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Separation and Detection of Monocrotophos by Chromatography Methods in Forensic Samples

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Abstract

Monocrotophos is a extremely hazardous organophosphate insecticide that is extensively used and easily accessible in India. Monocrotophos is also simply available in the marketplace and occasionally encountered in accidental /suicidal cases. Being also a persistent organic pollutant, it has banned in the U.S. and many other countries. In the present study we have encountered through a poisoning case and separation and detection of pesticides from viscera sample which have been a tedious task for forensic toxicologists. For routine forensic toxicological work, the detection of organophosphorus insecticides is achieved by thin layer chromatography (TLC) because of its simplicity and rapidity. In this case the pesticide was extracted from viscera by solvent extraction method and separated and detected by TLC, High-Performance Liquid Chromatography (HPLC) and Gas chromatography-mass spectrometry (GC-MS) methods. TLC is a treasured technique as a preliminary screening method to narrow the possible identities of unknown drugs in biological samples. We succeeded in separation of the encountered pesticide monocrotophos. It can also be separated by using a suitable mobile phase/stationary phase. HPLC offers a cost-effective technique with the ruggedness and reliability necessary for forensic testing and consequently is widely used in forensic laboratories today. GC-MS is one of the most generally used methods for the identification and quantification of forensic samples. As a "hyphenated" method, it trusts the separation power of a GC with the analyte specificity of a spectroscopic technique, providing highly specific spectral data on different compounds in a complex mixture often without prior separation. In this study, TLC, HPLC and GC-MS have been employed for the investigation of monocrotophos in viscera. Veteran forensic toxicologists trust on their own case involvement as well as the unique circumstances of each case under examination.

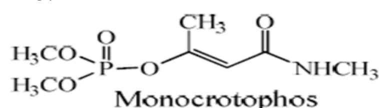
Keywords: Monocrotophos; Separation; Detection; TLC; HPLC; GC-MS

Introduction

Monocrotophos is classified by world health organization (WHO) is highly hazardous and has been responsible for deaths resulting from accidental or intentional exposure. Poisoning due to insecticides and pesticides has an important role in crimes all over the world. Toxicologists are dealing with maximum number of cases due to insecticides and pesticide poisoning involved in various types of crimes. Pesticides also cause much danger to the surrounding environments and other organisms. Since the study of insecticide residue poses an entirely different type of problem for the toxicologists because these residues are present in extremely small amount in heterogeneous materials including the biological materials. The significance of insecticide residue problem led to severe search for analytical methods for accurate and rapid analysis.

On July 16, 2013, children in Gandaman village in Bihar, one of the poorest states of India, fell violently sick after eating a school lunch. Twenty-three died, many within a few hours of eating. The children most likely died from an acute cholinergic syndrome due to intoxication with the pesti¬cide monocrotophos. The source of contamination is still unclear, but forensic analysis reported high levels of mono¬crotophos in the cooking oil used for preparing the school lunches, and a container previously used to store pesticides was found in the kitchen area. According to press reports [1], the school cook had complained about the colour and the smell of the newly delivered cooking oil, but the school principle negated the problem. It appears that the cooking oil was bought from a store owned by the husband of the school principal.

Monocrotophos (nuvacron, azodrin) is widely used in agriculture to protect crops from insects, similar to other organophosphorus insecticides, for example malathion, parathion, methyl parathion, fenthion, and methyl demeton. Consequently, characterization of this insecticide is necessary in forensic toxicology. The chemical structure of monocrotophos is;



Monocrotophos, dimethyl (E)-1-methyl-2-methylcarbamoyl vinyl phosphate, is a chemical belonging to the group of organophosphates. Many organophosphates are acetyl¬choline esterase inhibitors and cause an acute cholinergic syndrome through increased concentrations of the excita¬tory neurotransmitter acetylcholine in synapses and neu¬romuscular junctions (thus acting much like chemical weapons such as soman, VX, and sarin). Monocrotophos is insecticidal, but also highly toxic to other animals, including birds and mammals. Its use has been banned from the United States and subsequently from many Western countries, but it is still widely used in South America, as well as some African and Asian countries including India.

An organo phosphorous group has large application in the field of agriculture and is highly toxic for animal and human beings. Nowadays, monocrotophos is largely used for protection of crop. During 2007 Forensic Science Laboratory, Maharashtra (India) had detected 1500 cases due to organo phosphorous poisoning [2] out of which 275 cases of human poisoning was by monocrotophos. The increasing number of biological samples for poison detection need versatile aware and selective reagent.

Baumler and Ripprtain [3] have reported the use of palladium chloride for the detection of organophosphorous insecticides. Kawale *et al*. [4] have reported mercurous nitrate reagent and Joglekar *et al*. [5] have also reported mercuric nitrate-diphenyl carbazone reagent, which were used for the detection of derivatives of barbituric acid were further utilized for detection of organophosphorous and organothiophosphate insecticides.

Spectrophotometric method for estimation of monocrotophos [6] is also reported in literature. However, these reagent and method are not selective, hence highly selective and sensitive reagent is needed for monocrotophos estimation. Janghel [7] has reported spectrophotometric method for the determination of monocrotophos. The method is based on alkaline hydrolysis of monocrotophos to N-methylacetoacetamide followed by coupling with diazotized p-amino acetophenone in alkaline medium. The absorption maxima of the reddish-violet colored compound formed are measured at 560 nm.Chandegaonkar *et al*. [8] have reported detection of monocrotophos after TLC is possible by use of vanillin as reagent. The product from alkaline hydrolysis of monocrotophos reacts with vanillin to give a green compound. Chandegaonkar *et al*. [9] have also reported detection of monocrotophos by TLC using 5% sodium hydroxide solution then 5% benzil reagent.

Uma Maheswara Rao and Prameela Devi [10] have developed a simple, low cost, viable and sensitive enzymatic method for detection, separation and identification of an organophosphorus pesticide, monocrotophos from environmental samples. Gundi and Reddy [11] were studied the degradation of a widely used organophosphorous insecticide monocrotophos (dimethyl (E)-1-methyl-2-methylcarbamoyl vinyl phosphate) in two Indian agricultural soils at two concentration levels, 10 and 100 g^{-1} soil under aerobic conditions at 60% water-holding capacity at 28 ± 4 °C was studied in a laboratory. Ruin of monocrotophos in soils proceeded by hydrolysis with formation of N-methylacetoacetamide. Kulkarni *et al*. [12] have studied a new chromogenic spray reagent for chromatographic detection and identification of monocrotophos, an organophosphorus insecticide is described by HPTLC method.

Mee Kyung Kim *et al*. [13] have developed a quick, easy, cheap, effective, rugged and safe (QuEChERS) method for sample preparation was applied to determine seven organophosphorus pesticides (OPs) in stomach contents of poisoned postmortem animals. Rishi Pal *et al*. [14] have carried out a multi pesticide residues analysis for farmgate samples of okra fruits from four markets places of Meerut region. The objective of this study is to utilize the universal, rapid, precise and sensitive TLC, HPLC and GC-MS methods for the separation and detection of monocrotophos. Hence, the current investigation concluded by representing the significant analytical work that could be used for the separation and detection by the aforementioned chromatographic methods in pesticides/insecticides in forensic samples or in any confiscated materials.

Materials and Methods

Reagents and Chemicals

All of the solvents *i.e.* rectified sprit (Qualigens Fine chemicals, India), chloroform (S.D.Fine chemicals, India), Benzene (Rankem, India), hexane (Rankem, India), acetone (Glaxo Laboratories, India), cyclohexane (Loba Chemie, India), bromine (Ranbaxy, India), congo red (Merck, India), p-Chloranil (Loba Chemie, India), Fast blue B salt (Merck, India), sodium hydroxide (NICE, chemicals, India) sodium carbonate (NICE, chemicals, India) and reagents used were of analytical reagent grade. Deionized water was used to prepare all solutions. Freshly prepared solutions were always employed.

Preparation of Reagents

Chloranil Reagent: (i) 0.5 g of p-chloranil (2,3,5,6-tetrachloro cyclohexa-2,5-diene-1,4-dione) was dissolved in 100 mL of acetone. (ii) 0.25 g of sodium carbonate was dissolved in 100 mL deionized water.

Congo Red Reagent: 2 mg of congo red was dissolved in 100 mL of rectified sprit and 100 mL of deionized water and filtered using Whatmann No. 40 filter paper.

Fast Blue B Reagent: 1 mg of Fast blue B salt (o-dianisidine bis(diazotized) zinc double salt) was dissolved in 5% of sodium hydroxide solution.

Forensic Sample Preparation: Viscera were generally cut into small pieces and soaked well in chloroform at overnight then decanted. The decanted liquid was treated with charcoal and anhydrous sodium sulphate through Whatmann No.40 filter paper to remove fat, if any and kept in water bath to get concentration. The concentrated extract was screened for the presence of Monocrotophos using TLC, HPLC and GC-MS methods.

TLC Analysis

TLC is a rapid investigative tool by which results can be available with minimum time and can be handled with minimal operator training and cost for toxicological application. It is commonly used as a screening test for various pesticides, which is a reliable qualitative test and the test results are admissible as forensic evidence, in the court of law.

The plates were divided into 1.5 cm wide strips and solute spots were applied using a calibrated Hamilton syringe. Then, 2 μL of each organophosphorus insecticides and sample extract were spotted on the TLC plates as separate spots. The plates were developed in a closed glass sintered chamber containing developing solvents with 30 minutes saturation time (listed in Table 1), at 25-30 ^{o}C temperature.

The solvent in the chamber was allowed to reach the lower edge of the adsorbent though the spot points were not allowed to be immersed. The cover was put in place, and the system was maintained until the solvent ascended to a point 10 to 15 cm above the initial spots; this usually required about 30 to 90 minutes. The plate was removed from the developing chamber when the solvent reached solvent front and later was dried in air.

HPLC Analysis

Chromatographic identification was performed at room temperature (25 ± 1°C) with Perkin Elmer NFLR 0400, Flexar Quartenary -10 LC platform delivery system and a programmable UV-visible variable - wavelength was used with Kit-Flexar manual sample injector. The Chromatographic identification was achieved on a (250×4.6 mm) RP C_{18} column (Phenomenex, USA) having a 5 μm packing as a stationary phase. The elution was performed at a flow rate of 5 mL/min with UV detection at 254 nm. Samples (5 μL) were injected using Kit-Flexar manual sampler. The data acquisition and processing were carried out on Chromera software.

In order to evaluate an efficient and universal HPLC method, preliminary tests were performed with the objective to select adequate and optimal conditions. Parameters, such as optimal mobile phase, optimum pH, are preferably analysed by reverse phase columns and accordingly C_{18} column was selected. The concentration of methanol and acetonitrile were optimized to give symmetric peak with short run time based on asymmetric factor and peak area obtained.

GC-MS Analysis

The primary goal of the forensic drug examiner is the unequivocal identification of any controlled substance present in a forensic exhibit. Most forensic laboratories routinely employ GC/MS as the preferred method for this examination. The technique offers a rapid, semi-automated analysis of the sample and typically yields sufficient information to identify the compounds in question. However, the application of GC/MS for pesticide analysis does have its limitations.

GC-MS instrument chromatographic analysis was carried out on an Agilent 7820A GC system equipped with 5977E mass selective detector (MSD) with triple axis detector (TAD). It optimizes signal-to-noise by combining efficient ion collection and amplification with the elimination of neutral noise. DB-5 column width 0.25 μm film thickness (30 m × 0.25 mm I.D., Germany) was used for separation. Spitless injection was used and the carrier gas was helium at a flow rate of 1.2 mL/min. The data was acquired and processed by Agilent DA Express data analysis software and mass spectra libraries are screened for identification of pesticides and insecticides.

Results and Discussion

Like other organophosphorus insecticides such as malathion, parathion, methyl parathion, fenthion and methyl demeton, monocrotophos is widely used in agriculture sector to protect the crop from insects. Owing to their easy availability, they are often misused for homicidal or suicidal purposes.

Various methods have been reported in the literature for the detection and determination of monocrotophos insecticides, those includes, TLC [5], spectrophotometry [6,7], GC [13], etc. But all these methods are reported for pure compounds or for formulated products or for extract from water samples, grains etc and are highly susceptible to the impurities co extracted with insecticides from biological materials (viscera, blood, urine and stomach wash) in poisoning cases, however an elaborate clean up procedure is essential for their detection and determination in biological materials by above reported methods.

The TLC plates were removed from the developing chamber when the solvent reached solvent front and later were dried in air. One of the plates has been sprayed with p-chloranil reagent in which the sample and the control monocrotophos were only formed red color spot. The second plate has been sprayed with fast blue B reagent in which the Sample and the control monocrotophos were only formed yellow color spot.

p-chloranil test has been performed for the Sample and monocrotophos which were treated with 0.25% Sodium carbonate solution followed by 0.5% *p*-chloranil in acetone, the sample and the control monocrotophos (dimethyl (E)-1-methyl-2-ethylcarbamoyl vinyl phosphate) were only formed red color and other organophosphorus insecticides did not appear red color.

Hence TLC is the method of choice for the detection and determination of these insecticides in biological and non-biological materials in poisoning cases. TLC method can be successfully used for the separation, detection and determination of insecticide residues in biological and non-biological materials. Most TLC analyses have been carried out by traditional one-dimensional ascending development technique in a glass tank at ambient temperature. The analysis is performed on silica gel G particle size 15 m or high-performance silica gel layers including an organic binder but chemically bonded or impregnated layers.

As far as Forensic concern, TLC is frequently used as an inevitable screening tool. It is quick, easy to use, has a low cost, is relatively sensitive and can give a good degree of discrimination. Considering these problems; the TLC method was developed for the detection and identification of organophosphorus insecticide monocrotophos (nuvacron) in biological materials. The method is specific for monocrotophos only. Other organochlorine, carbamate and pyrethroid insecticide do not interfere.

Monocrotophos is a widely used and extremely dangerous insecticide also it is highly toxic orally as well by inhalation or absorption through skin. The acute dermal toxicity and severe poisoning will affect on central nervous system. Like other OPs, monocrotophos is potent cholinesterase inhibitor [15]. Its low cost and many applications will present a challenge to users looking for safer alternatives, or measures which will protect health [16]. Owing to its high poisoning capacity, characterization of this insecticide from toxicological point of is need for forensic laboratory.

The results of the present investigation are given in Table 1. It is observed that amongst the used solvents, the Benzene: Acetone (9: 1), Cyclohexane: Acetone: Chloroform (70: 25: 5) and Hexane: Acetone (8: 2) followed by bromination, debromination and spray congo red gave clearer and easily interpretable results (Figure 1).

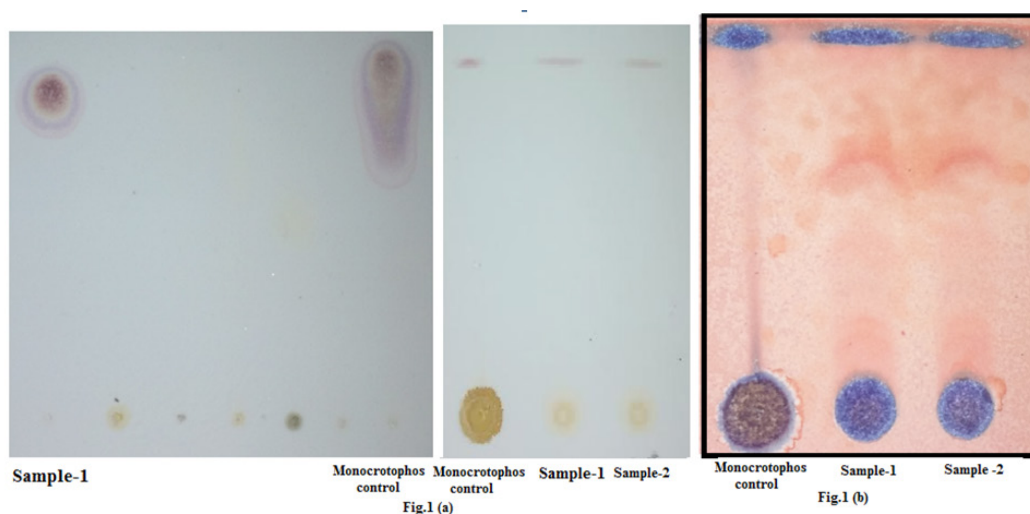


Figure 1: TLC chromatogram of Monocrotophos sample, System: Hexane: Acetone (4:1)
Figure 1 (a): Prior to spraying Congo red
Figure 1 (b): After spraying Congo red Monocrotophos from visceral exract Samples - 1 & 2

The reference standards and first portion of chloroforms extract were spotted on two different silica gel F_{254} TLC plates at a distance of 2 cm from base and elution was carried out up to 16 cm using two different mobile phases. After elution the plates were taken out and dried at room temperature. The TLC plates were visualized under ultraviolet light (254 nm) followed by spraying with developing reagents. Non-destructive procedure, such as the use of ultraviolet light (both 254 nm and 356 nm) was used for the localization of separated spots. Next, the plates were sprayed with three different chromogenic reagents (visualization reagent namely; congo red, choranil reagent, palladium (II) chloride.

Mobile phases	Rf	Observed Colour after bromination
Benzene: Acetone (9: 1),	0.75	Yellow, Violet
Cyclohexane: Acetone: Chloroform (65:25:5)	0.75	Yellow, Violet
Hexane: Acetone (8: 2)	0.75	Yellow, Violet

Table 1: The R_f values of monocrotophos on silica gel G UV_{254}

The R_f values in various solvents and the colors developed at each stage for monocrotophos is given in Table 1. After bromination Figures 1 and 1 (a) show the observed colors of yellow and violet. The yellow colour is fragmentation of carbamide ion of monocrotophos and violet color (R_f 0.75) is fragmentation of organophostate ion of the same. In Figure 1 (b) fragmentation of organophostate ions are present at base and top position in TLC plate as Dimethoate shows multiple spots from base line. The above mentioned three systems were recorded for the separation and detection of monocrotophos. For routine forensic toxicological work detection of organophosphorus insecticides is achieved by TLC because of its simplicity and rapidity. Other methods *viz.* GC, HPLC has been reported in the literature but there are limitations to their use in routine forensic work owe to the complex

matrix which may damage the columns. Besides TLC can be used to screen many samples in the time taken by these instrumental methods to screen one sample only. Hence TLC is the method of choice for screening biological samples.

The determination of the pesticide in different biological materials often faced with the problem of determining the minute quantity of insecticides mixed with large amount of extraneous material or intermixing. The technique of TLC has made a strong impact on analytical toxicology (Figure 2, 3 and 4).

In this study, mobile phases and with its composition, which have shown in Table 2 to separate and to detect monocrotophos. The relative retention times recorded for the studied insecticide is given in Table 3.

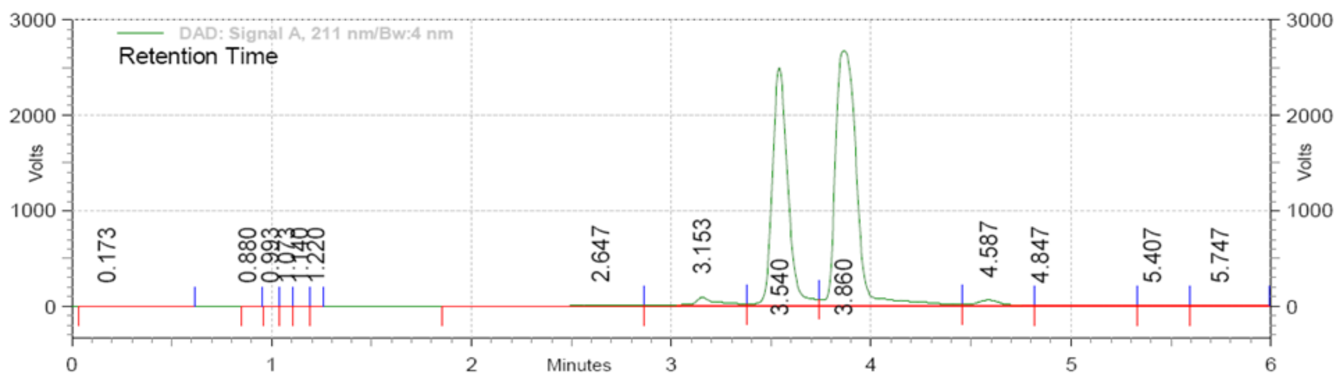


Figure 2: Chromatogram of the control monochrotophos in mobile phase A at λ_{max} 211 nm

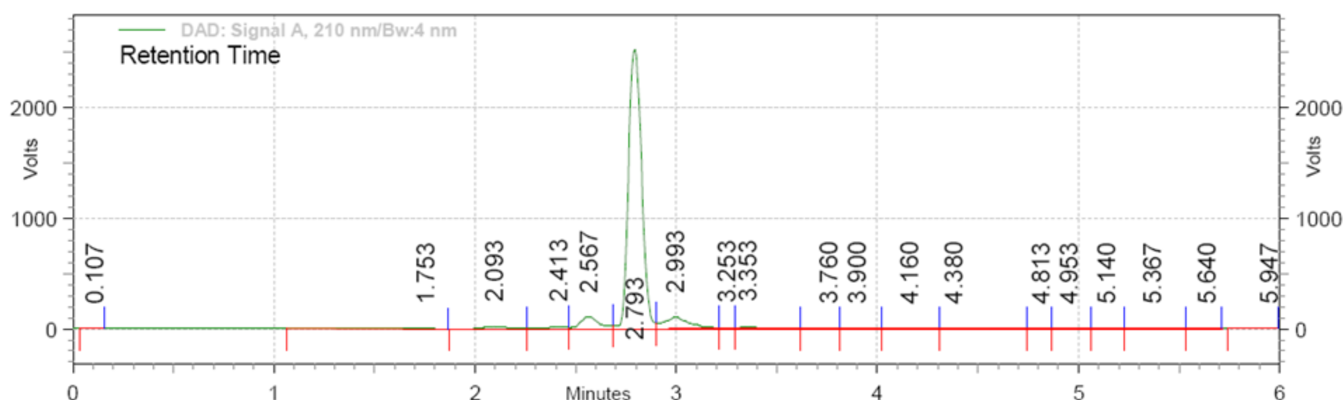


Figure 3: Chromatogram of the encountered forensic sample (monochrotophos) in mobile phase A at λ_{max} 210 nm

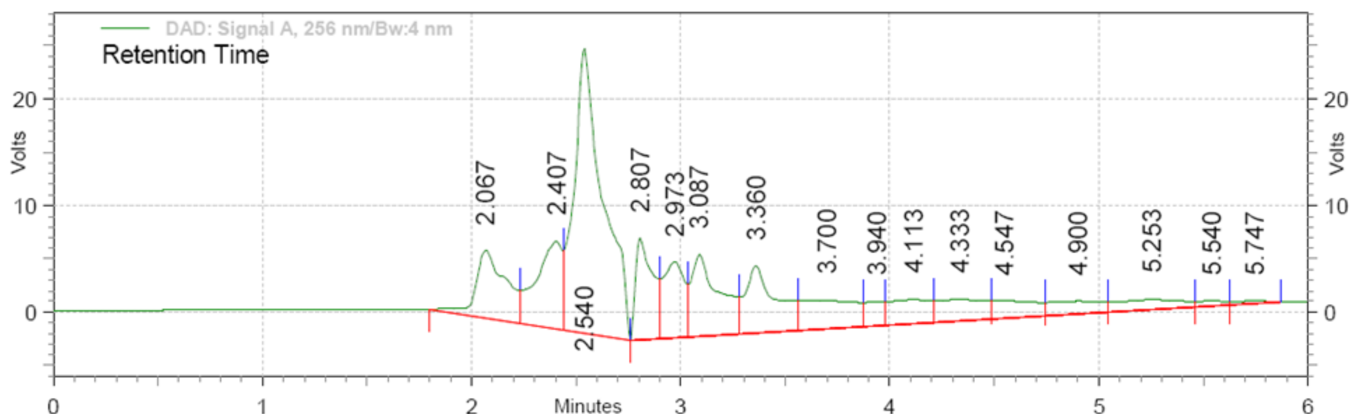


Figure 4: Chromatogram of the encountered forensic sample (monochrotophos) in mobile phase A at λ_{max} 256 nm

Mobile phases	Retention times (minute)		
	Monocrotophos at λ_{max} 211 nm	**Monocrotophosat λ_{max} 210 nm.**	**Monocrotophos at λ_{max} 256 nm**
A	3.54, 3.86	2.56, 2.79, 2.99, 3.25, 3.35, **3.76** and **3.90**	2.06, 2.40, 2.54, 2.80, 2.97, 3.08, 3.36, **3.7** and **3.94**

Table 3: The relative retention time for the studied monocrotophos at different λ_{max}

Various mobile phases were examined but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase methanol: water 60:40(v/v). The obtained chromatogram for this system shown in Figure 2 which has been observed at λ_{max} 211 nm. The retention time of monocrotophos was found to be, **3.54** and **3.86** mins, respectively with a good baseline.

In the obtained chromatogram for this system shown in Figure 3 which has been observed at λ_{max} 210 nm. The retention time of monocrotophos was found to be, 2.56, 2.79,2.99, 3.25, 3.35, 3.76 and 3.90 mins, respectively with a good baseline.

In the obtained chromatogram for this system shown in Figure 4 which has been observed at λ_{max} 256 nm. The retention time of monocrotophos was found to be, 2.06, 2.40, 2.54, 2.80, 2.97, 3.08, 3.36, 3.7 and 3.94 mins, respectively with a good baseline.

A good reliable separation has also been achieved with the mobile phase methanol: water 60:40 (v/v). The obtained chromatogram for this system is shown in Figure. 2. The retention time of monocrotophos was found to be, **3.54** and **3.86** minutes, respectively, indicates a good baseline. So, the HPLC chromatogram will be obtained with a good resolution with base line at the λ_{max} 211 nm which was the optimum wavelength in the UV detector. Limit of detection was 0.32 mg/kg.

Linking gas chromatography to mass spectrometry (GC-MS) to obtain and compare retention times from a standard to the unknown has also been used to provide compound identification. The oven temperature is programmed by holding at 100 ^{o}C to 270 ^{o}C at a rate of 10 ^{o}C per min. The injector temperature with split less mode was set at 250 ^{o}C. The carrier gas was the ultra high purity helium gas (Airgas) at a low rate of 1.2mL/min. Data analysis was conducted using Agilent DA Express data analysis software.

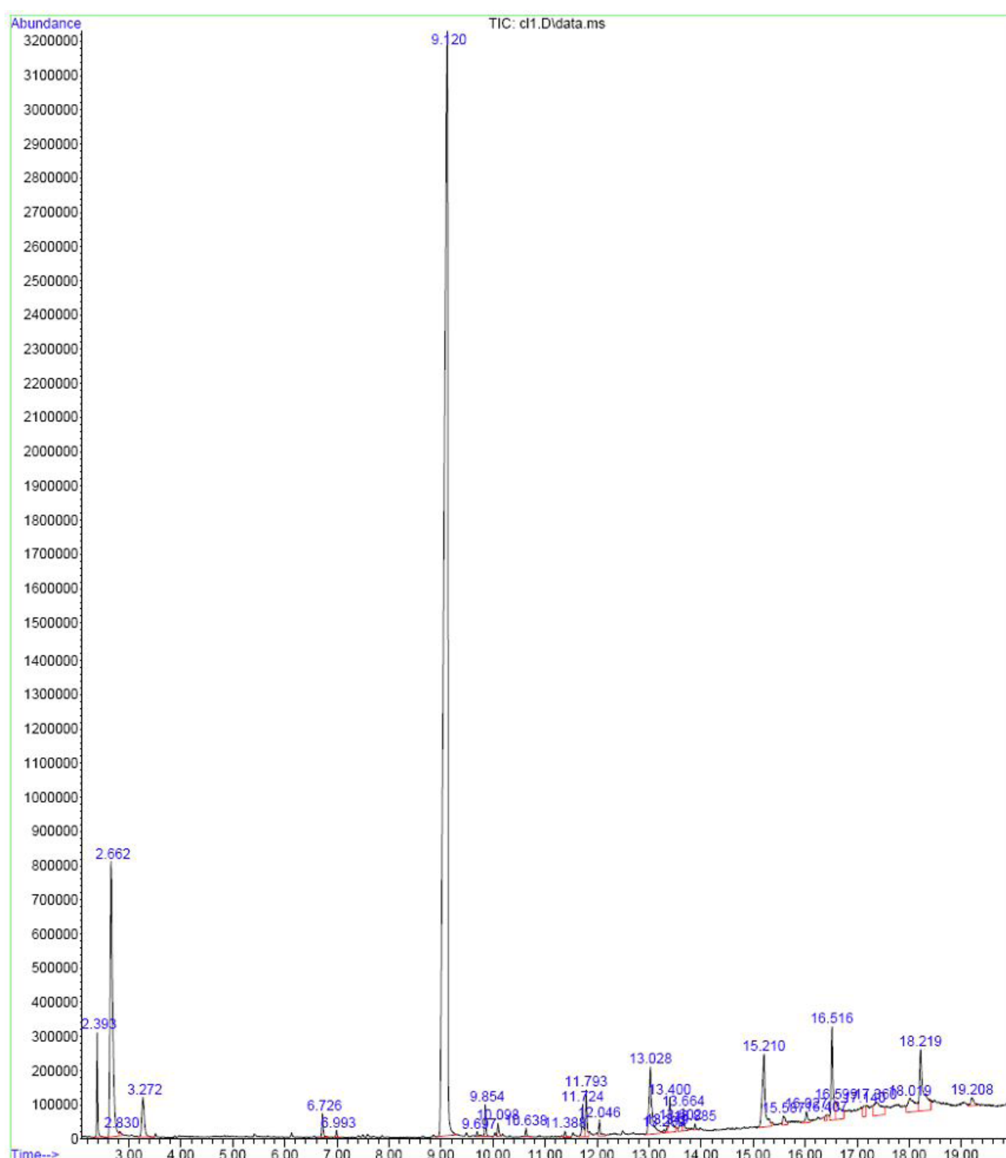


Figure 5: GC-MS chromatogram of monocrotophos (forensic) samples

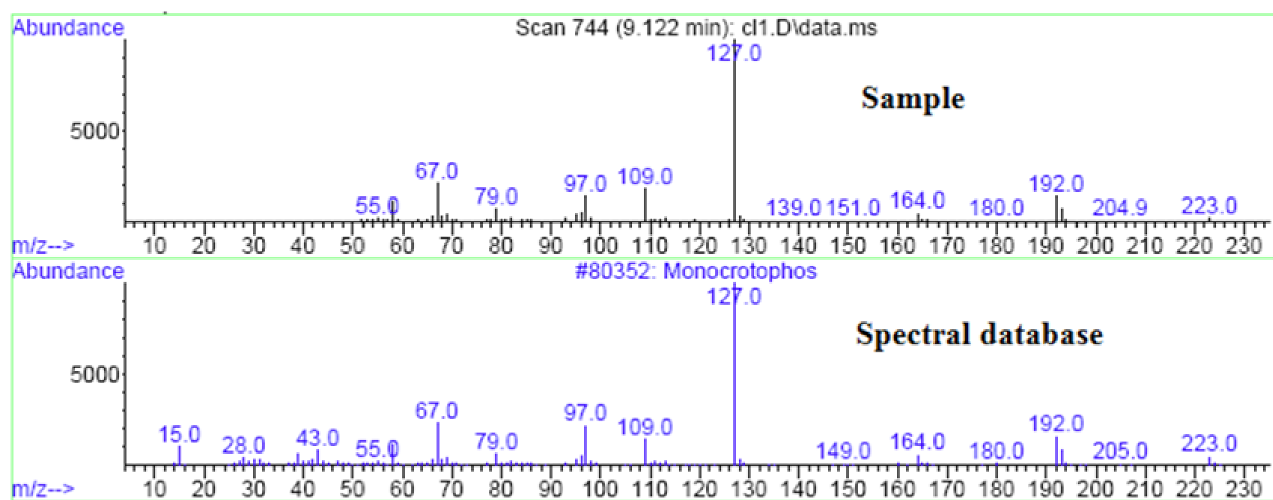


Figure 6: Mass spectra of monocrotophos (forensic) sample at retention time 9.12 minute

Figure 5 showed the typical gas chromatograph of monocrotophos in chloroform solution. Figure 6 showed the mass spectrum of the GC peak at **9.12** minute which showed major signals at the mass to charge ratio (m/z) 55, 67, 79, 97, 109 and 127. The GC peak at 15.542 minute was identified to be monocrotophos by the mass data library using Agilent DA Express data analysis software. Also, it has been matched with the mass spectral data base to prove the insecticide **monocrotophos**. Limit of detection was 0.32 mg/kg. Forensic scientists routinely depend upon GC/MS to differentiate individual compounds from complex mixtures. However, GC/MS has limitations. The experimental data presented in this work may provide useful information regarding the potential link between the forensic sample evidences and suspects [16].

Conclusion

Numerous features of separation methods are applicable to forensic science. The proper identification of forensic samples by the forensic examiner is of paramount importance. As the encountered samples become more complex, and compounds with similar molecular structures are submitted to the laboratory, it is imperative examiners have access to appropriate techniques that allow an identification of the sample under examination.

Because chromatography techniques are so versatile and can be used to determine so many different compounds, the technique is particularly well suited to the demands of a forensic laboratory. Both qualitative and quantitative information can be obtained, often with minimal sample preparation. Because only small volumes are needed for analysis, sample consumption can be minimized. Eluting fractions can be collected for further analysis an important consideration when dealing with trace evidence.

One of the organophosphorous insecticides, monocrotophos is increasingly being used in agriculture to control insects on a wide range of crops. Its ready access has resulted in misuse in many instances of homicidal and suicidal poisoning cases. This study describes a TLC, HPLC and GC-MS methods for the separation and detection of monocrotophos in forensic sample. Hence it can be routinely used for separation and detection of monocrotophos insecticides in toxicological case work.

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