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Review Paper

Forensic Science

EFFICIENT CHROMATOGRAPHIC APPROACH FOR THE ANALYSIS OF INSECTICIDES AND ECO-TOXICOLOGICAL ASPECTS

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ABSTRACT In today's rapid growing industries and technology, the frequent use of various insecticide in agriculture sector can be commonly encountered. Insecticides such as carbamate, organochlorine and pyrethroids helps protecting the crop from various insects, which ultimately leads to the better yield of agricultural products. But the main concern that comes out in such frequent practice is the adverse effects to the adjacent ecosystems. Especially, the very commonly used carbofuran, DDT, Cypermethrin and its adverse effects can be put forwarded as a potential example in this article. Apart from this, the scope of this article covers the mechanism of toxicity by insecticides to nontargeted organisms in the ecosystem, the recent developments in the analytical techniques considering HPLC and GC, the method of extraction and quantification of active component in sample. A comparison between GC and HPLC is done and the preferred instrumentation is concluded while considering the analysis of Insecticide. Scope of this review also covers the symptoms and treatment of insecticide poisoning.

KEYWORDS : HPLC, QuEChERS method, Insecticide analysis, PAM, Anticholinesterase activity, Insecticide toxicity, Comparison between HPLC and GC

1. INTRODUCTION

The frequent use of various Insecticides including Organochlorine, Carbamate and Pyrethroids can be commonly encountered in agriculture sector (Peng et al., 2008). Although these all chemicals help protecting the crops from different insects due to its toxicity, the remaining chemicals residue after the targeted or desired results still cause various adverse effects in the adjacent ecosystem (Huang & Cunningham, 1996). The application of Insecticides is commonly practiced by different individuals as it is helpful in various aspects. But the residues need to be degraded to prevent toxic effects to the nontargeted organisms. There are various techniques that are followed to degrade any toxic elements. The chemical method is fast resulting but is not cost effective and the use of chemical to the insecticide contaminated soil decreases the soil fertility and ultimately leads to form an unhealthy soil ecosystem. But Bioremediation stand out to be a best alternative. Using various microbes and plants for degradation activity is cost effective and eco-friendly at the same time. Various research has been conducted to conclude the efficiency of microbes as a degrading agent. Studies suggests the microbial degradation is very effective. But as it is a biological process, it takes time for the results to be obtained (Plangklang & Reungsang, 2009). However, microbial remediation and phytoremediation can be implicated combinedly in certain cases to boot up the process. Various instrumental analysis can be performed to monitor the process. HPLC, GC, ECD, LC, TLC, Spectrometry, Polarography are some of the commonly used instruments for qualitative analysis which can be attached to various detectors to get more accurate results in terms of quantity. Sample preparation for all these instruments is different. In further discussion the article will cover the HPLC analysis of concerned insecticide.

2. Classification of Insecticides

Insecticides are divided into 3 major groups viz. Organochlorine, Carbamate and Pyrethroids based on the chemical composition (Peng et al., 2008). There is also another group i.e., miscellaneous insecticides which includes spinosyns, benzoylureas etc. Insecticides are usually less toxic to plants and it specifically targets various insects. Methoxychlor, Toxaphene, Mirex, Lindane, Benzene hexachloride, DDT are the examples of Organochlorine pesticides. Similarly, Aldicarb, Carbaryl, Propoxur, Oxamyl, Carbofuran and Terbucarb are some examples of Carbamate. Allethrin, Resmethrin, Permethrin, Cyfluthrin etc comes under Pyrethroids.

3. Source of toxicity

As the insecticides or pesticides are precisely administered in the agricultural fields and industries or periphrastically by drifting and volatilization from the treated fields this can be the prime cause of pesticides or insecticides toxicity to environmental or the surrounding things like it can pollute the air, water ecosystem, soil as well as non-treated plants and animals. The appropriate measure of any insecticides which is used in the application to any agricultural fields never absolutely reaches to the target organisms and its maximum amount is spread into the environment. The unused insecticides can be the source of air contamination, soil contaminant and water ecosystem. Particularly the workers who works in the insecticides manufacturing organizations, in fields, executing of household pests and green house are especially affected by insecticide vulnerability (Mahboob, Al-Ghanim, Al-Misned, & Ahmed, 2014). The chances of risk are higher in the working area of the insecticides or pesticides at the time of manufacturing and management. Because during the manufacturing workers deals with the several dangerous chemicals including pesticides, crude materials and some other dangerous solvents so the opportunity of exposure is very high.

4. Mechanism of toxicity

Insecticides such as Carbamates, Organophosphates, organochlorines etc. are very harmful to non-targeted organisms such as birds, rodents, honeybees, fishes and other aquatic organisms by direct inhalation, ingestion or dermal absorption. Though it is not recognized as carcinogen still can be the source of genotoxic, carcinogenic, teratogenic and mutagenic effects (Acc. to WHO, 2019). These can be the source of acute and chronic toxicities by inhibiting acetylcholinesterase in synapses of Central Nervous System (CNS) which is the chief toxicological property of carbamate (Gupta et al., 2016). Several investigations concluded that, carbamate can be the source of significant decrease in isoenzyme- I and isoenzyme- II in mother and foetus (Das, Wdv, & Uss, 2013). Carbamate toxicity mechanism is established that unstable aggressive inhibition of acetylcholinesterase enzyme (AChE) in the central and peripheral nervous system (PNS). Carbamate disturbs the carbamylation of serine residue active site within carboxylesterase and butyrylcholinesterase enzymes. The inhibition of AChEase enzyme can be the source of successive

aggregation of acetylcholine in synapses which can break into choline and acetate residues (Das, Wdv, & Uss, 2013). Due to the stimulation of Ach receptor, there may be two types of acute clinical indications occurs, namely Nicotinic and Muscarinic. Nicotinic effects cause muscle weakness and tremors whereas muscarinic effects can damage cardiac, gastrointestinal and respiratory systems which results in obstruction of airways, increased salivation, defecation, bradycardia and gastroenteritis.

5. Effects of carbamate on aquatic ecosystem

Water bodies such as river, ponds etc are extremely exposed to carbamate toxicity as determined by its groundwater ubiquity score (GUS) index of 4.5, exhibit a comparatively high risk of being shifted from the targeted site to neighbouring water bodies after raining or by other means (Beklová, Dobšiková, & Pikula, 2003). Carbamate can be the source of acute and chronic toxicities to aquatic species by preventing of haematological, biochemical and enzymatic activities especially by lowering the levels of dissolved oxygen in water. carbamate can affect the aquatic species at various trophic levels, from algae to fishes. Carbamate acts as neuro-toxicant and acetylcholinesterase inhibitor in liver, brain and muscle of fish mainly (Ghazala et al., 2014). Carbamate vulnerability also undergo a practicable abnormality in fish such as change in the colour of body, fail to feed, loss of balance, reduction in the growth rate and reduction in the swimming performance (Ghazala et al., 2014). Heavy usage of insecticides or pesticides may result in the reduction of number of fishes and other aquatic species. Aquatic species receives exposure of pesticides by three modes i.e., direct absorption by skin (dermally), uptake of pesticides by means of gills (breathing) and intake of drinking contaminated water. The oxygen level reduces immediately due to killing of aquatic plants by usage of herbicides which exactly starts suffocation in fishes and reduce their production.

6. Effect of carbamate on soil and plant

Commercial management of carbamate residues can be quickly contaminating the soil ecosystem and plants by direct spraying, surface-runoff, flooding or accidental exposure. Carbamate uses results in life-threatening soil contamination which can brutally affect soil fertility, respiration, microbial biomass and diversity (Mohd-Nor et al., 2019). It absolutely inhibits dehydrogenases. It can affect the soil microbial activity also. The experiment determines local exposure and impact of insecticides by domestic applications. Thus, the extreme use of carbamate pesticides in agricultural soils results in ecological consequences.

7. Effect of carbamate on humans and other animals

Carbamate is organized to have extremely high mammalian toxicity. It may be dangerously lethal to invertebrates and birds. Environmental protection agency (EPA) recognizes it in the 'Toxicity category I' (Chapalamadugu & Chaudhry, 1992). This toxicity category I is very toxic category based on hazardous effects through oral and inhalation exposures. Various types of health problems including cancer, diabetes mellitus, respiratory disorders, neurological disorders, reproductive syndromes and oxidative stress are caused due to the direct exposure and handling of pesticides etc. carbamate basically causes acute toxicities in humans through accidental exposure whereas continuous exposures result in chronic toxicities. Due to daily contact with the pesticides increase the danger of diabetes (Lado-Abeal et al., 2000). Long-term exposure of carbamate pesticides to farmers, industrial workers and animals results in chronic toxicity. It includes dermal, endocrine, cytotoxic, mutagenic, reproductive, neurotoxic, genotoxic and dermal-skin problems. The direct exposure to the pesticides is the main reason of cancer all over the world. Basically, it enters the human body through inhalation, ingestion and dermal absorption (Gupta et al., 2016). Dermal exposure is less toxic to human as compared to inhalation and ingestion. High dose of carbamate vulnerability to human mainly cause weakness in muscles, headache, dizziness, sweating, dilated pupils, blurred vision, slurred speech, and muscle twitching.

8. Clinical Features of Acute Insecticide Poisoning Due to anticholinesterase activity

Insecticides such as Carbamates, Organophosphates, organochlorines etc. are the acetylcholine inhibitors. Within 1-2hrs of exposure the acute toxicity can be observed. Symptoms can be mentioned as muscarinic and Nicotinic. The Muscarinic symptoms such as Salivation, Lacrimation, Urination, Defaecation, Gastrointestinal Cramp can be termed as SLUDGE. Other acronym DUMBLE can be used as well, which stands for Diarrhoea, Urination, Miosis, Bronchorrhoea, Lacrimation, Emesis (Goel & Aggarwal, 2007).

Diagnosis can be done by measuring the butyry lcholinesterase activity or red cell cholinesterase. In such case we can pseudocholinesterase or Plasma cholinesterase can be considered. 30-50% of the normal cholinesterase in red cell can suggest the exposure and the symptoms can be observe when it falls to 20% (Nagami, Maejima, Nishigaki, & Natsukawa, 2015). Various factors can affect the level detection of pseudocholinesterase such as pregnancy, malnutrition, drug intoxication (morphine and codeine), neoplasm etc. Although the availability is high in case of pseudocholinesterase, the reliability is not up to the mark because of such fluctuation due to the above-mentioned factors (Eddleston, Szinicz, Eyer, & Buckley, 2002).

Treatment in the primary stage can be done by following the ABCD concept. The Airways, Breathing, Circulation and Distribution must be taken into consideration. Oxygen supply at initial stage is very important. To reduce the drug distribution present in stomach the patient must be positioned properly by keeping the head part lower that the abdominal part and can be placed left laterally. The further steps involve the application of various drugs. Drugs such as Atropine and Pralidoxime (PAM, 2-pyridine aldoxime methylchloride) can be commonly and effectively used (Shivakumar, Raghavan, Ishaq, & Geetha, 2006). Prior to this Diazepam can be used which can affect GABA receptors (Goel & Aggarwal, 2007) (Marrs, 2003). The high oxygen flow is necessary at all the stages. Atropine as an antagonist help increasing the heart rate and can be useful when the heart rate drops below 80/m. PAM application found to be very successful in various anticholinesterase functions (Buckley, Eddleston, Li, Bevan, & Robertson, 2011).

Exception in the use of PAM is there in the carbamate poisoning because of the short action period of carbamates (Goel & Aggarwal, 2007). The role of oxime in Carbamate poisoning is not clear. But in case of failure to receive the atropine, PAM can be given in order to increase the reception.

9. Application of HPLC or GC for Insecticide Analysis

HPLC which stands for High Performance Liquid Chromatography and GC is Gas Chromatography. These two can be deployed for the analysis of Insecticide. The concentration of insecticide before degradation and after degradation can suggest the efficiency of deployed microbes. However, we need to extract the active component before the separation process with the help of HPLC (He, Wang, Xu, & Liu, 2018). Although there are several sample extraction techniques developed such as Solid phase extraction, Liquid extraction, Soxhlet extraction, super critical fluid extraction etc., the latest technique is QuEChERS technique. QuEChERS stands for Quick, Easy, Cheap, Effective, Rugged and Safe (Chawla, Gor, Patel, Upadhyay, & Shah, 2020).

9.1. QuEChERS Method of Sample Extraction

QuEChERS technique is introduced by USDA scientists in 2003. This method is quick to execute and easy to perform, as it does not require automation and does not follow the laborious process. The process can be carried out with less sorbent and solvent and hence, it is a cheap process (He, Wang, Xu, & Liu, 2018). Various studies suggested its effectiveness and concluded it as a result-oriented method. It is also found to be capable of detecting verity of compounds including pH dependent and Polar compounds etc. The technique is carried out using Acetonitrile instead of chlorinated solvent.

Several QuEChERS methods are already been introduced and are mentioned as follows (Acosta-Dacal, Rial-Berriel, Díaz-Díaz, Bernal-Suárez, & Luzardo, 2021):

- Original Unbuffered Method
- European EN 15662 Method
- Mini-Multiresidue Method and
- AOAC Official 2007.01 Method

The commonly practiced methods i.e., EN and AOAC are somewhat similar, but there are some differences between these two methods. Sodium acetate is used in AOAC buffer extraction system. But in case of EN sodium citrate, sodium chloride, disodium citrate sesquihydrate can be used in buffer extraction system. The other difference is in the step of dispersive solid phase extraction. In case of EN it uses 25 mg PSA per ml of extract, wherein in case of AOAC method use 50 mg PSA per ml of extract (Acosta-Dacal, Rial-Berriel, Díaz-Díaz, Bernal-Suárez, & Luzardo, 2021).

General Procedure

Step-1: Sample Preparation and extraction

Here the homogenised sample will be taken in a centrifuge tube containing Magnesium Sulphate and any salt (NaCl or sodium Acetate/$C_2H_3NaO_2$). During the process of centrifugation, the Magnesium will help in creating a phage separation between acetonitrile and water layer. Basically, the compound or the intended sample will come to the acetonitrile side. The acetonitrile part can be taken for further clean-up process (Kim, Lee, Cho, & Choi, 2019).

Step-2: Sample extract Clean-up

The clean-up tube basically contains sorbents in different quantities. To remove extra water in the mixture of sample Magnesium Sulphate is added. For organic acid and polar pigments removal PSA is added. PSA stands for Primary/Secondary Amine (Kim, Lee, Cho, & Choi, 2019). Similarly, end capped C18 is added to remove lipid or sterol. Graphitized carbon block is used if the sample is suspected to have chlorophyll. Now this extract can be analysed using appropriate instrumentation.

There are various clean up methods already introduced, such as- liquid-Liquid partitioning, Column Chromatography, SPE, Chemical Clean up, Gel permeation/size exclusion chromatography etc (Kim, Lee, Cho, & Choi, 2019). Figure-1 is the diagrammatic representation of QuEChERS Method of Sample Extraction.

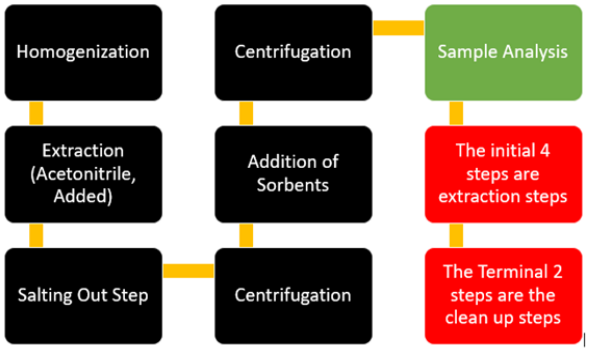


Figure-1

Step-3: Sample Analysis

9.2. Sample Analysis Using HPLC

HPLC which stands for High Performance Liquid Chromatography is suitable for non-volatile, Polar insecticides (Michel & Buszewski, 2002). In a successful study done by Monika M and her colleague, RP-HPLC was used to analyse pesticides in various food products. Another study done by Terry D uses HPLC to detect carbofuran and was well described.

Normal Phase (Polar Stationary Phase and Non-Polar Mobile Phase), Reverse Phase HPLC (Polar mobile Phase and Non-Polar Stationary Phase), Size exclusion (Separation done based on Size of Particle), Ion exchange Chromatography (Here the separation takes place depending on the solute affinity towards the stationary phase) (Y. Huang, Wang, Wang, Yin, & Tu, 2020).

The HPLC instrumentation and its working process depends on various parameters. Parameters such as the internal diameter of HPLC column (smaller the diameter smaller will be the sample loading and higher the sensitivity of instrument), Particle size of silica in the stationary phase (Smaller the size, surface area will be more), Column pressure (the fast result with less sample run time can be achieved through high pressure. UPLC is enhanced and modern instrument which can provide high pressure with good sensitivity)

Sample can be injected manually or automatically. The elution can be Isocratic or Gradient elution. The injected sample get separated in the column and detector such as UV detector can be used to detect the elements and the graph or chromatogram is recorded through a recorder which can be analysed further.

9.3. Sample Analysis Using GC

GC-MS (Gas chromatography-Mass Spectroscopy) is widely used in Petrochemical industries for Forensic analysis. Due to its sensitivity and accuracy, it is one of those commonly found analytical instruments in Forensic labs (Zuo, Yang, Huang, & Xia, 2013). Apart from petroleum products, we can use GC-MS in various chemical analysis as well. GC-MS is preferred when quick screening is required because the column separation is generally is faster in gaseous phase (Verzele, 1968).

Working Principle of GC

Separation of molecules based on their volatility and affinity towards stationary phase. Before analysis, the sample preparation is done (Reiser, 1966). Various sample preparation techniques are as follows:

Samples requiring GC-MS analysis could include heavy liquids, such as tars (it acts as solid), coal, rubber tires etc. In case like this, we can use **Pyrolysis MS** to convert the sample into the gaseous phase required for analysis by GC-MS. Pyrolysis-MS is an important technique in the overall analysis armoury and has been noted for its ability to analyse small amount of material with minimum sample preparation. Pyrolysis MS samples are heated and converted into gaseous phase which can be pass through GC columns (Fish & Crosby, 1968).

Second sample preparation technique **Thermal desorption mass spectroscopy (TDMS).** This technique involves collecting desorbed molecules from a surface. Here the surface temperature is increased. Then these individual components introduced into the GC-MS process (Meng, Zhao, Duan, Hao, & Guan, 2014). This method is generally used to analyse volatile organic compounds, commonly the source of order in the ambient air. Samples are collected using thermal

desorption tubes.

Another technique for preparing sample is **headspace GC-MS**. Here, the sample is heated to its boiling point and the vapours are collected which will be heated up to 100-200°C and then passed through the GC-MS (Snow & Slack, 2002).

The use of only GC or only MS will not give the accurate result. So, rather than using the common method (GC-FID) we can use GCMS to eliminate errors (Zure & Pinjari, 2019). For the analysis of compounds those are volatile, a purge and trap (P&T) concentrator system may be used to put the samples. The target analytes are separated by mixing the sample with water, then purged with inert gas (e.g. Nitrogen gas) into an airtight chamber, it is known as purging/sparging. The volatile compounds move into the headspace above the water and are drawn along a pressure gradient out of the chamber. The volatile compounds are drawn along a heated line onto a trap. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. Purge and Trap GC-MS is particularly suited to volatile organic compounds and aromatic compounds associated with petroleum. Mobile phase is mainly Hydrogen or Nitrogen that gets separated/detected at the detection port with suitable temperature programming. The results can be observed in the computer screen in the form of peaks.

Molecules when reach Mass spectrometer will be ionized and fragmented. These ionized molecules will be converted into electric signal by electron multiplier, which can be detected by the detectors. This process is completely automatic (Lundovskaya, Medvedev, Tsygankova, Volzhenin, & Saprykin, 2021). In a typical MS procedure, a sample, which can be solid, liquid or gaseous is ionized, for instance by bombarding it with electrons. It might cause a number of the sample's molecules to interrupt into charged fragments or just become charged without fragmenting. These ions are then separated consistent with their mass-to-charge ratio, for instance by accelerating them and subjecting them to an electrical or magnetic field, ions of an equivalent mass-to-charge ratio will undergo an equivalent amount of deflection (Lundovskaya, Medvedev, Tsygankova, Volzhenin, & Saprykin, 2021). The ions are detected by a mechanism capable of detecting charged particles, like an electron-multiplier. Results are displayed as spectra of the signal intensity of detected ions as a function of the mass-to-charge ratio. The atoms or molecules within the sample are often identified by correlating known masses (e.g. a whole molecule) to the identified masses or through a characteristic fragmentation pattern.

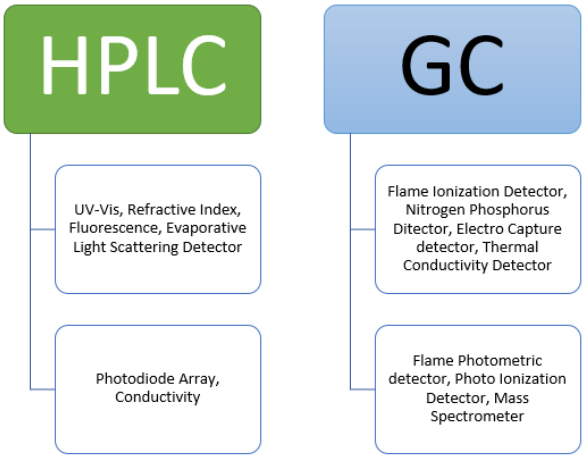


Figure-2

10. Comparison Between HPLC and GC For Insecticide Analysis

When we consider the *nature of sample* in case of GC, only the low molecular weight and volatile samples can be analysed. But HPLC can support the analysis of Volatile, Non-volatile and heavy molecular weight samples. So, the HPLC can be considered as a nature of sample independent or flexible method.

Next thing that we can take into account is the *mobile phase*. It is obvious that the use of a single inert gas is enough for GC as it just acts as a carrier gas which helps in the movement of sample. But contrasting situation in HPLC is- we need to select appropriate mobile phase. Because here the separation will take place considering the phase that is liquid and various factors such as polarity, molecular weight, solubility of sample in mobile phase plays an important role.

Considering the *Resolution* that can be achieved in the chromatogram, in case of GC- the similar volatility having nearly similar molecular mass can create interference in the chromatogram, which ultimately results a bad resolution. Similar thing can be observed in case of HPLC if there are molecules having similar polarity in the sample of interest.

Columns can be considered as a major part of any analytical instrument. In case of HPLC, the columns are usually short and wide. GC can be equipped with two types of columns (Capillary and packed columns). The capillary columns are optimised to work in a faster rate while giving a better resolution. So, GC can be a better option in such case.

The cost is high for analysis of sample in HPLC. In contrast, for the analysis of sample in GC the cost is low with respect to the HPLC analysis.

The *detector* can be attached for various purposes. Both GC and HPLC got multiple options and can be coupled with respective detectors according to the availability and need. GC can be coupled with MS, ECD, FID, TCD etc. and the HPLC can be coupled with the UV/Vis, RID, MS etc. Figure-2 represents the compactible detectors for HPLC and GC.

Figure below (Figure-3) is a diagrammatic representation of side-by-side analysis between HPLC and GC.

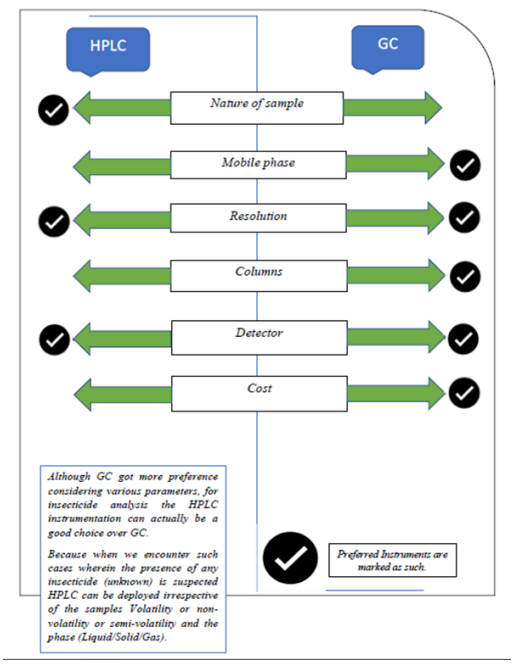


Figure-3

11. Bioavailability of Insecticide in fluctuating Weather Condition

Study concluded that, the bioavailability can be very less or can be high depending on various weather conditions. A study done by (Rajput, Kumari, Arora, & Kaur, 2018) suggested this fluctuation of weather can affect the bioavailability due to various reasons. The statistical information below suggests the Detection of various insecticide in Punjab state in different seasons. Considered Insecticides are Carbofuran, Atrazine, Parathion Methyl. The structures of these are given below and named as Figure-4,5,6 respectively.

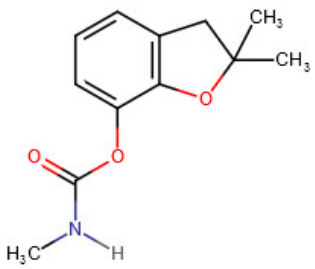


Figure-4

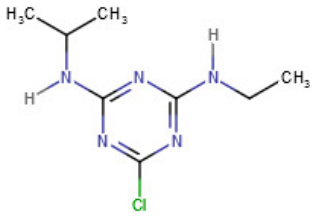


Figure-5

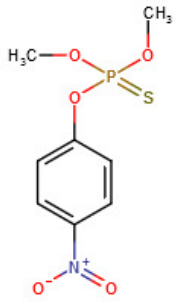
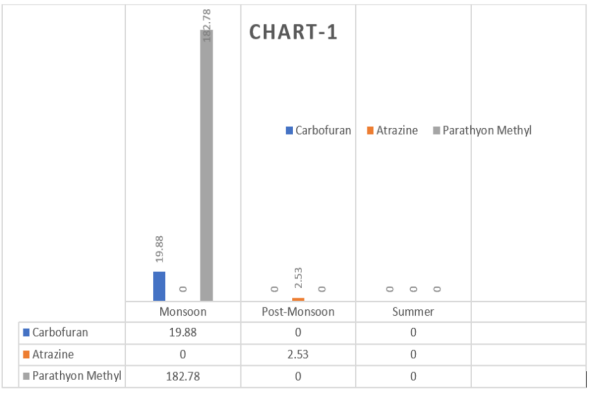
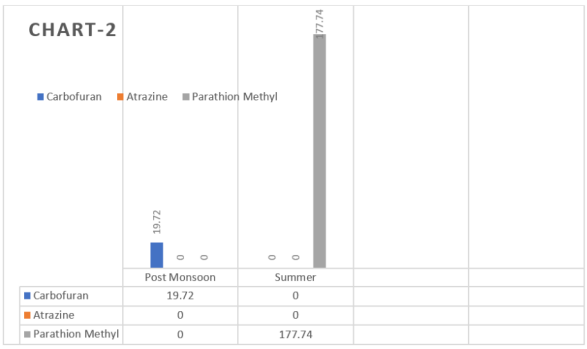


Figure-6



	Monsoon	Post-Monsoon	Summer
Carbofuran	19.88	0	0
Atrazine	0	2.53	0
Parathyon Methyl	182.78	0	0

Chart-1 represents the detected quantity (mg/L) of Carbofuran, Atrazine and Parathion Methyl using HPLC. Sample collection was done from a pond at Ajnala, Punjab (2016) in different seasons of the year 2016-17 (Rajput, Kumari, Arora, & Kaur, 2018).



	Post Monsoon	Summer
Carbofuran	19.72	0
Atrazine	0	0
Parathion Methyl	0	177.74

Chart-2 represents the detected quantity (mg/L) of Carbofuran, Atrazine and Parathion Methyl using HPLC. Sample collection was done from a pond at Manawala, Punjab (2016) in different seasons of the year 2016-17 (Rajput, Kumari, Arora, & Kaur, 2018).

The study done by (Rajput, Kumari, Arora, & Kaur, 2018) also suggested the maximum bioavailability of insecticides were encountered in the monsoon and the minimal is 0% or not detected in summers. The most encountered insecticide in Punjab was carbofuran, which is mentioned as 18.18% of the different verities of insecticides.

12. CONCLUSION

Considering the above discussion, we can say the use of insecticide is increasing day to day as it is beneficial for the food production. But in order to prevent the nontargeted toxicity we must have to consider various remediation techniques. Use of other suitable and biodegradable insecticide such as canola oil and baking soda must be encouraged more. The forensic analysis of such insecticide including the detection and quantification is very successful using various hyphenated instruments. However, we made a comparison between the GC and HPLC. Although GC overall can be a better option for sample analysis, considering insecticides analysis the HPLC instrumentation can actually be a good choice over GC. Because when we encounter such cases wherein the presence of any insecticide (unknown) is suspected HPLC can be deployed irrespective of the samples Volatility or non-volatility or semi-volatility and the phase (Liquid/Solid/Gas). Moving further, as of now the use of PAM is under controversy in carbamate poisoning. But other medical treatment can approach the same and can be a successful substitute in such case.

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