



THIN LAYER CHROMATOGRAPHIC DETECTION OF CHLORPYRIFOS IN HUMAN BIOLOGICAL FLUIDS USING LOW-COST CHROMOGENIC REAGENT

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ABSTRACT:

The present study aimed to develop a simple, rapid and low-cost thin-layer chromatography (TLC) method for the qualitative and semi-quantitative detection of chlorpyrifos (CP) in human biological fluids, namely blood and gastric aspirate samples obtained from poisoning patients. Chlorpyrifos residues were extracted from biological samples by liquid-liquid extraction using diethyl ether. Separation was performed on silica gel TLC plates using hexane-acetone (8:2, v/v) as the mobile phase. A 10% copper sulphate solution was employed as a chromogenic spraying reagent for visualization of TLC spots. The developed chromogenic reagent produced a distinct yellow coloured spot for chlorpyrifos with an R_f value of 0.86, corresponding exactly with standard chlorpyrifos. Among the tested agricultural chemicals, chlorpyrifos showed selective and intense colour development, whereas most other organophosphorus insecticides, pyrethroids, and herbicides did not produce visible colour under identical conditions. Repeatability studies demonstrated acceptable variation (± 0.01) in R_f values. Semi-quantitative analysis was carried out using sequential dilution studies and ImageJ-assisted image analysis. The limit of detection and limit of quantification were 3.8025 $\mu\text{g}/\text{spot}$ and 11.5229 $\mu\text{g}/\text{spot}$, respectively. Estimated chlorpyrifos equivalents in unknown blood and gastric aspirate extracts were 327.2416 $\mu\text{g}/\text{spot}$ and 168.9368 $\mu\text{g}/\text{spot}$, respectively. The developed TLC method provides a rapid, economical and preliminary screening approach for chlorpyrifos poisoning in clinical and forensic toxicology laboratories, particularly in resource-limited settings.

Keywords: Chlorpyrifos, Organophosphorus insecticides, Thin-layer chromatography, Chromogenic reagent, Copper sulphate, Biological fluids.

INTRODUCTION:

Chlorpyrifos is a chlorine-containing organophosphorus insecticide extensively used in agriculture for controlling harmful insects and pests. Although it improves crop productivity, chlorpyrifos is also highly toxic to several non-target organisms including soil biota, fish, birds, animals, plants, and humans [1-2]. In recent years, chlorpyrifos poisoning has emerged as an important public health concern due to its increasing involvement in accidental, suicidal,

and homicidal poisoning cases. Severe chlorpyrifos toxicity may result in respiratory depression, convulsions, paralysis, unconsciousness, urinary and bowel incontinence, and even death. Its toxic action primarily occurs through inhibition of acetylcholinesterase enzyme activity in the nervous system [3-4].

Several advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-

MS), high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and TLC-FID have been reported for chlorpyrifos detection in biological and environmental samples. Although these techniques provide excellent sensitivity and specificity, they are expensive, technically demanding, and often inaccessible in resource-limited clinical and forensic laboratories [5-9].

Thin-layer chromatography (TLC) remains one of the simplest and most economical analytical methods for separation and identification of drugs, pesticides, and toxic compounds. TLC offers advantages such as simplicity, rapidity, low operational cost, reliability, good reproducibility, and minimal instrumentation requirements [10-16]. The use of chromogenic spray reagents in TLC enables visualization of otherwise colourless compounds after chromatographic separation. Various chromogenic reagents have previously been developed for organophosphorus pesticides such as monocrotophos, profenofos, and glyphosate [17-19]. Mulani et al have studied thin layer chromatographic detection of Amitraz pesticide [20]. Ullah et al have reviewed number of research articles and reported about 35 specific chromogenic spray reagents which detect different types of pesticides [21]. Palladium chloride and mercurous nitrate reagents have also been reported for chlorpyrifos detection; however, palladium chloride is expensive and mercurous nitrate is highly toxic [22-25]. Futagamiet al have developed simple and rapid method for identifying 25 commonly used organophosphorus insecticides in human serum using 4-(4-nitrobenzyl) pyridine-tetraethylenepentamine reagent (NT reagent) and palladium chloride reagent [26].

Copper sulphate is an inexpensive and comparatively less toxic inorganic compound widely used in analytical chemistry and agriculture [27]. Previous studies have

demonstrated catalytic interactions between copper ions and chlorpyrifos, suggesting possible formation of copper-organophosphorus complexes [28-30]. This reagent has been applied by Clarke for identification of 25 sulphonamides by using simple thin layer chromatography [31]. Semi-quantitative detection of drugs and pesticides via thin layer chromatography coupled with digital image processing offers rapid, cost-effective alternative to standard densitometry [32-33]. However, no previous study has reported the use of copper sulphate as a chromogenic reagent for selective TLC detection of chlorpyrifos in biological specimens. Therefore, the present study was undertaken to develop a simple TLC-based qualitative and semi-quantitative method for chlorpyrifos detection in human blood and gastric aspirate samples using 10% copper sulphate solution as a chromogenic reagent. In addition to visual detection, digital image analysis using ImageJ software was employed for semi-quantitative evaluation of TLC spot intensity, estimation of chlorpyrifos concentration, and determination of analytical parameters such as calibration curve, limit of detection (LOD), and limit of quantification (LOQ).

MATERIALS AND METHODS

a. Chemicals and reagents

All the chemicals and solvents used were of analytical or chemically pure grade. Pre-coated TLC Aluminium plates (Silica gel 60 F₂₅₄, Merck Ltd., Darmstadt, Germany) were used for chromatographic analysis. Copper sulphate pentahydrate (CuSO₄·5H₂O) purchased from S.D. Fine Chem. Ltd., Mumbai, India. Standard control samples of various organophosphorus pesticides (Monocrotophos, Chlorpyrifos, Phorate, Profenophos, Dichlorvos, Quinalphos, Dimethoate, Ethion, Triazophos), pyrethroids (Cypermethrin, Deltamethrin, Transfluthrin, Allethrin, Permethrin) and herbicides (Paraquat dichloride, Glyphosate,

2,4-dichlorophenoxyacetic acid (2,4-D), Propaquizafop, Quizalofop ethyl,) were obtained from agrochemical suppliers.

Preparation of chromogenic reagent

A 10% copper sulphate solution was prepared by dissolving 10 gram of copper sulphate pentahydrate in 100 ml distilled water in a volumetric flask.

Collection of biological samples

Blood and gastric aspirate samples were collected from admitted poisoning patients with a clinical history suggestive of chlorpyrifos ingestion in the intensive care unit of a tertiary care hospital, Sewagram (India). Samples were forwarded to the toxicology laboratory for chemical analysis.

b. Extraction of chlorpyrifos from biological samples

Extraction from blood: Approximately 5 mL of blood sample was mixed with 5 mL of 10% sodium tungstate solution and 10 mL of 1 N sulphuric acid. The mixture was shaken for 10 minutes and centrifuged at $492 \times g$ (2000 rpm) for 5 minutes using a REMI R4C laboratory centrifuge. The supernatant was carefully transferred to a separating funnel and extracted with 10 mL of diethyl ether. The contents were gently shaken and allowed to separate into aqueous and organic layers. The upper organic (ether) layer containing the extracted chlorpyrifos was carefully collected and concentrated by evaporation under controlled conditions. The resulting residue was reconstituted in a suitable solvent for TLC analysis [35-36].

Extraction from gastric aspirate:

Approximately 10 mL of gastric aspirate was transferred to a separating funnel and extracted with 10 mL of diethyl ether. The mixture was gently shaken with intermittent venting and allowed to separate into aqueous and organic layers. The diethyl ether (organic) layer was

carefully collected. The extraction was repeated with another portion of diethyl ether, if sufficient sample was available, and the organic extracts were combined. The combined ether extract was evaporated to near dryness under a gentle stream of air/nitrogen at controlled temperature. The residue was reconstituted in an appropriate small volume of solvent for TLC analysis.

c. Preparation of standard chlorpyrifos solutions

Different concentrations of standard chlorpyrifos solution (1, 5, 10, 25, 50, 75, and 100 mg/mL) were prepared using standard chlorpyrifos and n-hexane as diluting solvent.

d. Thin-Layer Chromatography

Pre-coated silica gel TLC plates (10 cm × 10 cm) were used. 5 µl of each standard pesticide solutions and biological extracts were spotted using a micropipette. For organophosphorus insecticides and pyrethroids, the mobile phase consisted of hexane–acetone (8:2, v/v), whereas ethanol–water (7:3, v/v) was used for herbicides. The TLC chamber was saturated for 20 minutes before development. After chromatographic development, plates were dried at room temperature and sprayed with 10% copper sulphate reagent using a glass TLC sprayer. The overall procedure for detection of chlorpyrifos extracted from human biological fluids along with standard chlorpyrifos was repeated five times for validation of results. [Table 1] Retention factor (R_f) values were calculated using the standard formula:

$$R_f = \frac{\text{Distance travelled by the substance from the origin}}{\text{Distance travelled by the solvent front from the origin}}$$

2.5 Semi-quantitative analysis using ImageJ

TLC plates were photographed using a smartphone camera under standardized illumination and imaging conditions. The

captured images were analyzed using ImageJ software (National Institutes of Health, USA) for semi-quantitative estimation of chlorpyrifos. The image was first opened in ImageJ and converted to 8-bit gray scale. The image was subsequently inverted to facilitate visualization and densitometric analysis of the spots. Using the Rectangular Selection Tool, a rectangle of uniform dimensions was drawn around the first lane. The Analyze → Gels → Select First Lane command was then applied. The same rectangular selection was sequentially moved to each subsequent lane, and select Next Lane was used to define each lane.

After all lanes had been selected, the Analyze → Gels → Plot Lanes command was used to generate intensity profiles for the respective lanes. Each TLC spot appeared as a peak in the resulting intensity profile. The area under each peak was measured using the Wand (Tracing) Tool, providing an integrated pixel-area value corresponding to the intensity of the respective spot. For semi-quantitative estimation, a series of sequentially diluted chlorpyrifos standards were applied to the TLC plate at known concentrations ($\mu\text{g}/\text{spot}$). The corresponding integrated peak areas obtained from ImageJ were recorded. A calibration curve was constructed by plotting amount of sequentially diluted standard chlorpyrifos ($\mu\text{g}/\text{spot}$) against corresponding peak area (pixels).

Determination of LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the following equations:

$$\text{LOD} = \frac{3.3 \times \sigma}{S} \quad \text{and} \quad \text{LOQ} = \frac{10 \times \sigma}{S}$$

where σ represents the standard deviation of the lowest detectable concentration and S represents the slope of the calibration curve.

RESULT:

After spraying the developed TLC plates with 10% copper sulphate reagent, chlorpyrifos produced a distinct yellow coloured spot with an R_f value of 0.86. The R_f values and colour response of the residues extracted from blood and gastric aspirate exactly matched those of standard chlorpyrifos, confirming the presence of chlorpyrifos in the analyzed biological samples. Repeatability studies showed consistent R_f values with minimal deviation (± 0.01), demonstrating good reproducibility of the developed method [Figure 1 and Table 1].

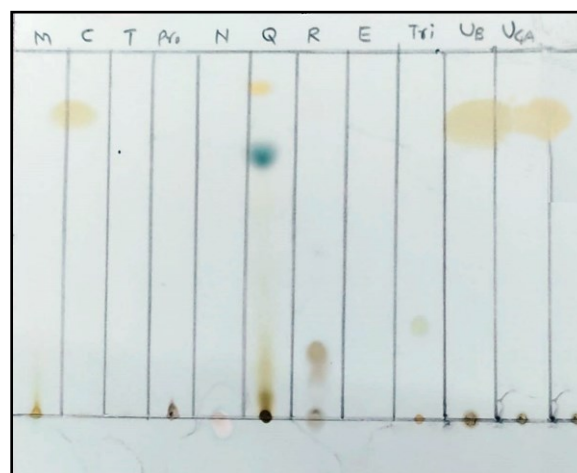


Figure 1: TLC plate image showing colour spot after spraying chromogenic reagent for organophosphorus insecticides where M= Monocrotophos, C=Chlorpyrifos, T=Thimet, Pro=Profenophos, N=Nuvan or dichlorvos, Q=Quinalphos, R=Roger or dimethoate, E=Ethion, Tri=Trizophos and UB= residue extracted from unknown blood & UGA= residue extracted from unknown gastric aspirate

Table 1: Method validation through repeatability

Number of trials	Rf value of standard chlorpyrifos	Rf value of blood extract	Rf value of gastric aspirate extract
1	0.86	0.87	0.86
2	0.86	0.86	0.87
3	0.86	0.86	0.86
4	0.86	0.86	0.86
5	0.86	0.86	0.86
Mean	0.86	0.86	0.86
±Deviation		0.01	0.01

Among the tested pesticides, chlorpyrifos, quinalphos, dimethoate, triazophos, and paraquat dichloride exhibited colour responses with copper sulphate reagent, whereas most other organophosphorus insecticides, pyrethroids, and herbicides showed no colour development. Chlorpyrifos produced the most distinct and selective yellow coloured spot [Table 2 and

figure 1,2,3]. It has been earlier reported that hexane-acetone (8:2, v/v) is the best developing solvent system for organophosphorus insecticides and pyrethroids insecticides due to their non-polar nature whereas developing system for herbicides is mixture of polar solvents [17-18,21].

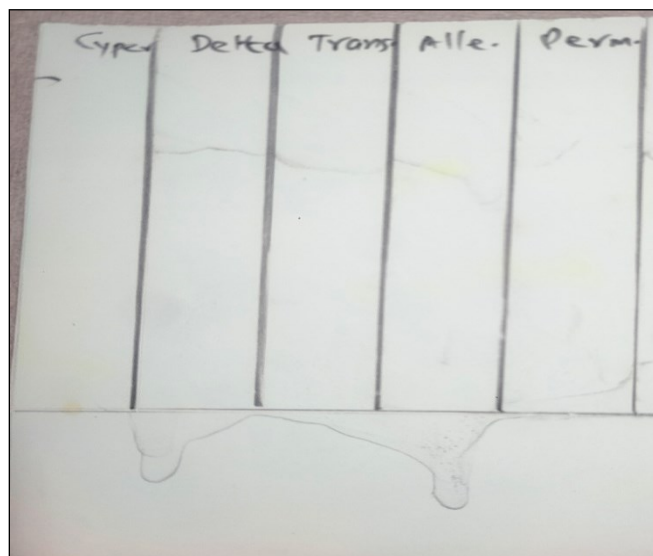


Figure 2: TLC plate image showing colour spot after spraying chromogenic reagent for pyrethroids where Cyper= Cypermethrin, Delta=Deltamethrin, Trans=Transluthrin, Alle=Allethrin, Perm=Permethrin

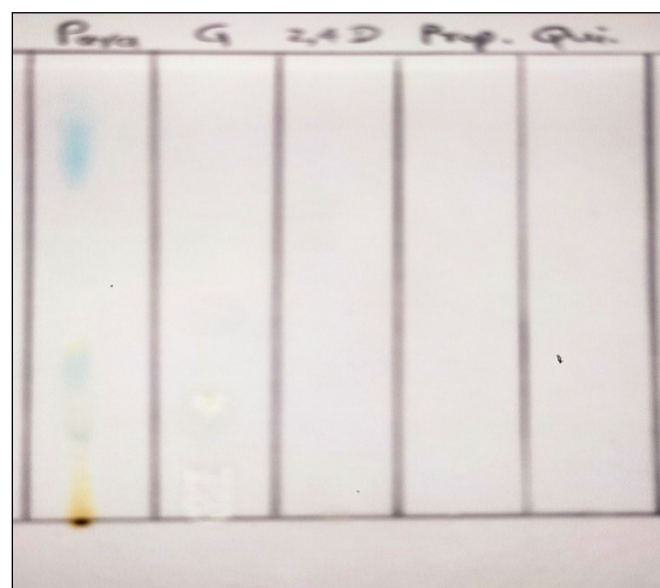


Figure 3: TLC plate image showing colour spot after spraying chromogenic reagent for herbicides where Para=Paraquat dichloride, G=Glyphosate, 2,4-D=2,4-Dichlorophenoxyacetic acid, Prop=Propaquizafop, Qui=Quizalofop ethyl

Table 2: Specificity assessment of developed chromogenic reagent against different pesticides and residue extracted from biological fluids

SN.	Compound	Pesticide class	Rf value	Colour response after spraying	Detection result
1	Monocrotophos (M)	Organophosphorus	-	No colour	Negative
2	Chlorpyrifos (C)	Organophosphorus	0.86	Yellow	Positive
3	Phorate or Thimet (T)	Organophosphorus	-	No colour	Negative
4	Profenophos (Pro)	Organophosphorus	-	No colour	Negative
5	Dichlorvos or Nuvan (N)	Organophosphorus	-	No colour	Negative
6	Quinalphos (Q)	Organophosphorus	0.9	Yellow-blue	Positive
7	Dimethoate or Roger (R)	Organophosphorus	0.14	Faint black	Weak
8	Ethion (E)	Organophosphorus	-	No colour	Negative
9	Triazophos (Tri)	Organophosphorus	0.26	Faint Yellow	Weak
10	Cypermethrin (Cyper)	Pyrethroid	-	No colour	Negative
11	Deltamethrin (Delta)	Pyrethroid	-	No colour	Negative
12	Transfluthrin (Trans)	Pyrethroid	-	No colour	Negative
13	Allethrin (Alle)	Pyrethroid	-	No colour	Negative
14	Permethrin (Perm)	Pyrethroid	-	No colour	Negative
15	Paraquat dichloride (Para)	Herbicide	0.76	Blue	Positive
16	Glyphosate (G)	Herbicide	-	No colour	Negative
17	2,4-Dichlorophenoxyacetic acid (2,4-D)	Herbicide	-	No colour	Negative
18	Propaquizafop (Prop)	Herbicide	-	No colour	Negative
19	Quizalofop Ethyl (Qui)	Herbicide	-	No colour	Negative
20	Residues extracted from unknown blood (U_B)	Organophosphorous	0.86	Yellow	Positive
21	Residue extracted from unknown gastric aspirate (U_{GA})	Organophosphorous	0.86	Yellow	Positive

Sequential dilution studies revealed progressive enhancement in spot intensity with increasing chlorpyrifos concentration and lowest visually detectable concentration was 5 mg/mL, corresponding to 25 μ g/spot [Figure 4]. Figure 5 and 6 shows the image processing for determination of peak area under spot of sequentially diluted chlorpyrifos for calibration plotting and unknown chlorpyrifos residues extracted from biological samples using ImageJ software. By plotting calibration curve, it is seen that a good linear relationship between chlorpyrifos concentration and spot peak area, represented by the regression

equation: $y=70.86x+2359$, where y is peak area and x is amount of chlorpyrifos (μ g/spot) [Figure 7]. The coefficient of determination (R^2) was found to be 0.952, indicating satisfactory linearity. Table 3 and table 4 also shows the peak areas, average area, standard deviation of sequentially diluted standard chlorpyrifos and peak areas under spot of unknown chlorpyrifos residues extracted from biological samples using ImageJ. It is observed that as the amount of chlorpyrifos increases, the area under the spot also increases.

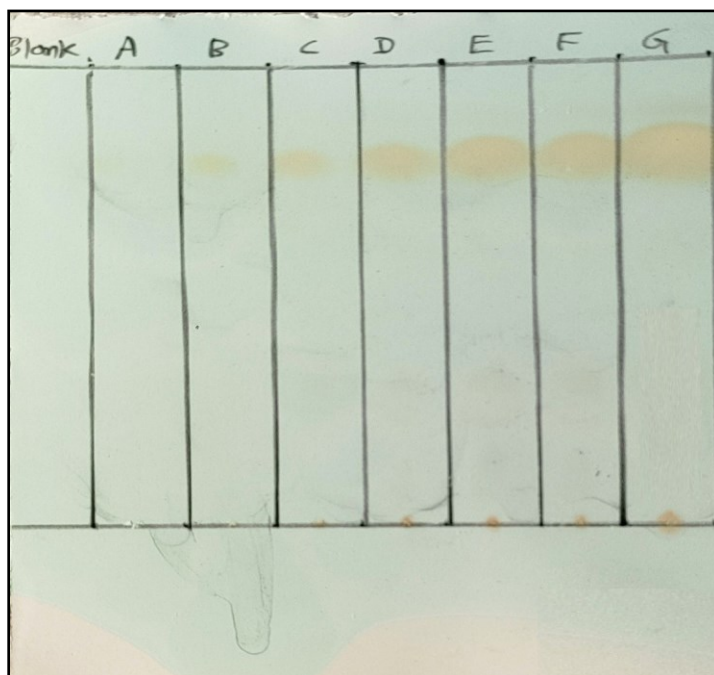


Figure 4: Picture of TLC plate results of sequentially diluted chlorpyrifos (where Blank=No sample, A=1 mg/ml, B=5 mg/ml, C=10 mg/ml, D=25 mg/ml, E=50 mg/ml, F= 75mg/ml, G=100mg/ml)

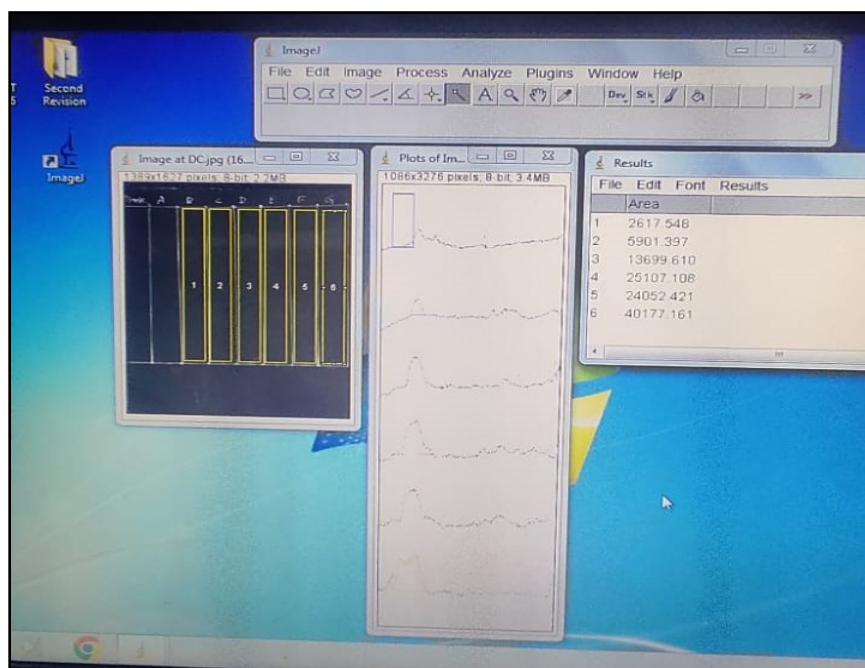


Figure 5: Image processing for the determination of peak area of sequentially diluted standard chlorpyrifos using ImageJ

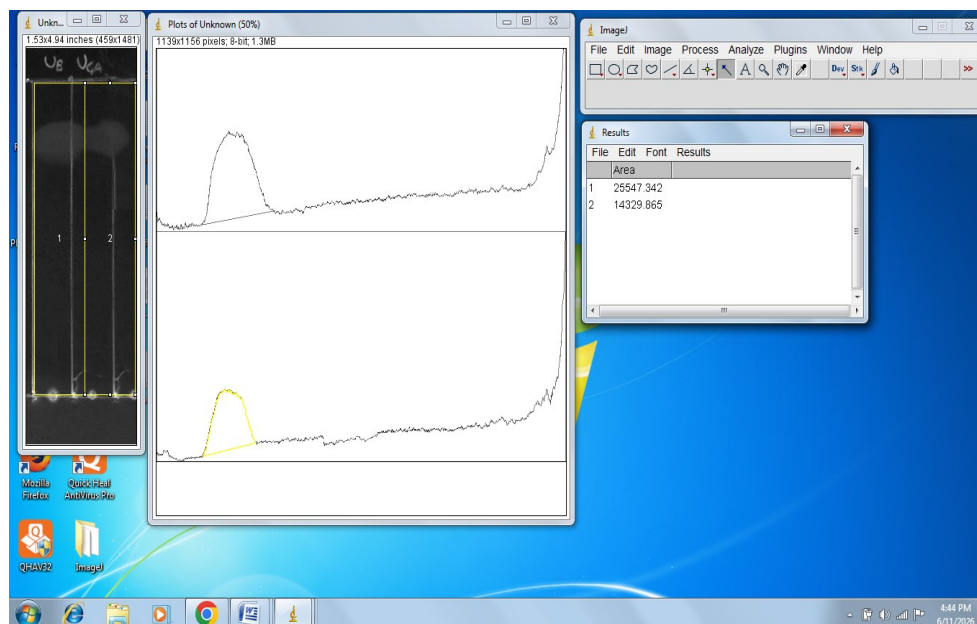


Figure 6: Image processing for the determination of peak area of chlorpyrifos residues extracted from unknown blood and gastric aspirate using ImageJ

Calibration curve-

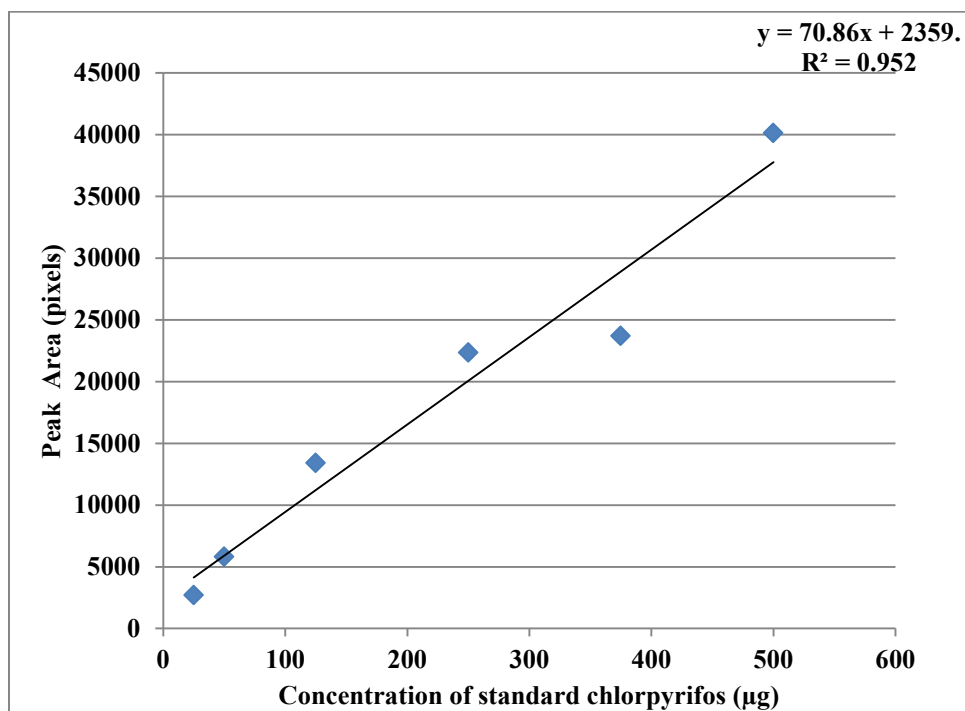


Figure 7: Calibration curve of amount of chlorpyrifos (µg/spot) against peak area (pixels)

Table 3: Determination of peak area of sequentially diluted standard chlorpyrifos using ImageJ

Concentration of Standard chlorpyrifos (mg/ml)	Amount per spot (µg)	Rf value	Peak area under the spot (Pixels)			Average area (Pixels)	Standard deviation (σ)
			Area 1 (Pixels)	Area 2 (Pixels)	Area 3 (Pixels)		
1	5	-	No peak	No peak	No peak	-	-
5	25	0.86	2780.548	2617.548	2707.669	2701.921	81.65185
10	50	0.86	5764.983	5901.397	5733.933	5800.104	89.08523
25	125	0.86	13751.024	13699.610	12728.246	13392.960	576.2329
50	250	0.86	23990.986	25107.108	17897.631	22331.908	3880.534
75	375	0.86	24827.836	24052.421	22198.844	23693.033	1350.84
100	500	0.86	40029.383	40177.161	40181.212	40129.252	86.51281

Table 4: Determination of peak area of unknown chlorpyrifos extracted from biological fluids using ImageJ

Unknown sample	Rf value	Peak area under the spot (Average area in Pixels)
Chlorpyrifos residue extracted from unknown blood (U _B)	0.86	25547.342
Chlorpyrifos residue extracted from unknown gastric aspirate (U _{GA})	0.86	14329.865

Calculations of LOD and LOQ-

From regression equation: $y=70.86x+2359$, slope of the calibration curve (s) = 70.86 and Intercept = 2359. The detectable concentration of standard chlorpyrifos by this method is found to be 5 mg/ml corresponding to 25 µg/spot and standard deviation (σ) related to this low concentration sample is 81.65185.

Hence, $LOD = \frac{3.3 \times \sigma}{S} = \frac{3.3 \times 81.65185}{70.86} = 3.8025 \text{ µg/spot}$ and

$LOQ = \frac{10 \times \sigma}{S} = \frac{10 \times 81.65185}{70.86} = 11.5229 \text{ µg/spot}$.

Calculations of estimated amount of chlorpyrifos in unknown blood and gastric aspirate samples-

To estimate the concentration from calibration curve, we use regression equation i.e.

$$y=70.86x+2359$$

where y=peak area and x=amount of chlorpyrifos (µg/spot)

On rearranging equation: $x = \frac{y-2359}{70.86}$

1) For unknown blood sample (U_B)-Peak area (y) = 25547.342 pixel,

Hence amount of chlorpyrifos(x) = $\frac{25547.342-2359}{70.86} = 327.2416 \text{ µg/spot}$

2) For unknown gastric aspirate sample (U_{GA})-Peak area (y) = 14329.865 pixel,

Hence amount of chlorpyrifos(x) = $\frac{14329.865-2359}{70.86} = 168.9368 \text{ µg/spot}$

The amount of chlorpyrifos in the blood extract was estimated as 327.2416µg/spot, whereas the gastric aspirate extract showed 168.9368µg/spot.

The present study shows that conventional TLC combined with 10% copper sulphate reagent can serve as a simple and economical approach for rapid detection of chlorpyrifos in biological specimens. The developed chromogenic reagent produced a distinct yellow coloured spot specifically for chlorpyrifos, which matched the poison residues extracted from blood and gastric aspirate samples. The observed agreement in Rf values, colour intensity, and spot morphology between standard chlorpyrifos and unknown extracts strongly supports the identification of chlorpyrifos in the analyzed specimens [26]. The selectivity of the developed reagent may be attributed to the interaction between copper ions and phosphorothioate-containing organophosphorus compounds. Chlorpyrifos,

quinalphos, dimethoate, and triazophos contain phosphorothioate groups capable of forming Cu–S coordinated complexes, resulting in coloured spot formation [30, 34] [Figure 8]. However, chlorpyrifos demonstrated the most characteristic and intense colour response, making the method particularly useful for targeted chlorpyrifos screening.

ImageJ based semiquantitative analysis significantly improved the objectivity of TLC spot evaluation compared with visual observation alone. The calibration curve exhibited satisfactory linearity ($R^2 = 0.952$), indicating that TLC spot density correlates reasonably

well with chlorpyrifos concentration. The slight deviation from linearity at higher concentrations may be due to saturation effects. The calculated LOD ($3.8025 \mu\text{g/spot}$) and LOQ ($11.5229 \mu\text{g/spot}$) suggest acceptable analytical sensitivity for preliminary toxicological screening applications. However, these values represent TLC plate detection sensitivity under the described chromatographic conditions. This method offers several advantages including low cost, minimal instrumentation requirements, rapid analysis, and ease of implementation in routine toxicology laboratories.

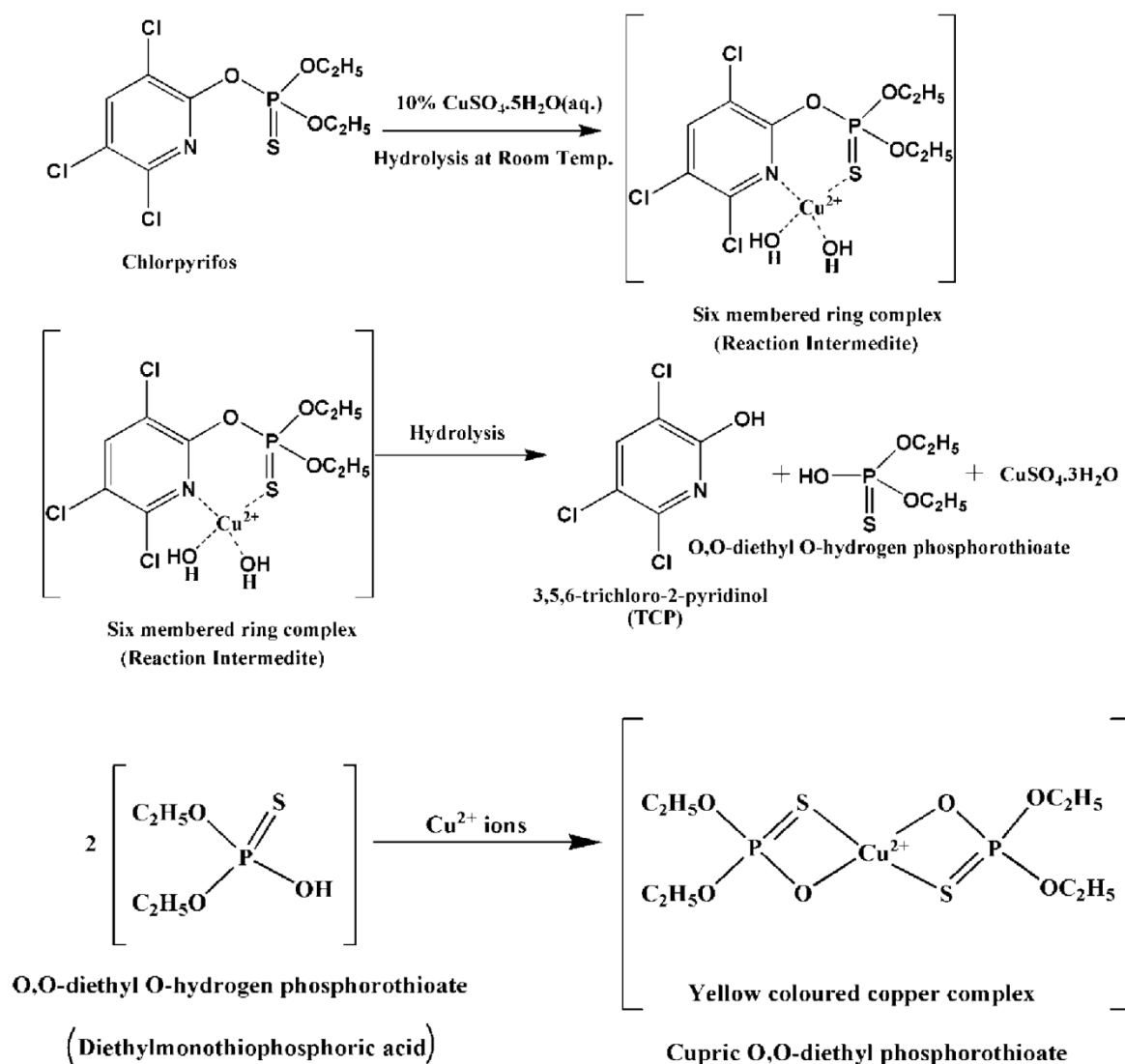


Figure 8: Illustration of the reaction between chlorpyrifos and copper sulphate solution

CONCLUSION

The present study successfully developed a simple, rapid and cost-effective TLC-based method for qualitative and semiquantitative detection of chlorpyrifos in human biological fluids such as blood and gastric aspirate. The use of 10% copper sulphate solution as a chromogenic reagent enabled selective visualization of chlorpyrifos as a characteristic yellow coloured spot. The developed method demonstrated

good repeatability, satisfactory selectivity, and acceptable analytical sensitivity for preliminary toxicological screening. ImageJ-assisted semiquantitative analysis further enhanced the applicability of the method by enabling estimation of chlorpyrifos concentration through TLC spot density measurements. The calculated LOD and LOQ values confirmed the utility of the method for detecting low

levels of chlorpyrifos under routine laboratory conditions. Because of its simplicity, affordability, and minimal instrumentation requirements, the developed method may be particularly valuable in resource-limited clinical toxicology to save the life of poisoning patients and forensic laboratories for rapid preliminary identification of chlorpyrifos poisoning.

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