. Vinothkumar*, R. Shanmugapriya and G. Arulkumar

Abstract

Field study was conducted to assess the harvest time residues of clothianidin 50 WDG in sugarcane at Tamil Nadu Agricultural University, Coimbatore during 2016 and 2017. Clothianidin was treated as soil drenching at 200 and 400 g a.i. ha^{-1} at the time of planting. Sampling was done at the time of harvest. The whole plant sampling was done and canes were chopped in to small pieces, homogenized separately in a mixer grinder and used for analysis. The validation of analytical method in UHPLC was performed for specificity, linearity, Limit of Detection (LOD), Limit of quantitation (LOQ), recovery, repeatability and ruggedness along with measurement uncertainty. Method was developed and validated with the good specificity to clothianidin. Linearity was obtained with R^2 value of 0.991 and with acceptable recovery range between 80 to 120 Percent. The LOQ and LOD values for clothianidin were 0.01 and 0.003 µg g^{-1} for sugarcane. Satisfactory repeatability ruggerdness was ensured. In the field experiment the harvest time residues of clothianidin 50 WDG applied at 200 and 400 g a.i. ha^{-1} at the time of planting as soil drenching were at below detectable level (BDL). Hence, the recommended dose of clothianidin 50 WDG (200 g a.i. ha^{-1}) at the time of planting as soil drenching will not leave any residues in the harvested cane.

Key words: Clothianidin, Sugarcane, Residues, UHPLC, Method validation

Introduction

Termites cause colossal losses to structures and agricultural crops in many parts of the world. Family Termitidae is the largest and economically the most important fungus growing termites which also create considerable problems in agriculture and forestry (Grace and Yates, 1999; Wong and Cheok, 2001; Cox, 2004; Peterson et al. 2006). Subterranean termites are the major problem attacking sugarcane crop from its germination through shoot emergence and finally it affects the quality of canes. At germination stage, the losses up to 90 – 100 percent have been recorded (Salihah et al. 1988). As many as 13 species of termite are reported to cause damage to sugarcane in India. Various control measures have been recommended for termites, the most effective being the use of insecticides (Sands 1977, James et al. 1990; Mill, 1992). Chaudhary et al. (1986) concluded that application of insecticides to cane setts at the time of planting was useful in controlling pest, although the duration of effectiveness varied among chemicals.

Clothianidin in small amounts exhibits excellent control of notorious insect pests belong to Isoptera, Hemiptera, Coleoptera, Thysanoptera, Diptera, Lepidoptera and Orthoptera in various crops. It has wide insecticidal spectrum, potent activity at low dosage, long term control effect, excellent systemic action, wide variety of application methods and high crop safety. Clothianidin binds in high affinity to the insect nicotinic receptors (Tomizawa and Casida, 2005). Interestingly, clothianidin shows an enhanced agonist efficacy relative to that of imidacloprid at the cholinergic neurons cultured from the central nervous system of third-instar *Drosophila* larvae (Brown et al., 2006). Clothianidin, (E)-1-(2-chloro-1, 3-thiazol-

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5-yl-methyl)-3- methyl- 2-nitroguanidine, is a new neonicotinoid insecticide. It had been discovered by the Agro Division of the Sumitomo Chemical Co., Ltd. (formerly Takeda Chemical Industries, Ltd.), and was co-developed with Bayer Crop Science (Uneme, 2011). This pesticide is highly effective in controlling hemipterous insects as well as coleopterous, thysanopterous, and certain lepidopterous pests (Chen, et al. 2005). The physical, chemical, biological, and toxicological property of clothianidin has been reported by Uneme (2011). Whereas reports on persistence, dissipation and harvest time residues on vegetables and agricultural crops are limited. Clothianidin and its four metabolites were analyzed in rice using high performance liquid chromatography (HPLC) with diodearray detection (DAD) (Chen et al. 2005). Drozdzyñski et al. (2008) has analyzed clothianidin residues in inflorescences of common horse chestnut using HPLC with tandem mass spectrometry (MS/MS). Dissipation of clothianidin in *Brassica chinensis* has been determined by Hou *et al.,* (2010) and Xie *et al.,* (2009) using HPLC and LC-MSMS, respectively. With this background, the studies were carried out to assess the harvest time residues of clothianidin in sugarcane.

Materials and Methods

Chemicals and reagents

The reference standard of clothianidin (99.9 % purity) was purchased from M/S Sigma Aldrich, Bangalore, India. Primary stock solutions of clothianidin (400 µg ml^{-1}) standard were prepared with C_6H_{14} (v/v) in a volumetric flask. An intermediate stock solutions of 100 µg ml^{-1} and 10 µg ml^{-1} were prepared from primary stock solution and working standards was prepared from intermediate stocks. The stock solutions were stored in the deep freezer at -20 °C and working standards were stored at 4ºC until further use. Acetonitrile (CH_3CN) of HPLC grade, sodium chloride (NaCl) and anhydrous magnesium sulphate ($MgSO_4$) of analytical grade were purchased from Merck India Ltd., Mumbai, India. NaCl and $MgSO_4$ were heated at 650 ºC for 4 h for activation and kept in desiccator until use. Primary Secondary Amine (PSA) (Bondesil 40 µm) and Graphitized Carbon Black (GCB) were purchased from M/s. Agilent technologies, USA. Type 1 water (or HPLC grade water) was harvested from Millipore water purification system.

Instrument parameters

The residues of clothianidin were estimated using Ultra High Performance Liquid Chromatography (UHPLC) (Shimadzu, Prominence i series 2030) equipped with auto sampler and Photo Diode Array (PDA) Detector (SPD-M20A). Chromatographic separation was achieved with reverse phase (C18 - Agilent) column of 250 mm length, 4.6 mm id, 5µ particle size in a column oven, at 40°C. The isocratic flow rate of 0.8 mL min^{-1}, with a mobile phase of CH_3CN and H_2O (70:30) was employed for separation. An injection volume of 20 µL was used and the total run time was 10 min. Residues of clothianidin were quantified by the comparison of peak height / peak area of standards with that of unknown or spiked samples run under identical conditions of operation.

Method validation and measurement uncertainty

The validation of analytical method was performed for parameters *viz.,* specificity, linearity, Limit of Detection (LOD), Limit of quantitation (LOQ), recovery, repeatability and ruggedness along with measurement uncertainty (SANTE, 2017). Sugarcane matrix matched standard was used for analysis. Specificity of the clothianidin was assessed by injecting standard solution six times at one concentration (0.05 µg mL^{-1}). The linearity study was performed by injecting five different

concentrations of standard of clothianidin between 0.005 to 0.40 µg mL^{-1} with three replications. Linear regression model was followed to compute LOD and LOQ. For linear calibration curve, the instrument response *y* is linearly related to the standard concentration *x* for a concentration between 0.005 to 0.4 µg mL^{-1} with five replications and expressed in the model $y = a+bx$. Hence, LOD and LOQ is calculated as $LOD = 3S_a/b$; $LOQ = 10S_a/b$. where, S_a is the standard deviation of the response and *b* is the slope of the calibration curve. Recovery studies were conducted to assess the validity of the present method. The homogenized untreated Sugarcane samples (10g) were spiked at five different concentrations *viz.,* 0.01, 0.05, 0.10, 0.20 and 0.40 µg g^{-1} of standard solution and replicated thrice along with untreated control. The control samples were analyzed and the result indicated that blank sample did not contribute any interference with the target compound. The percentage recovery was calculated by comparing the peak area of the spiked standards. Repeatability, reproducibility and ruggedness were estimated by spiking the sample at 0.01 µg g^{-1} level and replicated six times. Measurement uncertainty was calculated based on the procedure given by Ellison and Williams (2012) and Magnusson and Örnemark (2014).

Field trial and sampling

Field trial was conducted to study the harvest time residues of clothianidin on sugarcane variety Co 86032 at Semmedu village, Coimbatore District, Tamil Nadu, India, during January, 2016 - February, 2017. The crop was maintained well by adapting standard agronomic practices as per the recommendations of Tamil Nadu Agricultural University, Coimbatore. The experiment was laid out in randomized block design in a plot size of 100 m^2 and replicated thrice, including untreated control. The sugarcane plots were sprayed with clothianidin 50 WDG @ 200 and 400 g a.i. ha^{-1} at the time of planting. The target dose rate was mixed in required quantity of water and sprayed (using pneumatic knapsack sprayer by removing nozzle) over the planted setts in the furrows for the insecticide to spread thoroughly around the planting zone. The field chosen for conducting the experiment had no previous history of clothianidin application. Samples were collected at the time of harvest. Whole plant with leaf and stalk was uprooted during the course of sampling. Sampling of whole plant facilitated to determine the quantum of residues translocated from setts to stalk and leaf. The whole plants were brought to the laboratory, canes were chopped in to small pieces, homogenized separately in a mixer grinder and used for analysis. All the samples were stored in -20ºC until further use.

Extraction and cleanup

The residues were extracted by following the modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method (Anastassiades et al. 2003). A representative sample (Cane) of 10 g of the homogenized sample was transferred into a 50 mL centrifuge tube and mixed using a vortexer for one minute after adding 20 mL of CH_3CN. Four gram of anhydrous $MgSO_4$ and 1 g of NaCl were subsequently added, shaken well by vortexer and then centrifuged at 6000 rpm for 10 minutes. Nine milli liter of supernatant was transferred to test tube containing anhydrous sodium sulphate (Na_2SO_4) and 6 mL of supernatant aliquot was transferred into a 15 mL centrifuge tube containing 100 mg PSA, 600 mg anhydrous $MgSO_4$ and 10 mg GCB. The mixture was vortexed for 1 minute and then centrifuged for 10 minutes at 3000 rpm. The upper extract (4 mL) was transferred into a turbovap tube and concentrated to dryness under a gentle stream of nitrogen in a turbovap LV at 40° C. HPLC grade CH_3CN (1mL) was added to test tube, shaken well reconstituted 1 mL was transferred into a 1.5 mL glass auto sampler vial for analysis.

Determination of residues

The amount of residue was determined by comparing the sample response with the response of standard by using the formula,

$$\text{Residues in ppm} = \frac{A_S}{A_{Std}} \; x \; \frac{W_{Std}}{W_S} \; x \; \frac{IV_{Std}}{IV_S} \; x \; FV_S$$

Where, A_s - Area of the sample, A_{std} – Area of the standard, W_{std} - Weight of the standard injected in mg/kg, W_s - Weight of the sample in g, IV_{Std} - Injected volume of standard (µL), IV_s - Injected volume of sample (µL), FV_s - Final volume of sample (mL).

Result and Discussion

Method validation makes use of a set of tests that both test any assumptions on which the analytical method is based and establish and document the performance characteristics of a method, thereby demonstrating whether the method is fit for a particular analytical purpose (Thompson et al. 2002). Method validation was performed on parameters of specificity, linearity, LOD, LOQ, recovery, repeatability and ruggedness. Based on the results of the several preliminary studies, current method was developed. Best chromatographic separations were obtained following binary gradient of CH_3CN and H_2O in an isocratic flow of 70: 30 ratios. Clothianidin was eluted in 5.93 min at 270 nm. The specificity of clothianidin was assessed by injecting six times at 0.01 µg mL^{-1} level and calculated percent Relative Standard Deviation (RSD) based on area and retention time (RT) were 0.923 and 0.122 %. The RSD of the clothianidin standard in the instrument is comfortably below the acceptance limit of 5.0 and 2.0 percent RSD, respectively for area and RT (SANTE, 2017). A very good linearity was observed from 0.005 to 0.2 µg g^{-1} with correlation coefficient (R^2) of 0.991 for the target analyte clothianidin (Fig. 1 and Fig. 2).

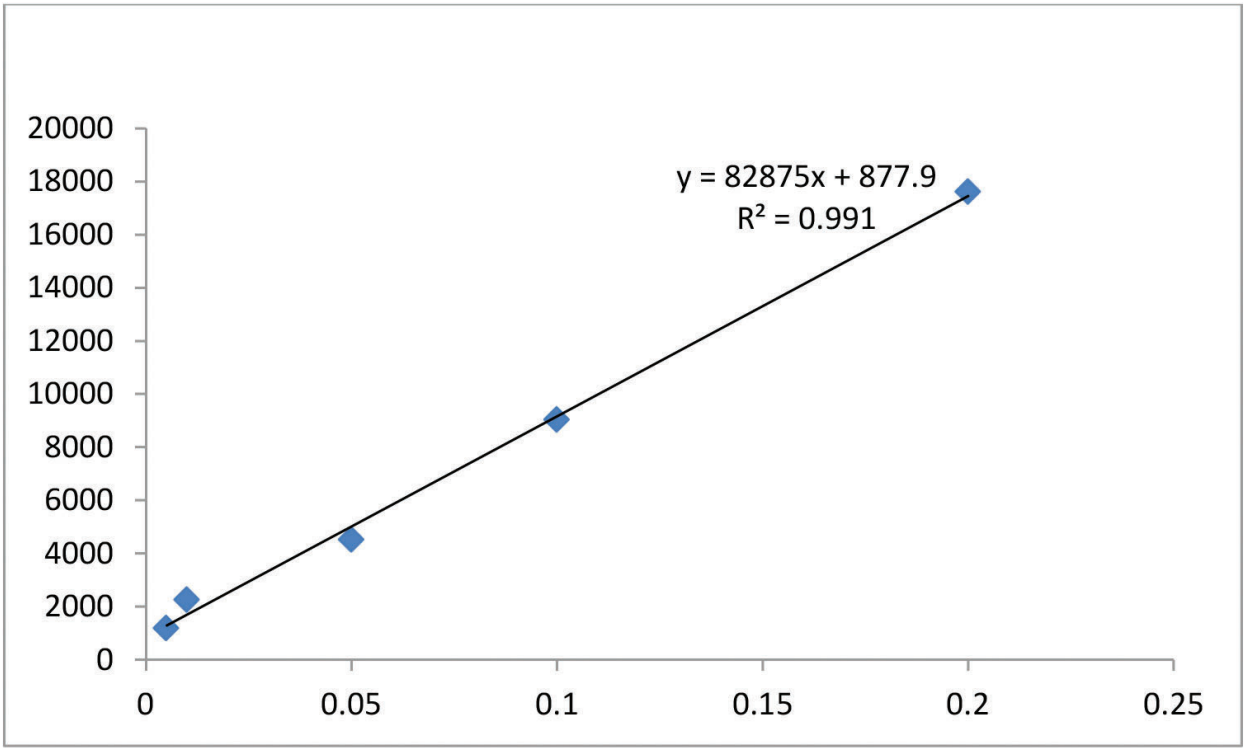

Fig. 1. Standard calibration curve of clothianidin

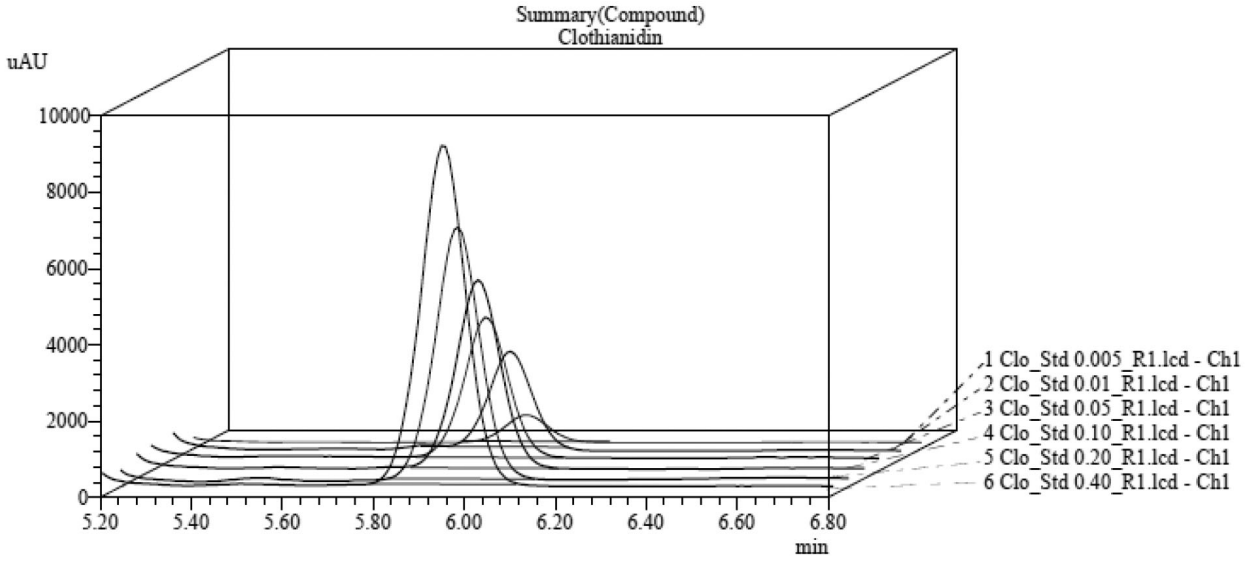

Fig. 2. Standard linearity chromatograms of clothianidin

The LOQ and LOD values for clothianidin were 0.01 and 0.003 µg g^{-1} for sugarcane. The mean per cent recoveries of clothianidin in sugarcane were 88.23, 86.44, 91.64, 87.80 and 94.15 with RSD percentage of 3.12, 4.53, 3.05, 2.70 and 2.91 when samples spiked at 0.01, 0.05, 0.10, 0.20 and 0.40 µg g^{-1}, respectively (Table 1; Fig. 3). Since, the recovery range comfortably recline between 60 to 140 per cent, the suitability of the method for residue analysis of clothianidin in sugarcane thus confirmed (SANTE 2017). RSD of the six replicate spiking, extraction and injections at LOQ level is 2.47 % indicating satisfactory repeatability. Ruggerdness was ensured by obtaining the RSD of 3.92 % in three replicates of recovery study at LOQ level each by changing the instrument column. The measurement uncertainty calculated in the present method is 11.64 percent for clothianidin at 0.01 µg g^{-1} in sugarcane. The harvest time residues of clothianidin 50 WDG at 200 and 400 g a.i. ha^{-1} applied at the time of planting as soil drenching were at below detectable level (BDL) in sugarcane. This may be due to the time gap between the application of insecticide and harvesting of the cane is more than 370 days by that time clothianidin is degraded by various environmental factors.

Present finding is in accordance with the results of Ramasubramanian (2013), who reported that, the recoveries of clothianidin in sugarcane soil

Table 1. Recovery percentage of clothianidin in sugarcane

Spiked level (µg g^{-1})	Percent recovery (%)			Mean ± SD	RSD
	R1	R2	R3		
0.01	90.79	85.32	88.59	88.23 ± 2.75	3.12
0.05	85.26	83.26	90.81	86.44 ± 3.91	4.53
0.10	89.08	94.62	91.23	91.64 ± 2.79	3.05
0.20	85.69	90.37	87.34	87.80 ± 2.37	2.70
0.40	94.68	91.19	96.59	94.15 ± 2.74	2.91

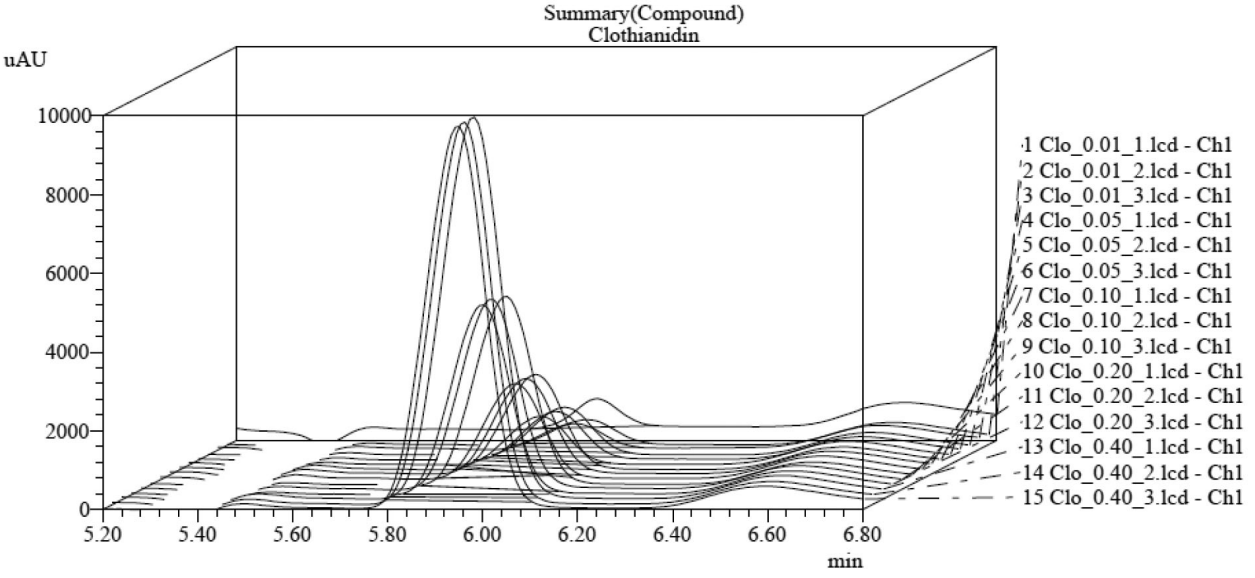

Fig. 3. Recovery chromatograms of clothianidin

were in the range of 93.19 to 95.43 percent at 0.01 – 0.1 µg/g fortification level in soil. The limit of quantification of the method was 0.01 µg/g in HPLC. Pratheeshkumar and Chandran (2016) reported that, the average recoveries obtained were 91.04 to 94.44 and 89.22 to 91.41 % for fresh and cured cardamom, respectively when fortified with 0.01, 0.05 and 0.1µg g^{-1} level of clothianidin. The LOD and LOQ in both fresh and cured cardamom were 0.005 and 0.01µg g^{-1}, respectively in UPLC-MS/MS. Recoveries between 92 and 102 percent, with relative standard deviations from 3 to 5 percent were obtained tomato samples fortified with clothianidin at three levels (Li et al. 2012) .

Half-life of clothianidin was 17.2 and 17.4 days at the single (50 ga.i./ha) and double doses (100 ga.i./ha), respectively in sugarcane soil (Ramasubramanian, 2013). The half-life of clothianidin in fresh and cured cardamom was 3.40 and 3.11 days at the lower dose and 3.42 and 3.45 days at the higher dose, respectively. The waiting periods of clothianidin on fresh and cured cardamom at the lower and higher doses were 18.41 and 22.09 days, and 21.16 and 27.54 days, respectively (Pratheeshkumar and Chandran, 2016). Concurrent recoveries were between 85.6 and 92.5%, with relative standard deviations ranging from 1.3 to 6.8 % at three fortification levels between 0.01 and 5.0 mg/kg. The half-lives in straw, paddy water and paddy sediment were found to be 1.9 - 4.9, 4.1 – 5.0 and 4.9 – 6.3 days, respectively (Zhang *et al.,* 2017).

To conclude, method was developed and validated in UHPLC with PDA Detector with the good specificity to clothianidin. Linearity was obtained with R^2 value of 0.991 and with acceptable recovery range. The LOQ and LOD values for clothianidin were 0.01 and 0.003 µg g^{-1} for sugarcane. Satisfactory repeatability ruggerdness was ensured. The harvest time residues of clothianidin 50 WDG at 200 and 400 g a.i. ha^{-1} applied at the time of planting as soil drenching were at below detectable level (BDL) in sugarcane.

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