CHAPTER 2

LITERATURE REVIEWS

2.1 Classification of pesticides

Pesticides can be classified into several subgroups using different criteria such as chemical structure, mode of action, potential to human health. For example, Table 2.1 was classified using persistence into environment.

Table 2.1 Classification of major pesticides according to persistence into environment

Pesticide type	**Example**
Persistent pesticides	Organochrolines: Hexachlorobenzene, Diedrin, Heptachlor epoxide, Dichlorodiphenyltrichloroethane
Non-persistent pesticides	OP insecticides, Carbamates, Pyrethroid insecticides, Dithiocarbamate fungicides, and Herbicides such as 2,4-D, Atrazine

Source: Modified from CDC, 2008a; CDC, 2008b; Greenhalgh, 1980

2.2 Non-persistent pesticides

OP, carbamates and pyrethroid were non-persistent pesticides. These compounds are break down quickly in the environmental. However, this study detected non-persistent pesticides due to have been more commonly used in recent day.

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Table 2.2 Symptoms and sign of OP pesticide poisoning

Mild	Moderate	Severe
Increased salivation	Excessive salivation	Symptoms for mild and moderate
Contraction of pupil of eye (miosis)	Small pupil with visual disturbance	Poisoning increased
Lacrimation (tears in eye)	Lacrimation, Sweating	Depressed consciousness or coma
Nausea	Vomiting, diarrhea	Reduced respriration
Headache	Headache, drowsiness	Cynosis
Weakness (of muscles) or tremor	Weakness increased, muscle tremor	Cardiac failure
Dizziness	Increased muscle tension	
	Disturbed gait (ataxia)	
	Bronchoconstriction and bronchial hypersecretion	
	Breathlessness increase	

Source: Modified from New Zealand Occupational Safety and Health Service, 2000.

2.2.1 Organophosphate pesticides poisoning

OP pesticides are wildly used in agricultural which are the toxic group of chemical warfare agents include nerve gases and poisons. OP pesticides affect the nervous system by phosphorylating the serine hydroxyl group located at the active site of cholinesterase

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enzyme (AChE) established a covalent bond, irreversible inhibiting. Therefore, enzyme unavailable for degrading the neurotransmitter called acetylcholine resulting in overstimulation of muscarinic and nicotine receptors (Fukuto, 1990). OP pesticides can increase acetylcholine at various sites caused increasing response in many organs and tissues resulting symptoms listed in Table 2.2. All OP pesticides share some common chemical properties. OP pesticides contain a central phosphorus atom with double bond to either sulfur or oxygen, R groups that are either ethyl or methyl in structure, and a leaving group (X) which is specific to the individual organophosphate. The general structure of OP pesticides appears in Figure 2.1.

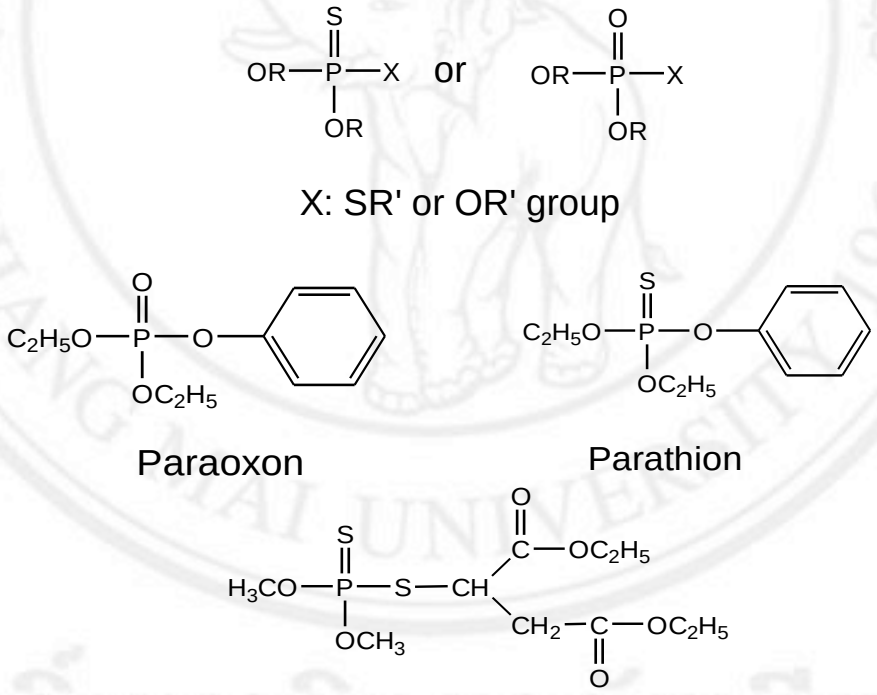

Figure 2.1 Typical structures of OP pesticides

Chemical changes of OP pesticides in the environment are same as in the human body. In human's liver, one chemical reaction is transformation at the double bond of the

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central phosphorus atom from sulfur to oxygen (oxon form) results the OP a more potent inhibited of cholinesterase enzymes. The another hand, metabolic reaction that decrease toxicity is hydrolysis of the OP yielding a dialkylphosphate and the leaving group in Figure 2.2 (National Pesticide Information Center, 2008a). The leaving group is specific to the parent compound of OP pesticides so can be measured as biomarkers of exposure. These metabolites are eliminated along urination (Barr and Needham, 2002).

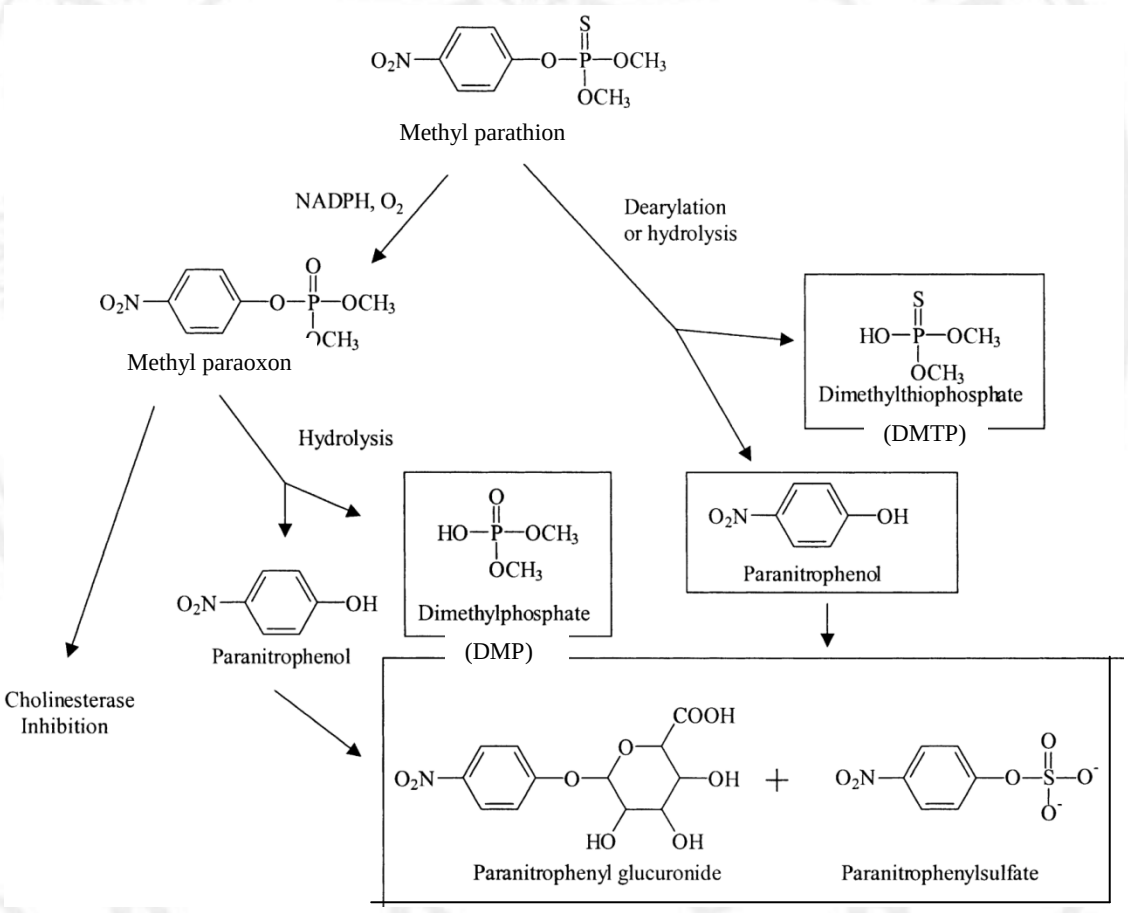

Figure 2.2 Metabolic pathway of methyl parathion in the body representative of organophosphate pesticide metabolism (Barr and Needham, 2002).

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2.2.2 Carbamate pesticides poisoning

Carbamates (also termed N-Methyl carbamate pesticides) are widely used in agriculture. The carbamate ester also reversible inhibited the acetylcholinesterase enzyme but the carbamyl-acetylcholinesterase combination dissociates more readily than OP pesticides (Fukuto, 1990; Reigart and Roberts, 1999) so carbamate poisonings tend to be shorter duration than OP. The signs and symtoms of OP poisoning based on excessive cholinergic stimulation unlike carbamate serious toxicity are central nervous system depression, as manifestation by coma, seizures, hypotonicity, and nicotinic effects including hypertension and cardiorespiratory depression.

Other serious signs are dyspnea, bronchospasm, and bronchorrthea with eventual pulmonary edema. N-methyl carbamates can be confirmed by analysis of urine for specific matabolites: alpha-naphthol from carbaryl, isopropoxyphenol for propoxur, carbofuran phenol from carbofuran, and aldicarb sulfone, sulfoxide, and nitrile from aldicarb (Reigart and Roberts, 1999).

2.2.3 The synthetic pyrethroid characterize

Pyrethroids have been used indoor such as in plant and storage protection, wood preservation, impregnation of wool carpets and textiles, disinfection and pest control. The exposed ways to human by food contamination, skin absorption, respiratory exposure (Leng *et al.*, 2003). The synthetic pyrethroid is derived structurally from the natural pyrethrins produced by certain species of the chrysanthemum plant. In Figure 2.3 showed the six insecticidal constituents of pyrethrum extract (Soderlund *et al.*, 2002).

A wide range of pyrethroid structures have resulted from development of agrochemical industrial and government or academic research laboratories. The

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interesting synthetic pyrethroids were them that are registered for use in the United States and many other parts of the words showed in Figure 2.4 (Soderlund *et al.*, 2002).

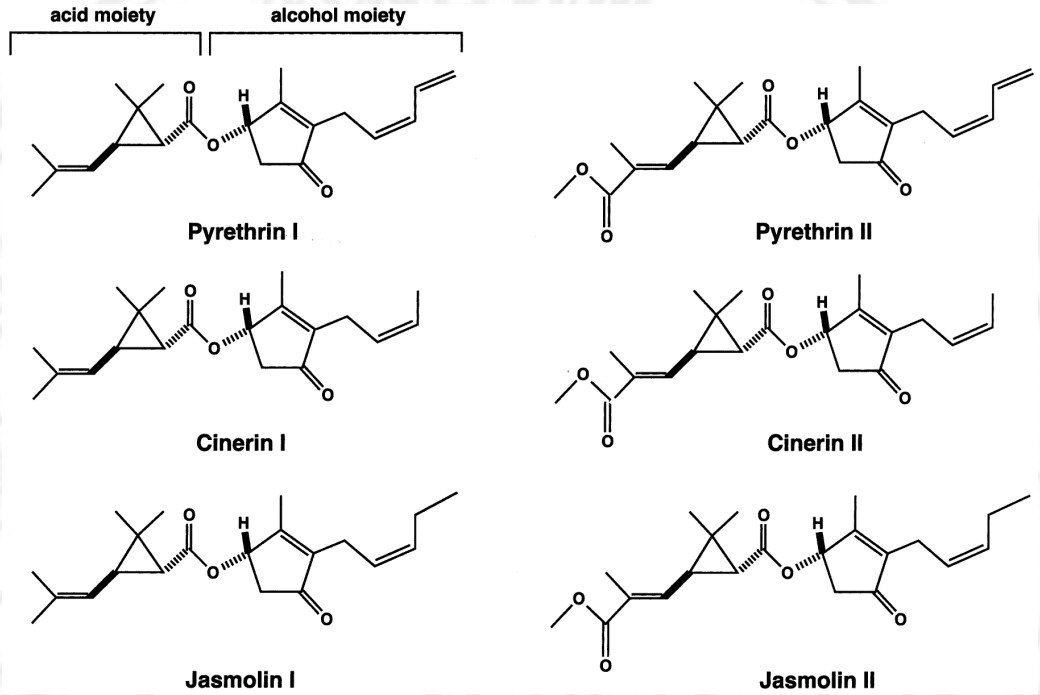

Figure 2.3 Structures of the six natural pyrethrins (Soderlund *et al.*, 2002)

There are two types of pyrethroid pesticides that differ in chemical structure and symtoms of exposure. Type I include allethrin, tetramethrin, resmethrin, d-phenothrin, bioresmethrin, and permethrin. Type II pyrethroid pesticides are, for example, cypermethrin, cyfluthrin, deltamethrin, cyphenothrin, fenvalerate, and fluvalinate. The systemic toxic effects on animals depend on the two structural types of pyrethroid pesticides that give rise generally to distinct patterns. Type I produce the so-called "T (tremor) syndrome" characterized by tremors, prostration and altered "startle" reflexes. Types II (Alpha-cyano) produce the so-called "CS (choreoathetosis/salivation)

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syndrome" with ataxia, convulsion, hyperactivity, choreoathetosis and profuse salivation being observed in experimental studies (National Pesticide Infromation Center, 2008b).

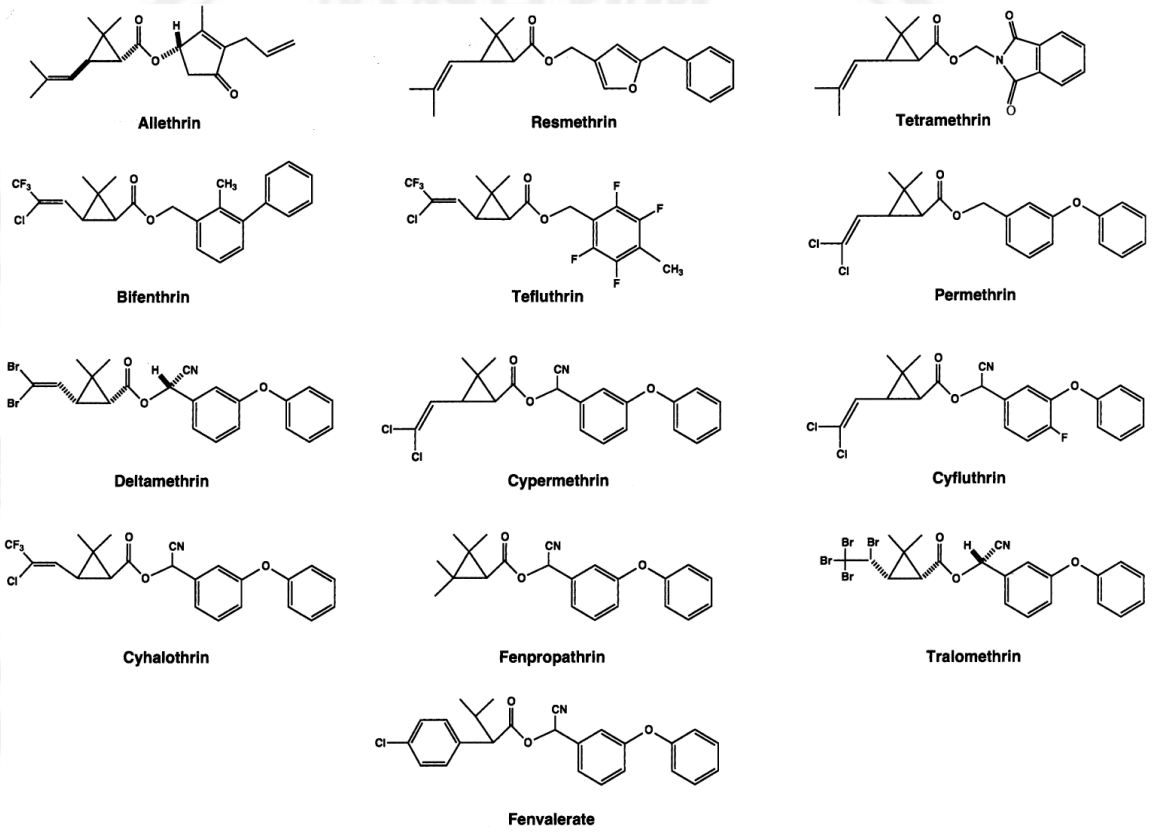

Figure 2.4 Structures of synthetic pyrethroids registered for use in the United States (Soderlund *et al.*, 2002)

The primary toxicity is interference of sodium ion (Na^+- K^+) channels in the nerve cell membrane. Electrical massages sent between nerve cells allow insect to generate a response, like movement. Pyrethrins affect the nervous system by leading to prolongation of the depolarizing after-potential, repetitive after-discharges and hyper-excitation that induce their neurotoxic potential (Aldridge, 1990; Narahashi, 1987). The pyrethroid inhibited the gamma-aminobutyric acid (GABA), type II that normally bind to

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ACh to product neurotransmission. Therefore, it is accumulation of ACh within the cholinergic synapses resulting neurotoxic of muscle tone in insect species.

It has been long believed that pyrethroid pesticides are less toxic to mammals because they are quickly broken down into inactive forms and pass from body in the urine and fees. However, several research publications gradually revealing the possible harmful effects in case of high dose exposures, symptoms of acute poisoning have been found muscle fasciculation, altered consciousness, dizziness, headache, nausea, anorexia, and fatigue (Aldridge, 1990).

Moreover, the US Environmental Protection Agency has classified permethrin as a potential carcinogen at high concentrations (California Environmental Protection Agency, 1992; Go *et al.*, 1999). In animal studies (Table 2.3), rats were oral toxicity of several pyrethroids that are currently registered for use in the United States. These compounds have acute oral LD50 values following administration in vegetable oils lying between 50 and 500 mg/kg and are therefore considered to be moderately toxic (EPA Category II).

In chronic exposure effects, Hallenbeck and Cunningham-Burns (1985) suspected that fenvalerate may cause lymph node and splenic damage as well as carcinogenesis. Repetto and Baliga (1996) demonstrated that pyrethroids have a suppressive effect on the immune system. Go *et al.* (1999) suggested that pyrethroids should be considered hormone disruptors on the estrogenic potential of pyrethroids in human breast carcinoma cells.

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Table 2.3 Acute oral toxicities of pyrethroids to rats

Compound	Strain[a]	Vehicle	LD50 (mg/kg)		Reference
			Male	Female	
Bioallethrin	S	Corn oil	395	410	(Furnax and Audegond, 1985a)
S-Bioallethrin	S	Corn oil	370	320	(Furnax and Audegond, 1985b)
Bifenthrin	S	Corn oil	70.1	53.8	(Freeman, 1982)
Lambda-cyhalothrin	A	Corn oil	79	56	(Southwood, 1985a)
		Aqueous suspension[b]	299	433	(Allen and Leah, 1990)
Cyfluthrin	W	Acetone +peanut oil	155	160	(Heimann, 1987)
		Corn oil	297	372	(Freeman, 1987)
Cypermethrin	S	Undiluted	7654	7180	(Rand, 1983)
Zeta-cypermethrin	S	Corn oil	134	86	(Freeman, 1989)

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Table 2.3 (continued) Acute oral toxicities of pyrethroids to rats

Compound	Strain[a]	Vehicle	LD50 (mg/kg)		Reference
			Male	Female	
		Corn oil	95	87	(Varsho, 1996)
Deltamethrin	S	Aqueous suspension[c]	>5000	>5000	(Myer, 1989)
Fenpropathrin	S	Corn oil	70.6	66.7	(Hiromori *et al.*, 1983)
Fenvalerate	S	Corn oil	~370[d]	~370[d]	(Bilsback *et al.*, 1984)
Esfenvalerate	S	Corn oil	87[e]	87[e]	(Bilsback *et al.*, 1984)
Permethrin	W	Corn oil	1200[e]	1200[e]	(Killeen, 1975)
		Undiluted	8900[e]	8900[e]	(Killeen, 1975)
Pyrethrum	S	Corn oil	710	320	(Gabriel, 1992)
Resmethrin	S	Corn oil	1695	1640	(Glomot, 1979)
Tefluthrin	A	Corn oil	21.8	34.6	(Southwood, 1985b)
		Sesame oil	99	157	(Audegond *et al.*, 1981a)
Tralomethrin	S	Aqueous suspension[f]	>1000 and <5000	>1000 and <5000	(Audegond *et al.*, 1981b)
Fenpropathrin	S	Corn oil	70.6	66.7	(Hiromori *et al.*, 1983)
Fenvalerate	S	Corn oil	~370[d]	~370[d]	(Bilsback *et al.*, 1984)

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Table 2.3 (continued) Acute oral toxicities of pyrethroids to rats

Compound	Strain[a]	Vehicle	LD50 (mg/kg)		Reference
			Male	Female	
Permethrin	W	Corn oil	1200[e]	1200[e]	(Killeen, 1975)
		Undiluted	8900[e]	8900[e]	(Killeen, 1975)
Pyrethrum	S	Corn oil	710	320	(Gabriel, 1992)
Resmethrin	S	Corn oil	1695	1640	(Glomot, 1979)
Tefluthrin	A	Corn oil	21.8	34.6	(Southwood, 1985b)
Tralomethrin	S	Sesame oil	99	157	(Audegond *et al.*, 1981a)
		Aqueous suspension[f]	>1000 and <5000	>1000 and <5000	(Audegond *et al.*, 1981b)
Fenpropathrin	S	Corn oil	70.6	66.7	(Hiromori *et al.*, 1983)

[a] Strain: A, Alderley Park; S, Sprague–Dawley; W, Wistar.

[b] Containing 0.5% hydroxypropylmethylcellulose + 0.1% polysorbate 80.

[c] Containing 1% methylcellulose.

[d] Exact LD50 not calculated; mortality less at higher doses.

[e] Combined value for males and females.

[f] Containing 0.25% carboxymethylcellulose + 0.2% polysorbate 80.

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2.3 Biological monitoring of pesticide exposure

Biological markers currently available for monitoring pesticides exposure in humans can be divided into three main groups:

- **Biomarkers of effect:** biomarkers that associated with possible health impairment or disease.
- **Biomarkers of dose or exposure:** an indicator of internal of internal dose that has resulted in absorption into the body.
- **Biomarkers of susceptibility:** biomarkers that acquired ability of an organism to respond specific substances.

2.3.1 Blood and salivary cholinesterase enzyme

The cholinesterase enzymes are biomarkers of effect for organophosphate and carbamate pesticides because these pesticides inhibit these enzymes. The ChE was divided according to substrates into two types, AChE and BChE. Both AChE and BChE have the capacity to hydrolyze acetylthiocholine chloride (ATC) but only BChE hydrolyses butyrylthiocholine chloride (BTC) (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, muscle and erythrocyte or red cell membrane whereas BChE is mostly found in liver, intestine, heart, kidney and lung (Prody *et al.*, 1987; Dave *et al.*, 2000). Molecular form of human RAChE is amphiphilic dimers but human PBChE is soluble tetramers (Massoulie *et al.*, 1993). Structure of human PBChE contains three interchain disulphide bridges to form specific three dimentional globular structure of monomer and also stabilize the dimeric structure. Proteolysis transforms the tetramer form to monomers, dimers and trimers containing free

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SH groups without affecting molecular weight or the catalytic activity (Grunwald *et al.*, 1997).

Each monomer (Figure 2.5), 20Å deep and narrow active site gorge lined approximately 55 residues. Active site gorge compose of 5 sub sites that complementary hydrolysis substrate. Peripheral anionic site hold substrate at the mouth of the gorge, choline binding site slides down substrate after that oxyanion hole site rotates the substrate from vertical to horizontal position. In acyl portion site, this site separates human BChE from human AChE because Leu286 and Val288 amino acids of human BChE catalysis of larger acyl group containing substrate such as BTC.

On the other hand, AChE that contains phenyl rings of Phe295 and Phe297 amino acids catalyze of shortest acyl group containing substrate such as ATC. The last site is catalytic triad that contains Ser198, His438 and Glu325 amino acids. His438 relays electrons from Glu325 to Ser198 and hydroxyl oxygen of Ser198 to become a nucleophil. Nucleophilic attact of this hydroxyl oxygen to ester bond of substrate leads the acyl-enzyme intermediate and free choline moiety. Then acyl group is hydrolyzed from Ser198 by nucleophilic attact of a water molecule activated by taking a proton from His438. Carbamates or OP can carry out last hydrolysis step very slowly and generaly is inhibited irreversibily (Ekholm and Konschin, 1999; Masson *et al.*, 2001; 2003).

Men had higher PBChE activity than women who were not taking oral contraceptives, and these women had higher PBChE activity than women taking oral

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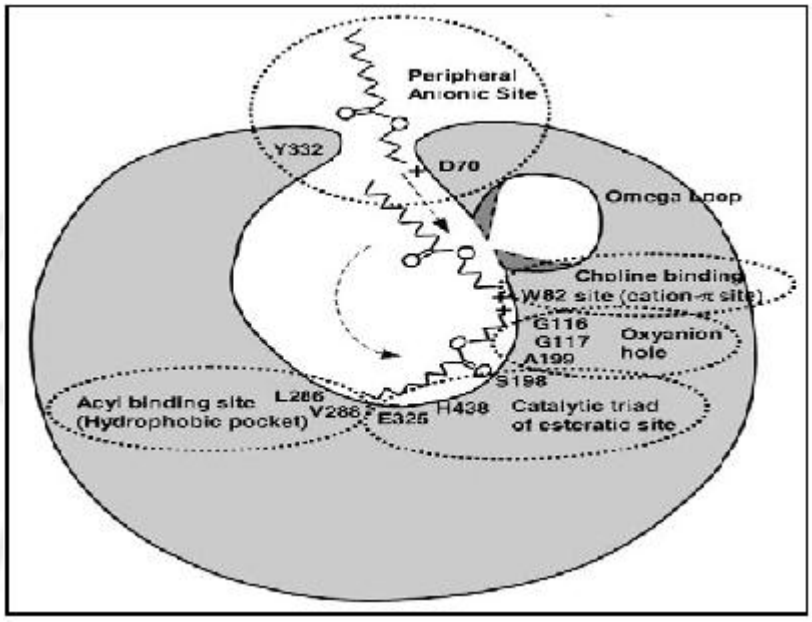

Figure 2.5 Schematic structure of cholinesterase active site of human PBChE monomer (Ekholm and Konschin, 1999; Masson *et al.*, 2001; 2003)

contraceptives. After 60 years old there was no inter-sex difference. Activity of RAChE from the oral contraceptive group was higher than in the other groups, men had the lowest activity, and there was an increase activity with age in both sexes until 60 years old (Sidell and Kaminskis, 1975).

The ChE can be also found in several body fluids like sweat, urine, tears and saliva (Yamalik *et al.*, 1990). Human saliva is comprised of both salivary AChE (SAChE) and salivary BChE (SBChE) that BChE activity constituted 70-90% of the whole ChE activity (Ueda and Yamaguchi, 1967). The origins of SChE are salivary glands, bacteria, oral tissues, and ingested substances (Chauncey *et al.*, 1953). Salivary flow rate not greatly affected to the ChE activities but changed to follow by diurnal variation. The previous research found SBChE activities at four a. m. were about three times as great as at four p. m. Moreover, males had approximately twice as much SBChE activities as

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females did [4.8 ± 2.4 (SD) mU/m1 and 2.2 ± 1.5 (SD) mU/m1, respectively]. Men who were stimulated saliva had little higher enzyme activities than in un-stimulated saliva, BChE activities increased in people who with gingival inflammation (Ryhanen *et al.*, 1983).

SChE analysis in rats that were orally intaked malathion doses of 100, 500 and 1500 ppm for 4 weeks. Plasma and saliva was measured antioxidant power and ChE activities. Those results found that plasma had antioxidant vitamins significant increase while saliva was not affected. ChE activities in rats after 28 days OP exposured were significantly decreased in both plasma and saliva, but not dose-dependent (Abdollahi *et al.*, 2004). Rat ChE activities in saliva, plasma and brain that were inhibited by specific inhibitors were measured. The previous study found that SChE activity was less than plasma or brain since saliva dilution greater than 180 to 1300 fold plasma and brain, respectively (Kousba *et al.*, 2003). Moreover, Borzelleca and Skalsky (1980) reported that rat SChE inhibition correlated with the PBChE and RAChE activity following carbaryl exposure.

People with periodontal disease had increased activities of enzyme because bacterial that accumulated in oral also could produce BChE (Chuncey, 1961). BChE could produce organic acids that destroyed both the hard and soft tissue (Makinen, 1966). Moreover, the periodontal therapy such as scaling, oral hygiene instructions, and surgical procedures could decrease SBChE activity (Yamalik *et al.*, 1991). Patients with Sjogren's syndrome had increased SChE activities because level of lymphocyte as origin of ChE was increased. Hydroxychloroquine, enable inhibiting enzyme activities, was used for therapy this illness by improving salivary flow rate (Dawson *et al.*, 2005). On the other hand, people with dementia (Breen, 2002) and Alzheimer's disease (Sayer *et al.*, 2004) had decreased SAChE activities compared with control.

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PBChE had positive correlation with RAChE levels ($r=0.45$ $p<0.05$) in healthy and OP exposed people. The workers of a pesticides factory have significantly lower of the ChE activities (plasma, $p<0.05$ and saliva, $p<0.01$, respectively) than healthy individuals. The workers always found clinical symptoms that correspond to OP-induced cholinergic effects and neurotoxicity such as weakness, headache and diarrhea (Abdollahi *et al.*, 1996). All results support the potential utility of saliva as a biomonitoring matrix for evaluating occupational and environmental exposure to OP and carbamate pesticides.

2.3.2 3-PBA Plasma and 3-PBA urine

3-PBA Plasma is biomarkers of dose or exposure for pyrethroid pesticides. Pyrethroid exposure biomornitoring is usually performed by analysis of its urinary metabolite after acidic hydrolysis of its glucuronide (Hardt and Angerer, 2003; Leng *et al.*, 2003; Abu-Qare and Abou-Donia, 2000; Baker *et al.*, 2004). The metabolite 3-PBA occurs after permethrin, cypermethrin and deltamethrin exposure and *cis*- as well as *trans*-DCCA after permethrin, cypermethrin and cyfluthrin exposure (Leng *et al.*, 2003). The 3-PBA metabolite is common metabolites for several pyrethroids and has been used as a maker of pyrethroid exposure by the CDC in the National Health and Nutrition Examination Survey study (Centers for Disease Control and Prevention, 2005).

Although urine is easier to collect but some studies found volunteer who had been exposed (orally) to cyfluthrin revealed that most (93%) of the urinary metabolites are excreted within 24 hr. (Leng *et al.*, 1997). Moreover, several studies have shown that there is no correlation between the metabolite concentration in urine (cis/trans-DCCA, Cis-DBCA, FPBA, 3-PBA) and the symptoms mentioned such as eye/skin/nose irritations, paresthesias, fatigue, Blurred vision, sweating and headache (Kolmodin-

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Hedman *et al.*, 1995; Wieseler *et al.*, 1998). In the other hand, Leng et.al. (1999; 2003) found a correlation between the half-life time (*t*1/2) of pyrethroids in plasma and the clinical findings. It follows that urinary biomarkers analyzing cannot be assessed cumulative doses. This observation led to an alternative biomarker for pyrethroid exposure.

It is well known that all xenobiotics in human will bind to protein called protein adduct that represent a much more persistence biomarkers than urinary metabolites, having half lives up to several months (Leng *et al.*, 1997). In several studies have been developed methods for 3-PBA plasma based on mass spectrometric analysis (Black and Noort, 2005; 2007; Noort *et al.*, 2002; Noort and Black, 2005). All studies are base on the basis of its chemical structure, it should not be expected that permethrin itself will react with proteins to give adducts. In this respect reasoned that the β-glucuronides of the two major carboxylic acid metabolites of permethrin, i.e., 3-PBA and Cl2CA (Figure 2.6) are electrophilic compounds that can form adducts with endogenous proteins (Bailey and Dickinson, 2003).

Conjugation to glucuronic acid ("glucuronidation") by UDP-glucuronosyl transferase- mediated transfer of a glucuronyl moiety of UDP-glucuronic acid to a nucleophilic site of a xenobiotic is one of the major Phase II detoxicification reactions. It renders the xenobiotic more polar, which facilitates its excretion. The glucuronidation reaction takes place predominantly in the liver. In case of glucuronidation of a carboxylic acid, potentially electrophilic acyl glucuronides result that can react with nucleophilic residues within proteins. Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished.

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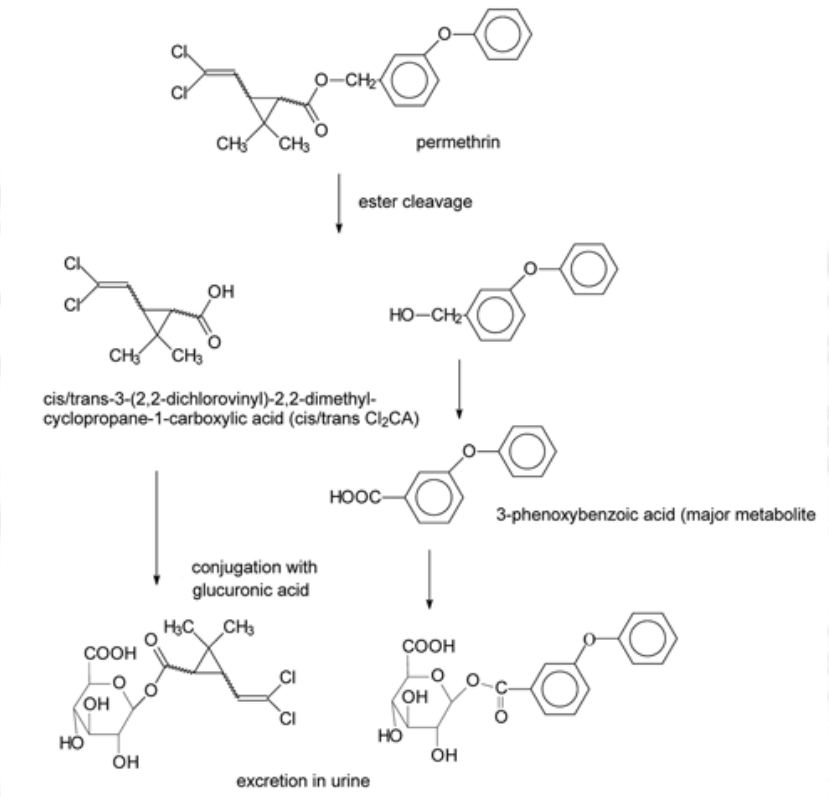

Figure 2.6 Metabolism of permethrin in mammals

According to the transacylation mechanism (Figure 2.7), nucleophilic sites in the proteins are acylated by the O-acyl glucuronide and consequently modified with the acyl moiety derived from the "original" metabolite. According to the glycation mechanism, an initial internal acyl migration occurs, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts (Grubb *et al.*, 1993; Smith *et al.*, 1990), which may eventually undergo a (slow) Amadori rearrangement (Figure 2.8). The adduct formation was describe the β-glucuronides of the two permethrin metabolites 3-PBA and Cl2CA with peptide model compounds and with albumin in human plasma.

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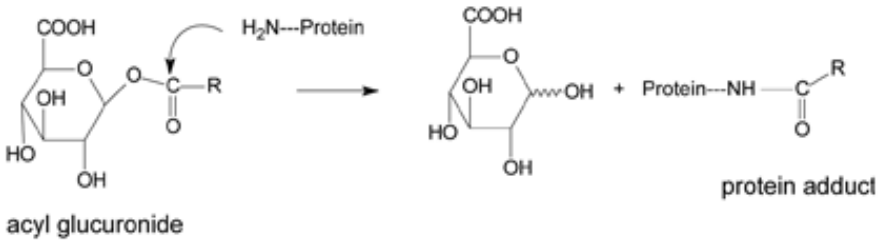

Figure 2.7 Adduct formation of acyl glucoronides with proteins by transacylation mechanism

In Noort *et al.*'s studies (2006; 2007; 2008), which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis/trans*-Cl2CA) will form persistent adducts to proteins. In the first year (2006) of the project the 3-PBA and Cl2CA glucuronide metabolites of permethrin have been successfully chemically synthesized. Moreover, they found the reaction of 3-PBA glucuronide adducts form boths of transacylation and the glycation mechanism. However, Cl2CA glucuronide could be from only in transacylation.

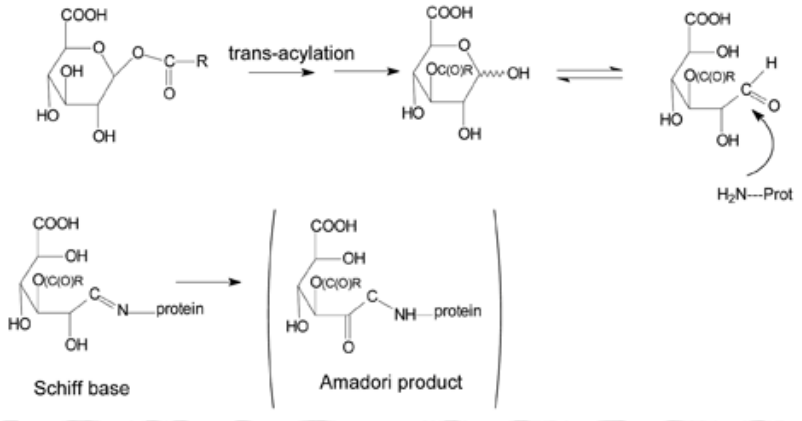

Figure 2.8 Adduct formation of acyl glucoronides via glycation mechanism

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After incubation of 3-PBA glucuronide with human plasma, it was founded that two peptide adducts could be detected. Their all identities have been assessed by means of 1H- NMR spectroscopy and liquid chromatography-tandem mass spectrometry. Moreover, the reactivity of these metabolites with various amino acids, peptides and albumin has been studied. The next year studied (2007) about the reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl2CA with model. That found that result in the adduct formation was ε-N-lysinyl adducts that formed both of transacylation and the glycation mechanism. When incubated human plasma with adduct formation found various ε-NH2-modified lysine derivatives. The last year (2008), found covalent binding was quantified by using [14C]-3-PBA glucuronide; >1.5% of total radioactivity was bound to proteins. It is envisaged that the obtained results can form a firm basis for the development of a protein adduct-based methodology for biomonitoring exposure to permethrin.

Following parent compound of pyrethroid pesticides in plasma studies, Leng and Gries (2006) determined parent compound in plasma compared metabolites in urine using two analytical methods. The solid-phase extraction and high-resolution gas chromatography–negative chemical ionization mass spectrometry (HRGC–NCIMS) were used for plasma analysis with a detection limit of 5 ng/L. For urinary metabolites were determined by gas chromatography– mass spectrometry (GC–MS). After acid hydrolysis and sample extraction with tert-butyl-methylether, the residue is derivatized with 1,1,1,3,3,3- hexafluoroisopropanol and analyzed by HRGC–MS with detection limit 0.1 µg/L.

Another prospective epidemiological study (Leng *et al.*, 2003) was determined indoor monitoring. The results of the biological monitoring are presented. Biological

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monitoring was performed in 57 persons before (T1) as well as 1 day (T2), 3 days (T3), 4 ± 6 months (T4), and 10 ± 12 months (T5) following a pest control operation (PCO) with pyrethroid containing products such as cyfluthrin, cypermethrin, deltamethrin or permethrin. Parent compound in plasma were measured by GC-ECD. The respective metabolites were measured in urine using GC/MS. For all cases the concentrations of parent compounds in plasma were found to be below the detection limit of 5 µg/l. With a detection limit of 0.2 µg/l of the investigated metabolites, the percentage of positive samples were 7% for cis-DCCA, 3.5% for trans-DCCA and 5.3% for 3-PBA before PCO. One day after PCO (T2) the percentage of positive samples increased remarkably for cis-DCCA (21.5%), trans-DCCA (32.1%) and 3-PBA (25%) showing significantly increased internal doses as compared to pre-existing values. This holds also true for T3, whereas at T4 and T5 the significant increase was no more present.

Not only parent compound in plasma but also 3-PBA metabolites in plasma were determined especially in pregnant women (Ostrea Jr. *et al.*, 2008; Ding *et al.*, 2004; Corrion *et al.*, 2005). The exposure of the pregnant woman to pesticides affected to the fetus including neurotoxin those effects to the rapid state of growth and development of its brain (Bruckner, 2000).

2.4 Methods for measurement of biomarkers

2.4.1 Cholinesterase enzyme activity measurement

Salivary and blood cholinesterase enzyme activities were measured as biomarkers of effect that modified from Ellman *et al.* (1961). However, the OP and carbamate pesticides can be used biomarker of exposure that measured metabolites in urine. The common dialkylphosphate metabolites of multiple OP pesticides are diethylphosphate

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(DEP), diethylthiophosphate (DETP), diethyldithiophosphate (DEDTP), dimethyl phosphate (DMP), dimethylthiophosphate (DMTP), and dimethyldithiophosphate (DMDTP) (Barr and Needham, 2002). One of the most accurate and precise method was lyophillization and chemical derivatization following by analysis using isotope-dilution gas chromatrography tendem mass spectrometry (GC-MS/MS).

The limits of detection (LODs) of the method were in the low ng/ml (parts per billion, ppb) to mid pg/ml range (part pertrillion , ppt) with coefficient of variation of 7-14% (Bravo *et al.*, 2004). This study used biomarkers of effect because would like to directly analyzed affection of cholinesterase inhibition and activity measurement is not multi-step, time-consuming, high-cost and require professional that is suitable for routine analysis.

2.4.2 Enzyme-linked immunosorbent assay (ELISA)

Immunoassay was used for detection of plasma 3-PBA and urine 3-PBA as biomarkers of exposure. The pyrethroids are one of the most heavily used pesticide classes in the world because it more likely safety than the others groups so that it is important to develop sensitive and rapid analytical techniques for environmental monitoring and assessment of human exposure to these compounds.

The methodology of plasma 3-PBA in this study was applied from urine 3-PBA analysis (Shan *et al.*, 2004). The current analytical method for urinary metabolites upon multistep sample cleanup procedures including acid hydrolysis, liquid–liquid extraction (LLE) or solid-phase extraction (SPE) and derivatization with high-performance liquid chromatography (HPLC) or gas chromatography (GC) with mass spectrometry (MS) (Schettgen *et al.*, 2002; Colume *et al.*, 2001; Leng *et al.*, 1997; Baker *et al.*, 2000).

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Although the instrumental methods are very sensitive for these metabolites, they can be expensive and relatively time-consuming and are not suitable for a routine and rapid analysis.

2.4.2.1 The step of ELISA development

The steps in development of ELISA was previously reported (Shan *et al.*, 2004; Tijssen, 1985) including design and synthesis of hapten, preparation of immunogens and coating antigens, immunization of mice, characterization of antibody, development and optimization of assay, validation, and optimization of assay.

2.4.2.2 Design and synthesis of hapten

Pesticides, 3-PBA, are small molecule with molecular less than 1,000 Dalton. They are non-immunogen in animal so that these molecules must first be covalently linked to a large protein called carrier protein such as bovine serum albumin (BSA) to make them immunogenic (Singh *et al.*, 2003). The hapten requires attaching to carrier protein at a functional group to selected site by linker arm. The most common functional group for hapten synthesis including –NH2, COOH, -OH, -SH, COOH in this study, which are usually attached to 3 to 6 carbon. In hapten design, it is important to consider the structure of hapten that similar to the target compound. The hapten must contain as similar geometric, size, and electric properties of the target compound as possible and characteristic moiety of the target molecule to facilitate the recognition by antibody (Szurdoki *et al.*, 2002).

The linker arm should connect at the right position on the target molecule as the position influence the selectivity of an assay. A major part of hapten molecule must be

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exposed to or easy recognized by antibody. The chemical used for linker arm may induce the immune system to produce antibody so that alkyl chains are preferable to minimize linker binding. However, normally, the length of linker arm has less effect on antibody affinity and specificity. The unique structure of individual target is required for synthesizing of hapten but not for group specific antibody. The hapten for use in the production of group compound specific antibody should be designed to the major part or similar part of group compound of the target molecule (Singh *et al.*, 2003).

2.4.2.3 Preparation of immunogen and coating antigen

In general, the large protein molecule is conjugated to hapten through covalent bonding between functional groups of hapten and side chain of amino acid (Colbert *et al.*, 1988; Alvarez *et al.*, 2003). Several difference protein including keyhole limpet hemocyanin (KLH), ovalbumin (OVA), and BSA have been used as carrier proteins. The selection of coupling method is dependent on the functional group of the hapten and protein carriers.

The reaction is carried out by conjugation method in water miscible solvent, in which the functional group is converted to an active from which then reacts with protein (Langone and Van Vunakis, 1975). The active hapten after centrifugation is added dropwised between activated form of the hapten and amino group of protein. Then the immunogen and coating antigen are purified by gel filtration or dialysis (Hao *et al.*, 2009).

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2.4.2.4 Production of antibody

Antibodies are glycoprotein of the immunoglobulin class produced by the immune system against foreign material such as pathogens or xenobiotics, and bind the target with high selectivity and affinity. Antibodies divide to five classes in higher mammals (IgA, IgD, IgE, IgG and IgM) that contained ~ 80% of IgG in human serum. In Figure 2.9 (A), IgG consists of two identical heavy (H) chains and two identical ight (L) chain. The inter- and intra-chain of disulfide bonds linked chains stabilities. The antigen-binding sites located at the amino terminal end of variable regions (V) on H (V_H) and L (V_L).

The variable regions of both chains are organized into complementarily determining regions (CDRs). In these regions vary the greatest amino acid sequences because it is the combination sites. The Fc (antibody fragment containing the crystallizable fragment), the lower part of the molecule, supported some biological effecter functions such as complement fixation. Not only the whole of IgG molecule can be used in immunoassay but also used fragments that contained the antigen binding sites such as F(ab), F(ab') and $F(ab')_2$ (Figure 2.9 (B)) (Abcam, 2012) .

Antibody can be produced in mammals such as goats, sheep, rabbit, mice and rat. First step of antibody production is immunization step which requires 3 to 4 subsequent injections subcutaneously or intramuscularly. Emulsification of immunogen with adjuvant such as complete Freund's adjuvant can be used for the following injection. For generating predominantly IgG subtype of antibody must be boosted at 3-4 weeks interval (Ercegovich, 1973). The immune system of animals evolved to recognize and eliminate foreign material, while preserving self.

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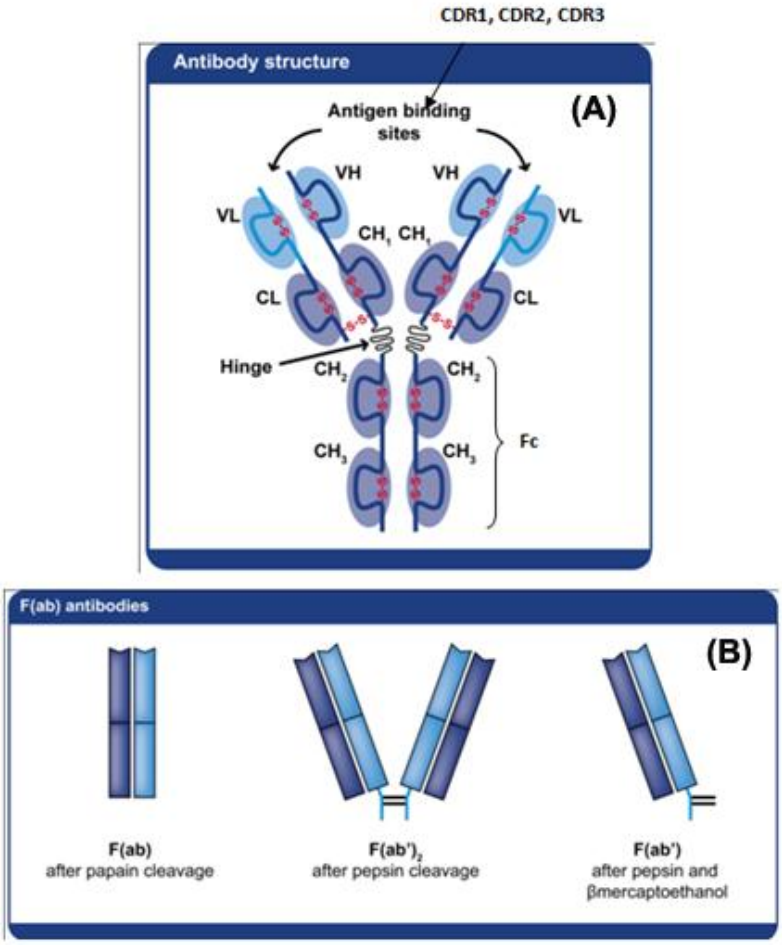

Figure 2.9 (A) Structure of IgG antibody and its fragments. V_L is the variable regic of light chain, V_H is the variable regions of heavy chain, C_L is the constant regions on light chain, and CH1, CH2, CH3 are the constant regions on heavy chain. (B) Fragments contained the antigen binding sites such as F(ab), F(ab') and $F(ab')_2$ (Abcam, 2012) .

The primary antibody response, usually IgM, generally has a lag period of 4-5 days, with a peak response at 10-14 days, following by a relatively quick decline. The secondary antibody response, usually IgG, is quicker, has a higher peak titer, and a slower decline. Antibodies are made by B-cells, which are stimulated by T cells. Each B

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and T cell is specialized to make/help make only one type of antibody specific to one type of epitope. However, in an immune response to an antigen many B and T cells are made that recognize most if not all of the epitopes that a given antigen presents. This assists the immune system in better eliminating the antigen. Therefore, when an animal is immunized its response is always polyclonal (Figure 2.10)

The reversible specifically antigen-antibody binding is controlled by structure and chemical interaction at the antigen-binding sites. The kinds of interactions are hydrophobic, ionic, H-bonding and van der Waals forces. The relative affinity of the antibody increases with the number of specific chemical interaction so the selectivity and sensitivity of immunoassay depend on the nature of binding process.

The antibodies divide to polyclonal antibody (pAb) and monoclonal antibody (mAb). The monoclonal antibody is antibodies that are identical because they were produced by one type of immune cell, all cones of a single parent cell. This antibody is more selectivity and sensitivity than pAb however this method is requiring professional person, time-consuming, sterile process.

Briefly, the mAb can be produced by five major steps: immunization, fusion, selection, cloning, and production. The animals are first injected with an immunogen preparation, and once a good humoral response is observed, an appropriate procedure is developed for screening the desired antibodies. The sera from test bleeds are used to develop and validate the screening procedure. When an appropriate screen has been established, the actual process of producing the hybridomas can begin. Several days prior to the fusion, the animals are boosted with a sample of the immunogen. The antibody secreting cells are then prepared from the spleen of the immunized animals, mixed with the myeloma cells, and fused.

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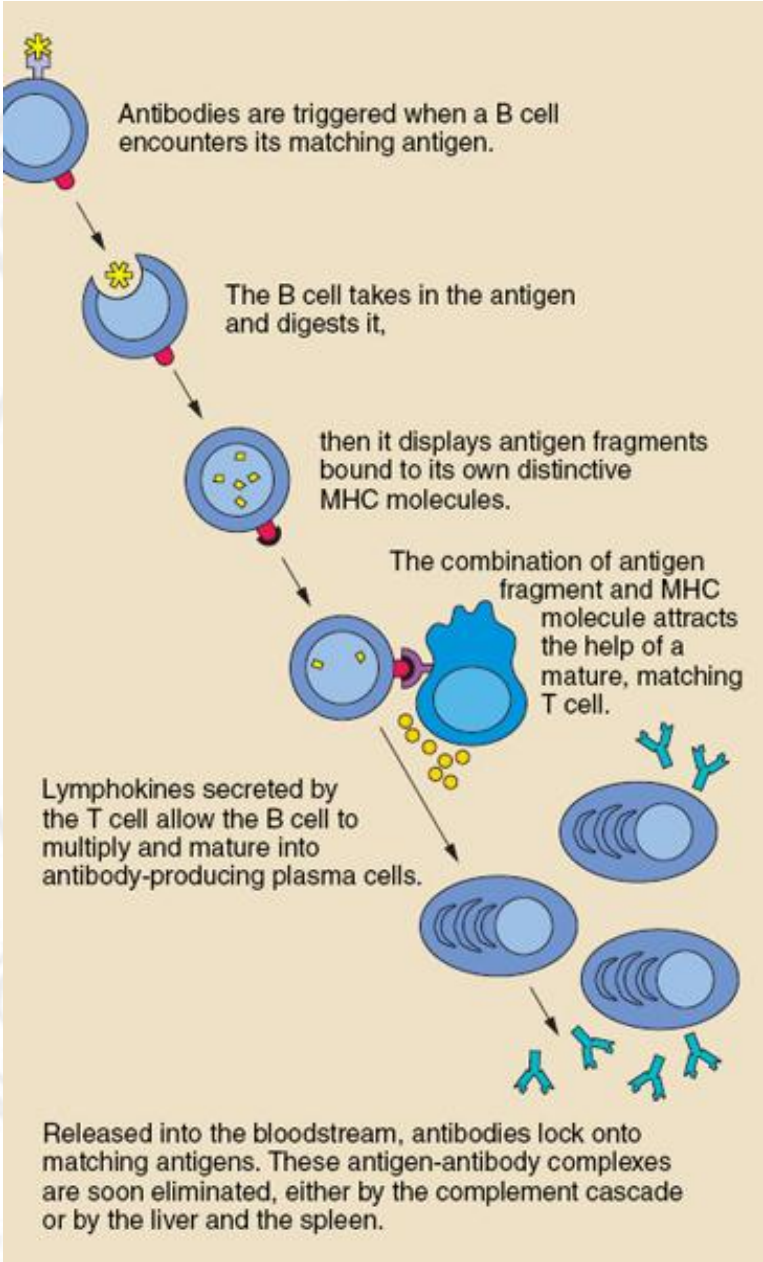

Figure 2.10 The principle of antibody production. B cell are triggered to mature into plasma cells that produce a specific kind of antibody when the B cell encounters a specific antigen (www.niaid.nih.gov)

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The hybridomas are diluted in selective medium and plated in multiwell tissue culture plates. After about one, a single hybridomas cell is cloned (Deshpapnde, 1996). The hybridoma cells can be propagated almost indefinitely in culture, and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules produced by a hybridoma cell line derived from a single hybrid cell are identical, and have the same binding properties; therefore the hybridoma technology guarantees the unlimited production of mAbs with constant characteristics (Hock *et al.*, 1995). However, this study was developed from urine 3-PBA ELISA (Shan *et al.*, 2004) that used polyclonal antibody and the results showed very sensitivity at the average IC50 was 1.65 ng/mL and only used small volume (50 µL of antiserum per well (1: 7000 in PBST) of antiserum from rabbits. The antigens using this method have to high purify before bind to antiserum (ELISA) so sample preparation (LLE or SPE) were developed.

2.4.2.5 Immunoassay formats

Immunoassay is a biochemical technique using the specific binding of an antibody to its antigen. The quantity of antigen or antibody can detect using a variety of methods. The most of common using is to label either the antigen or antibody. The labels may use an enzyme (enzyme immunoassay, EIA), radioisotopes such as I-125 (radioimmunoassay, RIA) or fluorescence (immunofluorescence assay, IFA). The types of ELISA most frequently used for analyzing pesticides are direct competitive ELISA (dc-ELISA) (Langone and Van Vunakis, 1975) and indirect competitive ELISA (ic-ELISA) (Figure 2.11).

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The dc-ELISA is a format where a constant amount of antibody is immobilized on the microplate usually by overnight incubation at 4°C or at room temperature. The analytes serially diluted in solution compete with the fixed amount of hapten labeled with an enzyme in an analyte-hapten mixer, for binding to the immobilized antibody. Binding of enzyme tracer to antibody depends on the concentration of analytes involved in competition reaction. The color is change after adding chromogenic substrate and intensity color is inversely proportional to the concentrations of analytes in the solution.

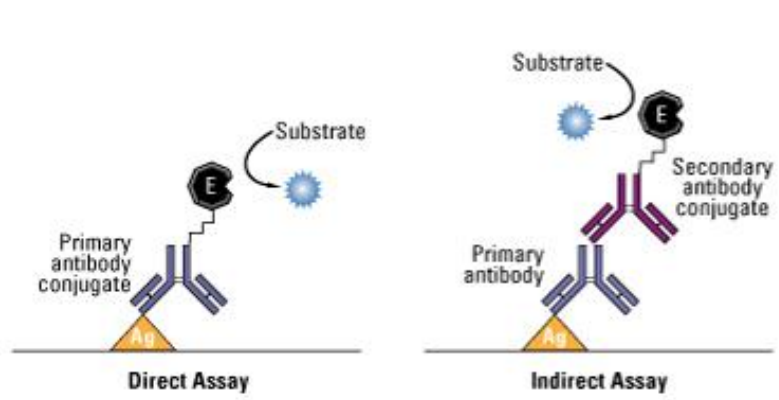

Figure 2.11 ELISA format (www.thermoscientific.com)

For the indirect ELISA, microplate is coated with fixed coating antigen concentration. Competition is performed by mixing fixed serum dilution with various concentrations of analyze solution. The analyte competes with the coating antigen immobilized on the plate for binding to the Abs. The amount of antibody bound to the coating conjugate is dependent on the amount of the free analyte. Then specific secondary antibody specific to the primary Ab conjugate with an enzyme will bind to the primary antibody.

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A chromogenic substrate is added and the color change is measure spectro photometrically. The increase in absorbance is inversely proportional to the amount of pesticides in solution. An advantage of immunoassay is simple sample clean up which is difference to conventional analyses that need very clean samples. The water-miscible solvents such as acetone, acetonitrile, or methanol are used of immunoassay to routine procedure for sample preparation. Many studies reported successfully analyze a variety of compounds from various matrices such as food (Botchkareva *et al.*, 2003), water (Hao *et al.*, 2009), soil, and urine samples (Shan *et al.*, 2004; Kim *et al.*, 2007). However, since unwanted interactions between co-extract and antibody cause matrix effect i.e. false negative or positive effect, sometimes a simple additional clean up step such as a solid-phase or solvent extraction step is required.

In this study, immunoassay was used for detection of plasma 3-PBA and urine 3-PBA as biomarkers of exposure. The pyrethroids are one of the most heavily used pesticide classes in the world because it more likely safety than the others groups so that it is important to develop sensitive and rapid analytical techniques for environmental monitoring and assessment of human exposure to these compounds. The methodology of plasma 3-PBA in this study was applied from urine 3-PBA analysis (Shan *et al.*, 2004).

The current analytical method for urinary metabolites upon multistep sample cleanup procedures including acid hydrolysis, liquid–liquid extraction (LLE) or solid-phase extraction (SPE) and derivatization with high-performance liquid chromatography (HPLC) or gas chromatography (GC) with mass spectrometry (MS) (Schettgen *et al.*, 2002; Colume *et al.*, 2001; Leng *et al.*, 1997; Baker *et al.*, 2000) Although the instrumental methods are very sensitive for these metabolites, they can be expensive and relatively time-consuming and are not suitable for a routine and rapid analysis.

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The immunoassay test is widely used in diagnostics, environmental monitoring, agriculture, and field or on-site testing of personnel. This assay provide rapid, sensitive, selective and costless analytical tools to determine trace chemicals such as agrochemicals and their metabolites as key urinary biomarkers of exposure (Shan *et al.*, 2004). More likely, the pyrethroid metabolite profile will vary from individual to individual. An assay detecting a single conjugate such as 3-PBA-glycine may not be sufficient for exposure monitoring. Therefore, the immunoassay that be more significant because it allows detecting all conjugation forms after a hydrolysis treatment, and it will complement conjugate specific assays.

Shan *et al.* (2004) used immunoassay method to detect urinary metabolites using adding antiserum (diluted 1: 10000) from immunized rabbits with 3-[4-(3-carboxy phenoxy) phenoxy] N-thyroglobulin ethylamine and coated ELISA plate with cAg**06** (0.5 μg/L) that gave an IC50 value of 1.65 ± 0.7 ng/mL with a lower detection limit of 0.1 ng/mL. The cross-reactivity didn't find from other metabolites. This method could eliminate the urine matrix effects by simple dilution (50X dilution).

The immune assay have been interesting since it is easier modification to give more sensitive method such as competitive chemiluminescent immunoassay (Ahn *et al.*, 2007) gave an IC50 value of 0.1 ng/mL with a lower detection limit of 0.01 ng/mL. Moreover, phage anti-immunocomplex assays (PHAIA) technology that used a polyclonal antibody to 3-phenoxybenzoic acid (3-PBA) to bind an anti-immunocomplex phage clone bearing the cyclic peptide CFNGKDWLYC. The assay setup with the biotinylated antibody immobilized onto streptavidin-coated magnetic beads significantly reduced the amount of coating antibody with 50% saturation of the signal (SC50) value of 0.2–0.4 ng/mL (Kim *et al.*, 2009).

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