CHAPTER 1

INTRODUCTION

1.1 Background of the Study

As per Food and Agriculture Organization (FAO) (1989), a pesticide could be any substance or combination of substances intended to destroy, avoid, or keep a check on any pest, including vectors of human or animal diseases, undesirable animal or plant species that could initiate harm during, or if not interfering with, the processing, packing, production, or marketing of agricultural commodities, food, wood and wood products, or animal feedstuffs, or which may be managed with the help of animals to keep a check on pests in or on their bodies. The term encompasses chemicals employed as growing controllers, desiccants, defoliants, agents to delay early fall of fruits, and the applied materials to crops either after or before harvest to avoid deterioration during the transport or storage stage. The term, however, does not include those substances that are employed as fertilisers, nutrients for plants and animals, food additives and animal drugs. The FAO in collaboration with the United Nations Environment Program (UNEP) (1990) has defined the term pesticide as those compounds that are developed to fight against the attacks of various pests and vectors on domestic animals, agricultural crops and human beings. The definitions mentioned above allow us to assume that pesticides contain toxic chemical agents (mostly organic compounds) whose release into the environment is intentional in a bid to fight crop pests as well as disease vectors.

In agriculture, employing pesticides has allowed substantial increase in crop yield per hectare of land. Many studies have helped to establish a correlation relationship between the amount of crop yields per hectare and the quantity of pesticides employed

per hectare (Hellar, 2002). Table 1.1 presents the use of pesticides and the corresponding crop yield in some countries. It is clear from the table that the greater the number of pesticides used, the higher the production per hectare. For example, Japan, which has the highest rate of pesticide use, up to 5.5 tons followed by The USA and Europe, 3.4 and 2.6 tons per hectare respectively So, these two have the highest production per hectare countries, while the African continent does not exceed 1.2 tons per hectare due to the low rate of pesticide use (0.1 tons per hectare).

Table 1.1: Pesticides Use and the Corresponding Crop Yield in Some Countries/Areas

Country/Area	Pesticide Use (kg/Ha)	Crop Yield (Ton/Ha)
Japan	10.8	5.5
Europe	1.9	3.4
USA	1.5	2.6
Latin America	0.2	2.0
Oceania	0.2	1.6
Africa	0.1	1.2

Source: Zacharia and Tano (2011)

Like dichlorodiphenyltrichloroethane (DDT) and other chemicals, pesticides have proved their worth in agriculture as well as in public health. Apart from reduction in the number of fatalities due to insect-borne diseases, economies were also seen to improve, and substantial increase in crop yields could be achieved. The use of insecticides has helped to save the lives of millions of people that could have otherwise been affected by insect-borne diseases (WHO, 2014) and hunger. It has been a decade since the use of pesticides gained wide popularity due to their effectiveness and potential economic benefits. Their roles in keeping a check on agricultural pests and disease courses have spurred their use in the agriculture sector significantly. In the agriculture sector, the pesticides' role is to safeguard crops and amplify production to meet the rising demand of the growing world population.

Even though pesticides, in agriculture and public health, offer various advantages as described above, their use generally leads to harmful environmental and public health effects. Amongst environmental contaminants, pesticides contribute substantially because of their high biological activity as well as toxicity (chronic and acute). Few pesticides that are thought to be selective in their modes of action are sometimes only limited to test animals. In general, pesticides are not considered safe for mankind, animals, birds and even the environment.

Though employing pesticides is inevitable, developed countries may have the required modern technology to increase crop yields. However, in developing countries, factors such as poverty, gradual decrease in usable areas and high population growth rate are likely to control the food production and consumption. Considering such a situation, pesticide use will more likely continue in the foreseeable future. So, the aim here should be to reduce undesirable environmental consequences due to pesticide residues in air, soil, food, human tissues, water and in the food chain. Efforts must be focused on striking a balance between both the benefits and negative impact associated with pesticide use. In 1951, Laug et al., first found organochlorine pesticide DDT in milk obtained from black American mothers. The risk to human health from pesticides contamination in general drew attention of many researchers. As such, various methods were designed to assess pesticide residues in water sources, human milk, agricultural products, and other biological fluids (Pirsaheb et al., 2015). Most of the applied pesticide permeating to soil and plants would certainly find their way towards water sources. Intensified monitoring programmers have been deployed due to concerns related to water quality supplies and agricultural products containing possible contamination with pesticides. However, various analytical techniques that are being developed and greater awareness regarding the need for extensive studies to keep a

check on biological and environmental contamination have helped to understand environmental effects of pesticides as well as their biological risk.

1.1.1 Classification of Pesticides

1.1.1.1 Classification of Pesticides Based on the Targeted Pest Species

In this type of classification, pesticides are named after the name of the corresponding pest in target as shown in Table 1.2, insecticides for insects, herbicides for weeds and rodenticides for rodents and so on.

Table 1.2: Classification of Pesticides Based on the Targeted Pest Species

Type of pesticide	Target organism / pest
Insecticides	Insects
Herbicides	Weeds
Rodenticides	Rodents
Fungicides	Fungi
Acaricides and Miticides	Arachnids
Molluscicides	Mollusks
Bactericides	Bacteria
Avicides	Bird pests
Virucides	Virus
Algicides	Algae

1.1.1.2 Classification of Pesticides Based on the Chemical Composition

In a chemical classification, the chemical nature of the active ingredients of pesticides determines the category. Classification of pesticides based on the chemical has been a useful tool for researchers associated with the field of pesticides and environment and those seeking additional details. This is because classification based on chemical will also provide some information regarding efficacy, knowledge of application, physical and chemical properties of the respective pesticides, the application rates, and precautions required during application. Based on chemical classification, the classification of pesticides could be done into four main categories,

namely organophosphorus, organochlorines, pyrethrin and pyrethroids and carbamates (Kaur et al., 2019).

Organochlorines pesticides are organic compounds such as OCPs have five or more chlorine atoms. In the agriculture and public health sectors, the first synthetic organic pesticides to be employed were organochlorines. Some of organochlorine pesticides chemical structures are shown in Figure 1.1. These organochlorine pesticides were broadly employed as insecticides to control a range of insects. Since they are resistant to most chemical and microbial degradations, their impact on the environment is a long-term residual effect. Organochlorine insecticides disrupt the nervous system, making the insect experience convulsions and paralysis, and its eventual death. DDT, endosulfan, lindane, aldrin, chlordane and dieldrin are some of the commonly employed representative examples of organochlorine pesticides.

1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) was a popular synthetic compound that was extensively employed in controlling insects in agriculture and disease vectors. In this group, (DDT) and its metabolites, p, p'-DDD and p, p'-DDE, are the most important compounds. In soil or foodstuff, even after many years of use, the metabolites could be found as residue. In occupational exposure, there is a direct exposure to these chemicals or indirect exposure could be due to consumption of foods comprising residues of these chemicals. Accumulation of these chemicals along the food chain has been noted and is fatal to humans. DDT was identified in different food commodities, e.g., poultry, animal products, human fat, fish, and milk (Kanja et al., 1986; Pirsaheb et al., 2015). The presence of DDT in the system could lead to premature births and even impact neurobehavioral functions (Longnecher et al., 2001).

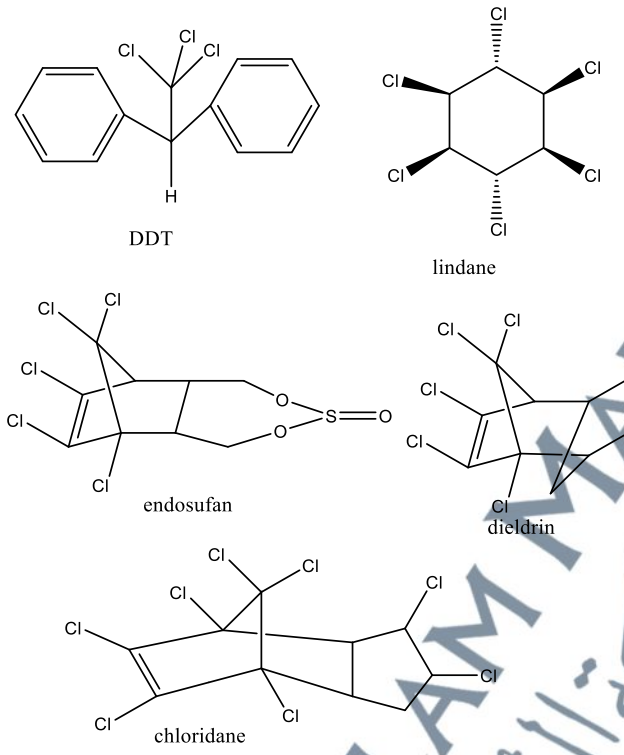


Figure 1.1: Chemical Structure of Some Organochlorine's Pesticides

Aldrin: Pure aldrin is characterized by a white crystalline solid structure, which is water insoluble but is soluble in many organic solvents. Aldrin remains stable during storage and is not affected by alkali and diluted acid but oxidizing agents, heat, concentrated mineral acids and various catalysts that could lead to decomposition (Tippawan, 1990). In construction work, it is employed as an ant killer, and compounds such as isodrin, heptachlor, dieldrin and endosulfan are highly insecticidal. Endosulfan has an environmental significance due to its apparent persistence and toxicity for non-target organisms such as fishes (Shetty et al., 2000).

Hexachlorocyclohexane (HCH) is present as isomers and α-HCH, ß-HCH, γ-HCH and δ-HCH are the most common isomers that are widely employed to control insect pest when dealing with agriculturally important crops, vegetables and seeds. Farmers commonly use Lindane (γ-HCH) as dust in the seed treatment. Under aerobic conditions like *Sphingomonas paucimobilis*, some microorganisms can degrade one or

more HCH isomers, even though it is a challenging task to biodegrade γ-HCH (Suar et al., 2004). As per a guideline (Geneva, 1967) issued with the World Health Organisation (WHO) to keep a check on the active ingredient in the technical product, technical HCH should have 12%–16%, w/w of the r-isomer. In pest control of foodstuffs, the odor associated with technical HCH was always seen as a problem. For example, potatoes are prone to taint problems when growing on land, which have been treated with HCH previously. In controlling locusts in crop fields and malaria-carrying mosquitoes in households, technical HCH has also been employed as an alternative to DDT (Tippawan, 2003).

Organophosphorous insecticides: As defined by Schrader's formula, organophosphorus insecticides include a phosphate group within their basic structural framework: Where R1 and R2 usually represent the methyl or ethyl groups (Figure 1.2). In some compounds, S can substitute the O in the OR3 group, whereas a wide diversity of forms is associated with the R3 group.

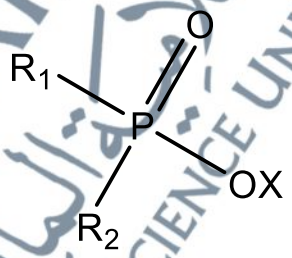


Figure 1.2: General Formula of Organophosphorus Pesticides

In general, these compounds are more toxic to invertebrates and vertebrates since cholinesterase inhibitors cause a permanent overlay of acetylcholine neurotransmitter in a synapse (Zacharia & Tano, 2011). As a result, nervous impulses cannot move across the synapse, leading to rapid twitching of voluntary muscles, and eventually paralysis and death. In the environment, several biological and chemical reactions can easily decompose organophosphorus, thus are not considered tenacious for the environment

(Martin, 1968). A few examples of widely employed organophosphorus insecticides are Dimethoate, Chlorpyrifos, malathion, parathion, glyphosate and diazinon (Figure 1.3).

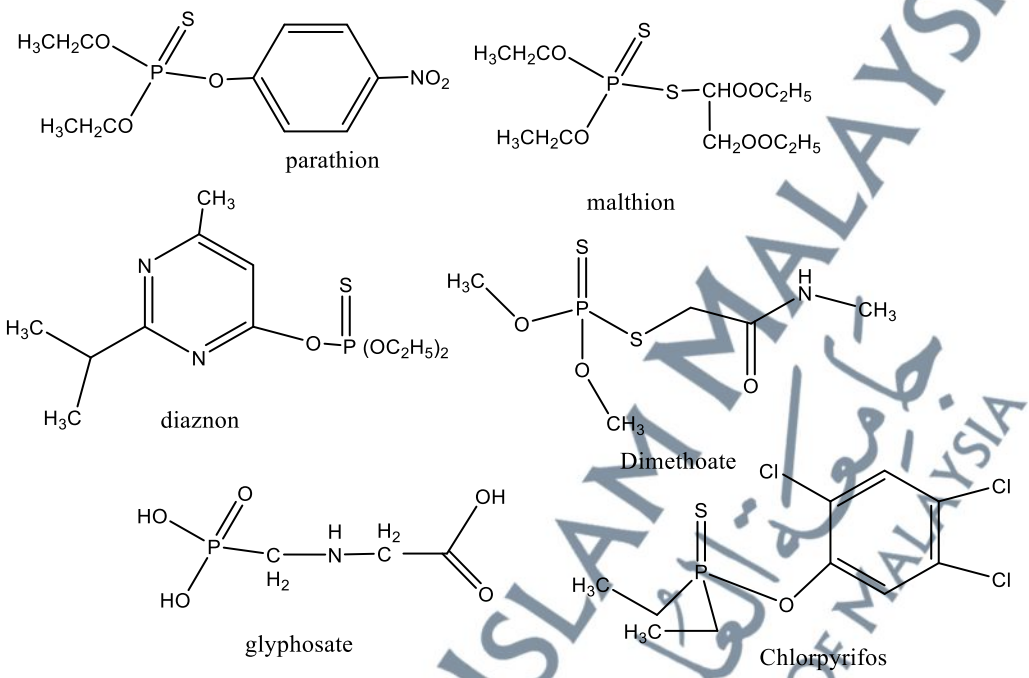


Figure 1.3: Chemical Structure of Some Organophosphorus Pesticides

Carbamates are organic pesticides derived from carbamic acid with the general formula shown in Figure 1.4.

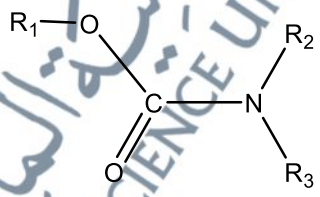


Figure 1.4: General Formula of Carbamates Pesticides

Where R1 represents the alcohol group, R2 signifies the methyl group and R3 is mostly hydrogen atoms. Fairly high insect and mammalian toxicities can be associated with both aryl carbamates and oxime as cholinesterase inhibitors. The cholinesterase inhibitions occurring in carbamates vary when compared with organophosphorus and species-specific and reversible characteristics can be associated with it (Drum, 1980,

Zacharia and James, 2011). Under this group, few of the widely employed insecticides include carbofuran, carbaryl and aminocarb (Figure 1.5).

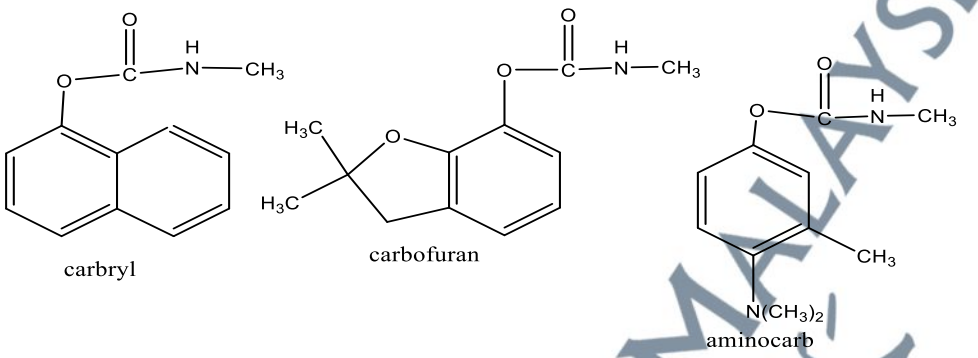


Figure 1.5: Chemical Structure of Some Carbamates Pesticides

Pyrethroids are also a kind of pesticides popular for their low mammalian toxicity, fast effect against insect pests and superficial biodegradation. Although effective insecticides are naturally occurring pyrethrins, their uses as agricultural insecticides are impractical due to rapid photochemical degradation. Naturally occurring pyrethrins' (pyrethroids) synthetic analogues were designed by altering the pyrethrin structure by bringing in a biphenoxy moiety and replacement of halogens with some hydrogens. This is done to impart not only stability but also keeping intact the basic properties of pyrethrins (Zacharia & Tano, 2011). Some of the most employed synthetic pyrethroids are cypermethrin, permethrin and deltamethrin Figure (1.6).

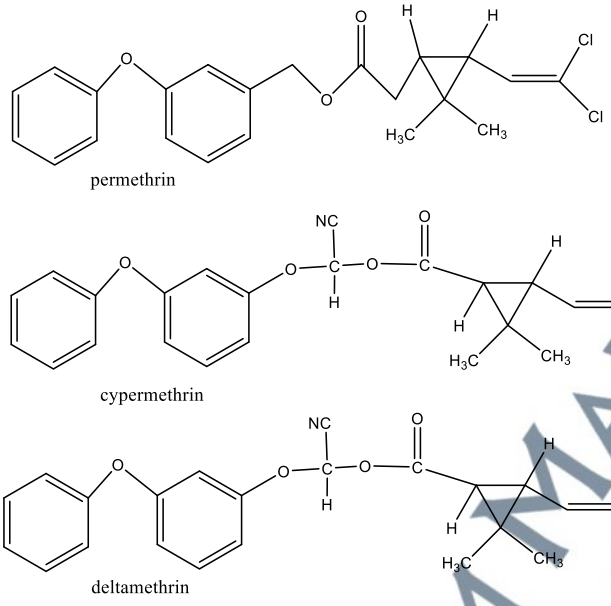


Figure 1.6: Chemical Structure of Some Pyrethroids

Other miscellaneous groups of pesticides: This group includes other peroxyacetic acids as well the herbicide 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) and bipyridyls with the herbicides diquat and paraquat (Figure 1.7).

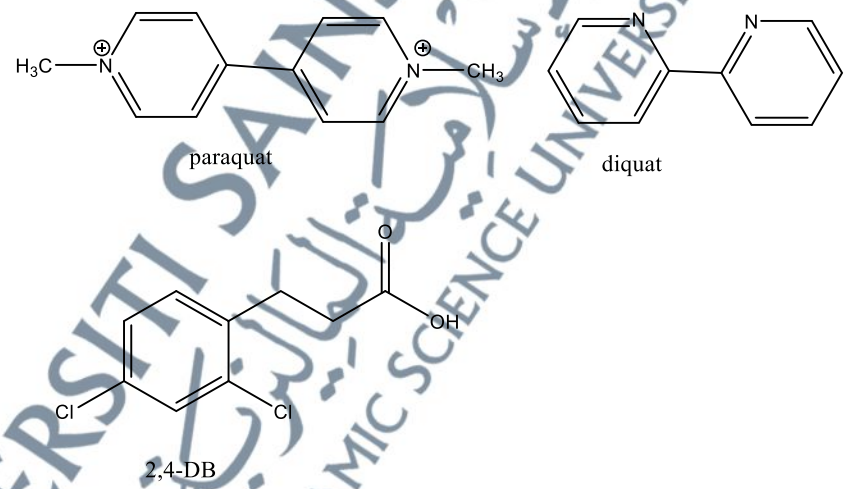


Figure 1.7: Chemical Structure of Some Miscellaneous Groups of Pesticides

Fungicides are pesticides that are utilized for controlling fungal infections in crops. They exist in either organic or inorganic forms. Examples of inorganic fungicides include malachite, Cu $(HO)_2$. $CuCO_3$ and Bordeaux mixture, Cu $(OH)_2$. $CaSO_4$.

Alternatively, organic fungicides include Carbendazim, oxine copper and benomyl (Figure 1.8) (Manahan, 2001).

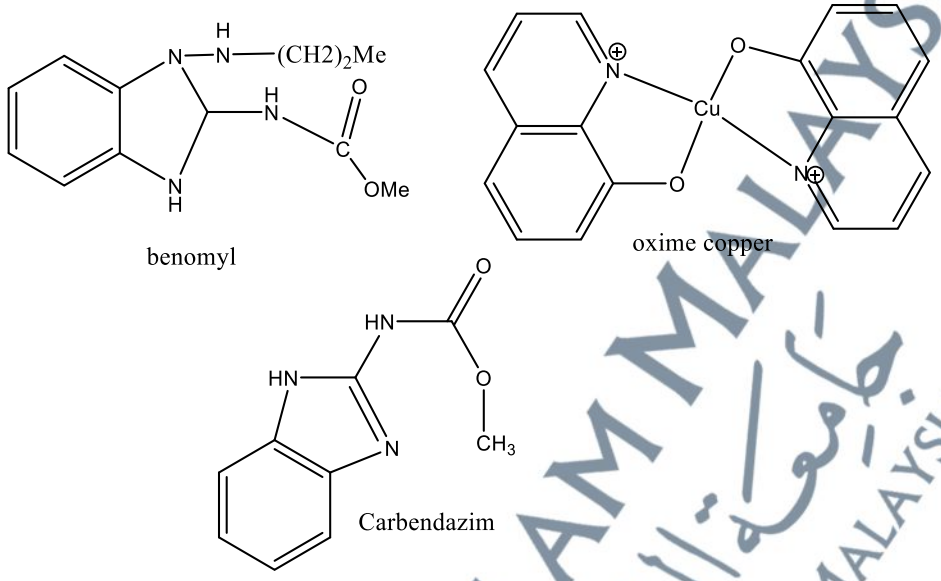


Figure 1.8: Chemical Structure of Some Fungicides

1.1.1.3 Other Minor Classes of Pesticides

1.1.1.3.1 Classification According to Activity Spectrum of the Pesticide

Pesticides are categorised into two groups for this classification: selective pesticides and broad-spectrum pesticides. Broad spectrum pesticides also include pesticides that are believed to affect a broad range of pests and even other organisms that are not usually the targets. Pesticides such as chlorpyrifos and chlordane mostly show toxicity to fish, pets, reptiles, and birds. On the other hand, selective pesticides refer to pesticides that only influence a specific group of pests. This means that other organisms experience little or no effect at all. A good example for this type of pesticide is herbicide 2,4-D, which effects broad-leaved but not on the grassy crops.

1.1.1.3.2 Classification of Pesticides According to the Mode of Formulation

1. Emulsifiable concentrates (EC) refer to fine suspensions of oil droplets in water that possess a milky color. Constant agitation is not needed before every application.
2. Wet table powders (WP) describe a suspension of fine particles in water. For these suspensions, constant agitation is needed before every application.
3. Granules (G) – For applications, granules are collected by mixing clay with the active constituents.
4. Baits – Preparation of the baits is done by mixing a food base with the active ingredient, which is especially utilized for rodent control.
5. Dusts (D) – Application of the dusts is done dry. They typically undergo mixing with carriers like talc, clay, or silica gel.
6. Fumigants – Fumigants are gaseous insecticides that are typically packaged under pressure before being kept as liquids. They can also exist in pellet or tablet forms that release gas upon mixture with water.

1.1.1.3.3 Classification of Pesticides According to Toxicity Level of Pesticides

The World Health Organisation (WHO) has developed a classification system that categorises pesticides based on the potential risks that they pose to human health when they accidentally get in contact with humans. For this classification, the pesticides are grouped into these classes: Class Ia = extremely hazardous; Class Ib = highly hazardous; Class II = moderately hazardous (Zacharia & Tano, 2011).

1.1.2 Heavy Metals

A heavy metal is a term that describes any kind of metallic element that possesses a relatively high density and poisonous or toxic even at low concentrations (Irwandi and Farida, 2009). Furthermore, heavy metals are generally not further metabolized into less harmful constituents, and they undergo accumulation upon their release (Akan et al., 2009). Mercury (Hg), arsenic (As), cadmium (Cd), thallium (TI), chromium (Cr), and lead (Pb) are examples of heavy metals that may cause health risks to individuals and animals due to their high toxicity as well as their likelihood to undergo bioaccumulation within the food chain despite their low concentrations (Suantak et al., 2011). Pollution of the environment with toxic heavy metals has become a worldwide predicament and it is mostly a result of different human activities (Gosh and Singh, 2005; Tandy et al., 2006). Industrial activities have caused the release of toxic trace metals like mercury, copper, cadmium, nickel, and chromium into the marine environment (Yedjou et al., 2012). Furthermore, there is an accumulation of heavy metals in the organs and tissues of aquatic organisms. Their integration and absorption are also dependent on the physiology and element of organisms as well as their environmental, chemical, physical, and biological conditions. It is therefore important to monitor the content of heavy metals for environmental and public health concerns, especially when it comes to food and agricultural products. Heavy metal contamination in food may result into serious health risks based on the type of element and the relative levels of this element. For instance, cadmium and mercury contamination may have an effect on the kidney and bring about chronic toxicity symptoms, which include poor reproductive capacity, weakened kidney function, tumors, hypertension, and hepatic dysfunction (Husna et al., 2014). Furthermore, lead contamination may result into liver damage and renal failure (Wibowo & Suhartono., 2014). Zinc, chromium, and copper lead to anuria, nephritis,

and extensive kidney lesions (Luckey and Venugopal, 1977; Mishra and Shukla., 2014). Even in extremely low concentrations, cadmium is highly toxic to aquatic organisms and possesses an accumulative polluting effect. It is therefore considered as one of the elements that possesses the most toxicity in the environment. Earlier researchers have observed the presence of considerably toxic levels of cadmium in different aquatic ecosystems (Audry et al., 2004; Chrastny et al., 2006). It has the tendency to behave as an endocrine disrupter, which means that it could interfere with certain biological functions such as growth, reproduction, osmoregulation, development, and the ability of fishes to cope up with stress. After entering the organs of freshwater fishes through their gills, it leads to significant metabolic alterations and biological system injuries at varying levels (Pratap and Bonga, 1990; Brown et al., 1984). After it enters the organs of freshwater fishes through their gills, cadmium has been reported to result into anaemia in different fish species for both low and high concentrations (Svanberg, 1976). The absorption of cadmium compounds is based on its solubility. Moreover, its accumulation mainly takes place in the kidneys. In humans, its biological half-life can range from 10 to 35 years (Sarkar et al., 2013).

Lead is also considered to be a non-essential heavy metal. Furthermore, lead poisoning is linked to mental damage. Lead also influences the hematologic, renal, and neurologic systems (Needleman et al., 1990; Hosseini et al., 2015). It is harmful because it also affects the central nervous system. Lead exposure also leads to the differential diagnosis of microcytic anaemia since this heavy metal increases the erythrocyte destruction rate and inhibits the synthesis of heme (Flora et al., 2006; Gautam et al., 2016). In the environment, common lead exposure sources include lead paint, soil or dust near highways or lead painted homes, airborne lead that comes from the combustion of petroleum products that contain tetraethyl lead, batteries, plumbing

leachates from solders or pipes, and lead from leaded chips (American Committee of Environmental Health, 2005). Heavy metal contamination has been linked to urban center discharges, sewage and waste leakages into rivers, and the entry of different agricultural chemicals, domestic wastes, and petroleum products into canals and rivers (M'onica et al., 2006).

1.1.2.1 The Toxic Effects of Heavy Metal and Pesticide Mixtures on Human Health

The risk to human body is increased by daily exposure to manifold toxicants. Heavy metals and pesticides, when combined, increase the severity of this danger. The toxicity of the mixture of pesticides and heavy metals is still not fully understood. Nonetheless, due to their interaction with one another and with the environment, these mixtures may have impulsive consequences (Wallace, & Djordjevic, 2020).

There is also a scarcity of information in the literature about cellular and molecular changes that occur in human cells and alter human genetics. Studies on animals revealed that the spermatogenic element was significantly lost, along with inefficiency and seminiferous epithelium, and a lack of germ cell maturation in rats due to the combined effect of Cd and diazinon (Adamkovicova et al., 2014).

The cytotoxicity of Cd/chlorpyrifos mixture resulted in lipid accumulation in hepatocytes, which increased hepatic toxicity (He et al., 2015). A recent study of patients with pancreatic cancer from various occupations who had been exposed to pesticides discovered that they had higher levels of Cd and Mn (Seo et al., 2019). However, the exposure to chemical mixtures at high concentrations, along with the impact of co-exposure to multiple toxicants, should be investigated further.

1.1.3 Method Development and Validation in HPLC Analysis

1.1.3.1 Principals and Instrumentation

High Performance Liquid Chromatography (HPLC) is a chromatography method commonly used for pesticide residue analysis in food and other fields (Torres et al., 1996). Analysts are divided between the stationary phase and the mobile phase after contact with the stationary phase in the HPLC column. Analytes in mixtures are separated by the liquid mobile phase under high pressure. The separation depends on the degree of interaction and affinity between the solute components and the stationary phase. The low affinity compound for the stationary phase will be isolated first. Regarding the existence of the stationary phase and the separation process, the mode of action of HPLC chromatography is defined as following; reverse phase (RP), normal phase, ion exchange and size exclusion chromatography (Snyder et al., 2012). Reversed phase (RP) HPLC separation is characterized by hydrophobic interactions between the stationary phase and the mobile phase, depending on the ratio and proportion of organic solvents in the mobile phase. With reversed phase chromatography, normal-phase chromatography operates in the opposite manner. The stationary phase of HPLC column is made of strong polar material such as silica gel, whereas the mobile phase consists of a mixture of polar and non-polar solvent polar solvent like isopropanol and hexane. Because of dipole-dipole interactions, the polar compounds retained by the stationary phase. In the ionic exchange technique, the contact is ionic in nature, and the solutes are separated as ions using an ion exchange resin or bound silica. In exclusion chromatography, the separation is based on the size and shape of molecules of the solutes in the mixture solution, the molecules with larger size are less retained by the stationary phase (Lindsay, 1992).

HPLC instrument consists of the following units: the mobile phase reservoir, high pressure pumps, injector, column, detector, and data analysis unit. The mobile phase in reverse phase chromatography is generally comprised of water or buffer mixed with one or more organic solvent with different percentages such as acetonitrile and methanol. The high-pressure pump is used to drive mobile phase mixture from reservoir into the column at a continual and constant flow rate and pressure. HPLC pumps are mainly two types: constant pressure and constant flow rate pumps. Constant flow rate pumps are used in most of current HPLC applications because it has less baseline noise and better retention precision (Lindsay, 1992). Prior to pumping, the mobile phase should be sonicated and filtered to remove air bubbles and particulate matter that can affect the pumping action and the column efficiency. The solvent elution can be performed either isocratically using a mobile phase with constant solvent ratio or gradient elution which involving increasing of the mobile phase strength throughout the separation process.

The sample is introduced into the HPLC system through the injector either by using conventional injection valves or automated injection devices. The automated injection devices which are extensively used modern systems to analyze up to one hundred sample. The automated injection devices enable loading the sample by syringe into the loop and mixing it with the mobile phase prior to entering to the column (Lindsay, 1992). The injector loop volume varies from 10 µL to 500 µL, however, 20 µL is the most used standard. The samples must be filtered before injection to avoid accumulation of any particulate matter that could affect the injector, column inlet or column packing efficiencies, therefore more obstacles can be experienced during HPLC system operation (Snyder et al., 2012).

The column is the most important part in the HPLC structure. It consists of a stainless-steel tube packed with regularly sized and shaped particles (polymer or silica)

known as stationary phase. The rule of the stationary phase material is to help retaining molecules on the column according to their chemical properties i.e., polar, non-polar, ionic, or neutral (Snyder et al., 2012). The silica stationary phase has active adsorption sites for silanol (Si-O-H) groups that are bound to an organic surface layer composed of C8 or C18. These organic surface layers exhibit great selectivity and sensitivity for a variety of chemicals. C18 is the most often used non-polar phase in octadecylsilane (ODS). The majority of HPLC systems contain a column oven to control the temperature of the column, and a guard column to safeguard the column by retaining any insoluble components in the injected sample extract.

Detection of the analysts is achieved by using an appropriate detector with low detection limit e.g., Ultraviolet UV, diode array (DAD), fluorescence, electrochemical (ECD, or mass spectrometry detectors (MS). For a detector, the most significant characteristics are sensitivity, linearity, selectivity, minimal dead volume, and easy use (Lindsay, 1992). Many chemical substances in the sample are known to absorb UV light at different wavelengths, and UV detectors are very popular due to their inexpensive cost and ability to monitor multiple wavelengths in the UV spectrum. A conventional UV detector can measure the absorbance of the compound at one wavelength, while a diode array detector (DAD) can measure the absorbance at several wavelengths and can also provide more identity for a particular sample component. With HPLC chromatograms, the various components' sensitivity to UV light absorption is recorded as an electrical signal.

The software is finally being utilized in the HPLC system to display the peak values (e.g., retention time, peak area, and peak height) and to guide instrumental development through control techniques (improving the validation for precision and accuracy). Additional features include using the software to make predictions and

decisions aimed to improve component's separation as a function of conditions (Snyder et al., 2012).

1.1.3.2 The Method Development

The ultimate goal of method development is to establish an experimental approach that enables the extraction and determination of the purpose of the experiment. In developing methodologies, there are a number of points to take into consideration, such as: the characteristics of the sample, the analysis objectives, and the accessibility of HPLC instruments. Essential data on the analyte 's properties, including chemical structure, UV spectrum and solubility, should be available. Before injection into the HPLC, the pretreatment for samples is required to avoid interferences and column damaging. The separation objectives must be specified whether one chemical, various chemical or all sample components need to be identified and determined. For example, it shall be determined whether a component needs single determination to be measured or qualitative analysis, where standardized detection is preferred (Snyder et al., 1988) for selecting an appropriate detector before starting method development. Following this, the development of the method should begin with a chromatographic separation step that required selecting a suitable HPLC method and optimizing the experimental conditions. Various approaches to the development of HPLC methods are offered.

Revers phase (RP) HPLC methods is often chosen as the first option. Because of the high-resolution, short time and improved and reproducible retention time, HPLC is increasingly considered to be best separation technology (Wang et al., 2003; Majors & Przybyciel, 2002). Separation is the main goal of HPLC routine methods and plans to resolve the sample components from each other. However, different types of chromatography are appropriate depending on their sample's composition. The

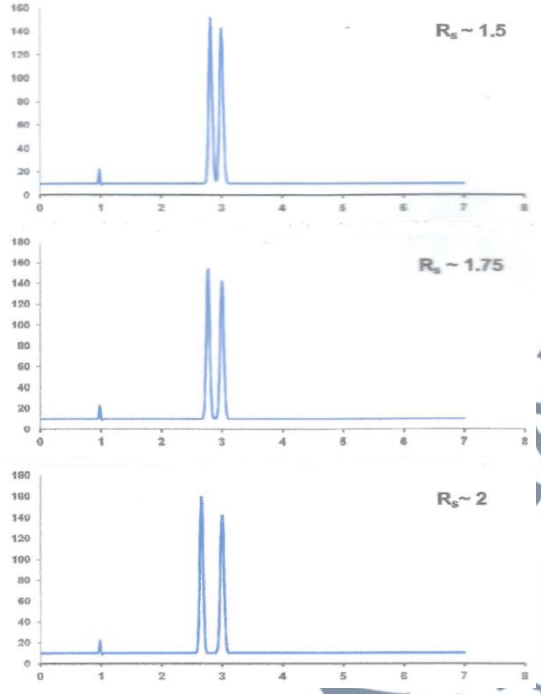
resolution is the parameter of measuring the separation quality. In fluid chromatography resolution (Rs) is used to measure the maximum distance between the two adjacent bands of chromatographic data (Dolan, 2002). It is usually stated as the following equation:

$$Rs = [2 (t_2 – t_1) / (w_1 + w_2)] \quad (1.1)$$

where t_1 and t_2 are the retention time of two neighboring peaks and W_1 and W_2 are peak width measured at baseline (Snyder et al., 1988, Sher Mohammed, 2012). Equation (1.1) can be utilized to calculate Resolution when the band peaks are completely split. Basically, because of difficulty in measuring the baseline bandwidth between overlapping peaks, the bandwidth at half-height of the peak ($W_1$and W_2) is the most excellent way for measuring Rs thus, Rs is defined by following equation:

$$Rs = [1.18 (t_2 – t_1) / (w_1 + w_2)] \quad (1.2)$$

For symmetrical peaks, both equations (1.1) and (1.2) provide roughly the same Rs value (1.5). This can be achieved in the case when the valley between two adjacent peaks touches the baseline only. For quantitative research use of this reference resolution as a minimum value is strongly suggested. However, during the method development of uncomplicated mixtures, some researchers propose values of 1.75 to 2.0 (Figure 1.9) (Snyder et al., 1988; Dolan, 2002).



Source: Sher Mohammed (2012)

Figure 1.9: HPLC Chromatograms Indicating Recommended Resolutions between Two Adjacent Peaks

Resolution can change because the column is overly used, or the experimental conditions fluctuate. There are three independent factors controlling the (Rs) which expressed by the following equation:

$$Rs = (1/4)\,(\alpha - 1)\,N0.5\,[k'/(1+k')] \quad (1.3)$$

where: (α) the separation factor of two bands on the column (or selectivity), (k') the capacity factor and (N) the plate number.

To achieve a required level of resolution, the selectivity factor (α) should be larger than one ($\alpha > 1$), the peaks must be maintained on the column ($1 < k' < 20$) and some minimum number of plates must be developed by the column (Lindsay, 1992; Snyder et al., 1988). These variables must be optimized to achieve the best conditions for a

specific separation (Sher Mohammed, 2012). Selectivity optimization can either be achieved by adjusting the column temperature, the stationary phase or the nature or composition of the mobile phase.

The capacity factor (k') can be managed by modifying the mobile phase composition. While, managing the column length, the particle size and/ or changing flow rate can be employed for controlling the number of theoretical plates (N). Practically, these factors (α, k' and N) are correlated, thus any change in one of these factors might lead to significant changes in the other. However, the retention factor (Rf) should be given the priority during the optimization process and then the conditions affecting selectivity and the theoretical plates should be optimized (Lindsay, 1992; Snyder et al., 1988).

Trial and error strategy should be used to optimize the experimental conditions such as column packing material, temperature, and mobile phase composition. Choosing a suitable stationary phase is the key point to achieve a sufficient separation factor (Fekete et al., 2009). Choosing of a bonded phase C18 is highly recommended for (RP) HPLC. Another criterion considered for column efficiency and separation with sufficient peak symmetry is a column with a minimum plate number (approximately 10,000). About 400 different reversed phase columns are commercially available, they differentiated by the selectivity of five hydrophobic solvent-column interactions (Fekete et al., 2009).

There are several elements affecting column efficiency and performance, involving particle size, performing temperature, duration, and mobile phase flow rate (Snyder et al., 1988; Wang et al., 2003). Temperature of the column has great effect on retention time controlling, selectivity enhancing and peak symmetry in liquid chromatography, but it may possibly cause challenges (Dolan, 2002). Temperature can

be varied (0-70 °C) to control selectivity, usually better resolution can be achieved at higher temperatures, typically in the range 25 to 60°C (Snyder et al., 1988).

Increasing the column length is not good choice since it increases the retention time and cause peak expansion. Decreasing the plate height equivalent of a theoretical plate by decreasing the particle size of the stationary phase, leading to increased HPLC column separation efficiency (Lindsay,1992). This can result in high back pressure if samples contain some particulate matter. Though the optimum flow rate is highly affected by the experimental conditions, particle size of 3 - 5 µm is suitable for performing flow rate of 1 mL/min. The retention and separation selectivity can also differ between column producers and production batches of usually identical columns from the same manufacturer (Dolan, 2002).

Successful selection of columns and mobile phase is a key point to develop simple and quick analytical HPLC procedure (Wang et al., 2003). As the mobile phase is responsible for passing the solute across the stationary phase, therefore it should be pure to avoid any material that can damage the stationary phase or instrument parts such as strong acids or bases and halides. For RP-HPLC, the first choice is the use of acetonitrile solvent for initial separation, followed by methanol. Lower pressures, slightly higher concentration of solvents as well as detection applicability in UV range 185 – 205 nm are the advantages of acetonitrile (Lindsay 1992; Snyder et al., 1988).

Following selection of the mobile phase is to choose the elution mode which mainly depending on separation space. For separation space (> 40%), gradient elusion mostly suitable. While separation space of less than 25 %, isocratic elution is suitable. The separation space is defined as (dt / tG) when dt is the last peak retention time (tn) minus the first peak retention time (t_1), and tG is the gradient time or run time (John Dolan, personal communication). Despite the gradient elution, isocratic elution is

preferred for simples containing of less than 10 elements because of its low cost (Schellinger & Carr., 2006).

The pH of the mobile phase has a significant impact on peak spacing, particularly on separation of acid or base sensitive compounds. The typical run time for HPLC method is between 10-20 min (Dolan et al., 1998; Snyder et al., 1988).

1.1.3.3 Principles of an Analytical Method Validation

A validation of a method tests the applicability of analytical methods. Validation is a structured and logical way of demonstrating the appropriateness of the developed method for assessing the analyte providing practical analytical data within required limits (Maldener, 1989). Method validation including sample preparation, review, and results evaluation. Applicability and specifications depend mainly on the target analyte, the analytical method used and procedure implementation area. In 2006, European Commission guidelines for adopting a method (SANCO/10232) established quality control protocols for pesticide residue analysis (European-Commission, 2006). The most common validation parameters for analytical methods are briefly explained below.

1.1.3.3.1 Selectivity and Specificity

Selectivity is to distinguish the wanted component from the interferences of other components in the sample. The term selectivity expresses the most critical parameter for method validation that describes accurate research measurements. The word selectivity is often used with the word specificity interchangeably. Currently, a major error is made by explaining it clearly if the approach is not very descriptive. Therefore, Thus, one should differentiate between the two words (Sher Mohammed., 2012).

Selectivity means the ability of the system to generate responses in the complex matrix for several analytes, and to distinguish the responses each other (Vessman, 1996). While specificity describes the process, which produces a response for a single analyte only. (IUPAC) proposed promoting the idea of selectivity and addressed the issue by voicing the notion that "specificity is the ultimate in selectivity" (Denboef & Hulanicki, 1983; Vessman et al., 2001). Therefore, these two concepts should be used carefully, and the selectivity must be considered as a key point in all applications of the analytical methods (Aboul-Enein, 2000).

Normally, the concept of selectivity is very common when using UV detection in HPLC analysis because it can identify several components that are present in a sample. A blank sample with and without the analytes should be used to test the selectivity against all matrix components. The method should separate these components or interferences with appropriate resolution (Rs > 1.5) (Maldener, 1989).

1.1.3.3.2 Accuracy and Precision

The accuracy of an analytical method identified as the similarity of the calculated value to the true value obtained. Practically no measuring method is optimal, because the real or actual value of any calculation cannot be precisely determined. Certified reference materials (CRMs) usually used to calculate the precision of the measurements that determine the difference between the predicted value and the true value, and then calculate the actual error size.

the accuracy and the precision are important quantitative parameters to evaluate the mistakes associated with the analysis process (Manoli and Samara, 1999). Therefore, identifying the sources of errors influencing the accuracy is necessary to

enable the analytical chemist to obtain a smart technique to minimize the effect of the errors.

There are three main sources of errors as the following: gross, systematic, and random errors. Gross errors which can destroy the developed method and performing a new one. Systematic errors or bias are constant errors when repeating the experiment under the same conditions. The best example of the systematic error sources are spectral interferences, and standard preparation Random errors are the errors which differ randomly when measurements are repeated under the same conditions. They are unavoidable and alleviating them takes a lot of care. Random errors influence precision while the accuracy is influenced by both random and systematic errors. Another parameter is called uncertainly which combines random and systematic errors. Uncertainly is used to specify a rational range within which the true value of the estimated quantity should lie (Miller and Miller, 2005; Currell, 2000). Calculation of uncertainty is mainly affected by sample preparation (Meyer & Majors, 2002).

An analytical method's precision is described as the degree of agreement between the individual tests acquired when the method is employed to different batches of a standardized sample. Usually, it is expressed as standard deviation (SD) or relative standard deviation (RSD percent) for more than five standard replicate measurements at varied concentrations, low, medium, and high concentration. Precision can be characterized by four types as the following: Repeatability or instrument accuracy is determined by a repetitive sample measurement to test the performance of the instruments. Instrumental measurements usually cause a random noise or error which can be tested with appropriateness tests but is difficult to eliminate. The second form is recurrence (intra-assay precision), which is measured by one researcher using the same conditions, performing sample analysis in one laboratory. The third form is intermediate

accuracy achieved under different operating conditions, using the same laboratory and analytical technique. Finally, reproducibility is the most critical form of precision when testing the same sample under different experimental conditions like a variety of laboratories, analysts, and instruments (Green, 1996).

1.1.3.3.3 Linearity and the Linear Range

An analytical procedure's linearity shows its capacity to produce results proportional to the analyte concentration in the sample, either directly or through mathematical transformation (Shabir, 2003, Chandran & Singh, 2007). A linear response is measured by testing a standard solution at least five different times within the predicted range of the analyte. The regression line equation ($y = ax + b$) is determined by the plotting of concentrations (x) against the response (y) (Caldas et al., 2009). The line of regression is supposed to associate certain distributed errors. The source of error from the calculated response is more than the error in preparing a concentration of samples. The correlation coefficient is frequently used to show the linearity of the regression line (Chandran & Singh, 2007). Nevertheless, there is an issue in the language used for linearity parameters according to various views in the literature. Five different phrases are used to describe linearity criterion, according to the authors, namely r, r^2, correlation coefficient, correlation coefficient with r correlation coefficient with r^2. Statistically, Pearson Product-Moment Correlation Coefficient (PMCC) is commonly expressed as r. In the case of a straight-line graph, the value r^2 is the same as the determination coefficient and represented as R^2 which is determined from the calibration curve regression line provided by Excel to R^2 as a decimal or as percentages if multiplied by 100. The r^2 value is always much less than r (Miller & Miller, 2005).

The $R^2(r^2)$ determination coefficient identifies the shift from the regression line as a percentage (Sher Mohammed., 2012). The linearity requirements are defined as a mixture of the r correlation coefficient and the R^2 (r^2) determination coefficient. In case of a biological samples HPLC assay, a correlation coefficient of more than 0.995 is usually considered appropriate (Arnoux and Morrison., 1992, Sher Mohammed., 2012), While for drug samples at low concentrations, the correlation coefficient should be ≥ 0.98 (Green, 1996). The linearity criterion for output of autosamplers is appropriate when r^2 is 0.998 or greater (Hall & Dolan, 2002; Shabir et al., 2007). Correlation coefficient r values > 0.99 are widely reported in analytical practices (Miller & Miller, 2005). The linearity description in case of autosampler performance is satisfactory when r^2 is equal 0.998 or more (Hall and Dolan, 2002; Shabir et al., 2007). However, calibration curves with r values greater than 0.99 are quite common in analytical practice (Miller & Miller, 2005).

Method range is the interval of appropriate accuracy, linearity, and precision between the upper and lower levels of an analyte concentration in the examined sample (Shabir, 2003; Chandran and Singh, 2007). Based on the results of the linearity study and the method's intended use, the range is measured on either a linear or nonlinear response curve (Green, 1996). It is preferable to plot the concentration or (log concentration) either against deviation from the regression line or against the concentration response ratio in order to avoid any confusion with the range determination process. A nonlinear calibration may be required in a certain analytical approach, but a linear form is chosen in the vast majority of cases (Chandran and Singh, 2007).

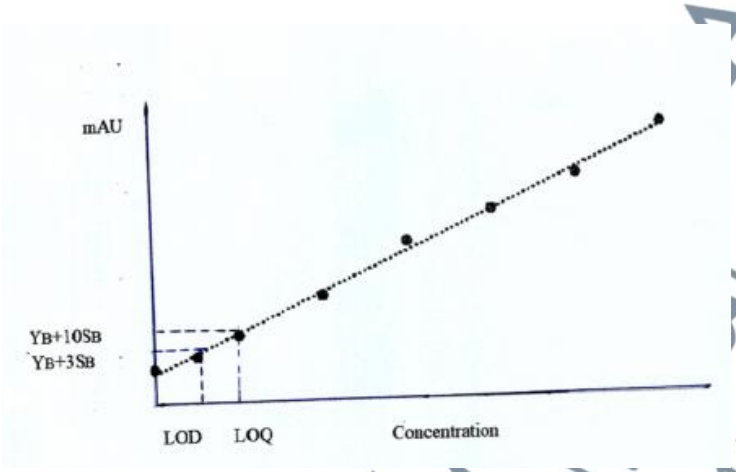
1.1.3.3.4 Detection Limit and Quantification Limit

Limit of detection (LOD) is the minimum analyte concentration that can be reliably detected and differentiated from zero level (or system noise level), but not quantifiable, while limit of quantification (LOQ) is the lowest concentration can be quantified with reasonable precision and accuracy under specified experimental conditions (Caldas et al., 2009). When evaluating LOD and LOQ values, a variety of methodologies are employed (Armbruster et al., 1994). LOD and LOQ are often determined based on the analyte's signal to noise ratio, which generates a response that is three and ten times higher than the system's noise level, respectively, when the analyte is detected. Another technique, on the other hand, uses the 3.3 SD / S relationship to assess LOD and the 10 SD / S relationship to measure LOQ, where SD is the standard deviation of detector response and S is the calibration curve slope. Many scholars prefer to use the value 3 for LOD measurements rather than the figure 3.3, which corresponds to an error probability of 7 percent rather than 5 percent, as it is more accurate (Oteef, 2008; Chandran & Singh, 2007).

The standard deviation can be considered as the standard deviation of replicated blank sample responses altering the response of peak area to concentration. In most chromatographic practices, this approach is not suitable because in most blank samples, the peak area response is not observable. Statistically, calculating the detection limit through the regression line of a low-level set of standard concentrations is the simplest approach (Miller and Miller, 2005; Oteef, 2008). The LOD exhibit signal (peak area) equivalent to the blank signal (YB) plus three standard deviations of the blank signal (3SB) as explained in the following equation:

$$\text{The peak area of limit of detection} = YB + 3SB \quad (1.4)$$

The calibration curve of low rang concentrations is plotted in using the Microsoft Excel. To measure the LOD peak area, the intercept and standard deviation of the regression line are replaced by YB and SB, respectively. The peak area of LOQ can be calculated in the same manner by replacing 3SB with 10SB (Figure 1.10).



Source: Miller and Miller (2005)

Figure 1.10: Calculating the LOD and the LOQ from the Calibration Curve Depends on the Standard Deviation of the Peak Area

1.1.3.3.5 Recovery

Quantitative analysis is used to exactly evaluate the quantity of the analyte. Determination of the method recovery is an important factor in validating the accuracy of the proposed method (Caldas et al., 2009). Recovery is a measure of an analytical method's effectiveness in isolating and quantifying the analyte in the examined sample after extraction and analysis. High recovery at the final measurement stage with low matrix interference is desirable. The IUPAC described recovery as "the proportion of the quantity of the analyte, present or added to the analytical portion of the materials examined, which is extracted and presented for calculation" (Thompson et al., 1999). Recovery experiments are usually curried out by analysis of a blank sample spiked with known concentration of the target analyte, checked at various levels of fortification for

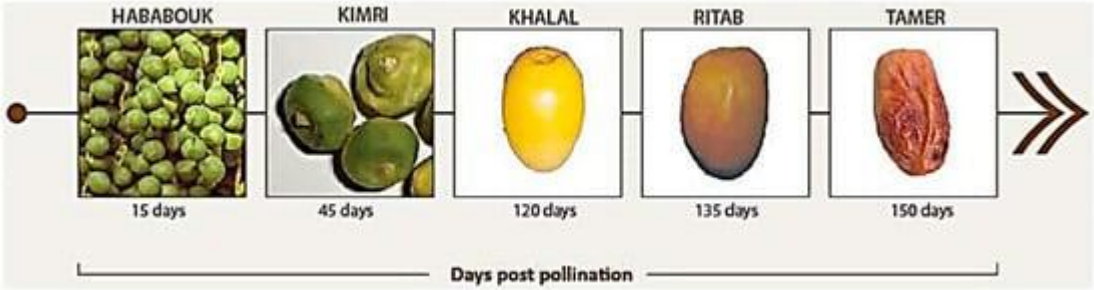
more than five replicates for each sample at various spiking levels. Recoveries in the range of 70 – 110 percent are most frequently appropriate (Linsinger, 2008). There are a multitude of factors that influence recovery values, including the sample matrix, sample preparation procedure, analyte of interest qualities, and concentration. Poor rates of recovery would probably be due to extraction, evaporation, adsorption and degradation of the analyte (LeDoux, 2011). Although the recovery rate may be greater than 100 percent in some cases, this could be owing to some ambiguity during extraction or interference in the HPLC analysis process. (Linsinger, 2008; Wieling et al., 1993). When optimization and validation of the proposed analytical method with a high recovery rate, real samples can be applied to quantitatively evaluate the interest analyte. In some laboratories the calculated recovery rates are used to correct values obtained from the real sample and others do not (Thompson et al., 1999).

1.2 Date Fruits

Many countries consider date palms fruits (*Phoenix dactylifera L.*) as a main food source. In the Near East and North Africa, they are considered as the main fruit and they are consumed in large amounts where they can come in the form of dried, fresh, molasses, and in other treated food products. Furthermore, this fruit is abundant in protein, carbohydrate, and minerals (Al-Showman and Fahad 1990, Kamal, and Fahad 2015). As a result of their high carbohydrate content (70–80 %), these fruits have found more utilisation as a sugar source. This is especially true for countries that practice ancient date fruits production. Date fruits are also considered as a good source of natural antioxidants due to the high content of various phenolic compounds in the fruits. These phenolic compounds include protocatechuic, vanillic, para hydroxy benzoic, coumaric, syringic, and ferulic (Al-Farsi et al., 2005; Eman Abdul Rahman., 2015). These

compounds have a significant effect on total antioxidant activity and may benefit human health as well (Besbes et al., 2009). Furthermore, date juice concentrates are excellent sources of ascorbic acid and tannins. There is also a strong relationship between the total flavonoids and phenolic contents in date fruits, which is suggestive of the notion that these compounds have a vital part in antioxidant activities (Kosanić et al., 2011). Because of their economic and religious importance, date fruits are considered as one of the most vital crops in the Kingdom of Saudi Arabia (KSA), Iran, Iraq, and western Arabic countries like Libya, Tunisia, and Algeria. Because of its high economic value and Date fruits are targeted by various species of pests that infest dates as they are being cultivated, this crop must be protected using a significant number of pesticides. The Ministry of Agriculture in KSA has registered Dicofol (DCF), Chlorpyrifos, and Amitraz (AZ) to control mites. These pesticides are used at a rate of 200 ml/100 L (Al-Rehiayani and Osman 2003, 2005). This results into the accumulation of pesticide residues on (or in) the date fruits during their harvest. Regrettably, to date there is very limited available data regarding the accumulation of pesticides residues in date fruits.

El-Saeid et al., (2010) conducted a study on three date fruit cultivars – Khalas, Nabout Seif, and Sukkari. The study was able to confirm that the fruits and seeds of the date fruits are contained with pesticide residues such as herbicides (Atrazine, Glyphosate,), OPPs (Dimethoate, chlorpyrifos), fungicides (Benomyl, Carbendazim), OCPs (Lindane, Dieldrin), and acaricides (Amitraz, Dicofol, Tourk, Abamectin). Abdallah. et.al., 2018 reported 42 pesticides in dates fruit marketed in Al-Qassim region, KSA using QuEChERS method and LC-MS/MS.



Source: Kamil et al., (2019)

Figure 1.11: Stages of Date Fruit Development Over Time

1.3 Problem Statement

Date palm fruits (Phoenix dactylifera L.) are primary food in many nations due to their nutritional content, antioxidant characteristics, and beneficial benefits on human health. Date fruits are consumed across the world, both for food and for medicinal purposes. Furthermore, recent studies have shown that dates fruits confer great benefits to the health of consumers, including the effects of high in fiber, high in disease-fighting antioxidants, may promote brain health by lowering inflammation and preventing plaques from forming in the brain, which is important for preventing Alzheimer's disease, and promote and ease natural labor for pregnant women when consumed during the last few weeks of pregnancy (Essa et al., 2016).

Date farming, like other agricultural commodities, is vulnerable to a variety of pests. Pesticides are frequently employed in practically every stage of date production, storage, and product manufacturing to ensure quality and food safety (Abdalla et al., 2018). Pesticides can be damaging to human health in addition to their beneficial effects on plants, and due to their widespread consumption, date fruits might be a potential source of pesticide exposure in humans.

While pesticides in agricultural products have been extensively monitored for many years, routine management of date fruits, one of the most popular commodities in

the Islamic world, has been less frequent. Maximum residue limits (MRLs) for approximately 450 pesticides in food have been established in the European Union (EU) by the directive 91/414/EEC, revised and applied by the regulation on maximum residue levels of pesticides in food and feed of plant and animal origin 396/2005/EC, to protect human health and regulate international trade.

Nonetheless, there has been an increase in scientific inquiry and public concern in recent years over the existence and control of trace-level multi-residues of pesticides in date fruits. As a result, robust, sensitive, and selective analytical methodologies for pesticide residue analysis in date fruits are required. Today, one of the most widely used methods for pesticide residue analysis in various food matrices is the quick, easy, cheap, effective, robust, and safe (QuEChERS) approach, which was initially published by Anastassiades et al., (2003).

QuEChERS is a sample extraction and clean-up process used to prepare the sample for gas or liquid chromatography. The QuEChERS technology is used to extract pesticides from foods having a water content of more than 80%. Because date fruits have a low water content of < 20%, there was a need to modify the QuEChERS methodology in terms of the components involved in extraction and clean-up conditions, as well as pesticide chromatographic analysis.

Pesticide residue analysis is often performed in several steps. First, target analytes are extracted from the sample matrix using organic solvents. Second, crucial clean-up procedures, then liquid or gas chromatographic (LC or GC) analysis, which is often followed by detection step.

Matrix effects are key causes of ambiguity in chromatographic analysis (Zrostlikova & Hajslova, 2003). Depending on the pesticide and the matrix, co-eluting

matrix components may be responsible for false-negatives, false-positives, or inaccurate quantification (Taylor, 2005).

Pesticides residue analysis procedures require a high degree of precision and accuracy, which associates with a sufficient removal of co-extractives generating matrix effects (Kruve, et al., 2008). Even though several approaches for various food matrices have been reported in literature, pesticide residue analysis remains an analytical difficulty (Botitsi, et al., 2011; Fernandez-Alba, et al., 2008). This is especially true for extremely tough matrices like date fruits. Although many efforts have been made to develop methods for pesticide residue analysis in date fruits, particularly employing HPLC-DAD, sample clean-up and solvent usage remain unsatisfactory.

In HPLC-DAD, the advantages of high-performance chromatography (HPLC) to identify pesticides multiresidue were merged with low-cost quick simultaneous sample analysis under reproducible conditions, high automation, and the possibility of multi-detection.

The large quantities of consumed dates can cause higher risk of pesticides, which is especially true in children and other susceptible individuals. Therefore, there is a need to establish safe washing methods for pesticide removal from date fruits. This current study will therefore validate some safe techniques of washing treatments. So that the impact on consumer's health can be reduced.

However, due to the increased size of cities and their intersection with palm tree farming areas as well as highways, real ways, oil fields, and chemical industries, recently published data from Medal East showed an increase in some dangerous heavy metals contents in date fruits, such as arsenic (As) and lead (Pb), which were found higher than the maximum tolerable daily intake (EDI) in date fruit samples from some locations in KSA.The calculated hazard risk index predicted that both AS and Pb may

have a possibility of health risk to a consumer's health (Aldjain, et al., 2011; Salama et al., 2019).

Heavy metals can cause serious threat to animals and human beings because of their high toxicity and tendency to bioaccumulate in the food chain even at low concentrations, (Suantak et al., 2011). Therefore, monitoring of pesticide residues and heavy metals in the imported dates into the Malaysian market is necessary due to the large amounts of date fruits consumed.

1.4 Objectives of this Study

In the present work, the study will focus on the following:

1. To develop and optimize the QuEChERS extraction and clean up methodology for determination of the selected pesticides (dimethoate, carbendazim and chlorpyrifos) in date fruits samples.
2. To develop and optimize of HPLC- DAD analysis method for separation and analysis of the selected pesticides from date fruits.
3. To investigate of the suitable solvent systems and the optimization conditions to reduce the pesticides content in date fruit with minimum effect on its nutritive value.
4. To develop and optimize of a sensitive digestion procedure for date matrix to determine some heavy metal's contents in the examined date fruit samples using GFAAS and this will include:
    a. Determination of health hazard index HHI of the heavy according to the measured concentration.
    b. Determination the effect of water washing on heavy metals contents in date fruits.

1.5 Scopes of the Study

1. The study focused on QuEChERS modification and optimization of date fruit matrix extraction and cleanup, the factors, and responses for optimization of the extraction and cleanup analysis were compared using plackett-Burman(P-B) screening design.
2. A successful HPLC-DAD Chromatographic method for isolation and quantification of the target pesticides in date fruit matrix was developed and validated via linearity, sensitivity, accuracy and precision.
3. The developed method was successfully applied in extraction and determination of examined pesticides in date fruits using isocratic elution and simple mobile phase methanol/water (95: 5) 5mM ammonium formate.
4. Health hazard index (HHI) for examined pesticides was calculated.
5. Suitable washing methods were studied and optimized for pesticide removal to achieve a high percentage of removal of the examined pesticides from date fruit with a minimum effect on the nutrition value of dates.
6. A sensitive digestion method for determination of some essential and non-essential heavy metals (Fe, Cu, Ni, Pb, As, Co, Al and Sn) in date fruits was developed and optimized regarding the digestion mixture ratio, temperature, and duration.
7. The developed digestion method was successfully employed for determination of heavy contents in date samples.
8. Health hazard index of heavy metal was calculated.
9. Effect of water washing on heavy metals content of date fruits was evaluated.