
**UNIVERSITI PUTRA MALAYSIA**

# *RESIDUE LEVELS OF CHLORPYRIFOS IN LEAF MUSTARD (BRASSICA JUNCEA (L) COSS.) IN RELATION TO SAMPLING TIME*

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# RESIDUE LEVELS OF CHLORPYRIFOS IN LEAF MUSTARD (*Brassica juncea (L) Coss.*) IN RELATION TO SAMPLING TIME

By

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## DEDICATION

This dedication is to mummy and daddy, Kai Ling, Sun Hoi, Sind Hoi, Kah Hin, Aunt Kah Kee, Moi Jin, Kok Yong, Benson, Kim Guan, Jaron, May Yin, Sze Yee, Hui San, Yoke Mee, Sheue Yann, Yah Hui, See Jian, Emily, Vince, Ai San and Constanne. Thanks for your love and support.



**"Time is precious.**

**You can't own it, but you can use it.**

**You can't keep it, but you can spend it.**

**Once you've lost it, you can never get it back.**

**Use your time wisely."**

## ABSTRACT

An experiment was conducted in the netted greenhouse at the share farm in Universiti Putra Malaysia Bintulu Campus to study leaf residue levels of the insecticide chlorpyrifos used to control insect pests in leaf mustard (*Brassica juncea (L.) Coss.*). There were 3 treatments in 4 replicates, 0.46 kg/ha, 0.92 kg/ha and an untreated control. Leaf samples for residue analysis were harvested at 8, 14 and 20 days after treatment. Chlorpyrifos residue levels in the leaf samples were determined by HPLC (Model-Jasco), fitted with an Inertsil ODS-3 (5 μm, 4 mm x 150 mm length) column with PDA-wavelength detector. Insect damage assessment was determined at 13 and 18 days after treatment to evaluate effectiveness of the treatments. Insect damage was significantly different between the control and insecticide treatments. Results obtained indicate that chlorpyrifos residue levels in leaf mustard were not detected in all samples at 8, 14 and 20 days after treatment. This suggests that a single application of chlorpyrifos at 3 weeks after planting should not leave any residues in the leaves harvested after 4 weeks from planting.

## ABSTRAK

Satu eksperimen dijalankan dalam sebuah rumah jaring di ladang kongsi Universiti Putra Malaysia Bintulu Kampus untuk mengkaji sisa-baki racun serangga chlorpyrifos dalam daun sawi (*Brassica juncea (L.) Coss)*. Terdapat 4 replikasi dan 3 rawatan, iaitu 0.46 kg/ha, 0.92 kg/ha dan kawalan tanpa rawatan. Sampel daun yang diguna dalam analisis sisa dituaikan pada 8, 14 dan 20 hari selepas rawatan. Sisa chlorpyrifos dalam sampel daun telah ditentukan dengan menggunakan HPLC (Model-Jasco), dilengkapi dengan kolum Inertsil ODS-3 (5 μm, 4 mm x 150 mm panjang) bersama dengan pengesan PDA-multiwavelength. Penilaian kerosakan serangga ditentukan pada 13 dan 18 hari selepas rawatan untuk menilai keberkesanan rawatan. Kerosakan serangga adalah nyata, berbeza antara kawalan dan rawatan racun serangga. Keputusan yang diperolehi menunjukkan sisa chlorpyrifos dalam daun sawi adalah pada paras yang tidak dapat dikesani dalam semua sampel pada 8, 14 dan 20 hari selepas rawatan. Ini menunjukkan bahawa rawatan chlorpyrifos sekali pada 3 minggu selepas menanam tidak akan meninggalkan sebarang sisa racun berkenaan pada daun yang dituai pada atau selepas minggu ke-empat selepas menanam.

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## APPROVAL SHEET

I certify that this research project reported entitled "Residue Levels of Chlorpyrifos in Leaf Mustard (*Brassica juncea (L) Coss.*) In Relation to Sampling Time" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Bioindustry Science in the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus.

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mg/ kg/ day = milligram per kilogram per day

mg/ kg = milligram per kilogram

ml/ min = milliliter per minute

mg/ L = milligram per liter

kg/ ha = kilogram/ hectare

ppm = part per million

Kcal = Kilocalorie

mPa = miliPascal

mg = milligram

µg = microgram

cm = centimeter

% = percentage

mm = millimeter

µm = micrometer

$m^2$ = meter cubic

ml = milliliter

µl = micro liter

g = gram

m = meter

# CHAPTER 1

# INTRODUCTION

Leaf or Chinese mustard (*Brassica juncea (L) Coss.)* is one of the main vegetables consumed in Malaysia. It is also planted and consumed widely in other countries (Siemonsma and Piluek, 1996). It has been cultivated in Malaysia for many years and covers an area of approximately 4285 ha (DOA, 2002). The vegetable contains numerous nutrients including the antioxidants, vitamin E, beta carotene and vitamin C that can contribute towards a healthy cardiovascular system. Leaf mustard production in Malaysia contributes a significant amount of income to farmers who sell the vegetable in the domestic market.

During its growth process, leaf mustard gets damaged by many insects and diseases such as army worms, mites, *Plutella* sp., cutworms, aphids, leaf roller, leaf rot and soft rot (DOA, 2002). Different pesticides are applied to control insect pests of leaf mustard, which include organophosphates and pyrethroids (DOA, 2001). Chlorpyrifos is one of the most-widely used active ingredients for pest control in many vegetable crops. It is a Class II insecticide which is moderately toxic to humans. The maximum residue limit (MRL) of chlorpyrifos is 0.1 mg/kg (FDR, 2005). However, farmers have misused chlorpyrifos resulting in residue levels above the permitted MRL. This had been reported in vegetables such as cabbage, cauliflower, tomato, chili and mustard (Mukherjee, 2002).

Concern over toxic residues in fruits and vegetables and their toxic potential risk to human health, persistence and tendency to bioaccumulate has led to the development of many methods for monitoring pesticides compound (Štajnbaher and Zupančič-Kralj, 2003). The analytical methods vary depending on the type of the sample and the purpose of analysis. High performance liquid chromatography (HPLC) is one of the most common methods for pesticides residue analysis.

It is used for separation of mixed components and qualitative or quantitative analysis. In view of the widespread use of chlorpyrifos in leaf mustard, studies are needed to determine residue levels over time following application and established appropriate time of application to ensure residue levels at harvest are below the internationally MRL.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Leaf mustard

Leaf mustard is from the Cruiceferae family, and gets its name from the characteristic cross-shaped flowers of the genus *Brassica*. The species of mustard most often grown commercially is *Brassica juncea* which is of oriental origin and known as Chinese or leaf mustard. *Brassica juncea* is an annual with green leaves, sometimes with a whitish bloom. Leaf mustard is one of the more popular vegetables consumed by Malaysians. The common local names of leaf mustard are "Sawi Hijau", "Sawi Bunga" and "Choy Sam". In 2002, the total local production was approximately 131,000 metric tonnes from a cultivated area of 4,285 hectare. In terms of economic value, the contribution of leaf mustard production in gross income per hectare is RM 14, 000 or RM 1.00 /kg (DOA, 2002).

### 2.1.1 Crop characteristics

The life cycle for leaf mustard is around 27-30 days after sowing and yields range from 14,000-16,000 kg/ha (DOA, 2002). The stem is small, slender or big and compact and green or light green in colour. The leaves are upright, dark green in colour and with small, yellow flowers. Seeds of leaf mustard are brown in colour, roundish and small with a 1000 seed weight of 2 g. The seeds must be mixed with a fungicide before sowing to prevent fungus disease (DOA, 2001). Leaf mustard requires a lot of water for fast growth. Therefore, during hot days plants must be irrigated twice per day

(Siemonsma and Piluek, 1996). The temperature requirement is around 23-35 °C and are suitable to plant in soils with a pH range from 5.0-6.0 (DOA, 2002). Leaf mustard is rich in nutritive value, comprising of vitamins, minerals, protein, dietary fibre and carbohydrate (Table 1) (Siemonsma and Piluek, 1996; DOA, 2002).

### 2.1.2 Weed control

Weeds problems occurring during the early growth phase of leaf mustard can be managed by minimum tillage methods. With minimum tillage, weeds controlled before sowing the crop. It is best to till the soil to about 5 cm depth in order to encourage weed seed germination thereby depleting the available weed seed bank. Then, a non-selective herbicide such as glyphosate can be sprayed shortly before sowing. The advantages of minimum tillage is that it allows for better timing for crop establishment as there is no need to wait for suitable conditions for land preparation (RADA, 2006). Besides, the problem of soil erosion is reduced as residual vegetative matter is generally present and this improves in water retention in the soil (RADA, 2006). In addition, it allows the use of marginal lands as there is little soil disturbance and reduction in cost for land preparation. However, minimum tillage still has disadvantages where weed infestation can become a major problem.

### 2.1.3 Pest and disease problems

In practice, application of a pesticide is often the only means of reducing a pest population rapidly (Matthews, 1984). Good practices use of pesticides which are more active to the target species than to non-target species, by improved timing of application

and by increasing the proportion of the applied dose that reaches the target organism (Matthews, 1984). Effective control measures must be based on a clear understanding of the behaviour of the target species (WHO, 2004). In selecting a pesticides and the

Table 1: Nutritional composition of leaf mustard (For every 100 g edible portion) (DOA, 2002)

| Content | Amount |
|---|---|
| Energy | 29.0 Kcal |
| Moisture | 91.1 g |
| Protein | 2.2 g |
| Carbohydrate | 3.3 g |
| Fibre | 0.4 g |
| Ash | 1.5 g |
| Calcium | 138.6 mg |
| Phosphorus | 83.0 mg |
| Iron | 1.3 mg |
| Sodium | 12.4 mg |
| Potassium | 471.5 mg |
| Beta carotene | 2957.0 µg |
| Vitamin B1 | 0.09 mg |
| Vitamin B2 | 0.27 mg |
| Niacin | 0.28 mg |
| Vitamin C | 89.0 mg |

appropriate formulation, consideration should be given to its biological effectiveness against the pest concerned, susceptibility of the target organism, methods of application, and its safety to humans, its toxicity to non-target organisms, and the registration status of the pesticide for the required use (WHO, 2004). In leaf mustard, different pesticides are used to control different pests and pathogens (Table 2).

Table 2: Pest and disease management in leaf mustard (DOA, 2002)

| Pest / Disease | Symptom | Pesticides used |
|---|---|---|
| Army worm (*Spodoptera litura*) | Presence of holes in the leaves and shoots. | cypermetrin, trichlorfon, fenvelerate |
| Mites (*Phllotreta sinuata*) | Presence of fine holes in the leaves. | malathion, acephate, cypermetrin |
| Plutella (*Plutella xylostella*) | Presence of transparent holes in the leaves. | *Bacillus thruringiensis* acephate, chlorfluazuron |
| Cut worms (*Agrotis ypsilon*) | Stem is cut off at the base. | trichlorfon, chlorfluazuron, fenvelerate |
| Aphids (*Aphid* sp.) | Leaves become crinkled, collapse and the plant dies. | cypermetrin, profenofos, dimethoate |
| Leaf roller (*Phytomyza* sp.) | Presence of lines on the leaf surface caused the plant wilts and die. | |
| Leaf rot (*Corticium solani*) | Leaves rot and plant dies. | quitozene, benomyl |

## 2.2 Pesticide residues

Pesticides are diverse and omnipresent. Approximately 1400 pesticides are used worldwide (Wilson and Otsuki, 2004). Pesticides are necessary and essential for pest control in agricultural production. But, the health risks from pesticide residues in the diet have become a public concern. Poisoning from pesticides has been estimated to affect between 500,000 to 2.9 million people worldwide annually, causing fatalities in 1 % of the cases or more than 10,000 deaths per year worldwide (Moore, 2002). This has lead to strict regulations on maximum permissible residue limits in foodstuffs (Rissato *et al.*, 2005).

Food is a very basic need. It is important to ensure food is safe, accessible and affordable to all people at all times. In Malaysia food safety is something which many are not familiar with (Yanasekaran, 1999). Food safety in Malaysia is not taken seriously by all parties. The pesticides used are not controlled properly largely due to limited or lack of proper enforcement. Hence, many farmers use pesticides beyond the recommended harvest interval thus causing contamination of crops (Yanasekaran, 1999). There was increased public awareness on pesticides contamination in food when cabbages from Cameron Highlands were rejected by Singapore in 1998 because of high pesticides content (Yanasekaran, 1999). There was some action by authorities to check for pesticides residue levels in vegetables. However, there are mounting concerns about the long-term environmental impact of vegetable production in the Cameron Highlands, focusing particularly on the heavy use of pesticides and the associated health hazards,

build-up of resistance and contamination of the environment (Mazlan and Mumford, 2005).

### 2.3 MRLs and ADI

Good pesticides application practices can reduce the risk to human health whereby residue levels are avoided or maintained below the legal limit at harvest. With respect to safety maximum residue levels (MRLs) permitted, are primarily intended to ensure pesticides are used correctly. MRLs are defined as the maximum concentration of pesticides residue (expressed as milligrams of residue per kilogram of food /animal feed) likely to occur in or on food and animal feed with the use of pesticides according to Good Agricultural Practices (GAP) (PRC, 2006). Each country sets MRLs on food imports and these are imposed as regulatory standards at the border (Wilson and Otsuki, 2004). The MRL of chlorpyrifos is 0.1 mg/kg (FDR, 2005).

A complete review of data based on the biochemical, metabolic, pharmacological and toxicology properties of the pesticides derived from experimental studies is important to establish the acceptable daily intake (ADI) of a pesticide residue (Wilson and Otsuki, 2004). The ADI (computed as mg/kg/day) is the amount of chemical which when consumed every day of an individual's lifetime will not have any detrimental effect (PRC, 2006). ADI for chlorpyrifos is 0.01 mg/kg/day (Kamrin, 1997).

## 2.4 Chlorpyrifos

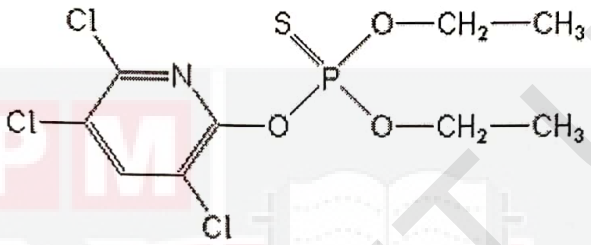
The number of organophophorus (OPs) insecticides registered in various parts of the world has increased rapidly, and has now reached about 250 (Cabras, 2003). At present, they are the most widely marketed insecticides with 37.2 % market share (Cabras, 2003). Organophosporus pesticides are esters, amides or thiol derivatives of either phosphoric acid or thiophosphoric acid. The majority now in use, such as chlorpyrifos, azinphos-methyl and malathion, contain the thiono moiety (Cooper, 2002).

Chlorpyrifos (O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl) phosphorothioate) is an organophosphorus pesticide of moderate mammalian toxicity and active against a wide variety of agricultural and public health arthropod pests (WHO and FAO, 1975). It was introduced by the Dow Chemical Company in 1965 and is marketed under the names Dursban and Leursban. Application methods include soil-incorporation or directed uses by bark and foliar treatments (Montemurro *et al.*, 2002). It is used in agriculture, horticulture, viticulture and forestry and in a wide range of crops such as corn, rice, cereal, ornamental plants, fruits and vegetables (FAO, 2004).

### 2.4.1 Chemical and physical properties

Chlorpyrifos is moderately toxic following acute oral, dermal and inhalation exposures and is classified in toxicity category II for all exposure routes (USEPA, 2000). Technical chlorpyrifos is amber to white crystalline with a mild sulfur odor (Kamrin, 1997). Molecular weight of chlorpyrifos is 350.6 (WHO, 2002). The melting points are 41.5-44 °C and vapor pressure is 2.5 mPa (Kamrin, 1997). The $LD_{50}$ value of

chlorpyrifos is 135 mg/kg (FAO, 2004). It is a stable compound in neutral and acidic aqueous solutions, although the stability decreases as pH increases (Montemurro *et al.*, 2002). Chlorpyrifos is practically insoluble in water (2 mg/L) and soluble in most organic solvents such as acetone, xylene, and methylene chloride (Montemurro *et al.*, 2002). The structure of chlorpyrifos:




Chlorpyrifos is moderately persistent in soils. The half life of chlorpyrifos in soil is usually between 60-120 days but can range from two weeks to over one year depending on the soil type, climate, and other conditions (Cooper, 2002). Adsorbed chlorpyrifos is subject to degradation by UV light, chemical hydrolysis and soil microbes (EXTONET, 1999; Kamrin, 1997).

The concentration and persistence of chlorpyrifos in water will vary depending on the type of formulation. For example, a large increase in the concentration of chlorpyrifos in water occurs when emulsifiable concentrate (EC) and wettable powders (WP) enter through run-off or spray drift (Cooper, 2002). With granules and controlled release formulations the concentrations are lower than both EC and WP, but may persist longer (Kamrin, 1997). Volatilization is probably the primary route of loss of chlorpyrifos from water (Kamrin, 1997; Hamilton and Crossley, 2003).

### 2.4.2 Toxicology

Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant. One of the severe effects for humans is the acute toxicity. Chlorpyrifos, an irreversible inhibitor of cholinesterase (ChE) and acetylcholine esterase (AChE), is one of the most widely used organophosphate insecticides in the US (Zhao *et al.*, 2006). Sufficient inhibition of AChE in the central and peripheral nervous systems causes excessive accumulation of acetylcholine which in turn results in neurotoxicity. Inhibition of ChE is believed to be the most sensitive effect in all animal species evaluated and in humans, regardless of route or duration of exposure (Zhao *et al.*, 2006).

Chlorpyrifos can not only cause neurotoxic effects through inhibition of cholinesterase activity, but can also cause other systemic toxicities, such as maternal toxicity in treated pregnant dams and their foetuses, although the developing foetuses are not considered as more sensitive to chlorpyrifos than the adult animal (Zhao *et al.*, 2005). Chlorpyrifos has also been shown to cause inhibition of brain DNA (Dam *et. al.*, 1998), protein synthesis (Whitney *et al.*, 1995) and changes in brain RNA concentration (Johnson *et al.*, 1998). Subacute chlorpyrifos exposure caused inhibition of acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BuChE), and a significant reduction in the platelet uptake of 5-Hydroxytryptamine (5-HT) in rats (Sachanal *et al.*, 2001). Chlorpyrifos has been recently shown to have mutagenic potential in mouse somatic and germ cells (Donya, 1998).

### 2.4.3 Chlorpyrifos residues in farm produce

Residues remain on plant surface for approximately 10-14 days (Kamrin, 1997). The pre-harvest interval is 7 to 14 days (EPA, 2004). However, some farmers harvest vegetables before the pre-harvest interval (Mazlan and Mumford, 2004). Therefore, the vegetables and fruits may contain residue of the insecticide above the prescribed MRLs and become a health hazard to consumers. Presence of chlorpyrifos above the prescribed tolerance limit in vegetables has been reported (Mukherjee, 2002).

Limit of detection (LOD) for chlopryrifos is 0.05 ppm in fruits and vegetables (MFQPA, 2000). A national survey of pesticides and their metabolites in the US found that the primary chlorpyrifos breakdown product, 3, 5, 6-trichloro-2-pyridinol was the second most commonly detected chemical in food (Cox, 1995). Chlorpyrifos is regularly detected in fruits and vegetables in the UK. The residue level in one sample of Spanish celery exceeded the MRL of 0.05 mg/kg (Anon, 1998). Between 1990 and 1992, residues of chlorpyrifos have been found in tomatoes, oranges, peaches, cherries, banana, apples, broccoli, tea, grapes, orange juice and several types of processed oriental foods including noodle soup (Cox, 1995). Moreover, chlorpyrifos residues had also been found in meat and dairy products following a pour on treatment to control flies (Cox, 1995). Chlorpyrifos residues have also been detected in selected baby foods (USFDA, 2003).

### 2.4.4 Degradation of chlorpyrifos over time

The degradation of the pesticides follows a logarithmic curve (Emden and Service, 2004). The persistence of residue is measured in terms of half-life which is the time taken for residue levels to fall by half. Many factors influence the degradation of pesticides including application factors, pesticide properties and weather conditions. The half-life and degradation of chlorpyrifos and other pesticides were closely related to application times, rate and weather (Kumar *et al.,* 2004; Zhang *et al.*, 2006). As chlorpyrifos is a volatile insecticide (Kamrin, 1997) thus water evaporating from the leaf surface will carry dissolved volatile residues away into the air (Emden and Service, 2004). In addition, rainfall will wash the residue off surfaces to a greater extent (Emden and Service, 2004). Oxidation and radiation catalyzed by ultraviolet also plays an important role in the degradation of pesticides (Kamrin, 1997; Emden and Service, 2004). Besides, different chemical formulations of chlorpyrifos have different rate of degradation over time. For example, the dissipation of chlorpyrifos in orange fruits for an EC was fast during the first phase and became much slower during the later period, while residue levels of chlorpyrifos from microencapsulates (ME) remained almost constant for 65 days and then began to decrease (Montemurro, 2002).

## 2.5 Chlorpyrifos residue analysis

Numerous studies have been reported on chlorpyrifos residue analysis (Racke *et al.*, 1996; Guardino *et al.*, 1998; Montmurro *et al.*, 2002; Sardar and Kole, 2005) including methods for residue analysis in vegetable crops (Mukherjee, 2002; Ngan *et al.*, 2005: Zhang *et al.*, 2006). The most frequently used analytical methods were gas

chromatography and high performance liquid chromatography (Ahmed, 2001; Topuz *et al.*, 2005; Zhang *et al.*, 2006; Mauldin *et al.*, 2006).

High performance liquid chromatography (HPLC) is now being extensively used in pesticide chemistry and related areas where chemicals of interest are frequently of low polarity or thermally unstable for GC separation (Torres, 1996). HPLC is a useful technique for trace quantities of polar or termolabile compounds where selectivity and sensitivity of the more commonly used detectors may in some cases be improved by the use of suitable derivation techniques (Galera, 2006). Carbamates, organophosphorus, ureas, phenols, phenoxy acids, and synthetic pyrethroids, are polar and/or thermally labile and thus are not directly amenable to GC determination (Muszkat and Aharonson, 1986). The main advantages of post-column derivatization are that the analytes are separated in their original form, without the need for a complete derivatization reaction (assuming reproducibility) and the reaction products need no stability for a long time (Galera, 2006). The diverse methods used to determine pesticides in food samples by HPLC have been well documented by Bushway (Torres, 1996).

A HPLC system requires a solvent delivery system in which the mobile phase is pumped through the column (Sørensen *et al.*, 1999). The columns used are generally made of stainless steel which can withstand the applied pressure. The commercially available stationary phases are selective in retaining any molecular structure (polar, non-polar, ionic, or neutral). When the solvent is pump through at high pressure, a small volume of sample is deposited on the top of column. The pesticide distributes itself

between the stationary phase and mobile phase as the solvent moves through the column. The pesticides with higher affinity for the stationary phase will exit last. A fraction collector is needed for preparative techniques and a computer system can be used for both control of the system and treatment of the analytical data (Sørensen *et al.*, 1999).

HPLC methods for the determination of pesticide residues in fruits and vegetables employ reversed-phase chromatography with $C_{18}$ or $C_8$ columns and aqueous mobile phase, followed by UV absorption, UV diode array, mass spectrometric or fluorescence detection (Torres, 1996). In the reversed-phase columns the mobile phase is polar (usually water with methanol or acetonitrile) and the bonded phase is hydrophobic, such as $C_{18}$. Non-polar component are not suitable because they are strongly retained on reverse-phase columns, while polar compounds are only slightly retained. Reverse-phase columns are suitable for the separation of both nonionic and ionic compounds on a single column (Muszkat and Aharonson, 1986). Reversed-phase chromatography is mainly based on the distribution of analytes between solutions on the stationary phase and the more hydrophilic mobile phase, involving hydrophobic interactions or the analytes equilibrium distribution between these two phases (Sørensen *et al.*, 1999). The obtainable separation of analytes is principally determined by the characteristics of the mobile phase.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Introduction

This study comprised of two parts: (1) A field study to evaluate effectiveness of insecticide treatments, and (2) Analysis of treated plants for the insecticide residues.

## 3.2 Field study

This study was conducted in a netted greenhouse in the field in University Farm at Universiti Putra Malaysia Bintulu Campus starting from 19 August 2006 until 31 October 2006.

### 3.2.1 Land preparation

The study area was first sprayed with a post-emergence herbicide to control the weeds on the vegetable beds. All weeds were slashed with a motorized mower and rotovated with a power tiller. The soils were left undisturbed and weeds allowed growing for about three weeks. The glyphosate, a broad spectrum, non-selective systemic herbicide was sprayed to control the weeds using a RB 15 Knapsack sprayer fitted with a fan nozzle. The sprayer was calibrated to deliver a spray volume of 700 liters/ha. The herbicide was applied at 4.2 liters a.i./ha.

### 3.2.2 Planting of leaf mustard

There were 12 totals of beds each of which was 8 m long by 1.5 m wide. The leaf mustard seeds treated with Thiram fungicide were drill sown in four rows on each bed. A total of 250 g of leaf mustard seeds was used. The beds were irrigated using the sprinkler system for about 15-20 minutes everyday as required. Fertilizer application was carried out once a week for 3 weeks and later twice a week for 1 week. A 10 ml of suspension of foliar fertilizer (20-20-20+ Biostimulant) with 2 ml of wetting agent mixed in 4 liters of water was used at each application.

### 3.2.3 Application of treatments

The chlorpyrifos insecticide (Cobra) was applied 3 weeks after sowing when the leaf mustard was at growth stage 15 (4 to 5 leaves) according to the BBCH scale (Stauss, 1994). Insecticide treatments were applied with a spray volume of 482 liters water/ha. There were 3 treatments: 0.46 kg chlorpyrifos / ha, 0.92 kg chlorpyrifos / ha and an untreated control. Wetting agent (0.1 %) was added to the spray mixture to enhance chlorpyrifos performance. The experimental of layout was a Randomize Complete Block Design (R.C.B.D.) with 4 replicates. Effectiveness of treatments was determined by scoring for insect damage at 13 and 18 days after treatment using 50 cm x 50 cm quadrant. The insect damage assessment was based on the number of leaves damaged within each of two quadrant samples per plot.

### 3.2.4 Crop sampling for yield and residue analysis

Leaf samples were taken at 8, 14, and 20 days after chlorpyrifos application, by collecting about 100 g of leaf at random from each of the treatment plots. All samples were placed into plastic bags and taken to the laboratory for residue extraction as described below. The yield of the leaf mustard was recorded by harvesting and weighing all plants within 1 m x 1 m quadrant in each of the treatment plots.

## 3.3 Determination of chlorpyrifos residues

### 3.3.1 Extraction of chlorpyrifos from leaf samples

The 4 replicates samples for each treatment were pooled to form 2 samples for each of the treatments. Each of the pooled 200 g samples were macerated and homogenized in a blender. An aliquot of 30 g was weighed into a 250 ml conical flask and mixed with 100 ml of acetonitrile and homogenized for 4 minutes. Then, 50 g of anhydrous sodium sulfate was added to absorb the excess water. The mixture was allowed to equilibrate for 4 minutes and filtered through a 12 cm filter paper into another conical flask. The extract was further treated with 50 g of anhydrous sodium sulfate and filtered again. The extract was transferred to a rotary evaporator and evaporated in a fume cupboard. The dried residue was re-dissolved in 5 ml of acetonitrile and filtered through a 0.45 μm PTFE filter into specimen bottle. The sample bottles were capped tightly and wrapped with aluminium foil and kept in a freezer for HPLC analysis (Arrebola *et al.*, 2003).

### 3.3.2 Preparation of chlorpyrifos standard solution

Chlorpyrifos standard (0.015 g) was dissolved in 50 ml of acetonitrile in a volumetric flask and kept in the freezer. Working standard solutions were prepared by volumetric serial dilutions.

### 3.3.3 Residue analysis

HPLC Model-Jasco was performed on a LC Net II data acquisition apparatus, gradient pump, Auto-sampler, oven column and PDA-multiwavelength detector. Chlorpyrifos standard was obtained from Riedel-de-Haën with a purity of 99.2 %. Solvents (acetonitrile) used in the study were HPLC grade and all inorganic reagents were laboratory grade (glacial acetic acid and anhydrous sodium sulfate).

The samples were analyzed by HPLC Model-Jasco (Japan) fitted with an Inertsil ODS-3 (5 μm, 4 mm x 150 mm length) column, using gradient elution from 100 % solvent A (water: acetonitrile: glacial acetic acid, 90: 10: 0.5) to 100 % solvent B (acetonitrile: water: glacial acetic acid, 90: 10: 0.5) over 30 minutes at a flow rate of 1.5 ml/min. Samples of 10 μl were injected. Detection was by a PDA-multiwavelength detector set at 300 nm absorbance. Nine samples of chlorpyrifos standards (30 ppm) were also injected for identification and qualification of residues (Racke *et al.*, 1996).

## 3.4 Data analysis

Data were analyzed by ANOVA using Statistical Analysis Software (SAS) for applied science where there are significant differences; means were spared the range test (LSD).

# CHAPTER 4

# RESULTS

## 4.1 Crop Yield

The ANOVA for the effect of treatments on yield of leaf mustard showed no significantly differences among treatments (Table 3, Appendix 1). In terms of weight, there was no loss in yield of leaf mustard although there was insect damage (Table 4 and 5).

Table 3: Effect of insecticide treatments on yield of leaf mustard

| Treatment | Leaf yield (g/plot) |
|---|---|
| Untreated control | 524.1 a |
| Chlorpyrifos (0.46 kg/ha) | 468.9 a |
| Chlorpyrifos (0.92 kg/ha) | 422.7 a |
| S.E. | 120.2 |

Means with the same letter are not significantly different at p= 0.05 (LSD).

### 4.2 Insect Damage Assessment

Data on insect damage assessment at 13 days after treatment were significantly different (Table 4, Appendix 2). The insect damage assessment was based on the total number of leaves damaged within two quadrant samples of size 0.25 $m^2$. The degree of damage for untreated control was similar to the treatment with chlorpyrifos 0.46 kg/ha. Treatment with 0.92 kg/ha chlorpyrifos was very effective in reducing insect damage in leaf mustard compared to treatment with 0.42 kg/ha chlorpyrifos.

Table 4: Assessment of insect damage at 13 days after treatment

| Treatment | No. of damaged leaves[1] (per 0.25 $m^2$) |
|---|---|
| Untreated control | 63 a (1.1662) |
| Chlorpyrifos (0.46 kg/ha) | 23 ab (0.8191) |
| Chlorpyrifos (0.92 kg/ha) | 9 b (0.4203) |
| S.E. | — (0.1687) |

Means with the same letter are not significantly different at p= 0.05 (LSD).
[1] Data were transformed to log (x + 1) prior to statistical analysis. Values within parenthesis are log (x + 1) transformed data.

Results obtained from insect damage assessment at 18 days after treatment were still significantly different between the untreated control and insecticide treatments (Table 5). Treatments with 0.46 kg/ha and 0.92 kg/ha chlorpyrifos were equally effective in reducing insect damage.

Table 5: Assessment of insect damage at 18 days after treatment

| Treatment | No. of damaged leaves[1] (per 0.25 $m^2$) |
|---|---|
| Untreated control | 112 a (1.4448) |
| Chlorpyrifos (0.46 kg/ha) | 23 b (0.7361) |
| Chlorpyrifos (0.92 kg/ha) | 12 b (0.4445) |
| S.E. | — (0.2601) |

Means with the same letter are not significantly different at p= 0.05 (LSD).
[1] Data were transformed to log (x + 1) prior to statistical analysis. Values within parenthesis are log (x + 1) transformed data.

### 4.3 Residue levels of chlorpyrifos in leaf mustard after treatment

Analysis of chlorpyrifos residues were determined by HPLC at retention time of 25.74 minutes (Table 6, Figure 1). Residues of chlorpyrifos were not detected at 8, 14 or 20 days after treatment in untreated and treated samples (Table 6) as proven by the chromatograms for leaf samples collected at 8, 14 and 20 days after treatment (Figure 2, 3 and 4).

Table 6: Detection of chlorpyrifos residues in leaf mustard

| Treatments | Chlorpyrifos rentention time (min) | | | Mean (± S.D.) |
|---|---|---|---|---|
| | 8 DAT | 14 DAT | 20 DAT | |
| Standard | 25.54 | 25.81 | 25.87 | 25.74 ± 0.47 |
| Untreated control | ND | ND | ND | – |
| Chlorpyrifos (0.46 kg/ha) | ND | ND | ND | – |
| Chlorpyrifos (0.92 kg/ha) | ND | ND | ND | – |

DAT = Days after treatment
ND = Non-detected

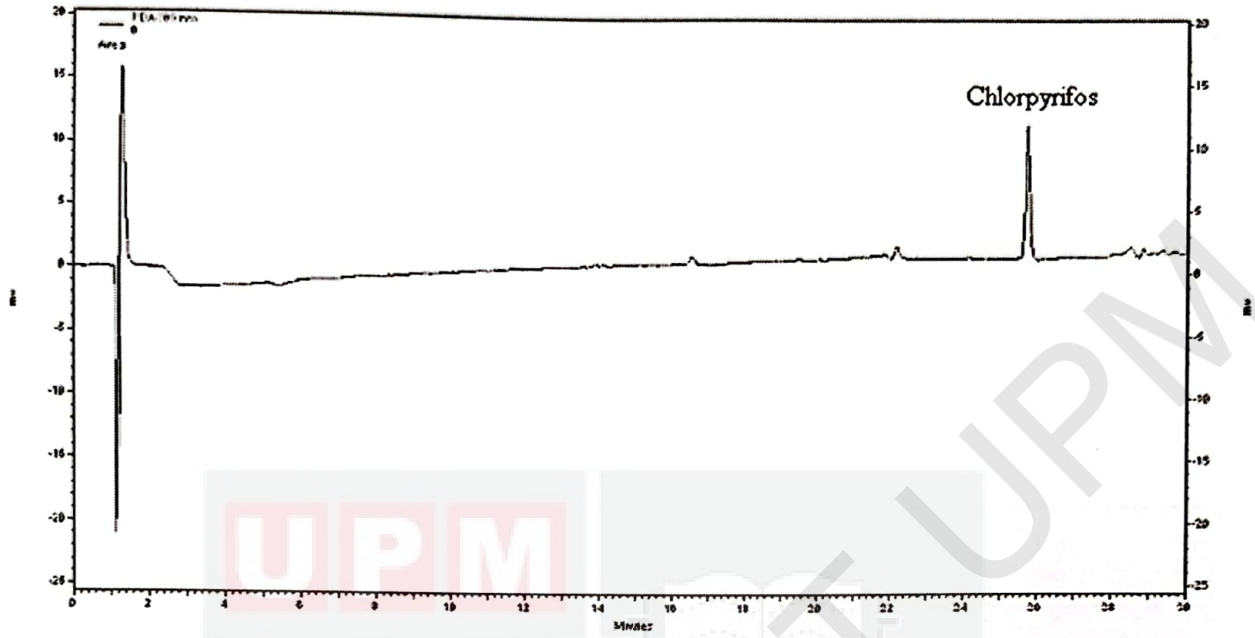



Figure 1: Chromatogram of chlorpyrifos standard (10 µl, 30 ppm) (Detection limit 0.05ppm)

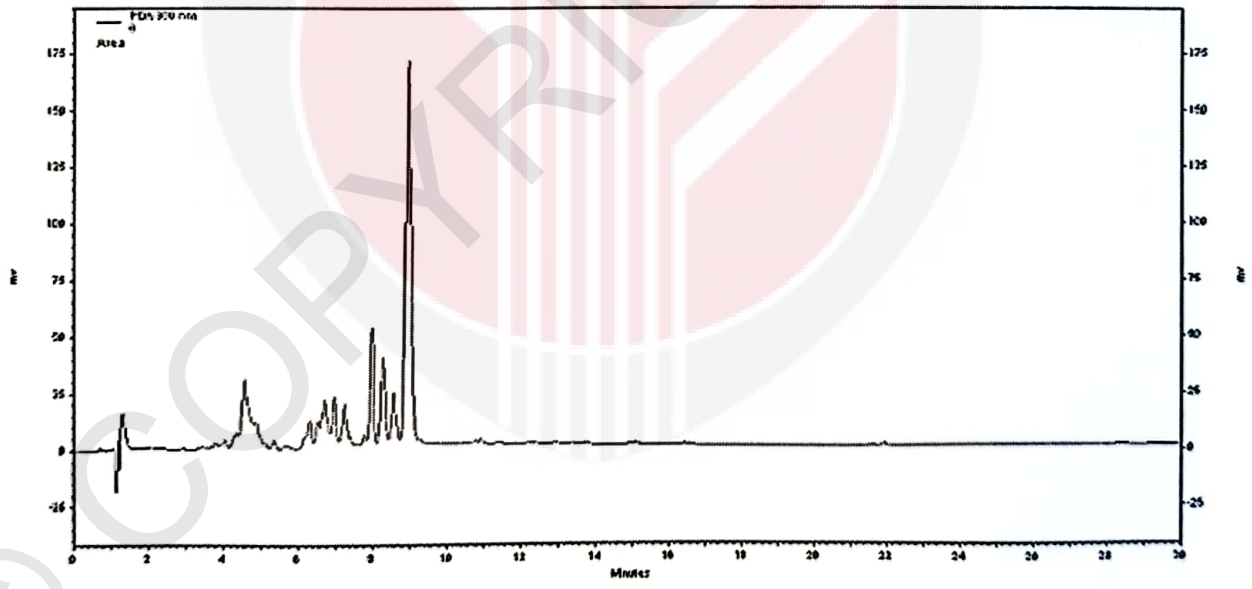



Figure 2: Chromatogram of leaf extracts from untreated control plots at 8 days after treatment

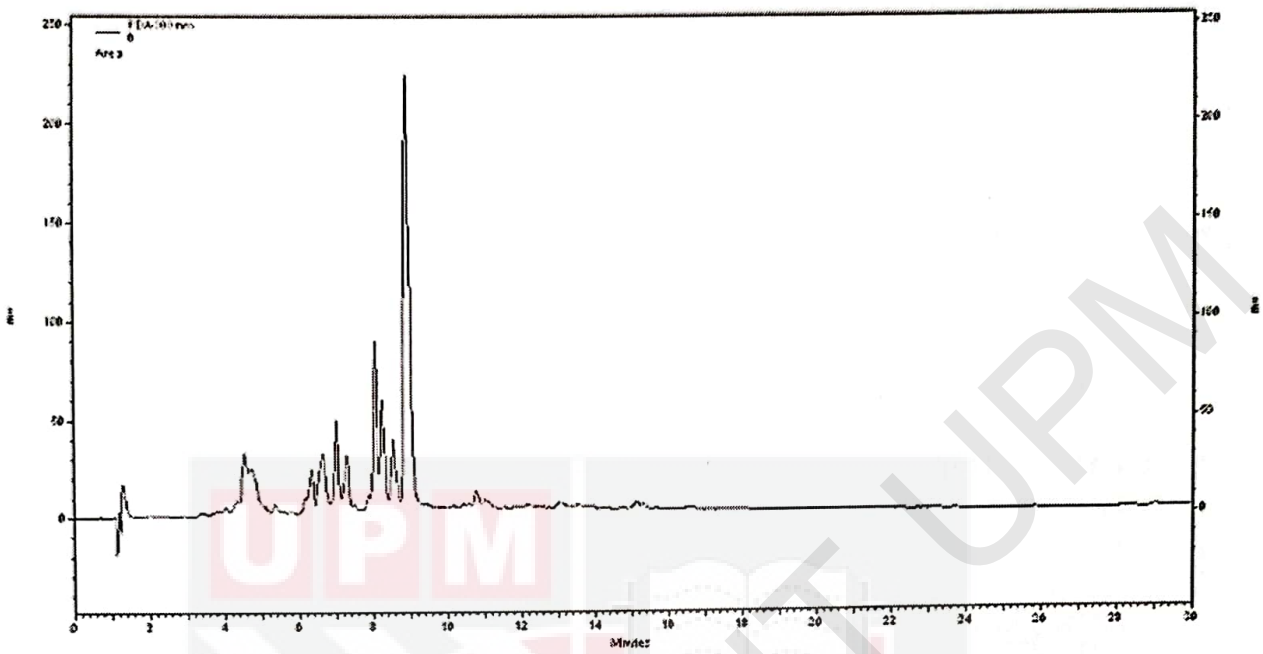


Figure 3: Chromatogram of 0.46 kg/ha chlorpyrifos treated leaf extracts at 8 days after treatment

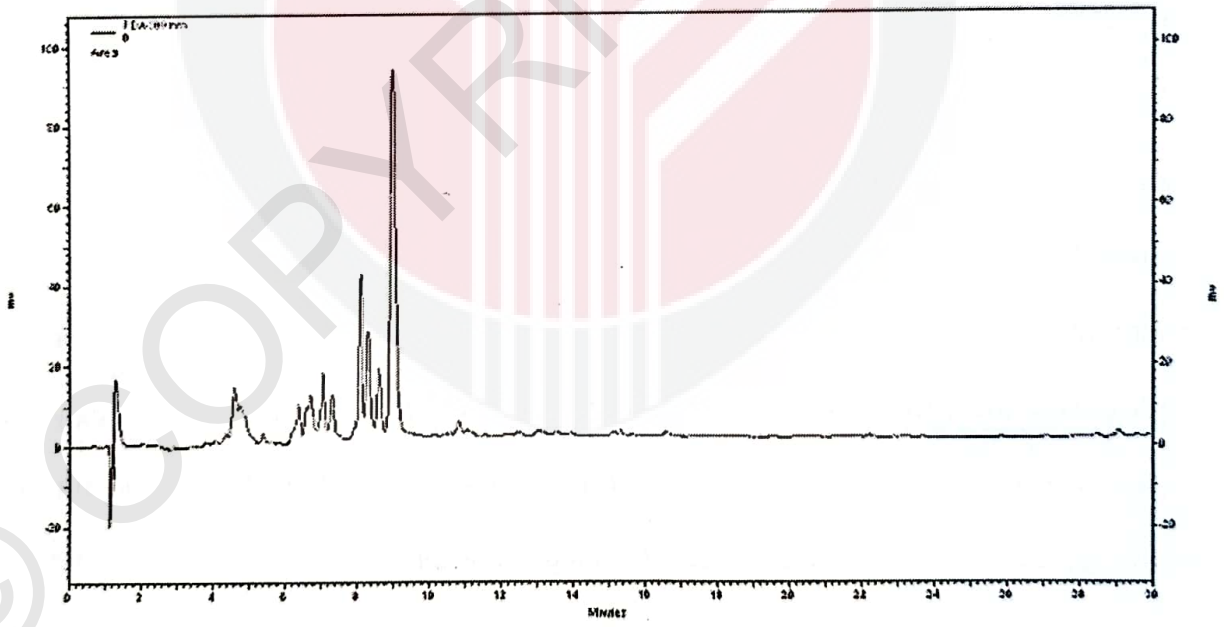


Figure 4: Chromatogram of 0.92 kg/ha chlorpyrifos treated leaf extracts at 8 days after treatment

# CHAPTER 5

# DISCUSSION

Effect of insecticide treatments on yield of leaf mustard was not significantly different among untreated control and insecticide treatments (Table 3). The reason may be because the leaf mustard was planted in a netted greenhouse, thus there was no severe insect damage. Mean of leaf yields were between 422.7 to 524.1 g/plot. There was no loss in leaf yield in terms of weight.

However, the results of insect damage assessment to leaf mustard at 13 days after treatment were significantly different between the untreated control and chlorpyrifos treatments, with numbers of leaf damaged ranging from 63 to 9 (Table 4). The number of damaged leaves in the untreated control and treatment with 0.46 kg/ha chlorpyrifos were similar. However, assessment of insect damage at 18 days after treatment in both insecticide treatments were significantly lower compared to the untreated control (Table 5). Treatments with 0.42 kg/ha and 0.92 kg/ha chlorpyrifos were equally effective in reducing leaf damage. The chlorpyrifos treatments were effective in reducing insect damage. Both treatments were effective in preventing loss in quality and total yields. On the order hand, 0.46 kg/ha chlorpyrifos would be more economical to recommend for farmers since both treatments were equally effective in reducing insect damage at 18 days after treatment.

Residue of chlorpyrifos standard was detected at 25.74 minutes retention time (Figure 4). Residues analysis of leaf extracts for all treatments were not detected at 8, 14 or 20 days after treatment (Table 6). All the chromatograms for untreated control and treated samples were similar (Figure 2, 3 and 4). The reason may be due to the degradation of residue levels with time (Kumar *et al.*, 2004). Moreover, the residues on plant surfaces may also be degraded by radiation (Emden and Service, 2004) and wash out by rainfall (Zhang *et al.* 2006). The results suggest that the chlorpyrifos residue levels in leaf mustard were far below the detectable level of 0.05 ppm (MIFQPA, 2000). This study shows that chlorpyrifos when applied at normal recommended doses, will not exceed the MRL of 0.1 mg/kg (FDR, 2005) at the recommended pre-harvest interval (7 days) (Zhang *et al.*, 2006). The results indicate that the leaf mustard was safe for consumption. However, further research should be carried out to ensure safety under conditions where repeated application is practiced.

# CHAPTER 6

# CONCLUSION

A single application of chlorpyrifos at 0.46 kg/ha or 0.92 kg/ha was effective in reducing damage. At 18 days after treatment both treatments were equally effective. Chlorpyrifos residues were not detected in all treated samples at 8, 14 or 20 days after treatment. The residue levels of chlorpyrifos in leaf mustard did not exceeded the MRL and thus the leaves were safe for consumption.

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# APPENDICES

Appendix 1

Table 7: ANOVA for effect of insecticide treatments on yield of leaf mustard

| Source | df | Sum of Squares | Mean Square | F value | Probability |
|---|---|---|---|---|---|
| Replicate | 3 | 19050.15 | 6350.05 | 0.219 | n.s. |
| Treatment | 2 | 20598.25 | 10299.13 | 0.357 | n.s. |
| Error | 6 | 173230.55 | 28871.76 | | |
| Total | 11 | 212878.95 | | | |

n.s. = Not significant at p= 0.05

Appendix 2

Table 8: ANOVA for assessment of insect damage at 13 days after treatment

| Source | df | Sum of Squares | Mean Square | F value | Probability |
|---|---|---|---|---|---|
| Replicate | 3 | 1.1143 | 0.3714 | 6.527 | * |
| Treatment | 2 | 0.2252 | 0.1126 | 1.979 | n.s. |
| Error | 6 | 0.3416 | 0.0569 | | |
| Total | 11 | 1.6811 | | | |

n.s. = Not significantly at p= 0.05
* = Significant at $p < 0.05$

Appendix 3

Table 9: ANOVA for assessment of insect damage at 18 days after treatment

| Source | df | Sum of Squares | Mean Square | F value | Probability |
|---|---|---|---|---|---|
| Replicate | 3 | 2.1171 | 0.7057 | 5.216 | * |
| Treatment | 2 | 0.1454 | 0.0726 | 0.537 | n.s. |
| Error | 6 | 0.8121 | 0.1353 | | |
| Total | 11 | 3.0745 | | | |

n.s. = Not significantly at p= 0.05
* = Significant at $p < 0.05$

## PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Residue Levels of Chlorpyrifos in Leaf Mustard (*Brassica juncea (L) Coss.*) In Relation to Sampling Time" by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus and published in the form approved by the Faculty.



GOH WEI LING

Date: 07/05/07