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## Determination of organophosphate insecticide (Chlorpyrifos) in cabbage, cauliflower and capsicum by high performance liquid chromatography

Rama Mohana Rao Chilumuru<sup>1\*</sup>, Cyril Arun Kumar Lakkineni<sup>2</sup> and Chandra Bala Sekharan<sup>3</sup>

<sup>1</sup>Department of Bio-Technology, VSR & NVR College, Tenali, Andhra Pradesh, India

<sup>2</sup>Department of Zoology, VSR & NVR College, Tenali, Andhra Pradesh, India

<sup>3</sup>Department of Pharmaceutical Biotechnology, Medarametla Anjamma Mastan Rao College of Pharmacy, Narasaraopet, India

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### ABSTRACT

*Chlorpyrifos is a crystalline organophosphate insecticide. A simple and rapid reverse phase high-performance liquid chromatographic method using UV detection was developed and validated for the analysis of chlorpyrifos in selected vegetable samples like cabbage, cauliflower and capsicum under isocratic conditions. A Kromosil C18 analytical column (150 mm × 4.6 mm I.D., 5 µm particle size) and a mobile phase constituting acetonitrile: 1 mM phosphate buffer (85:15 v/v, pH 4.5 adjusted with orthophosphoric acid) were used. The flow rate was 1 ml/min and the analyses performed using ultraviolet detector at a wavelength of 230 nm. The method response was linear over the concentration range of 2 to 12 µg/ml. The developed method was validated as per standard guidelines. Validation of the developed method demonstrated system suitability, sensitivity, accuracy, precision and selectivity of proposed method. This rapid and simple method was successfully applied in the estimation of chlorpyrifos in cabbage, cauliflower and capsicum. The results indicated that the chlorpyrifos residues were observed in all the samples and the concentration was found to be above the limit of maximum levels (0.05 mg/kg) recommended by the European Union Regulations 396/2005.*

**Keywords:** Pesticides, organophosphate, High Performance Liquid Chromatography, Chlorpyrifos, Quantitative analysis, Vegetable samples.

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### INTRODUCTION

Pesticides are substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. The most common use of pesticides is as plant protection products, which in general protect plants from damaging influences such as weeds, diseases or insects [1]. Pesticide management tools are often considered by farmers to be effective, easy to use and inexpensive. Environmental impact on wild life, increased soil erosion, and contamination of surface water and ground water due to pesticides is enormous.

Prolonged dietary exposure to pesticides may pose health problems such as respiratory, memory disorders, dermatological conditions, cancer, neurological deficiencies, miscarriages and birth defects [2]. The Food Quality Protection Act became law in 1996 requiring that all existing pesticide tolerances (maximum residue limits) be re-evaluated for risk using a much stricter set of scientific standards. The Office of Pesticide Programs of the

Environmental Protection Agency has to reassess all existing chemical tolerances by 2006, with checkpoints to mark its progress every 3 years. Entire classes of pesticides that have been used to protect crops since the early 1950's potentially could be removed from the growers' arsenal during this reassessment process [3]. Banning a pesticide that has few substitutes can have several effects. It can increase the total quantities of pesticides used and accelerate increased resistance of insects, fungi, and bacteria. Also, it can affect the fruits or vegetables, cosmetic appearance; raise costs for users switching from the banned pesticides; reduce income for producers in certain regions; and reduce yields and storability, thereby increasing food costs [4].

Chlorpyrifos, chemically known as O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, is a crystalline organophosphate insecticide. In agricultural, it remains one of the most widely used organophosphate insecticides [5]. It acts on the nervous system of insects by inhibiting acetylcholinesterase. It is moderately toxic to humans and chronic exposure leads to neurological effects, developmental disorders, and autoimmune disorders [6]. Detailed survey of literature for chlorpyrifos revealed several methods have been reported for the assay of chlorpyrifos residues in different fruits, vegetables and water samples. These analytical techniques include HPLC with UV detection [7-15], liquid chromatography-tandem mass spectrometry [16,17], HPTLC [18-21], gas chromatography-mass spectrometry [12,16,22], gas chromatography with electron capture detection [10,13,23,24], spectrophotometry [25], reflectance near-infrared spectroscopy [26], chemiluminescence assay [27], immunoassay [28] and capillary electrochromatography [29]. Only few methods have been reported for the determination of chlorpyrifos residues in cabbage, cauliflower and capsicum. The methods adopted include HPLC with UV detection [11,13], capillary electrochromatography [29] and gas chromatography with electron capture detection [13,30].

The methods reported for analysis of chlorpyrifos residues in cabbage, cauliflower and capsicum suffered disadvantage of use of an internal standard, less sensitive, lack of selectivity, use of expensive detectors, long time for analysis and tedious procedures. Considering this drawback, there was a need to develop more advantageous methods for its determination in cabbage, cauliflower and capsicum. For this reason, an attempt has been made to develop a simple, sensitive, cost effective and reliable HPLC with UV detection for estimation of chlorpyrifos residues in cabbage, cauliflower and capsicum with out the use of internal standard.

## MATERIALS AND METHODS

### Experimental

#### Apparatus

1. High Pressure Liquid Chromatography was performed with an isocratic High Pressure Liquid Chromatography system (Shimadzu HPLC class VP series, Shimadzu Corporation, Kyoto, Japan) with two LC-10 AT, VP pumps, variable wavelength programmable UV/Visible detector SPD-10A, VP, CTO-10AS VP column oven, SCL-10A, VP system controller. A 20  $\mu$ L Hamilton syringe was used for injecting the samples. Data were analyzed by using PEAK software.
2. Double beam UV-VIS spectrophotometer Model UV-VIS 2301 (Tech-comp limited, Hong Kong, Japan) was used for spectral studies.
3. Degassing of the mobile phase was done by using ultrasonic bath sonicator (Loba Chemie Pvt. Ltd. Mumbai, India)
4. Samples were weighed by using Denver electronic weighing balance (Denver instruments, Colorado, USA.).
5. Kromosil C18 analytical column (150 mm  $\times$  4.6 mm I.D., 5  $\mu$ m particle size), under reversed phase chromatographic conditions, is used for the chromatographic analysis of chlorpyrifos.

#### Chemicals and solvents

The chemicals and solvents used for the preparation of mobile phase are of analytical reagent grade and HPLC grade, respectively. Acetonitrile and orthophosphoric acid were purchased from Merck Specialities Pvt. Ltd., Mumbai, India. Disodium hydrogen phosphate is from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India. Milli-Q-water was used throughout.

#### Mobile phase

The mobile phase consists of a mixture of acetonitrile and 1 mM phosphate buffer (pH 4.5) in the ratio of 85:15 *v/v*. The phosphate buffer (1 mM) was prepared by dissolving 178 mg of disodium hydrogen phosphate in water and diluted to 1000 ml with water. The pH was adjusted to 4.5 with orthophosphoric acid. Before use, the mobile phase

is filtered through a 0.45 µm Millipore membrane filter and degassed with ultrasonic bath sonicator for 15 minutes. The mobile phase is also used as the diluent for the preparation of stock standard solution.

#### **Stock standard solution**

The bulk sample of chlorpyrifos was obtained from Modern Insecticide Limited, Punjab, India. A stock standard solution containing 1 mg/ml of chlorpyrifos was prepared in diluent.

#### **Collection and storage of vegetable samples**

Vegetable samples such as cabbage, cauliflower and capsicum were directly collected from different local vegetable markets at Guntur, Tenali, Ongle and Vijayawada of Andhra Pradesh, India. The samples were labeled and brought to the laboratory. All the samples were stored at -20°C until analysis.

#### **Chromatographic conditions**

Column	:	Kromosil C18 analytical column (150 mm × 4.6 mm I.D., 5 µm particle size)
Flow rate	:	1 ml/min
Column temperature	:	25°C
Injection volume	:	20 µl
Detection wavelength	:	230 nm

#### **Assay of chlorpyrifos**

Working standard solutions equivalent to 2 to 12 µg/ml of chlorpyrifos were prepared by appropriate dilution of the stock standard solution with the mobile phase. Twenty µL aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 ml/min. The peaks were recorded at 230 nm.

#### **Extraction of chlorpyrifos residues from vegetable samples**

The pesticides such as chlorpyrifos residues were extracted from vegetable samples with optimized extraction method as described by Hussain and Samia [31]. Twenty grams of vegetable sample was taken and 20 ml of distilled water was added. The mixture was left undisturbed for 15 minutes, after which 50 ml of acetonitrile was added and the sample was homogenized by crushing in a pestle and mortar. The sample was filtered by suction. To the remaining residue on the filter, 20 ml of acetonitrile was added and again the sample was homogenized and filtered by suction. Both filtrates were combined together and the volume was increased to 100 ml by adding acetonitrile. From this solution 20 ml of sample was taken and 10 g of NaCl and 20 ml of 0.5 M phosphate buffer was added and shaken. The solution was left undisturbed for removal of the aqueous layer. The organic layer was dried over anhydrous sodium sulphate (Sdfine-Chem limited, Mumbai, India). The dried sample was reconstituted with mobile phase for the analysis of chlorpyrifos by using the proposed method.

#### **Assay chlorpyrifos residues in vegetable samples**

The extract prepared from vegetable samples, as described in above section "Extraction of chlorpyrifos residues from vegetable samples", was further diluted appropriately with the mobile phase for the analyses of chlorpyrifos residues by the proposed method. For this purpose, the vegetable sample solution was injected into HPLC system in duplicate. The chromatograms were recorded. The area under the peak was calculated. The concentration of chlorpyrifos in the vegetable samples was calculated using the corresponding calibration curve or corresponding regression equation.

## **RESULTS AND DISCUSSION**

#### **Method development**

Reversed phase high performance liquid chromatography (RP-HPLC) method is widely used for routine analysis of analyte of interest. In this study, we attempted to develop a new HPLC method with UV detection for the assay of chlorpyrifos in the vegetable samples that is more suitable in terms of time of analysis, tailing, sensitivity, cost and mobile phase.

To optimize the method, various mobile phase compositions under isocratic conditions were tried in preliminary tests. The mobile phase containing 1 mM phosphate buffer (pH 4.5) in the ratio of 85:15 v/v with flow rate 1ml/min

was found to be the suitable mobile phase for achieving the goal of interest. The selected mobile phase gave sharp and baseline resolute peak for the chlorpyrifos at 230 nm. Kromosil C18 analytical column (150 mm × 4.6 mm I.D., 5 μm particle size) column with column temperature set at 25 ± 1° C provides efficient and reproducible separation and analysis of chlorpyrifos. Under the optimized chromatographic conditions, the retention time of chlorpyrifos was 6.81 minutes.

#### Method validation

Method validation includes all of the procedures required to demonstrate that a method to quantify the concentration of chlorpyrifos is reliable for the intended application. The proposed HPLC method was validated for parameters like system suitability, linearity, sensitivity, selectivity, precision and accuracy.

#### System suitability studies

The system suitability was studied by performing the experiments and looking for changes in retention time, peak area, peak asymmetry and theoretical plates. Five injections of the standard solution of the chlorpyrifos were injected for this purpose. The results are presented in Table 1. The percentage relative standard deviation values were in the range of 0.259-0.961%. The values obtained (%RSD < 1.0) demonstrated the suitability of the system for the analysis of the chlorpyrifos.

Table 1: Results of system suitability studies

Parameter	Mean value	% RSD
Retention time	6.81	0.961
Peak area	9836	0.357
Theoretical plates	7365	0.452
Peak asymmetry	0.96	0.259

#### Selectivity

Selectivity of the HPLC proposed method was assessed by examining peak interferences from other substances present in the vegetable samples and components of mobile phase. This was done by comparing the chromatograms of blank and vegetable sample extract with the pure drug. It was observed that none of the peaks appears at the same retention time of drug (Figures 1–5). This confirmed the specificity of the proposed method. In addition, the well shaped peaks also signify the selectivity of the proposed method.

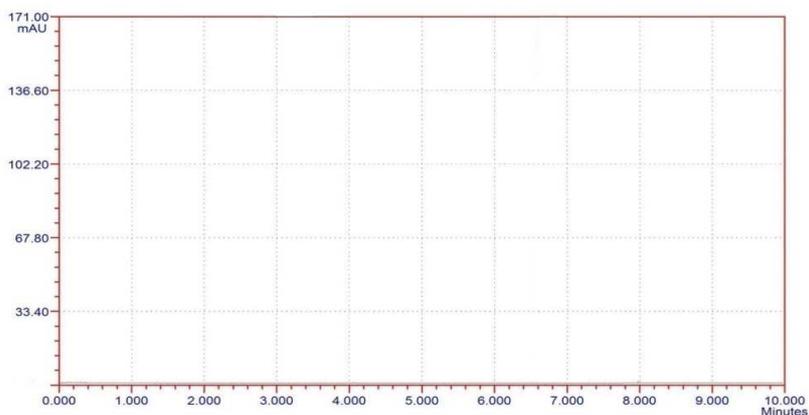


Figure 1: Chromatogram of mobile phase blank

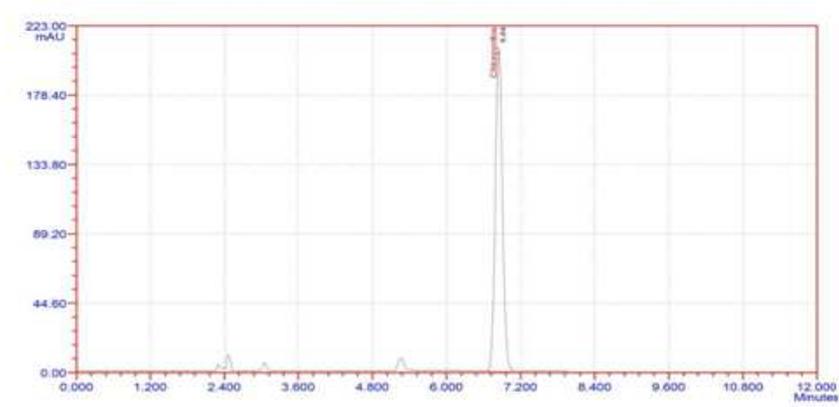


Figure 2: Chromatogram of pure chlorpyrifos

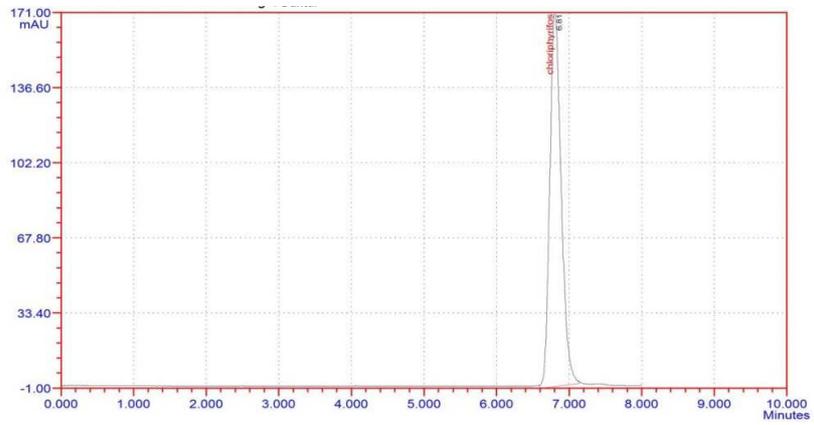


Figure 3: Chromatogram of chlorpyrifos in cabbage

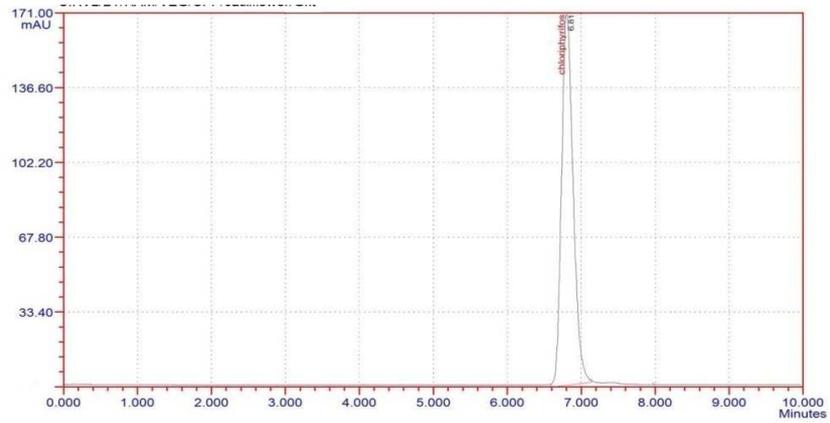


Figure 4: Chromatogram of chlorpyrifos in cauliflower

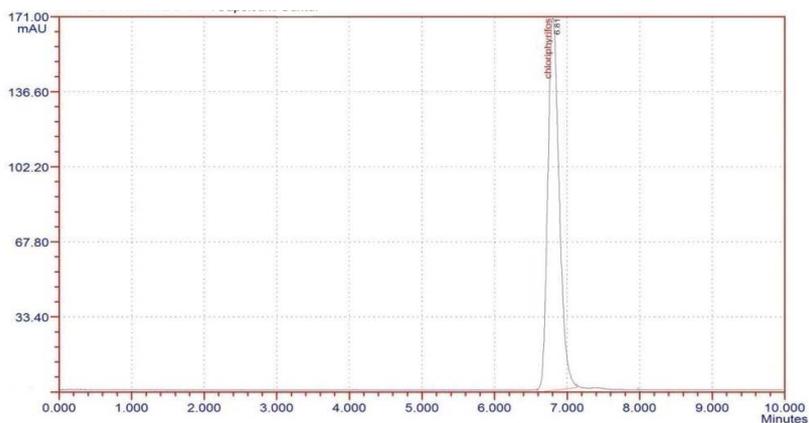


Figure 5: Chromatogram of chlorpyrifos in capsicum

### Linearity

The linearity of the proposed methods was determined by constructing calibration curves. A calibration curve was constructed using the least squares method by plotting the peak area vs concentration of chlorpyrifos. The Beer's law limit, slope, intercept and regression coefficient values (Figure 6) of the proposed methods were calculated using least square regression analysis. The linearity was found in the range of 2-12  $\mu\text{g/ml}$ . The high value of the regression coefficient ( $> 0.99$ ) and the small value of the intercept of the regression equation proved the linearity of the proposed method. The concentration against peak area plot of the proposed method was illustrated in Figure 6.

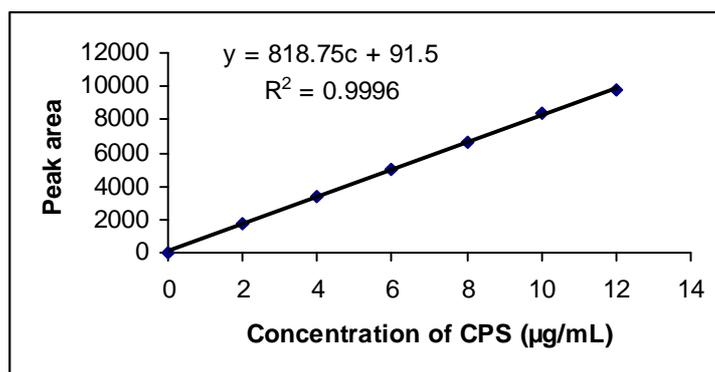


Figure 6: Calibration curve for chlorpyrifos

### Sensitivity

The sensitivity of the proposed HPLC method was evaluated by determining the limit of detection (LOD) and limit of quantitation (LOQ). The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated from  $kSD/b$  where  $k = 3$  for LOD and 10 for LOQ. SD is the standard deviation of the responses of the minimum detectable chlorpyrifos concentration, and  $b$  is the slope of the calibration curve. The low values of LOD (0.80  $\mu\text{g/ml}$ ) and LOQ (1.50  $\mu\text{g/ml}$ ) indicated the adequate sensitivity of the proposed method.

### Precision

Precision of the proposed HPLC method was assessed by injecting five standard solutions of chlorpyrifos with fixed concentration (12  $\mu\text{g/ml}$ ). The peak areas were determined. Relative standard deviation of the peak area was then calculated to represent precision. The relative standard deviation was found to be 1.059%, which proves that the proposed method is adequately precise.

### Recovery studies

Accuracy of the proposed HPLC method was assessed by recovery studies through standard addition method. In the standard addition method known quantities of chlorpyrifos at three different concentration levels (50, 100 and

150%) were supplemented to the standard solution previously analyzed. The solutions were once again analyzed by the proposed method. The percentage recoveries for the chlorpyrifos were calculated and are presented in the Table 2. Recovery studies showed the proposed method to be adequately accurate and suitable for the determination of the chlorpyrifos.

**Table 2: Accuracy of the proposed method (Recovery studies)**

Level (%)	Concentration of chlorpyrifos ( $\mu\text{g/ml}$ )			Recovery (%)	Average recovery (%)
	Fixed	Spiked	Recovered		
50	4	2	5.98	99.66	99.40
100	4	4	7.94	99.25	
150	4	6	9.93	99.30	

#### Application of the proposed method for the determination of chlorpyrifos in vegetable samples

The proposed HPLC method was successfully applied for the determination of chlorpyrifos in the selected vegetable samples (cabbage, cauliflower and capsicum). The results were summarized in Tables 3. The vegetables such as cabbage, cauliflower and capsicum for the present study were collected from the different local markets at Guntur, Tenali, Ongole and Vijayawada. The concentration of chlorpyrifos in cabbage samples were in the range of 4.58-9.84 mg/kg (Table 3). The highest concentration was observed in sample collected from Ongole and lowest in Tenali sample. The chlorpyrifos concentration of cauliflower and capsicum were found to be in the range of 12.57-18.25 mg/kg and 4.27-5.36 mg/kg, respectively (Table 3). Highest concentration of chlorpyrifos was observed in cauliflower and capsicum samples collected from Tenali and low concentration in samples collected from Guntur.

**Table 3: Assay of chlorpyrifos in selected vegetable samples**

S.No	Sample	Vegetable	Collection area	Concentration of chlorpyrifos (mg/kg)
1	Sample -1	Cabbage	Guntur	5.63 $\pm$ 0.67
2	Sample -2	Cabbage	Tenali	4.58 $\pm$ 0.35
3	Sample -3	Cabbage	Vijayawada	6.37 $\pm$ 0.76
4	Sample -4	Cabbage	Ongole	9.84 $\pm$ 0.31
5	Sample -5	Cauliflower	Guntur	10.52 $\pm$ 0.35
6	Sample -6	Cauliflower	Tenali	13.67 $\pm$ 0.46
7	Sample -7	Cauliflower	Vijayawada	18.25 $\pm$ 0.22
8	Sample -8	Cauliflower	Ongole	12.57 $\pm$ 0.47
9	Sample -9	Capsicum	Guntur	4.27 $\pm$ 0.38
10	Sample -10	Capsicum	Tenali	5.36 $\pm$ 0.92
11	Sample -11	Capsicum	Vijayawada	4.71 $\pm$ 0.87
12	Sample -12	Capsicum	Ongole	5.02 $\pm$ 0.32

#### CONCLUSION

A simple and rapid high performance liquid chromatography with UV detection method was developed for the estimation of chlorpyrifos. The developed method was validated for linearity, selectivity, sensitivity, precision and accuracy. From the validation data, the developed and validated HPLC method was found to be linear, sensitive, precise, specific, reproducible and cost-effective. Recovery of chlorpyrifos from cabbage, cauliflower and capsicum by using acetonitrile was found to be efficient. The method was successfully employed for the quantification of chlorpyrifos in cabbage, cauliflower and capsicum. The chlorpyrifos residues detected in all the selected vegetable samples (cabbage, cauliflower and capsicum) were above maximum residue limits established by European Union Regulations 396/2005 (0.05 mg/kg) [32].

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