DETECTION OF CARBAMIC PESTICIDE USING A BUTYRYLCHOLINESTERASE-BASED BIOSENSOR

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THIS DISSERTATION IS SUBMITTED AS A PART OF REQUIREMENT TO OBTAIN DEGREE OFBACHELOR OF SCIENCE WITH HONOUR

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ABSTRACT

The biosensor provided a new and alternative tool for pesticides analysis that is sensitive, fast and inexpensive compared to the conventional methods for determination of carbamate pesticide using amperometric butyrylcholinesterase (BChE) biosensor. In this study, the objective is to immobilize butyrylcholinesterase enzyme (BChE) based on chitosan (CHIT) onto glassy carbon electrode (GCE) through cross-linking immobilization method. The BChE biosensor was fabricated by firstly depositing CHIT film on glassy carbon electrode (GCE) and then attaching BChE on CHIT film using glutaraldehyde as a cross-linking agent. CHIT was selected as BCHE immobilization matrix due to its excellent mechanical strength and biocompatibility. The methylene blue was used as a redox mediator that shuttle electron transfer in BChE biosensor. Optimal conditions were pH 7, scan rate of 0.03 V and 10 s for BChE/CHIT/GCE to determine peak response of carbaryl inhibition in 0.2 M PBS-0.1M KCl solution in the presence of 0.4 mM BTCl as substrate. The inhibition of carbaryl as one of carbamate compound that has high toxicity showed a decline in response of peak current BChE/CHIT/GCE by reducing the immobilized BChE activity and thiocholine products. The lowest detection limit of carbaryl on BChE/CHIT/GCE was 10 $\times 10^{-11}$ ng/ml.

Keywords: Biosensor, Pesticides. Butyrylcholinesterase. Chitosan. Methylene blue

ABSTRAK

Biosensor ini menyediakan alat baru dan alternatif untuk analisis racun perosak yang sensitif, cepat dan murah berbanding dengan kaedah konvensional untuk menilai kadar racun perosak karbamat menggunakan butyrylcholinesterase amperometrik (BChE) biosensor. Dalam kajian ini, objektif adalah untuk memegunkan enzim butyrylcholinesterase (BChE) berdasarkan kitosan (CHIT) ke elektrod karbon berkaca (GCE) melalui kaedah rangkaian silang. Biosensor BChE telah dibuat bermula dengan mendepositkan CHIT pada elektrod karbon berkaca (GCE) dan kemudian mencantumkan BChE pada CHIT menggunakan glutaraldehid sebagai ejen silang. Metilena biru digunakan sebagai pengantara redoks yang dapat mempertingkatkan pemindahan elektron dalam BChE biosensor. pH 7.0, kadar imbasan 0.03V pada 10s telah terpilih sebagai keadaan yang optimum untuk BChE/CHIT/GCE untuk menentukan gerak balas kemuncak kesekatan carbaryl ke dalam larutan 0.2 M KCl PBS-0.1M dalam kehadiran 0.4 mM BTCl. Perencatan carbaryl sebagai salah satu sebatian carbamate yang mempunyai ketoksikan yang tinggi menunjukkan penurunan dalam sambutan puncak BChE semasa / CHIT /GCE dengan mengurangkan aktiviti BChE terpegun dan thiocholine produk. Had pengesanan carbaryl pada BChE/CHIT/GCE adalah 10 x10-10 ng /ml.

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LIST OF SYMBOL, UNITS, AND ABBREVATIONS

A	ampere
BChE	butyrylcholinesterase enzyme
BSA	bovine serum albumin
BTCl	butyrylthiocholine chloride
C	carbon
CE	counter electrode
CHIT	chitosan
CM	carbamate
CMs	carbamates
CV	cyclic voltammetry
GCE	glassy carbon electrode
LD_{50}	Lethal Dose, 50% /median lethal dose
ng/ml	nanogram per milliliter
Pt	platinum
RE	reference electrode
V	volt
WE	working electrode

CHAPTER 1

INTRODUCTION

1.1 Background Study of Pesticides

Pesticides have been utilized over years that designed to control or destroy any pests, undesirable plants (weeds), fungi, insects and microorganisms like bacteria and viruses. In agriculture, farmers frequently use pesticides in order to protect their crops and seeds before and after harvesting (Sassolas *et al.*, 2012). However, nowadays pesticides are not only greatly used in agriculture but their rapid increase consumption is also found in nonagricultural applications such as industrial vegetation control (roadways, railroads), public health, or grass management (Trojanowicz, 2002). Broad consumption of pesticides in both agriculture and non-agriculture contribute to environmental chemistry issues as the residue of pesticides may move into the food chain via air, water, and soil (Sassolas *et al.*, 2012). In addition, pesticides are considered as the most life-threatening environmental pollutants as they stimulate accumulation and cause long-term consequence on living organisms (Shaoqin *et al.*, 2013).

Pesticides wide utilization draws great public attention and concern due to its carcinogenic and cytotoxic features that can cause health problems in animals and humans and even towards ecosystems. Among pesticides, the pesticides that are most utilized namely carbamic because of its prominent insecticidal activity and relatively low persistence (Arduini *et al.*, 2006). Carbamate pesticide is the most acutely poisonous landscape pesticides. It is the main cholinesterase (ChE) enzyme inhibitors affecting nervous system. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two types of natural cholinesterase (ChE) enzymes. Acetylcholinesterase (ChE) enzyme plays crucial role in living organisms as a key enzyme primarily in central nervous system.

Function of acetylcholinesterase (AChE) is the catalytic saponification of the neurotransmitter acetycholine that initially bound to the active site of the enzyme and then rapidly hydrolyzed (Trojanowicz, 2002). Carbamate pesticides may interfere with the AChE enzyme and prevent the removal of acetylcholine.

The identification and detection of pesticides are typically based on gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) due to their sensitivity and reliability. However, these traditionally methods permit several limitations such as time-consuming for pesticides extraction, extract cleaning and solvent substitution. Moreover, these conventional methods need highly trained personnel, expensive equipments and not suitable for on-site analysis (Shaoqin *et al.,* 2013). Electrochemical biosensor developments are very useful as they analyze and detect pesticides rapidly compared to classical method. Enzyme-inhibition-based methods are one of the fundamental of pesticide biosensors that have quick response time, low cost, simple, and high sensitivity. Among these, butyrylcholinesterase (BChE) biosensor based on the inhibition action of pesticides on BChE is considered as one of the best alternative (Arduini *et al.*, 2006). The quantification of enzyme-inhibition-based biosensor involves initial enzymatic action determination, incubation of biosensor in a solution that contains pesticides and ultimately measurement of residual activity after exposure of the enzyme in immobilized form to an inhibitor (Shaoqin *et al.*, 2013).

1.2 Biosensor and Principles

The term biosensor is defined as an analytical device that converts a biological recognition event to an electrical signal that can be measured. The biological or recognition materials include tissues, microorganisms, organelles, cell receptors, enzymes, antibody, nucleic acids, or DNA (Rai and Duran, 2011) (Figure 1.1). The transducer part of the sensor is used to transfer and converts biological event or signal from output domain to a measurable signal that is electrical one. The transducer part of the sensor also can be called as a detector, sensor, or electrode but mostly the term transducer is chosen to avoid confusion. Potentiometry, amperometry, voltammetry and conductometry are sensing mode in electrochemical transducer that frequently used to measure the output signal from the biorecognition domain (Mostafa, 2010).

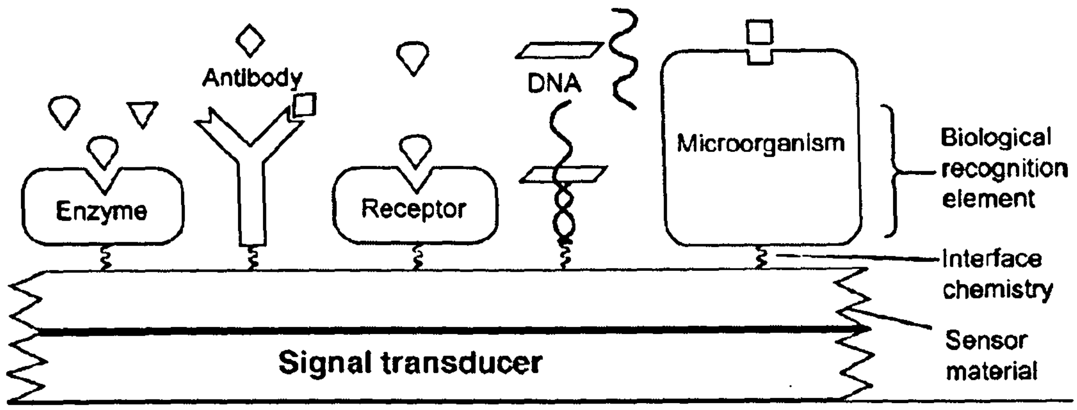


Figure 1.1 Schematic representation of biosensor (Mostafa, 2010).

The measurement of potentiometry is based on the potential difference between either an indicator or reference electrode or two reference electrodes that divided by a permselective membrane and there is no current flow between them. The reaction between the indicator and reference electrodes generates potential difference proportional to the logarithm of the ion activity or gas fugacity (concentration). The most common potentiometric devices are ion-selective electrodes (ISE) (K^+, Cl^-, Ca^{2+}, and F^-) and gas electrode (CO_2 and NH_3). Amperometric sensors measure the current resulting from the electrochemical oxidation or reduction of an electroactive species by holding a fixed potential at working electrodes (Pt or Au). The current produced are usually proportional to the bulk analyte concentration. Conductometric measurements involve the resistance determination of a sample solution between two parallel electrodes (Thévenot *et al.*, 2001).

Electrochemical biosensor have electrochemical cell constituting three types of electrodes which are working electrode (WE), reference electrode (RE) and auxiliary or counter electrode (CE) as shown in Figure 1.2. Reference electrode refers to a standard hydrogen electrode. However, using electrode with hydrogen gas is not very suitable due to its explosive properties. Ag/AgCl and saturated-calomel electrodes are examples of commonly used reference electrodes. The role of CE is as a cathode whenever the WE is functioning as an anode and vice versa. The CE usually has a bigger surface area compared to the WE. Fabrication of CE is frequently made of electrochemically inert substances such as platinum (Pt), gold (Au), or carbon. The WE can function as either cathodic or anodic depending on the reaction on the WE is a reduction or an oxidation. There are several types of WE including GCE, screen printed electrode (SPE), Pt electrode, Au electrode, silver (Ag) electrode, carbon nanotube paste electrode and others (Koyun *et al.,* 2012).

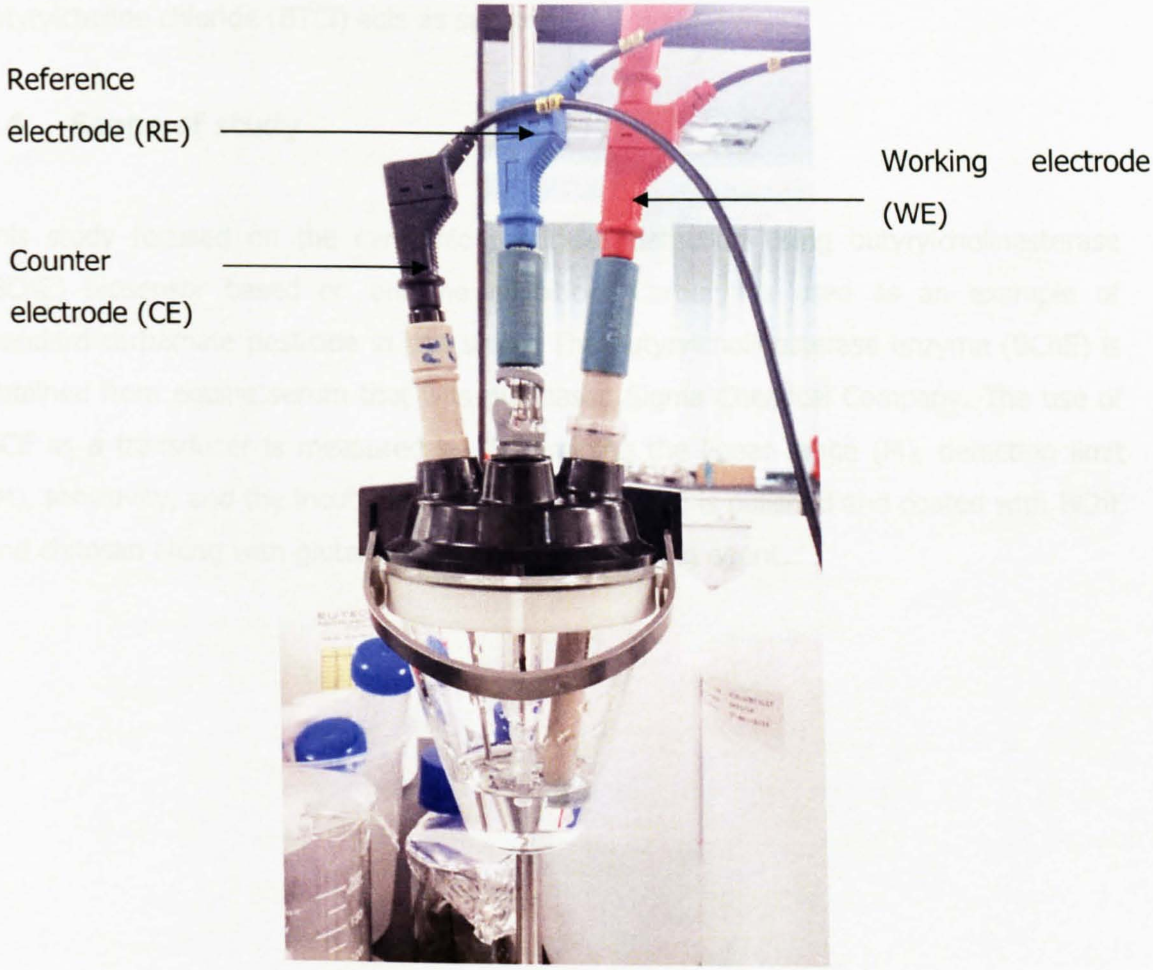


Figure 1.2 Electrochemical cell comprising WE, RE and CE.

1.3 Objectives of the study

The main objective of this study is to immobilize butyrylcholinesterase enzyme (BChE) based on chitosan (CHIT) onto glassy carbon electrode (GCE) through cross-linking immobilization method. The purpose of this study is to determine the selectivity, sensitivity, and detection limit of bare glassy carbon electrode (GCE). The significance of this study is to detect the lowest concentration of carbamic pesticides using bare glassy carbon electrode (GCE) based on the butyrylcholinesterase enzyme (BChE) inhibition.

The hypotheses of this study is the catalytic peak current of BChE/CHIT/GCE decreases in the presence of carbamate pesticide as an BChE inhibitor competes with butyrylcholine chloride (BTCl) acts as substrate.

1.4 Scope of study

This study focused on the carbamic pesticides detection using butyrylcholinesterase (BChE) biosensor based on enzyme inhibition. Carbaryl is used as an example of standard carbamate pesticide in this study. The butyrylcholinesterase enzyme (BChE) is obtained from equine serum that was purchased Sigma Chemical Company. The use of GCE as a transducer is measured by determining the linear range (M), detection limit (M), sensitivity, and the incubation time. The bare GCE is polished and coated with BChE and chitosan along with glutaradehyde as a crosslinking agent.

CHAPTER 2

LITERATURE REVIEW

2.1 Pesticides

2.1.1 Definition and History of Pesticides

According to the Federal Insecticide, Fungicides, and Rodenticide Act (FIFRA), pesticides refer to any substance or mixture substances designated for destroying, combating, controlling, or mitigating any pest including insect, rodent, fungus, weeds and nematodes, aquatic plant or animal life or viruses, bacteria or other microorganisms. Another definition of pesticides under FIFRA also defined as any substance or mixture of substances that utilized for plant regulator, defoliant, or desiccant (Marrs and Ballantyne, 2004).

Sulphur was the first known pesticide to be used. By the seventeenth century, sulfate as an insecticide was extracted from natural products that extracted from tobacco leaves and later two more pesticides that are natural, pyrethrum and rotenone were developed (Rathore and Nollet, 2012). In 1880s, copper fungicides, a mixture of copper sulphate and calcium hydroxide are introduced and are still utilized to some extent. There are three historical stages of pesticides production (Zhang *et al.,* 2011). The first stage was before 1870s comprising of natural pesticides, sulfur in ancient Greece, the second stage was the era of inorganic pesticides usage (1870s-1945) and the third stage (1945) where the era of organic synthetic pesticides began (Marrs and Ballantyne, 2004 ; Zhang *et al.,* 2011). In 1939, dichlorodipenyltrichloroethane (DDT) was the first introduced modern synthetic insecticides belong to organochlorine pesticides that have chemically combined chlorine and carbon. It was widely used pesticide in the world against pests and illness or during the Second World War (1940-

1945) such as to combat malaria, yellow fever and other insect-vectored diseases. Nevertheless, in 1960s, the usage of DDT was banned as it was found that DDT was preventing birds from reproducing, making birth defects in animals and humans that was a vast menace to biodiversity written in the book, the Silent Spring by environmentalist Rachel Carson. Each year, about 2.5 millions of pesticides are utilized and have increased 50-fold since 1950 (Rathore and Nollet, 2012). Table 2.1 below shows the chronology the development of pesticide in the world (Stephenson and Solomon, 1993).

Table 2.1 Chronology of Pesticides Development

Period	Example	Characteristics
1800s-1920s	Early organics, nitrophenols, and naphthalene	Lacked specificity and were toxic to user and non-target organisms
1945-1955	DDT and chlorinated organics	Persistent, resistance and good agricultural properties
1945-1970	Carbamates, organophophorus compounds, cholinesterase inhibitors	Lower persistence, toxic and have some environmental problem
1970-1985	Biological pesticides, synthetic pyrethroids	Lack of selectivity, resistance and costs
1985-now	Genetically engineered organisms	Have problems with mutation and disruption of ecology

Pesticides are classified into three main classes, which are fungicides, herbicides, and insecticides. Organophosphate and carbamate pesticides are both classified as insecticides. The insecticides generally divided into four major categories. The first category is organochlorines or chlorinated hydrocarbons (DDT, aldrin, dieldrin and chlordane) that break down chemically very and can remain in the environment for a long time. The second and third are belongs to organophosphates and carbamate compounds. Organophosphates are very toxic to humans but do not remain in the environment for long time. Parathion and malathion are examples of these pesticides. Carbamate compounds (carbaryl, aldicarb, carbofuran) are believed highly toxic to humans. The fourth one is known as pyrethroids. In 1973, the first synthetic pyrethroids that have high toxicity to insects with low mammalian toxicity. Cypermethrin is an example of pyrethroids (Rathore and Nollet, 2012).

The organochlorine caused a revolution in the efficacy of pesticides especially to combat the malaria mosquito and other disease vectors in humans. The advantages of organochlorine are inexpensive and long-lasting control of crop pests. Examples of organochlorine pesticides are DDT, aldrin and lindane that belongs to dichlorophenylethanes, chlorinated cyclodienes and hexachlorocyclohexanes respectively.

2.1.2 Effect of Pesticides to Human Health

Pesticides have been used widely in agriculture and food control industry for pest control and high crop production. However, not all pesticides are equally risky and not everyone is equally at risk. Acute and chronic effects are two wide categories of health consequences caused by pesticides. Acute effect is a short-term effect that appear instantly (at once) or very soon after pesticides exposure and chronic effect refers to long-term that may manifest themselves many years and their origins are much difficult to trace (Rathore and Nollet, 2012).

Pesticides can enter via four routes of exposure. They are skin (dermal), lungs (inhalation), mouth (oral) and eyes. Chronic effects to certain pesticides can cause several effects like birth defects, toxicity to a fetus, tumors (benign and malignant), and disorders in blood or nerve including reproduction effects. Poisoning to pesticides can cause mild irritation in skin, coma, or even death. Sensitivity of individuals towards the pesticides is varying depends on different degrees of the chemicals (Lorenz, 2009). For example, mild effects from organophosphates manifest in the form of nausea, vomiting, diarrhea, abdominal pain and salivation. Moderate poisoning includes various effects like diminished muscle strength, motor incoordination, hypo or hypertension, dsypnea and muscle twitching. Coma, paralysis (respiratory and muscular), convulsion, cyanosis and extreme muscle weakness and hypersecretion are resulted from severe manifestation of pesticides. Furthermore, pesticides can cause a range of serious consequences on human health including lung damage, endocrine dysfunction, reproductive dysfunction, nervous system damage, and cancer.

Pesticides exposure are primarily separated into three categories that are occupational exposure (spray operators and farm), accidental exposure and pesticides residues found in food. Common exposure to pesticides can occur through home and garden use, through indirectly spray drift and through residues in household, dust, food and water (Fenner-Crisp, 2001). Occupational exposure is still can occur regardless the strict regulations and the utilization of safer pesticides in the developed countries (Rathore and Nollet, 2012).

2.2 Carbamate Pesticides

2.2.1 History of Carbamates

Development of carbamates (CMs) is arisen into commercial pesticides in the 1950s. During the middle of 19^{th} century, physostigmine was the first carbamate compounds that extracted from Calabar beans of the perennial plant namely *Physostigmavenenosum* that ordinarily found in tropical West Africa (Gupta, 2011). In 1956, carbaryl (sevin) was introduced as the first type of carbamate pesticides utilized as insecticides. It is applied as a spraying powder against mites in winegrowing and other various crops. Carbaryl is the most heavily utilized insecticide among all carbamates (Patnaik, 2007). In fact, carbaryl may be used as DDT substitute and it can decrease environmental pollution because it does biodegrade and does not accumulate in the ecosystem (Pesticides, man and biosphere book). Aldicarb (temik) is used directly into soil to prevent mites, aphids, and nematodes. It is used on soybean, cotton, peanuts, potatoes, and other crops. Another type of carbamate is carbofuran (furadon) that sprayed on the ground to control insects in fruits, vegetables, and tobacco and forest trees. In 1966, methomyl (lannate) was introduced as a broad-spectrum acaricide to control ticks and spiders in vegetables, fruits, and cotton (Patnaik, 2007).

2.2.2 Structure of Carbamate Compounds

Carbamate pesticides have been utilized broadly to control and eliminate pests and fungus in lawns, gardens, and agriculture to protect fruits, vegetables, and crops. Carbamate compounds are esters of carbamic acid and their general formula is shown below where $R_1$and R_2 are hydrogen, methyl, ethyl, propyl or other groups of short chain alkylsand R_3 is phenol, naptyl ring or other cyclic hydrocarbons or oxime derivatives (Figure 2.2) (Singh, 2012).

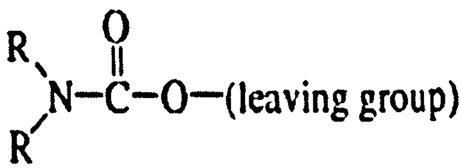
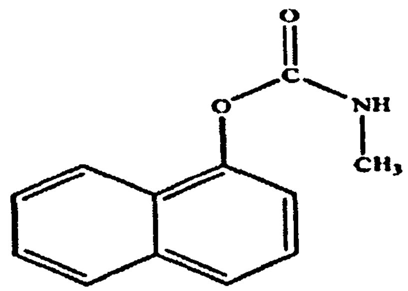


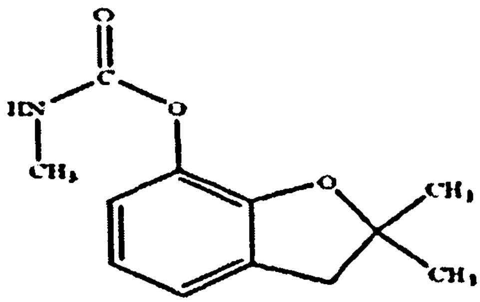
Figure 2.1 General structure of carbamates (Singh, 2012).

Generally, pure carbamate pesticides are odourless white crystalline solids of low pressure with variable, high melting point and typically have low water solubility that can be readily dissolved in polar organic solvents like ethanol, methanol, and acetone. However, carbamate pesticides have poor solubility in non-polar organic solvents, for instance petroleum or hexane. Most of carbamic pesticides are averagely soluble in solvents like benzene, xylene, chloroform, and toluene (WHO, 2009).Carbamates insecticides classified into three major groups, which are,

1. Group I: Contains N-methyl carbamate esters of phenols, compounds with a hydroxyl group attached directly to a phenyl or napthyl ring. For example, carbaryl (1-napthyl N-methyl carbamate).
2. Group II: Contains N-methyl and N-dimethyl esters of heterocyclic phenols *i.e.* carbofuran (2, 3-dihydro-2, 2-dimethyl benzofuran-7-yl N-methyl carbamate).
3. Group III: Comprises oxime (the OH group of that has been carbamylated). Aldicarb is an example of this group (Singh, 2012).



(a) Carbaryl



(b) Carbofuron

Figure 2.2 Molecular structures of some carbamate compounds (Singh, 2012).

2.2.3 Toxicity of Carbamate Compounds

Within carbamate insecticides, aldicarb possesses higher toxicity compared to other groups of carbamate insecticides as it synthesized to mimic the ACh structure (Gupta, 2011). Aldicarb is a systemic oximecarbamate insecticide that applied into the soil that has both contact and oral activities. Its IUPAC name is 2-methyl-2 (methylthio) propionaldehyde-O-methylcarbamoyloxime that has chemical formula, $C_7H_{14}N_2O_2S$ (Roberts and Hutson, 1999).

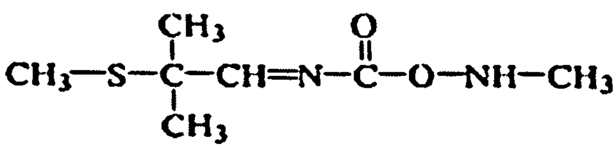


Figure 2.3 Chemical structure of aldicarb (Patnaik, 2007).

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