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Screening for pesticides (to develop a scheme for testing)

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Forensic Science

Roseny Augustina binti Ismail

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150, Kubang Kerian, Kelantan
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CERTIFICATE

This is to certify that the dissertation entitled

"Screening for Pesticides (to Develop a Scheme for Testing)"

is the bonafide record of research work done by

Ms. **Roseny Augustina binti Ismail**

during the period **November 2003** to **February 2004**

under my supervision .

Signature of Supervisor: 

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Date: **29th January 2004**

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Abstract

Pesticide poisoning is an important cause of morbidity and mortality in many countries in the world. Pesticides are substances or mixture of substances for controlling pest. Because of its hazardous property for killing the pests, pesticides are also always harmful for human beings if there are not used wisely and carefully. There are still many types of pesticides that are available in the market although many types of them had been banned to be produced or to be sold. For the purpose of pesticide screening, classification of pesticides is based on their chemical properties. This project focuses on pesticides which are classified as organochlorine hydrocarbons, chlorinated phenoxy acids, organophosphorus pesticides, carbamate pesticides and paraquat. In this project, pesticides from each class were added to an artificial biological sample of stomach content.

Then, the pesticides in the stomach contents are extracted with various types of solvents and mixture of solvents. In each step, the adjustment of pH is so important because the solubility of pesticides in solvents may be depending on the pH of the sample. By following this method of extraction, at the end of the project the best solvent and the suitable pH for extracting pesticides in biological samples will be known. After extracting the stomach contents, all fractions of the organic layer that are collected in each extraction step, will be evaporated and the residue will be subjected to thin layer chromatography (TLC) for identification of pesticides. Beside identification of pesticides by using

TLC, identification of paraquat will be made separately and estimated quantitatively.

Chromatograms of TLC of different pesticides will be developed using different solvent systems according to their chemical nature. The suitability of spray reagent to a particular class of compounds is also a main concern in this project. This is to confirm the presence of pesticides in extracted solvents. The R_f value of the samples and R_f value of the standards will be compared and pesticides that have extracted will be identified. The presence of pesticides in a particular solvent will indicate that the pesticides are soluble in that solvent. Then, the solvent that extracted most of the pesticides will be concluded as the best solvent to use for extraction.

At the end of this project, a simple scheme of extraction will be developed and this scheme can be used for screening pesticides in pesticide poisoning cases especially when the pesticide is unknown. The scheme is useful for the usage of laboratories which lack of modern automated instrument for screening pesticides.

Introduction

Pesticides, as defined by the Federal Insecticide, Fungicide and Rodenticide Act include "(1) any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest [insect, rodent, nematode, fungus, weed, other forms of terrestrial or aquatic plant or animal life or viruses, bacteria or other organisms, which the Administrator declares to be a pest]" and "(2) any substances or mixture of substances intended for use as a plant regulator, defoliant or desiccant" (Rose et al., 1999). Pesticide is a generic name for a variety of agents that are classified more specifically on the basis of the pattern of use and organism killed (Ecobichon, 1994). Major agricultural classes of pesticides are insecticides, herbicides and fungicides (Ecobichon, 1994).

Over the centuries, humans have developed many ingenious methods in their attempts to control the invertebrates, vertebrates and microorganisms that constantly threatened the supply of food and fiber (Ecobichon, 1994). The use of pesticides to control the harmful organism is very effective and causes the supply of food and fiber increase. Therefore, pesticides are very useful to avoid agricultural losses due to insect damage. In spite of beneficial use of pesticides, they are harmful to human being where they are not been used appropriately. The target species are not only the organism affected by pesticides because sometimes pesticides also act on the humans in a harmful manner. Basically, pesticides control pest by blocking cellular process mechanically by inhibiting

metabolic systems, disruption of protein synthesis and enzyme, disruption of enzyme process, denaturing proteins, interfering hormone system, disruption of nervous system, or by inhibiting photosynthetic mechanism in plants.

Because of the hazardous effect of pesticides on human being, pesticides also can be known as toxic substances. Similar to other toxicants, exposure to pesticides can be classified into two, namely chronic and acute exposures. Both types of exposure can lead to pesticide poisonings. Whether the poisonings are acute or chronic exposure, there is still a need for screening methods for suspected pesticide in poisoning cases. Because pesticides include such a diversity of chemical types that it is not surprising that their toxicities cover a very wide range of signs and symptoms (Fysh and Whitehouse, 1986). For example, as little as 100 mg of mevinphos or nicotine may cause death, whereas 1.5 kg of the herbicide aminotriazole is unlikely to endanger human life. Even within particular class of pesticides the lethal dose may vary considerably (Fysh and Whitehouse, 1986).

In Malaysia, there are many cases of pesticide poisonings. Population can easily gain access to the pesticides because pesticides are sold widely in agricultural shops as well as in ordinary hardware shops. Due to the poisoning cases occurring, a simple and an effective method for screening the pesticides in poisoning cases have to be developed. Although there are a number of separate methods available for the detection of common pesticides, a simple and an efficient method of extraction of commonly used pesticides have yet to be developed. With the facilities available, there is no way that these

compounds to be detected in body fluids such as blood or urine as the levels of poison will be very low. Thus, it should be possible to develop a scheme to detect these compounds in stomach contents in the event of non absorbed poison still remaining in stomach.

This project focuses on detection of pesticides in stomach contents. The project is mainly to develop a simple and an effective method of extraction of most commonly used pesticides and for pesticides which are available in market. In this project, extraction is done using various solvents and mixture of solvents for extraction of various classes of pesticides. As stated before, the major agricultural classes for pesticides are insecticides, herbicides and fungicides. Further, they can be divided into many groups of compounds depending on the chemical nature. Sub-classes for insecticides are chlorinated hydrocarbons, organophosphorus insecticides, carbamates and alkaloidal insecticides and sub-classes of herbicides are chlorinated phenoxyacetic acids, substituted ureas, triazine herbicides, uracil herbicides and quaternary ammonium compounds (Fysh and Whitehouse, 1986). Pesticides that are under investigation in this project are mainly insecticides and herbicides from classes of chlorinated hydrocarbons, organophosphorus insecticides, carbamates, chlorinated phenoxyacetic acids and quaternary ammonium compounds.

Insecticides and herbicides that have been used as samples in this project are endosulfan, methylchlorophenoxyacetic acid (MCPA), malathion, chloropyrifos, fenthion, demethoate, propoxur and paraquat. The chemical

classification of these pesticides is stated in the table 1. All these pesticides were added to the stomach content which was artificially made and then was extracted using various solvents and mixture of solvents. The organic layers with the extracted compounds were evaporated to obtain residues of the pesticides. The residues were then subjected to thin layer chromatography (TLC) for identification. Thin layer chromatography was used in this project because this technique represents a cheap, simple and quick method for screening pesticides (Fysh and Whitehouse, 1986). In spite of advantages in using thin layer chromatography (TLC), it suffers from the disadvantage that there is no single system and a location method to cover all the compounds of interest (Fysh and Whitehouse, 1986) and the lack of sensitivity. In this project, identification for paraquat was not done using thin layer chromatography because the method gave less favourable results (Akintowa, 1984). Therefore, determination of paraquat was done using spectrophotometric determinations which involved the reaction of paraquat with 3% solution of sodium dithionite in 0.3M sodium hydroxide (NaOH) which is freshly prepared. The absorbance of the resulting blue cation measured at 460nm and 397nm can be used as a measure of the paraquat concentration.

Organochlorine insecticides have been used extensively from mid-1940s to the mid-1960s in all aspects of agriculture and forestry, in building and structural protection and in human situations to control a wide variety of insect pests (Abd. Majid, 1996). Their well-known properties (low-volatility, chemical stability, lipid solubility, slow rate of biotransformation and degradation) made

this compounds to restricted uses (Abd. Majid, 1996). Nowadays, because of its persistence in the environment, accumulated concentrations within various food chains and the magnification of biologically active body bioburdens in many wildlife species that interfered with the reproductive success of some species, organochlorine pesticides are banned in most countries (Abd. Majid, 1996). From 1992 to 1994, there are still many product containing organochlorine compounds available in Malaysia and that is listed in Pesticides Board of Malaysia but now only endosulfan is still legally sold in the market.

Table 1: Chemical classification of pesticides

Pesticides	Chemical classification
Endosulfan	Organochlorine (chlorinated hydrocarbons)
MCPA	chlorinated phenoxyacetic acids
Malathion, chloropyrifos, fenthion, demethoate	Organophosphorus ester
Propoxur	Carbamates
Paraquat	Quaternary ammonium compounds

Chlorophenoxy herbicides, which include 2,4-D, 2,4,5-T, MCPA and silvex are plant regulators (Rose et al., 1999). These are characterized structurally by the presence of a simple acid, which is esterified onto a phenyl ring with one to three chlorine substitutions (Rose et al., 1999). During Second World War, considerable effort was directed toward the development of effective, broad-spectrum herbicides in both United States and the United Kingdom with a view to both increasing food production and to finding potential chemical warfare

agents (Kirby, 1980; Ecobichon, 1994). The Chlorophenoxy compounds including their acids, salts, amines and esters were first commercially available products evolving from the research in 1946 (Ecobichon, 1994). One of the most popular herbicides developed is 2,4-D which was used in combination with 2,4,5-T as a component of Agent Orange during the Vietnam War (Rose et al., 1999).

Organophosphorus ester insecticides were first synthesized in 1937 by a group of German Chemist led by Gerhard Schrader at Farbenfabriken Bayer AG (Ecobichon, 1994). After realizing that organochlorine insecticides introduced problems and became more widely appreciated, insect pest control began to rely on more on the anticholinesterase active organophosphorus and carbamate ester pesticides (Ecobichon, 1986). Before organophosphorus compounds were used as insecticides, under the management of Nazis in Second World War, some of these compounds were developed as potential chemical warfare agents (Ecobichon, 1994). VX, Soman and Sarin are among the strongest nerve agents (Miki et al., 1999). Although it is true that all of the organophosphorus esters were derived from "nerve gases", a fact that media continually emphasizes, is that the insecticides used today are at least four generations of development away from those highly toxic chemicals (Ecobichon, 1994).

The first pesticidal carbamic acid esters were synthesized in the 1930s and were marketed as fungicides (Ecobichon, 1994). Since these aliphatic esters possessed poor insecticidal activity, interest lay dormant until the mid-

1950s when renewed interest in insecticides having anticholinesterase activity but reduced mammalian toxicity led to the synthesis of several potent aryl esters of methylcarbamic acids (Ecobichon, 1994). The synthesis and commercialization of the carbamate pesticides has been in progress since the 1950s (Ecobichon, 1986). Nowadays, carbamates are mainly esters of methylcarbamic acids (Lima et al., 1995).

Paraquat (1,1'dimethyl,4,4'bipyridyl) is a non selective contact herbicide (Akintowa, 1984). Paraquat was first synthesized in 1882, but its pesticidal properties were not discovered until 1959 (Haley, 1979; Ecobichon, 1994). The common paraquat salts are all apparently fully ionized and experiments have shown that anions, chloride, sulphate, methylsulphate etc., do not affect the toxicity of paraquat (Fletcher, 1974). Paraquat is a widely used herbicide in attempted suicides through oral ingestion to cause paraquat intoxication (Lin, 1990; Hsu et al., 2003).

Review of Literature

There are many researches that had been done involving pesticide analysis because the issue of pesticide will never stop and it has always been a health issue, an environmental health issue, an occupational health issue and a public health issue. Therefore, analysis for pesticides is so important to detect the contamination of pesticides in any matter, biological or environmental. The development of any analytical method is useful for the purpose of reference in analysis of pesticides.

Fysh and Jones (1979) and Fysh and Whitehouse (1986) had introduced the basic scheme for pesticides extraction in which the solvent used was ether. The scheme is always been a reference for pesticides analysis. In the past few years, researches on pesticides mainly focused on the analysis of pesticides using automated equipments and using methods that do not need an extraction of the samples. These new methods of analysis will give better and quick results. For example, there is research done by Agustina et al. which analyzed pesticides using stop-flow fluoroimmunoassay (1997). This research was basically developed as an analytical method to determine the presence of 2, 4-dichlorophenoxyacetic acid in orange and grape juice. Determination of phenylurea herbicides in environmental waters by using an application of on-line coupling of solid-phase extraction to liquid chromatography (Martin-Esteban et al., 1997) is another research done in the past. In another analysis using automated equipment was the detection of 2, 4-dichlorophenoxyacetic

acid using a fluorescence immunolyzer (Rogers et al., 1997). Besides that, many other researches had been carried out such as removal of atrazine and four organophosphorus pesticides from environmental water by diatomaceous earth-remediation method (Fernandez Hernando et al., 2000). Another example in the development of pesticide analysis was the kinetic study of degradation of chlortoluron (a phenylurea herbicide) in water disinfection process (Losito et al., 2000). Francoise Parrot et al. (1995) had done a research in determining glyphosate containing herbicides by using a routine aminoacid analyzer in glyphosate herbicide poisoning cases. It was an ion exchange chromatography procedure, using automatic amino acid analyzer, which was routinely carried out and the equipment was used for analysis of amino acids in biological fluids (Francoise et al., 1995). In their work, they used serum as a biological sample. In a case report of organophosphate poisoning after disulfoton ingestion, the organophosphate was extracted with hexane and was analyzed with gas chromatography equipped with alkali flame thermionic detector sensitive to phosphorus (Futagami et al., 1995).

According to researches that had been carried out previously and also the case reports, we know that there are many analytical methods for screening pesticides which are mostly involving modern equipment. Although the usage of automated equipments in screening the pesticides is the rapid and cost-effective method, the classical method that involving the extraction of the sample is still very important in a developing country like Malaysia because most laboratories in Malaysia are not well-equipped with such modern

equipment. In a developing country, in which the economy is based on agriculture, free availability of pesticides, poverty related socioeconomic problems, lack of adequate protective clothing, and limited treatment facilities contribute to the high morbidity and mortality (Fernando, 1995). There is a lack of easily accessible information on pesticides related ill news in the countries of Asia-Pacific Region (Fernando, 1995). This scenario is common to Malaysia also. In this country, population is not openly informed about the hazards of pesticides. Furthermore, there is a lack of expertise and a lack of facilities in laboratories that can handle pesticide poisoning cases. Therefore, it is important that a simple scheme for analysis of pesticides has to be developed. This project is basically based on the classical method of pesticide analysis.

Objective of the Study

The objective of this research is to develop an efficient and a simple extraction scheme to screen the pesticides in biological specimens. The method of analysis will involve the extraction of biological sample for determining pesticide identification. The scheme that will be created should be able to extract as many types of pesticide as possible and should be able to detect them quickly. This project also aims at the fact which solvent is most suitable for a particular class of pesticides.

Another objective of this work is to fulfill a need for a rapid analytical method for pesticide screening because pesticide poisoning is a major health concern globally. In the case of pesticide poisonings, a rapid analysis method for detection and evaluation of pesticides in biological fluids is so important to confirm the diagnosis, to monitor the patient and to evaluate the effectiveness of elimination procedures such as gastric lavage, forced diuresis and hemodialysis.

Materials and Methods

Materials:

1. Equipments:
    1.1. Mortar
    1.2. pH meter
    1.3. Water bath (was set to 95 °C)
    1.4. UV spectrophotometer
    1.5. TLC plate and TLC apparatus

    The plate was activated for 20 minutes in 70 °C before loading the sample.

2. Glassware:
    2.1. Volumetric flask
    2.2. Separatory funnel (100ml)
    2.3. Small funnel
    2.4. Conical flask (250ml)
    2.5. Beaker (50ml)
    2.6. Beaker (100ml)
    2.7. Test tube

3. Material used:

    3.1. Rice

    3.2. Pesticide

        3.2.1. Endosulfan

        3.2.2. MCPA

        3.2.3. Malathion

        3.2.4. Chloropyrifos

        3.2.5. Fenthion

        3.2.6. Demethoate

        3.2.7. Propoxur

        3.2.8. Paraquat

    Endosulfan and paraquat was bought at 'Pertubuhan Peladang Negeri Kelantan' with the other pesticides is get from chemical store of School of Health Science.

4. Chemicals:

    4.1. Petroleum ether

    4.2. Diethyl ether

    4.3. Methyl tertiary butyl ether

    4.4. Chloroform

    4.5. Hexane

    4.6. Ethyl acetate

    4.7. Cyclohexane

4.8. Acetone

4.9. Chloroform

4.10. Bromine

4.11. O-toluidine

4.12. Potassium Permanganate

4.13. Bromophenol Blue

4.14. Silver nitrate

4.15. Sodium dithionite

4.16. Sodium bicarbonate solid

4.17. Anhydrous sodium sulfate

5. Reagents:

5.1. 2M hydrochloric acid

6ml of hydrochloric acid in a stock aolution was diluted into 100ml with distilled water

5.2. 1% solution of O-toluidine (chromogenic reagent)

1ml of O-toluidine was diluted to 100ml with 95% ethanol.

5.3. 1% solution of potassium permanganate (acidified) (chromogenic reagent)

1g of potassium permanganate was diluted to 100ml with 0.25M sulphuric acid.

5.4. Solution of bromophenol Blue (chromogenic reagent)

0.05g of bromophenol Blue was dissolved in 10ml acetone. Then, this solution was diluted to 100ml with 1% of silver nitrate solution

5.5. 1% silver nitrate solution

1g of silver nitrate solid is diluted to 100ml with acetone:water mixture (1:3)

5.6. 3% solution of dithionite in 0.3M sodium hydroxide

3g of sodium dithionite was diluted to 100ml with 0.3M sodium hydroxide.

5.7. 0.3M sodium hydroxide

2.4g of sodium hydroxide was diluted to 200ml with distilled water

5.8. Standard for paraquat

- 250µg/ml paraquat

  0.1ml of paraquat in a stock solution (concentration of stock paraquat is 25% w/w) was diluted to 100 ml with distilled water.

- 1.0µg/ml paraquat standard

  1ml of 250µg/ml prepared paraquat was diluted to 250ml with distilled water.

- 2.5µg/ml paraquat standard

  2.5ml of 250µg/ml prepared paraquat was diluted to 250ml with distilled water.

- 4.0µg/ml paraquat standard

  4ml of 250µg/ml prepared paraquat was diluted to 250 ml with distilled water.

- 5.0µg/ml paraquat standard

  5ml of 250µg/ml prepared paraquat was diluted to 250 ml with distilled water.

Methods:

1. Preparation of the sample

Cooked rice was ground using a mortar and the ground rice was placed in a beaker. 500ml of distilled water was added to the beaker. 2M of hydrochloric acid was added into the beaker containing the mixture of rice and added distilled water and adjusted the pH of the sample to 3.5 as natural stomach contents are acidic, it was decided to maintain the acidity in the prepared stomach contents.

To prepare the samples, eight types of pesticides were added to a 100ml volumetric flask. The pesticides were endosulfan, MCPA, malathion, chloropyrifos, fenthion, demethoate, propoxur and paraquat. All pesticides except paraquat were not added in specific volume but paraquat was added in specific quantity, 0.1ml from 250µg/ml prepared paraquat because estimation for paraquat will be made quantitatively using spectrophotometer. Other pesticides will be detected qualitatively using thin layer chromatography (TLC). Added pesticides were mixed thoroughly with the stomach contents to obtain a uniform sample. Total volume of the sample was 100ml and the final concentration of paraquat was approximately about 2.5µg/ml in the sample.

2. Extraction of the sample

Extraction of the pesticides in the sample was made separately by following two schemes. The schemes are shown in the Figure 1 and Figure 2. The first scheme is basically the extraction of the pesticides in the sample by using a single solvent in every step of extraction whereas the second scheme is the extraction of the pesticides using a mixture of two solvents. Before starting the extraction, the prepared sample was filtered using a filter paper to remove any solid in artificial stomach contents that would interfere with the extraction processes.

i. Extraction with petroleum ether

To start the extractions following the first scheme, 20ml of homogenized prepared sample was measured and was placed in a separatory funnel. Then, about an equal volume of petroleum ether (boiling point 40-60 °C) was added to the same separatory funnel. The mixture was shaken thoroughly for the pesticides in the sample to be extracted to the petroleum ether layer. After shaking the mixture there were two layers in the separatory funnel in which the lower layer was aqueous layer and the upper layer was petroleum ether. The aqueous layer was collected in a beaker and the petroleum ether layer was collected in a conical flask. Petroleum ether layer was passed through small funnel with cotton wool and anhydrous sodium sulfate to remove any traces of water in this layer before collecting in a conical flask. After that, the previous

aqueous layer was extracted again with petroleum ether. The aqueous layer was collected in the beaker and was kept for the next step of extraction. The petroleum ether layer was collected and combined with the previous collected petroleum ether layer. All the organic layers are needed to be passed to anhydrous sodium sulfate in every step of extraction, to remove traces of water.

ii. Extraction with diethyl ether

For the next step in the extraction, the aqueous layer from the previous step of extraction was placed in a separatory funnel. Then, about an equal volume of diethyl ether was added to the aqueous layer and was shaken thoroughly. There were two layers seen in separatory funnel where the lower layer was the aqueous layer while the upper layer was the diethyl ether layer. The aqueous layer was collected in a beaker while the upper layer was collected in a conical flask. Again, the collected aqueous layer was then extracted with diethyl ether. After shaking well the mixture of the aqueous and the diethyl ether, the lower layer was collected in a beaker whereas the upper layer was collected in a conical flask. The diethyl ether layers were combined.

Basically, in real cases of poisoning, the extraction of the synthetic organic pesticides from biological materials, such as stomach contents and food residues is complicated by the fact that certain groups of pesticides are either acid or base labile (Fysh and Jones, 1979). For example, the substituted ureas are hydrolyzed by dilute acid or alkali and substituted *N*-methylcarbamates decompose in alkaline media (Fysh and Whitehouse, 1986). The

organophosphorus insecticides are also hydrolyzed in alkali and certain members of the class (azinphosmethyl, diazinon and malathion) are also unstable in acid (Fysh and Whitehouse, 1986).

By referring the scheme that had been introduced by Fysh and Jones in book of 'Forensic Toxicology' (1979) and scheme that introduced by Fysh and Whitehouse in book of 'Clarke's Isolation and Identification of Drugs' (1986), the collected aqueous layer from the previous extraction was adjusted to pH 2 with 2M hydrochloric acid. After adjusting the pH, the aqueous layer was placed in the separatory funnel and about an equal volume of diethyl ether was added into it. The mixture of acidified aqueous layer was shaken thoroughly to make soluble the pesticides in the diethyl ether layer. Then, two layers were seen in the separatory funnel. The lower layer was the aqueous layer and was collected in a beaker while the upper layer was the diethyl ether layer and was collected in a conical flask. The collected diethyl ether layer was combined with previous diethyl ether layer before adjusting the pH to 2. After that, the collected aqueous layer was extracted once again with diethyl ether and was shaken well. Method for collection of aqueous layer and diethyl ether layer was the same as the previous collection. Thus, all the diethyl ether fractions were combined.

iii. Extraction with methyl tertiary butyl ether

For the next step of extraction, the previously collected aqueous layer was divided into two equal portions. One portion was kept for estimation of paraquat

using spectrophotometer whereas the other portion was adjusted to pH 7 with sodium bicarbonate solid. After adjusting the pH to 7, the aqueous layer which was 10ml was placed in a separatory funnel and was extracted with a volume of methyl tertiary butyl ether with a volume equal to that of the aqueous layer. After extracting the aqueous layer, the lower layer, which was the aqueous layer, was collected in a beaker while the methyl tertiary butyl ether layer was collected in a conical flask. Then, the extraction with methyl tertiary butyl ether was repeated for a better extraction of the pesticides. The collected aqueous layer was kept for the next step of extraction and the methyl tertiary butyl ether layer was combined with the previously collected methyl tertiary butyl ether layer.

iv. Extraction with chloroform

Then, the previous aqueous layer from above step was adjusted to pH 2 with 2M of hydrochloric acid and after that, it was placed in a separatory funnel and about an equal volume of chloroform was added to the separatory funnel for extraction. The mixture of acidified aqueous layer and chloroform was shaken thoroughly for a few minutes. Then, two layers would be seen in the separatory funnel. The lower layer was the chloroform layer and was collected in a conical flask. Again, the collected aqueous layer was extracted with chloroform about an equal volume as the aqueous layer. After shaking the mixture, the aqueous layer was discarded and the chloroform layer was combined with the previous chloroform layer.

v. Extraction with the mixture of petroleum ether and hexane

After completing the extraction using the first scheme, extraction process was started again and the second extraction process followed the second scheme. The prepared sample was again measured 20ml and was placed in a separatory funnel. Then, about an equal volume of mixture of petroleum ether and hexane (1:1) was added to the same separatory funnel for extraction. The mixture of sample and the extraction solvents was shaken thoroughly. After shaking the mixture there were two layers in the separatory funnel in which the lower layer was aqueous layer and the upper layer was the mixture of petroleum ether and hexane layer. The aqueous layer was collected in a beaker and the mixture of petroleum ether and hexane layer was collected in a conical flask. After that, the previous aqueous layer was extracted again with the mixture of petroleum ether and hexane. The aqueous layer was collected in the beaker and was kept for the next step of extraction. The organic solvents were collected and combined with the previously collected organic solvent layer.

vi. Extraction with the mixture of diethyl ether and hexane

For the next step of extraction, the aqueous layer from the previous step of extraction was placed in a separatory funnel. Then, about an equal volume of a mixture of diethyl ether and hexane (1:1) was added to the aqueous layer and was shaken thoroughly. There were two layers seen in separatory funnel where the lower layer was the aqueous layer while the upper layer was the organic

solvents layer. The aqueous layer was collected in a beaker while the upper layer was collected in a conical flask. Again, the collected aqueous layer was then extracted with a mixture of diethyl ether and hexane. The mixture of diethyl ether and hexane layer was combined with the previously collected organic solvent layer.

The next step of extraction, the collected aqueous layer in the beaker was adjusted to pH 2 with 2M hydrochloric acid. After adjusting the pH, the aqueous layer was placed in the separatory funnel and about an equal volume of a mixture diethyl ether and hexane (1:1) was added into it. The mixture of acidified aqueous layer and the extraction solvents was shaken thoroughly. Then, two layers was seen in the separatory funnel. The lower layer was the aqueous layer and was collected in a beaker while the upper layer was the mixture of diethyl ether and hexane layer and was collected in a conical flask. The collected organic layers were combined with previous diethyl ether layer before adjusting the pH to 2. After that, the collected aqueous layer was extracted once again with a mixture of diethyl ether and hexane (1:1) and was shaken well. Method for collection of aqueous layer and organic layer was the same as the previous collection. This organic layer was combined with mixture or organic layer which extracted the pesticides before adjusting the pH to 2.

vii. Extraction with the mixture of methyl tertiary butyl ether and chloroform

For the next step of extraction, the previously collected aqueous layer was divided into two equal portions. One portion was kept for estimation of paraquat