CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

1.1.1 Pesticides in Thailand

The widespread uses of pesticides from agricultural and public health use have caused severe environmental pollution and potential health hazards including acute and chronic cases of human poisonings. The kinds of pesticide compose of fungicide, herbicide and insecticide. The major market shares comprising over 80% of all imported pesticides are the herbicide and insecticide. Major insecticides can be divided into four main categories including organochlorine, organophosphate, carbamate, and synthetic pyrethroid.

Pesticides most frequently imported for agricultural use included glyphosate (herbicide), carbofuran (carbamate insecticide), methamidophos (organophosphate insecticide), 2,4-D sodium (herbicide), atrazine (herbicide), methyl parathion (organophosphate insecticide), alachlor (herbicide), and chlorpyrifos (organophosphate insecticide) (Alternative Agricultural Network, 2003; Thailand Department of Agriculture, 2003).

The aim of this study concentrated in the widest using non-persistent pesticides that used in agricultural area and in home. In Thailand, organophosphate and carbamate pesticides are widely used in order to boost fruits products and other high-value crops (Kunstadter *et al.*, 2001). Farmers have been increased consumption of pesticides in their

crops for high yield production. Not only they often overused pesticides, but also often mix pesticides themselves creating a "cocktail" of several chemicals without considering their synergistic effects since they believed it will better protection production from insects or diseases (Poapongsakorn *et al.*, 1998).

Chiang Mai province is located in the northern region of Thailand. Although the province has only a small percentage of cultivated area, it has produced large amounts of agricultural products. Moreover, farmers in Chiang Mai province spend more money on both pesticides and fertilizers than farmers in any of the other provinces in northern Thailand (The 1st Office of Agricultural Economics, 2007; Chiang Mai Office of Agriculture Economics, 2007). Several studies found farmers in Chiang Mai province exposed to organophosphate pesticides by detecting their metabolites in urine (Panuwet *et al.*, 2004, 2008).

Whereas the synthetic pyrethroids in Thailand are found in many commonly used consumer home and garden pesticides for treating problems with mosquitoes, caterpillars, ants, spiders, garden worms, wasps, cockroaches, aphids and other insect infestations. Moreover, the national programs currently used synthetic pyrethiods such as deltramethrin, cypermethrin and permethrin for controlling mosquito vectors of malaria and duegue diseases using fogging and impregnated mosquito net in endemic areas (WHO, 1995). Moreover, some studies found consumers in case of school students from Chiang Mai province exposed to pyrethroid pesticides using detection in urine (Panuwet *et al.*, 2009). Normally, all pesticide biomonitoring has focused primarily on measuring concentrations of chemicals and their metabolites in urine, as well as cholinesterase (ChE) activity in blood (Wilson *et al.*, 1996).

1.1.2 Organophosphate and carbamate pesticide statement and significance

The ChE can be divided according to substrates into two types, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Both AChE and BChE have the capacity to hydrolyze acetylthiocholine chloride but only BChE hydrolyses butyrylthiocholine chloride (Lassiter *et al.*, 1998). Their tissue-specific distribution or origin sources are also different from each other. AChE is known to be abundant in brain and muscle whereas BChE is mostly found in liver, intestine, heart, kidney, lung and body fluid (Prody *et al.*, 1987; Ryhanen *et al.*, 1983). Both of AChE (in red blood cell) and BChE (in plasma) are also being in blood (Stefanidou *et al.*, 2003).

The primary mechanism of action of OP and carbamate pesticides is the inhibition of ChE activity. Acetylcholinesterase (AChE) catalyses the acetylcholine (ACh) neurotransmitter to its constituent components of acetic acid and choline (Sayer *et al.*, 2004). OP pesticides such as chlorpyrifos inhibit AChE through their active oxon metabolites with subsequent accumulation of ACh within the cholinergic synapses, resulting in a wide range of neurotoxic effects (Mileson *et al.*, 1998). However, the activity of butyrylcholinesterase (BChE) becomes more important in scavenging OP and carbamate inhibitors before they reach AChE (LaDu *et al.*, 1990; Mattes *et al.*, 1996).

Moreover, there was a statistically significant decrease in plasma BChE activity among 90 Thai applicators for the organophosphate pesticides at Rachaburi province in the high-exposure period that compared to the low-exposure period. There were statistically decrease in AChE and BuChE activities in the high-exposure period ($P < 0.001$) compared to the low-exposure period in 90 individuals occupationally exposed to OPs comparing 30 controls. Moreover, the relation between PBChE activity and

symptoms such as dizziness, headache and nausea showed decease significant evidences (Jintana *et al.*, 2009).

OP and carbamate pesticide biomonitoring has focused primarily on measuring concentrations of chemicals and their metabolites in blood and urine as biomarkers of exposure, as well as cholinesterase (ChE) activity in blood as a biomarker of effect (Wilson *et al.*, 1996). However, the present study focused on biomarkers of effect by measuring the activity of ChE in a process which is not multistep, time consuming, high cost and requires a professional to carry out routine analysis.

However, biomonitoring of blood is invasive and can be time-consuming (Wilson *et al.*, 1996). For this reason, biomonitoring of saliva has recently been explored as a practical and feasible alternative to blood (Nigg *et al.*, 1992; Pichini *et al.*, 1996; Lu *et al.*, 1997; Denovan *et al.*, 2000; Henn *et al.*, 2006). ChE can also be found in saliva comprised of both salivary AChE (SAChE) and salivary BChE (SBChE). The SBChE activity constitutes about 70–90% of the total ChE activity in unstimulated whole saliva (Yamalik *et al.*, 1990).

Numerous enzymes with a molecular mass of approximately 14.3–480 kDa have been found in saliva. They include amylase, invertase, maltase, carbonic anhydrase, urease, oxidases, catalase, proteolytic enzymes, lipase, phosphatases, lysozyme, and hyaluronidase. Enzymes in the oral cavity originate from several sources such as the major and minor salivary glands, bacteria, oral tissue, and ingested substances (Chauncey *et al.*, 1953). The basic functional form of AChE and BChE are an amphipathic dimer and a globular tetramer with a molecular mass of approximately 160 kDa and 340 kDa, respectively (Rosenberg *et al.*, 1984; Haupt *et al.*, 1966).

Although BChE and AChE are large molecules, they are able to cross the cell membrane of the salivary glands and then excrete into the oral cavity. In another study, AChE and BChE were found in the peripheral autonomic ganglia of the parotid gland (Koelle *et al.,* 1950). However, the parotid ChE represented 60% of the activity of all enzymes in saliva. Oral total ChE was found to be of both glandular and bacterial origin, with the parotid gland activity contributing about 1%.

However, in the present study we were aware of the poor oral/dental hygiene among the studied groups, and saliva sample collection was performed after rinsing the mouth with drinking water five times. Then biggest source of BChE activity should be the salivary gland. The collection methods for unstimulated whole saliva such as a disposable plastic pipette and a cotton-wool roll working well for collections is faster, easier, and more comfortable for participants. However, in study of Henn *et al.* (2006) found that the majority of visits (86%), participants preferred the cotton-wool roll. Moreover, cholinesterase activities measured in the pipette-collected samples and the cotton wool-collected samples were significantly correlated ($r= 0.41$, $P < 0.01$, $n=98$). Therefore, the cotton wool didn't find problems with adhesion of molecules, including cholinesterase, to cotton-wool swabs.

Moreover, some researchers testing on animals also used SChE as a biomarker of the effect of OP and carbamate pesticides. For instance, rat SChE inhibition was related to blood ChE inhibition when incubated with an OP pesticide (Kousba *et al.,* 2003). Additionally, rat SChE activity decreased after exposure to malathion in vivo. For human pesticide exposure assessment, the SChE activity in a group of pesticide factory workers was lower than the activity in healthy controls (Abdollahi *et al.,* 1996). Various

reported studies confirmed that SChE excreted from the salivary gland was contained in the saliva.

However, SChE has approximately 1000 times lower activity than blood ChE (Kousba *et al.*, 2003). The inhibition of BChE or AChE through OP exposure would be much more complete in saliva than in blood. Therefore, a sensitive method for detecting the relatively low activity of enzymes in saliva needs to be developed in order to find a correlation between SChE and blood ChE.

1.1.3 Synthetic pyrethroid pesticide statement and significance

The synthetic pyrethroids are derived structurally from the natural pyrethrins that originate from the botanical insecticide pyrethrum, an extract obtained from the flowers of Chrysanthemum cinerariaefolium. Synthetic pyrethroids including cypermethrin, deltamethrin and permethrin are registered for use in the United States and many other parts of the world and account for more than $1.5 billion in pesticide sales (Davies *et al.*, 2007). Synthetic pyrethroid pesticides in Thailand have been also widely used as household products for controlling vectors such as flies, cockroaches, ants, and ticks. Importantly, national programs currently employ pyrethroids such as deltamethrin, cypermethrin and permethrin for controlling mosquito vectors of malaria and dengue diseases in endemic areas using techniques such as fogging and impregnated mosquito bednets (WHO, 1995). Moreover, pyrethroids such as cypermethrin have been widely used to boost agricultural production (Panuwet *et al.*, 2008).

After humans are exposed to pyrethroids, the parent compounds measured in plasma have a half-life of 2.5-12 hr (Eadsforth *et al.*, 1988; Woollen *et al.*, 1992). The parent compounds are hydrolyzed by an esterase enzyme to 3-phenoxybenzyl alcohol or

3-phenoxybenzaldehyde. These compounds are rapidly converted to 3-phenoxybenzoic acid (3-PBA). The 3-PBA is conjugated to glucuronic acid that renders the xenobiotic more polar and facilitates its excretion in urine. In mammals, pyrethroid esters are mostly eliminated in urine (93%) during the first 24 hr (Leng *et al.*, 1997). Total 3-phenoxybenzoic acid (3-PBA), has been measured in human urine as a biomarker of exposure (Ahn *et al.*, 2011; Egeghy *et al.*, 2011).

Other metabolites such as *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid that derive from parent compounds such as cyfluthrin, permethrin, and cypermethrin (Olsson *et al.*, 2004) are also formed but were not measured in this study. Moreover, several studies have shown that there is no correlation between the concentration of the urinary metabolite and the symptoms mentioned (Kolmodin-Hedman *et al.*, 1995; Leng *et al.*, 1998; Wieseler *et al.*, 1998).

The disadvantage to measuring 3-PBA in the urine is that it is considered a biomarker of acute exposure, since the metabolite is so rapidly eliminated. On the other hand, it is well known that protein adducts of xenobiotics measured in plasma are persistent biomarkers having half lives up to several months (Phillips, 2002). 3-PBA-glucuronide has also been shown to form protein adducts (Noort *et al.*, 2008).

Two mechanisms of adduct formation from glucuronidation of carboxylic acids have been demonstrated. In the transacylation mechanism, acyl glucuronides that are potentially electrophilic can react with nucleophilic residues in proteins. According to the glycation mechanism, an initial internal acyl migration occurs and also reacts with amino groups of the protein, leading to Schiff base adducts or Amadori rearrangement (Grubb *et al.*, 1993). The most likely protein in humans for adduction of acyl glucuronides is albumin due to its abundance (60%) in blood (Presle *et al.*, 1996).

Analytical methods for the detection of pyrethroid metabolites such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) with mass spectrometry (MS) are expensive (Leng *et al.*, 1997; Baker *et al.*, 2000; Colume *et al.*, 2001; Schettgen *et al.*, 2002). Immunoassay is a biochemical technique using the specific binding of an antibody to its antigen. The quantity of antigen or antibody can be detected by labeling either the antigen or antibody.

In this study, we analyzed by competitive enzyme immunoassays, the antigen in the sample competed for limited antibody binding sites with an antigen mimic conjugated to a carrier protein. The bound antibody was detected by reaction with a secondary antibody labeled with an enzyme. The resulting signal was inversely proportional to the antigen (3-PBA) concentration. An enzyme-linked immunosorbent assay (ELISA) is a suitable and easy application in the field. Moreover, the ELISA method provided higher sample throughput with lower cost as compared to the GC-MS method such as for permethrin (Chuang *et al.*, 2010).

Acute toxicity such as headache, dizziness, nausea, irritation of the skin and nose, and paraesthesia can occur when exposed to an overdose of pyrethroids (He *et al.*, 1988; He *et al.*, 1989). In the case of chronic toxicity, the pyrethroid fenvalerate may cause lymph node and splenic damage as well as carcinogenesis (Hallenbeck and Cunningham-Burns, 1985) and pyrethroids have a suppressive effect on the immune system (Repetto and Baliga, 1996). Permethrin has been classified as a potential carcinogen at high concentrations by the U.S. EPA (California Environmental Protection Agency, 1994; United States Environmental Protection Agency, 1989). Pyrethroids may be considered hormone disruptors from studies on their estrogenic potential in human breast carcinoma cells (Go *et al.*, 1999).

Because of the potential toxicity, the U.S. EPA set a Reference Dose (RfD) of 0.25 mg/kg/day for both acute and chronic dietary exposures to permethrin (Reregistration Eligibility Decision for Permethrin, 2007). The World Health Organization has set a limit of 0.3 mg/L (300 ng/mL) as a guideline for permethrin in drinking water when it is applied to water for mosquito control (The World Health Organization Guidelines for Drinking-water Quality, 2006). The Agency for Toxic Substances and Disease Registry (ATSDR) determined Minimum Risk Levels (MRLs) for oral exposures to technical grade permethrin of 0.3 mg/kg/day for acute oral exposures (up to 14 days) and 0.2 mg/kg/day for intermediate durations (15-364 days) (Toxicological Profile for Pyrethrins and Pyrethroids, 2003). Because of the potential health implications, it is important to monitor populations in order to form strategies to minimize exposure.

Thus in this part, we opted to determine both plasma and urinary 3-PBA in populations consisting of consumers and farmers in Thailand utilizing an immunoassay to monitor 3-PBA in urine (Ahn *et al.*, 2007) that had been further adapted and validated to measure 3-PBA in plasma following hydrolysis of adducts. To our knowledge this is the first study to assess 3-PBA protein adducts in humans.

1.2 Research objectives

Objective 1: To develop a salivary cholinesterase assay and its application in pesticide exposure assessment.

The specific objectives are as following:

(1) To develop human salivary cholinesterase assay.

(2) To apply developed methods to detect activities of BChE and AChE in saliva among large groups of consumers and farmers in agricultural area.

(3) To examine the relationship between activities of BChE and AChE in saliva and blood in volunteers.

Objective 2: To develop of sample preparation step and sensitivity ELISA method that can detect plasma 3-PBA and applied in pesticide exposure assessment.

The specific objectives as following:

(1) To develop sample preparation step and sensitivity ELISA method that can detect plasma 3-PBA.

(2) To apply developed methods to detect plasma 3-PBA among large groups of consumers and farmers in agricultural area.

(3) To examine the relationship between plasma 3-PBA and urine 3-PBA in volunteers.

The present study was done following to conceptual frame work that showed in Figure 1.1.

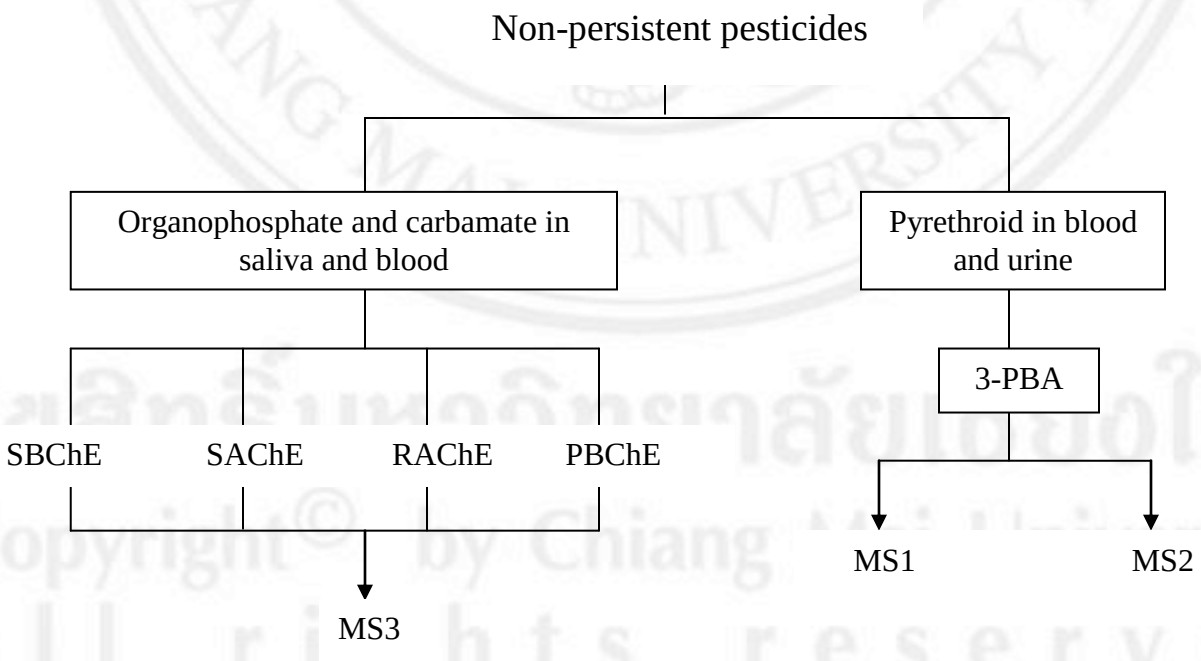

Figure 1.1 Conceptual framework of the present study

1.3 Definitions

Polyclonal antibody was produced by two New Zealand white rabbits that were immunized with hapten 3-[4-(3-carboxyphenoxy) phenoxy] N-thyroglobulin ethylamine) and could be detected antigen (3-PBA) in samples.

Sensitivity was a low LOQ and high accuracy when applied to measure concentration in samples with low variability (%CV) i. e. by the intra-assay for within-day for %CV, and between-day for all samples.