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Development and Validation of the RP-HPLC Method for the Analysis of Chloropyrifos

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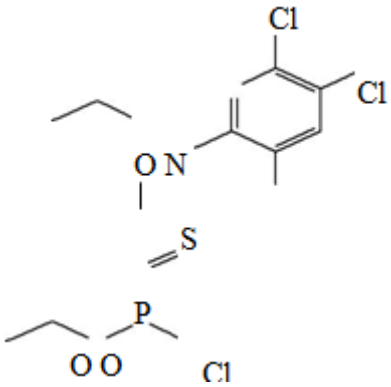
Abstract: ***A simple, selective, precise and accurate High Performance liquid Chromatographic method for the analysis of Chloropyrifos in its formulations was developed and validated in the present study. The mobile phase consist a mixture of Acetonitrile and Potassium Dihydrogen Orthophosphate in the proportion 70:30 and adjust the p^H to 6.8. This was found to give sharp peak of Chloropyrifos at a retention time of 6.780 min. HPLC analysis of Chloropyrifos was carried out at a wave length of 289 nm with a flow rate of 1.0µL/ min. the linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999 in the concentration range of 12mg to 72mg. The linear regression equation was y = 63577x + 14260. The developed method was employed with a high degree of precision and accuracy for the analysis of Chloropyrifos. The method was validated for accuracy, precision, robustness, ruggedness, specificity. The precision, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is better for the quantification of Chloropyrifos.***

Keywords: Chloropyrifos, RP-HPLC Method development, Validation.

1. Introduction



($C_9H_{11}Cl_3NO_3PS$)

IUPAC NAME: [*O***, *O*-Diethyl *O*-3, 5, 6-trichloropyridin-2-yl] phosphorothioate**

Chloropyrifos is a crystalline organophosphate insecticide. It was introduced in 1965 by Dow Chemical company and is known by many trade names including **Dursban** and **Lorsban**. Chloropyrifos was a well-known home and garden insecticide, and at one time it was one of the most widely used household pesticides in the US. It acts on the nervous system of insects by inhibiting acetylcholinesterase. Chloropyrifos is moderately toxic and chronic exposure has been linked to neurological effects, developmental disorders, and autoimmune disorders. Exposure during pregnancy affects the mental development of children, and the use in homes in the U.S. has been banned since 2001. In agricultural use, it remains "one of the most widely used organophosphate insecticides", according to the United States Environmental Protection Agency (EPA).

Chloropyrifos is produced via multistep synthesis from 3-methylpyridine, eventually reacting 3, 5, 6-trichloro-2-pyridinol with diethyl thiophosphoryl chloride. Chloropyrifos acts on pests primarily as a contact poison, with some action as a stomach poison. It is available as granules, wettable powder, dust able powder and emulsifiable concentrate. The crops with the most intense Chloropyrifos use are cotton, corn, almonds, and fruit trees including oranges and apples. Chloropyrifos is normally supplied as a 23.5% or 50% liquid concentrate. The recommended concentration for direct-spray pin point application is 0.5% and for wide area application a 0.03 – 0.12% mix is recommended [1-5].

In flash back most foodstuffs and environmental samples which are Chloropyrifos analysis is usually carried out using gas chromatography (GC) with flamephotometric detection or nitrogen phosphorous detection[6-8] and HPLC with UV detector were carried out[9-10]. But in the present study the RP-HPLC method described here is simple, sensitive, and reproducible for Chloropyrifos determination in Formulations with low background interference. An attempt has been made to develop and validate to ensure their accuracy, precision and other analytical method validation parameters as mentioned in various gradients. One method reported for the HPLC determination based on the use of a C-18 column, with a suitable mobile phase, without the use of any internal standard. For pesticide formulation the proposed method is suitable for their analysis with virtually no interference of the usual additives present in pesticide formulations.

a) Instruments

Waters HPLC 2-2695 series consisting pump, Auto sampler, UV-Visible detector, Thermostat column compartment connected with Waters (alliance) Empower software

b) Methodology

Following conditions are carried out for the HPLC method.

Chromatographic Conditions

Column	:	RP-Select B C18, 250 mm X 4.6 mm,
Flow rate	:	1.0 ml /min
Wavelength	:	289nm

Column	:	30°C
Injection volume	:	20 µl
Run time	:	15 minutes
Diluent	:	Mobile phase
Elution	:	Isocratic
Needle wash	:	Water: Acetonitrile 90:10 (v/v)

c) Preparation of Mobile phase:

The content of the Mobile phase was prepared from filtered and degassed mixture of Phosphate buffer (3.0 g of potassium hydrogen phosphate in 1 liter water and pH was adjusted to 6.80) and Acetonitrile in the ratio of 30:70 v/v.

d) Preparation of Diluents (Solvent)

HPLC grade Acetonitrile is used as a diluent and measured accurately 100 ml of Acetonitrile (HPLC grade) by using standard flask.

e) Preparation of Chloropyrifos Standard Stock solution:

Weighed and transferred 48 mg of CHLOROPYROFOS into 25 ml volumetric flask then added 20 ml of diluent and Sonicated for 10 minutes and make up with diluent.

f) Preparation of Sample Solution

The fortified soil after 10 minutes sonication sample is extracted with Whatman filter paper 1 room by using Acetonitrile as a solvent. 10 ml of Chloropyrifos extract solution diluted to 100 ml with diluent.

g) Procedure

20 µl of blank solution, placebo solution and six times of Standard solution were Injected. Disregard peaks due to blank and placebo.

System suitability requirements from SST solution:
Tailing factor : NMT 2.0
Theoretical Plates : NLT 2000

h) Precision (Repeatability):
Preparation of precision solution
10 ml of standard stock solution diluted to 100 ml and make-up to volume with diluent. The same procedure is repeated for remaining six preparations. % RSD for the RT and Area are tabulated in Table-1.1.

Table 1.1: Precision Study of Chloropyrifos

S. No	Method Precision		System Precision	
	Retention Time	Area	Retention Time	Area
1	6.999	3090404	6.765	3057975
2	6.999	3086568	6.765	3033832
3	6.999	3090358	6.783	3067389
4	6.965	3055341	6.781	3061265
5	6.865	3067345	6.778	3068945
6	6.943	3078390	6.779	3065638
Average	6.962	3078068	6.775	3062507
Standard Deviation	0.053	14192.07	0.008	5872.344
% Relative	0.76	0.46	0.12	0.19
standard deviation				

Acceptance Criteria:
The %RSD of areas from six preparations precision level should not be more than 2.0%.

i) Accuracy
The accuracy of the test method was demonstrated by preparing recovery samples (i.e. test sample with known quantities of 50%, 100% and 150% of target concentration). Results are tabulated in Table-1.2.

Table 1.2: Accuracy Study of Chloropyrifos

Accuracy	Std.	50%Spike	100%Spike	150%Spike
Trial-1	3073123	1557645	3071213	4588754
Trial-2	3098324	1541276	3070875	4587542
Trial-3	3076345	1540542	3070764	4589658
Average	3082597.333	1546487.667	3070950.667	4588651.333
Amount Recovered	100	50.168	99.622	148.857
% of Recovery	100	100.337	99.622	99.238

j) Linearity
The Linearity of detector response was demonstrated by prepared solutions of 25% to 150% level of the target. Results are tabulated in Table-1.3.

Preparation at 25% to 150% Level:

Table 1.3: Linearity study of Chloropyrifos

Concentration (mg/25mL)	*Linear solutions (%)*	*Stock solution taken in (mL)*	*Diluted to volume (mL) with diluent*	*Area*
12	25%	2.5	100	771864
24	50%	5	100	1552840
36	75%	7.5	100	2325508
48	100%	10	100	3072698
60	125%	12.5	100	3809329
72	150%	15	100	4589065

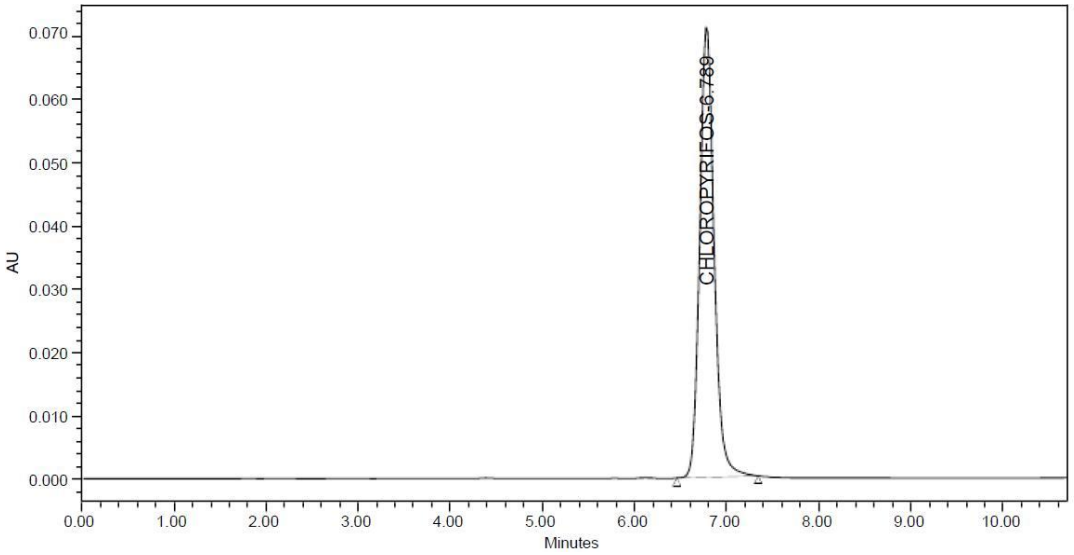


Figure 1.1: Chromatogram of Chloropyrifos

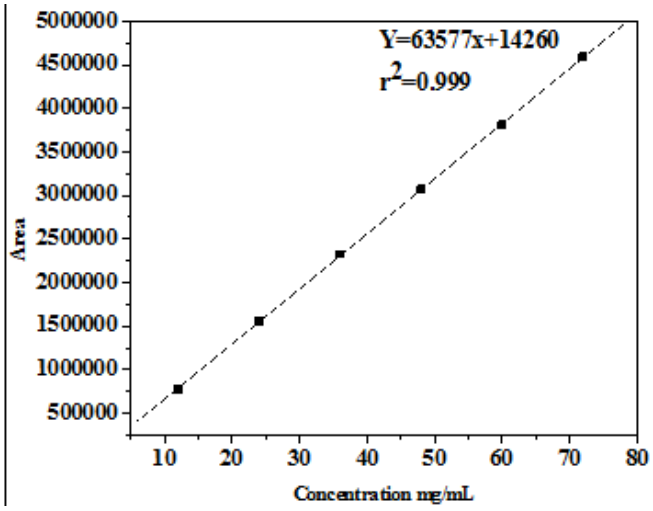


Figure.1.2: Linearity of Chloropyrifos

The correlation coefficient shall not be less than 0.998

k) Assay

10 ml of standard stock solution dilluted to 100 ml and make- up to volume with diluent. Repeated the same procedure for remaining preparations.

Table 1.4: Assay Study of Chloropyrifos

Standard-1	3058956
Standard-2	3059898
Standard-3	3058756
Average weight	3059203
Sample-1	3061256
Sample-2	3060321
Average weight	3060789
Label Claim	48 mg
Standard weight	48
Sample weight	100
Standard factor	0.0001
Sample factor	0.0001
Standard purity	99.6
Avg. wt.	100
Amount	47.83 mg
%assay	99.65%

l) Ruggedness

The ruggedness of test method was demonstrated by carrying out precision study in six preparations of sample on a single batch sample by different analysts. The results of the intermediate precision study are tabulated in Table-1.5.

Table 1.5: Rugge dness Study of Chloropyrifos

.S.No.	RT	Area
1	6.925	3074578
2	6.928	3079845
3	6.934	3080568
4	6.932	3081154
5	6.937	3082165
6	6.935	3082211
Average	6.932	3080087
Standard Deviation	0.005	2849.805
%RSD	0.07	0.09

m) Robustness

The robustness of test method was demonstrated by carrying out flow variation ± 10%, i.e. (0.9 ml to 1.1 ml/min), buffer variation ±10% i.e. (35:65 - 45:55) and the results are tabulated in Table-1.6.

Table-1.6: Robustness Study of Chloropyrifos

S. No:	RT	Area
Buffer-1	12.625	2954604
Buffer-2	8.937	3019427
Flow-1	7.535	3411119
Flow-2	6.262	2793294

n) LOD
LOD = 3 x STDEV / SLOPE
0.3048

o) LOQ:
LOQ = 10 x STDEV / SLOPE
0.9236

p) Specificity Studies

The sample was analyzed in Specific conditions i.e. in 0.1M

HCl Acidic Medium, 0.1M NaOH Basic Medium and Heat variation, UV- light range Variation. Then RT and Area were tabulated in Table-1.7.

Table 4.7: Specificity Study of Chloropyrifos

Specificity	RT	Area
Acid	6.835	179250
Base	6.835	179250
Heat	6.792	1882178
UV-light	6.792	1882178

Table 4.8: Performance calculations, detection characteristics precision and accuracy of the proposed method for Chloropyrifos

Parameter	*RP-HPLC Method*
Retention time (t) min	6.789
Theoretical plates (n)	9562
Plates per meter (N)	38248
Height equivalent to theoretical plate (HETP)	0.026
Linearity Range (µg/ml)	0-80
Peak asymmetry	1.15
LOD (µg/ml)	0.3048
LOQ (µg/ml)	0.9236
Regression equation (y* =bc+a)	
Slope (b)	63577
Intercept (a)	14260
Correlation coefficient (r^2)	0.9999
Method Precision Relative Standard Deviation (%RSD)	0.46
System Precision Relative Standard Deviation (%RSD)	0.19

2. Results and Discussion

The appropriate wavelength in UV region has been selected for the measurement of active ingredient in the proposed method. This method was validated by linear fit curve and all the other parameters were calculated.

Parameters Fixation

In developing methods, systematic study of the effects of various parameters was undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

a) Mobile phase Characteristics

In order to get sharp peaks and base line separation of the components, carried out number of experiments by varying different components like percentage of Organic phase in the mobile phase, P^H of the aqueous phase, total P^H of the selected mobile phaseand flow rate by changing one at a time and keeping all other parameters constant. The optimum conditions obtained were included in the procedure proposed.

b) Detection Characteristics

To test whether Chloropyrifos has been linearly eluted from the column, different amounts of Chloropyrifos were taken and analyzed by the above mentioned procedures. The peak area ratios of component areas were calculated and the values are graphically represented in Fig.1.2. The linear fit of the system was illustrated graphically. Least square regression analysis for the method was carried out for the slope, intercepts and correlation coefficient. The results are presented in table-1.3.

c) Performance Calculations

To ascertain the system suitability for the proposed method, a number of statistical values have been calculated with the observed readings and the results are recorded in Table-1.8.

d) Method Validations

The UV absorption maximum for Chloropyrifos was fixed at 289 nm respectively. As the final detection was made by the UV absorption spectrum, each method was validated by linear fit curve.

e) Precision

The Precision of the method and system was ascertained separately from the peak area ratios obtained by actual determination of a fixed amount of pesticide. The percentage of relative standard deviation and percentage of errors (at 0.05 and 0.01 confidence limits) were calculated for Chloropyrifos and readings presented in Table-1.1. The Precession of the assays was also determined in terms of dilution variation in the peak areas for a set of pesticide solution was calculated in terms of %RSD and the results are presented in Table-1.4.

f) Accuracy

To determine the accuracy of the proposed methods, different amount of Technical grade (Rallis India Ltd. 98%w/w) samples of Chloropyrifos within the linearity limits were taken and analyzed by the proposed method. The results (%RSD error) are recorded in Table-1.2.

g) Ruggedness

The ruggedness of test method was demonstrated by carrying out precision study in six preparations of sample on a single batch sample by different analysts. The results of the intermediate precision study are recorded in Table-1.5.

h) Robustness

The robustness of test method was demonstrated by carrying out flow variation ±20%, i.e. (0.9ml - 1.1 ml/min), buffer variation ±10% i.e. (35:65 and 45:55). Results of this study are recorded in Table-1.6.

i) Specificity Studies

The Specificity Studies are carried out by varying specific conditions studied Heat, UV- light range and in Acidic, Basic medium. The results are recorded Table-1.7.

3. Conclusion

The method was found to be accurate and precise, as

indicated by recovery studies close to 100 and % RSD is not more than 2.The summary of validation parameters of proposed RP-HPLC method is given in tables. The simple, accurate and precise RP-HPLC method for the determination of Chloropyrifos as Technical grade and formulation has been developed. The method may be recommended for routine and environmental analysis. It is concluded that the analytical method is validated and could be used for routine analysis.

4. Acknowledgements

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