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HPLC Method Development and Validation of Chromafenozide in Paddy

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Abstract A simple and efficient HPLC–UV method was developed and validated for determination of chromafenozide in paddy as there was no previous report on record in this regard. The residue analysis method of chromafenozide, its dissipation and final residue in paddy along with soil were also studied after field treatment. Residues of chromafenozide were extracted and purified from paddy and soil followed by liquid/liquid partitioning, chromatographic column and determination by HPLC equipped with PDA detector. The separation was performed on a Phenomenex Luna RP C_{18} (250 × 4.6 mm i.d, 5 μm particle size) column at room temperature. The mean accuracy of analytical method were 94.92 %, 95.38 %, 94.67 % and 96.90 % in straw, grain, soil and field water respectively. The precision (repeatability) was found in the range of 1.30 %–9.25 % for straw/grain, 1.27 %–11.19 % in soil; 1.0 %–9.25 % in field water. The precision (reproducibility) in straw/grain was ranging from 2.2 % to 12.1 %, in soil it from 2.0 % to 11.7 %. The minimum detectable concentration was 0.01 mg kg^{-1}. The degradation of chromafenozide formulation in rice, soil and water was determined and results showed that chromafenozide as wettable powder formulation degraded with the half-lives of about 4.4 and 2.9 days in paddy plant and soil respectively for double recommended dose. The results indicated that the developed method is easier and faster then could meet the requirements for determination of chromafenozide in paddy.

Keywords Chromafenozide · HPLC · Residue · Half-life · Paddy

India is the second largest producer of food grain next to China. But one of the major constraints of crop production is the problem of pest attack under tropical agro-climatic condition of India where the population of pest may increase drastically. In India the agricultural losses due to pests have recently been estimated to be Rs. 6, 89,400 annually (Dhaliwal et al. 2004). Pesticides are generally widely used in rice cultivation, often resulting residues in rice grains (Tsochatzis et al. 2010). Therefore pesticides play an important role in food production to meet the food grain demand of the second largest populated country in the World. But one of the major drawback of pesticide use is that residue may remain in food above Maximum Residue Limits (MRLs). This could cause hazards to consumers as well as environment (Resgalla et al. 2007). So the controlled monitoring of pesticide is very essential and to do so, it is necessary to have an analytical procedure to estimate the residual level of pesticides. There has been a continuous search for pesticides with minimum environmental impact including residual problems.

Chromafenozide, developed by Nippon Kayaku Co. Ltd and Sankyo Co. Ltd. Japan, IUPAC name: 2′-tert-butyl-5-methyl-2′-(3, 5-xyloyl) chromane-6-carbohydrazide, Formula: $C_{24}H_{30}N_2O_3$, is a white crystalline solid, belonging to the dibenzoylhydrazine group of pesticides (Fig. 1). N-tert-Butyl-N, N′-dibenzoylhydrazine and its analogs are nonsteroidal ecdysone agonists that exhibit insect molting hormonal and larvicidal activities (Minakuchi et al. 2003).

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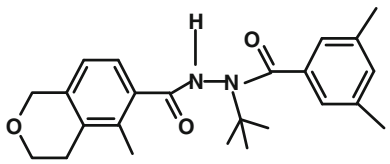

Fig. 1 The structure of chromafenozide

Chromafenozide is found to be significantly potent against various lepidopteran insects and at the same time almost non-toxic to non-lepidopterous species. As chromafenozide has a low toxicity profile in mammals and non-target organisms, it would be an ideal agent for Integrated Pest Management (IPM) (Yanagi et al. 2006). It is registered for use in Japan on various crops. HPLC is the method of choice for the analysis of chromafenozide because of its versatility, precision, and relatively low cost (Escarpa and González 2001; Tsao and Yang 2003).

In this work, a simple, fast and efficient HPLC–UV method was developed for the determination of chromafenozide in paddy. To obtain efficient preconcentration with good reproducibility and accuracy, liquid/liquid partition and chromatographic column system were applied. Finally, the proposed procedure was validated. The parameters involved were calibration and linearity, limits of detection and quantification, precision (repeatability and reproducibility), accuracy (recovery). The present work was also designed to investigate the residues of chromafenozide formulation in paddy and soil so as to determine the interval between spraying and harvest required for the safe use of this crop and to prevent any health problem to consumers. This would help to establish adequate monitoring of the residue of this newly introduced insecticide and its judicious incorporation in pest management strategies in rice fields.

Materials and Methods

Field trials were carried out during Kharif 2007 at Experimental Research Field, Department of Agricultural Chemicals, BCKV, Mohanpur, West Bengal, India. A Randomized Block Design (RBD) was used, with three replications for each test. Treatments were carried out during August–October, 2007; using a Knapsack sprayer. Chromafenozide commercial formulation was applied at two recommended doses of 100 g a.i/ha i.e. T_1 and 200 g a.i/ha i.e. T_2 with untreated control T_3. To study the dissipation pattern, paddy and soil samples were collected randomly from each treated plot as well as from the control plot at 0 (2 h), 1,3,7, 15, 30, 45 days after insecticide application and up to 15 days for water. Soil, Grain and straw samples were also collected at time of harvest. During the experiment, the average rainfall was 5.6 mm, and the maximum and minimum average temperatures were 32.3 and 24.7°C respectively.

Chromafenozide analytical standard (99.4 %) was supplied by Nippon Kayaku Co. Ltd, Japan. Chromafenozide 80 WP supplied by M/s PI Industries Ltd., Gurgaon. Distilled water was filtered through Milli-Q apparatus (Millipore, USA). HPLC grade acetonitrile was obtained from E.Merck, India. Other solvents used for extraction were distilled prior to use. Florisil of 60–100 mesh was purchased from Spectrochem (India). Other reagents were used of analytical grade (SRL, India). Stock standard solutions of the active ingredients were prepared in acetonitrile.

Chromafenozide was analyzed using a SHIMADZU-LC-10AT HPLC system equipped with Photodiode Array Detector (PDA).A Phenomenex-Luna 5 μ reverse phase (RPC_{18}) column (4.6 × 250 mm) was employed. The mobile phase of acetonitrile: water (7:3) was used to determine the chromafenozide. The flow rate was 1 mL min^{-1}, and injection volume 20 µl. UV detection was performed at 224 nm.

Soil: Soil was crushed in a hammer mill and sieved to obtain same size granules; 50 g soil was soaked overnight in a 250 mL conical flask in a mixture of water and acetonitrile 20:80 (v/v) 100 mL. Subsequently, the extracts were shaken for 1 h on a mechanical shaker and filtered through Whatman no. 1 filter paper. Filtrates were evaporated with rotary vacuum evaporator at 40°C. The concentrate obtained was transferred to separating funnel containing 50 mL of 5 % sodium chloride, followed by liquid–liquid partitioning with dichloromethane for three times at the volume of 40, 30 and 30 mL respectively. Organic layer was collected, concentrated and then dissolved in 5 mL of hexane and transferred to a chromatographic column (300 × 30 mm (id), packed with florisil) which was washed with 5 mL of hexane prior to use. The column was washed with 20 mL hexane/ethyl acetate (17:3, v/v) and the elute was discarded. Chromafenozide was eluted from the column with 50 mL of dichloromethane. The extract was evaporated to dryness and transferred quantitatively with acetonitrile to a 10 mL volumetric flask to be used for injection in the HPLC.

Grain/Straw: 20 g of rice/straw sample was chopped and soaked for overnight with 100 mL mixture of water and acetonitrile 20:80 (v/v). The mixture was filtered through Whatman no.1 filter paper and the residue was rinsed thrice with 50 mL of the fresh solvent. The combined filtrate was concentrated in a rotary vacuum evaporator at 40°C. The concentrate was transferred into a 250 mL separatory funnel and washed with 50 mL of 5 % NaCl aqueous solution and subsequently with 60, 60 and 60 mL of dichloromethane. Dichloromethane layer was collected

through 20 g of anhydrous Na_2SO_4 and concentrated in a rotary vacuum evaporator at 40°C. The residual concentrate was dissolved in hexane and transferred into the florisil column which was washed with 5 mL hexane prior to use. The column was washed with 20 mL of hexane/ethyl acetate (17:3, v/v) and then elute was discarded. Chromafenozide was eluted from the column with 50 mL of dichloromethane and then evaporated to dryness. Final volume was made up to 10 mL with acetonitrile to determine the chromafenozide in HPLC.

Water: 200 mL of field water sample was taken and filtered through Whatman no. 1 filter paper. The filtrate was partitioned with 50 mL of 5 % NaCl aqueous solution and 60, 60 and 60 mL in a separating funnel. Dichloromethane layer was collected through anhydrous Na_2SO_4 and evaporated to dryness in a rotary vacuum evaporator at 40°C. Final volume was made up to 10 mL with acetonitrile for injection in the HPLC.

The target pesticide chromafenozide was identified by retention time. Linearity range, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ) were evaluated for the analytical methodology. Recovery was assessed using spiked blank samples. The samples were extracted, purified and analyzed using the method above.

The first-order rate of degradation for chromafenozide in paddy and soil was determined with the following equation:

$$C_t = C_0 e^{-kt} \quad (1)$$

where C_t is the concentration of pesticide remaining in substrate (mg kg^{-1}) after t (days), C_0 is the initial concentration of chromafenozide (mg kg^{-1}), and k is the rate of degradation (d^{-1}).The half-life ($t_{1/2}$) of chromafenozide for each substrate was calculated using Eq. (2):

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

Results and Discussion

High performance liquid chromatography with reversed—phase column has proven to be a good option for chromafenozide determination as no derivation step is needed. Chromatographic separation in C_{18} columns provides good results. The selection of wavelength was performed by DAD (λ = 200–280 nm). The detection at 224 nm offers suitable chromatograms for quantification of chromafenozide in real samples. Under the chosen conditions, chromafenozide showed a retention time of 7.5 min, allowing a complete separation of its signal from those of foreign substances present in the samples.

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample (Francotte et al. 1996). Standard calibration curves of chromafenozide standard and chromafenozide in different matrices of paddy were constructed by plotting concentrations against peak areas. Good linearity was achieved in the concentration range of 0.03 to 10.0 mg kg^{-1} (0.03, 0.05, 0.1, 0.5, 1.0 and 10.0 mg kg^{-1}). The parameters obtained by the selected chromatographic conditions for chromafenozide (standard) calibration correspond to: $y = -31.91 + 45815x$, $R^2 = 1.0$ where y = peak area, x = chromafenozide concentration (mg L^{-1}), and R = correlation coefficient. The R^2 value was above 0.999 in straw, grain, and soil and field water shown in Table 1.

Limits of detection (LOD) and quantification (LOQ) were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method. In this study, LOD and LOQ of chromafenozide are 0.01 mg/kg and 0.03 mg/kg irrespective of substrate.

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. Intra-day and inter-day precisions of the method determined for chromafenozide in paddy plant or straw, grain, soil and field water according to FDA guidance for bioanalytical method validation. To determine intra-day accuracy and precision, three replicate analyses of each substrate were carried out at each of the five concentrations (0.03, 0.10, 1.0, 5.0 and 10.0 mg kg^{-1}). Inter-day accuracy and precision were assessed by the analysis of six samples (two samples per day at each level of five samples on three consecutive days). For each extraction there are three injections. The precision around the mean value must not exceed 15 % of the C.V. (%) except for LOQ concentration level where it must not exceed 20 % of the C.V. (%).The intra-day and inter-day precision values of the assay method are shown in Tables 2 and 3. The precision of the method was calculated as the relative standard deviation-coefficient of variation (C.V.) of the concentrations determined in all replicates. The intra-day C.V. (%) for chromafenozide was below 11.19 %. All inter-day C.V. (%) were below 11.76 %.

Table 1 The slope, intercept and correlation coefficient (R^2) of calibration curves of chromafenozide in different matrix

Matrix	Slope	Intercept	R^2
Straw	43003	1216.0	0.999
Grain	44720	571.01	1.000
Soil	43287	855.70	1.000
Water	44962	100.88	1.000

Table 2 Intra-day accuracy and precision of chromafenozide determination at five concentration levels (n = 3)

Substrates	Spiked level (mg kg^{-1})	Mean detected concentration (mg kg^{-1})	SD	Accuracy (%)	Precision C.V. (%)
Straw	0.03	0.0277	0.0025	92.3	9.02
	0.10	0.0944	0.0051	94.0	5.42
	1.00	0.9633	0.0473	96.3	4.91
	5.00	4.8867	0.1793	97.7	3.66
	10.0	9.7900	0.2594	97.9	2.65
Grain	0.03	0.0273	0.0025	91.1	9.25
	0.10	0.0957	0.0075	95.6	7.84
	1.00	0.9760	0.0511	97.6	5.23
	5.00	4.9333	0.2517	98.6	5.10
	10.0	9.8933	0.1290	98.9	1.30
Soil	0.03	0.0259	0.0029	86.3	11.19
	0.10	0.0957	0.0042	96.0	4.37
	1.00	0.9653	0.0280	96.5	2.90
	5.00	4.8767	0.0643	97.5	1.32
	10.0	9.8600	0.1249	98.6	1.27
Water	0.03	0.0281	0.0026	93.6	9.25
	0.10	0.0973	0.0057	97.3	5.85
	1.00	0.9757	0.0339	97.5	3.47
	5.00	4.9100	0.1308	98.2	2.66
	10.0	9.8967	0.1050	98.9	1.06

Accuracy is the measure of how close the experimental value is to the true value and it was measured by analyzing the percentage of recovery of the target pesticide in the extract of different substrates. The blank samples were spiked with different amounts of standard compound before extraction. The accuracy determined at each concentration level must be within ±15 % of respective nominal value except at LOQ concentration level where it

Table 3 Inter-day accuracy and precision of chromafenozide determination at five concentration levels (n = 6)

Substrates	Spiked level (mg kg^{-1})	Mean detected concentration (mg kg^{-1})	SD	Accuracy (%)	Precision C.V. (%)
Straw	0.03	0.0265	0.0028	88.3	10.56
	0.10	0.0937	0.0065	94.0	6.93
	1.00	0.9500	0.0608	95.0	6.40
	5.00	4.8267	0.1922	96.6	3.98
	10.0	9.7333	0.3786	97.3	3.89
Grain	0.03	0.0267	0.0029	89.0	10.86
	0.10	0.0933	0.0099	93.0	10.64
	1.00	0.9567	0.0751	95.7	7.84
	5.00	4.8000	0.2646	96.0	5.52
	10.0	9.8333	0.1528	98.3	1.55
Soil	0.03	0.0272	0.0032	90.6	11.76
	0.10	0.0920	0.0098	92.0	10.65
	1.00	0.9567	0.0503	95.6	5.25
	5.00	4.8377	0.2009	96.7	4.14
	10.0	9.6800	0.2022	96.8	2.08
Water	0.03	0.0283	0.0029	93.3	10.35
	0.10	0.0967	0.0058	97.0	5.97
	1.00	0.9733	0.0462	97.3	4.74
	5.00	4.9000	0.1732	98.0	3.53
	10.0	9.8167	0.2363	98.1	2.40

Table 4 Residues (mg kg^{-1} ± SD) and Half-life ($t_{1/2}$) of chromafenozide in paddy, field soil and field water

Dose (g ha^{-1})	Time (days)	Residues(mg kg^{-1} ± SD)		
		Paddy plant	Field Soil	Field water
100 (T_1)	0	0.52 ± 0.047	0.20 ± 0.095	0.07 ± .01
	1	0.44 ± 0.026	0.15 ± 0.05	0.04 ± 0.01
	3	0.32 ± 0.092	0.07 ± 0.026	ND
	7	0.15 ± 0.061	BDL	–
	15	ND	ND	–
	$t_{1/2}$ (days)	3.9	2.0	–
	R^2	0.998	0.99	–
200 (T_2)	0	0.82 ± 0.076	0.32 ± 0.075	0.12 ± .01
	1	0.65 ± 0.090	0.24 ± 0.026	0.05 ± 0.01
	3	0.38 ± 0.075	0.14 ± 0.031	BDL
	7	0.27 ± 0.085	0.06 ± 0.030	–
	15	ND	ND	–
	$t_{1/2}$ (days)	4.4	2.9	–
	R^2	0.924	0.99	–
Control (T_3)	ND	ND	ND	ND

ND not detected

must be within ±20 % of the nominal value (Pathak et al. 2007). The intra-day and inter-day accuracy values (recovery percentage) of the test method are shown in Tables 2 and 3. The average recoveries for chromafenozide were found 94.92 %, 95.38 %, 94.67 % and 96.90 % in straw, grain, soil and field water respectively, which are very satisfactory.

The robustness of an analytical method is defined as the measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability during normal usage (Michail et al. 2007). For the determination of the method's robustness a number of chromatographic parameters, such as both column package and size, mobile phase composition, flow rate and detection wavelength, were varied to determine their influence in the quantitative analysis. Finally, the robustness of the method was also assessed. Minor modifications of the initial mobile phase isocratic (from 65 to 75 % acetonitrile instead of 70 %) had no effect on the peak resolution of the compounds, even when using a second Luna C18 column of the same dimensions. Therefore, this HPLC method can be regarded as accurate, precise and robust (Parejo et al. 2004).

Initial deposits, half-lives and regression equation of chromafenozide in paddy plant and soil are presented in Tables 4. The results show that the residues of chromafenozide in rice plant and cropped soil decreased progressively with time irrespective of application rates. The residue level decreased to below detectable limit on the 15th day in paddy and soil samples irrespective of any doses. In the untreated control (T_3), chromafenozide residues were not detected. The half-life values calculated from the best-fit lines of the logarithm of residual concentrations versus time period, suggested first order reaction kinetics in the dissipation of chromafenozide residue. The study revealed that the dissipation rate was independent of initial deposit.

As it is evident from the analytical data Table 4 average initial deposits on 0 (2 h) day of chromafenozide residues on paddy plant were found to be 0.52 and 0.82 mg kg^{-1} for T_1 and T_2 respectively which were dissipated to 0.15 and 0.27 mg kg^{-1} in 7 days after application. In 15 days after application the residues were reached to below detectable limit for both the treatments. The percentages of dissipation recorded on 15th days were 81.37 % and 74.73 % respectively. The average initial deposit in soil were 0.985 mg/kg and 1.64 mg/kg on 0 (1 h) days for T_1 and T_2 respectively. No residues were found for both treatment in 15 days after application. It was dissipated up to 71 % and 67 % on 7th day for T_1 and T_2 respectively. Table 4 also describes the kinetic data of chromafenozide dissipation in paddy plant. The dissipation of chromafenozide in paddy plant followed the first-order kinetics with the half-life values varying from 3.9 to 4.4 days irrespective of any doses and the dynamics could be described by the equation $y = 2.722–0.077 \times$ and $y = 2.871–0.068 \times$ for T_1 and T_2 respectively. Figure 2 shows the residue of chromafenozide in paddy plant samples over the testing time period. No residue was detected in control samples. No chromafenozide residue was found in harvested samples of straw, husk and grain.

The residue of chromafenozide in field soil over the testing time period shown in Fig. 3. The average initial deposits of chromafenozide in cropped soil were 0.20 and

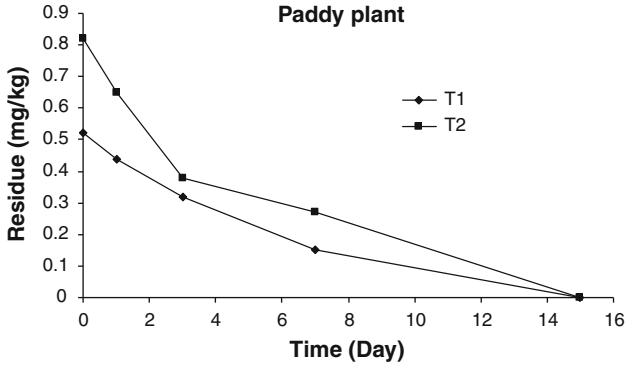

Fig. 2 The degradation curve of chromafenozide in paddy plant

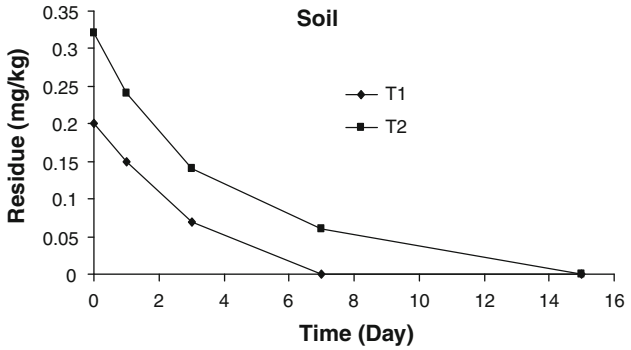

Fig. 3 The degradation curve of chromafenozide in soil

0.32 mg $kg^{-1}$for T_1 and T_2 respectively which were dissipated to 0.06 mg kg^{-1} in 7 days after application for T_2 and below detectable limit for T_1. In 15 days after application the residues were below detectable limit for T_2. The percentage of dissipation recorded on 7th day was 79 % for T_2. The dissipation of chromafenozide in cropped soil followed the first-order kinetics with half-life values varying from 2.0 to 2.9 days according to the application rate. The estimated residues were given in the Table 4. The degradation dynamics could be described by the equation $y = 2.312–0.153 \times$ and $y = 2.485–0.103 \times$ with corelation coefficient $R^2 = 0.990$. A sharp decline of residues was observed for chromafenozide in soil also.

In case of field water the initial deposits were found to be 0.07 and 0.12 mg kg^{-1} corresponding to the doses (Table 4). Within 3 days the initial deposits of chromafenozide went below detectable limit irrespective of doses.

Chromafenozide is a newly introduced insect growth regulator used for controlling lepidopteran insecticide. The present work was designed to investigate the residues of chromafenozide in/on paddy to determine the interval between spraying and harvest required for the safe use of this crop. On the basis of the above findings it may be concluded that, a novel HPLC method having high reproducibility and sensitivity for the determination of chromafenozide in paddy was developed in this study. The method was validated over a concentration range of 0.03–1.00 mg kg^{-1} ($R^2 > 0.9970$) and it offers good accuracy and precision for monitoring the full kinetic profile of chromafenozide in paddy plant or straw, grain, field soil and field water. The advantages of our method are small sample volume (20 μL), short time of analysis and a simple sample extraction and clean-up procedure.

Chromafenozide residue on plant was below the detectable limit at 15 days for recommended doses. No residue was detected in harvested samples of husk, grain or straw as well as control samples irrespective of the doses. There are currently no established Codex, Canadian or Mexican maximum residue limits (MRLs) for residues of chromafenozide in/on plant or livestock commodities. Japan has established tolerances: a minimum MRL of 0.2 ppm chromafenozide in/on rice (GAIN Report 2007). The MRL value of Chromafenozide in paddy in India also has not yet been established. But considering the above findings it might be concluded that Chromafenozide may not pose any residual toxicity problem in paddy as the residue found always below the detectable level after 15 days of application of the insecticide.

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