Analysis of diazinon pesticide using potentiometric biosensor based on enzyme immobilized cellulose acetate membrane in gold electrode

Mashuni1*, L O A N Ramadhan1, M Jahiding2, Herniati1
1Department of Chemistry, Faculty of Mathematics and Natural Sciences, Halu Oleo University (UHO), Kendari, Sulawesi Tenggara, 93231
2Department of Physics, Faculty of Mathematics and Natural Sciences, Halu Oleo University (UHO), Kendari, Sulawesi Tenggara, 93231

*E-mail: mashuni2696@yahoo.com

Abstract. Biosensor for analysis of diazinon pesticide using Potentiometric transducer has been developed. The basic element of this biosensor was a gold electrode modified with an immobilized acetylcholinesterase enzyme layer formed by entrapment with glutaraldehyde crosslinked-cellulose acetate. The aim of the research is to determine the composition of glutaraldehyde crosslinked-cellulose acetate in the gold electrode which provide optimum performance of biosensors of diazinon pesticide analysis on characterization include a range of concentration, sensitivity, and detection limit. The results showed the composition of the cellulose acetate 15% and glutaraldehyde 25% that obtain optimum performance in the measurement of diazinon pesticide with a range of working concentration of 10-6 ppm to 1 ppm, the value of sensitivity 20.275 mV/decade and detection limit 10-6 ppm. The use of cellulose acetate provides highly sensitive devices allowing the efficient analysis of pesticides. The response time of electrode is on the measurement of pesticide diazinon with concentration variation of 10-6 ppm to 1 ppm with response time is about 5 minutes.

1. Introduction
In the last few decades, the use of pesticide was dominated by two types of insecticide, i.e. carbamate and organophosphate compound. Both compounds were used a lot in the agricultural process because of its low stability in the environment, compared to organochlorine compound [1]. The pesticide is a chemical material which is used to control the growth of pests, diseases, and weeds. The intensive use of pesticides in agriculture, especially organophosphate, has left a little residue in plants and been a problem for environment and health of living creatures, especially human [2]. Pesticide of diazinon is one of the insecticides from the group of organophosphate, used a lot in an agricultural business to eradicate the pests. Along with the intensive use of pesticide, the analysis of pesticide residue is very important to do because of its high toxicity and its serious risk for the health of human and environment [3].

The analytical method of pesticide residue used a lot is by using Gas Chromatography (GC) and High-Pressure Liquid Chromatography (HPLC). When GC or HPLC are used to detect the samples, many pretreatment procedures including extraction, purification, and concentration are required before analysis and thus, GC or HPLC method shows some disadvantages, such as being troublesome, time-
consuming, and costly. Moreover, it needs requirement of solvent that has to be used in extraction. Therefore to overcome the weaknesses, a new method in the analysis of pesticide residue is used, i.e. by using a biosensor, whose application is more simple and cheap [4]. However, the biosensor method possesses some advantages, such as the simple operation of the sample preparation, low cost, and it needs only 25 min to complete the analytical process. Therefore, the potentiometric enzymatic membrane biosensor is likely to play an important role in OPs detection for agricultural products in the future [5].

A biosensor is an analytical tool which is a combination of biological component and physical transducer to detect a targeted compound. Biosensor consists of three elements, i.e. biological element, transducer, and electronic system processing a signal. One of the biological elements commonly used in designing is enzyme immobilized in a transducer. One of the enzymes that can be used in the biosensor is acetylcholinesterase [6]. The efficiency of carbamate and organophosphate as a pesticide and their toxicity to human and animals is caused by the ability of inhibiting enzyme group of a hydrolase, called esterase. The enzyme of acetylcholinesterase (AChE) is very important for central nerve system in human and insects [7].

In this research, the biosensor of pesticide of diazinon by immobilizing enzyme of acetylcholinesterase (AChE) in the material of cellulose acetate (CA) membrane and glutaraldehyde (GA) membrane functioning as a bifunctional reactant in gold wire. The success of a biosensor, few of them, depends on the ability of an enzyme of being bound in the surface of biosensor and still being active during the desired application, as well as the role of glutaraldehyde (GA) solution as an agent of bifunctional reactant between enzyme and membrane. Its mechanical strength and stability of membrane cellulose acetate are the reason of the user in this research.

2. Methods

2.1. Chemicals

The materials used in this research are: active material of pure diazinon pesticide, acetylcholinesterase (AChE) enzyme from Electrophorus electricus (electric eel) (Type C3389), acetylthiocholine chloride (ATCh) substrate (Type A5626), glutaraldehyde 25% from Aldrich-Sigma, cellulose acetate with Mr = 61000 g/mol, Na$_2$HPO$_4$.12H$_2$O, NaH$_2$PO$_4$.H$_2$O, KCl, acetone p.a (E. Merck), ethanol p.a (E. Merck), Gold wire, bronze wire, platinum wire, silver wire, tin wire, silica gel, parafilm and distilled water.

2.2. Procedure

Preparation of the phosphate buffer solutions of NaH$_2$PO$_4$. H$_2$O (A) and Na$_2$HPO$_4$. 12H$_2$O (B). Next, A and B were each made into phosphate buffer solution pH 8.0. A standard substrate solution of acetylthiocholine chloride was made in a solvent with phosphate buffer solution pH 8.0 at the concentration of 1 x 10-3 M. After that, diazinon pesticide solution 1 ppm -10-6 ppm was prepared by diazinon pesticide which then be diluted using ethanol in a flask. KCl 0.1 M solution was prepared by diluting 0.7445 gram of KCl in 100 ml quads. AChE enzyme from Electrophorus electricus Sigma 1.17 mg with the activity of 500 UN was diluted in a solvent with 9.0 ml buffer phosphate pH 8.0 and 1 ml KCl 0.1 M [8]. Cellulose acetate (CA) 15% were made by scaling 1.5 g CA, and then being diluted in 10 ml acetone. Glutaraldehyde solution (GA) 25% used in this study was produced from Aldrich-Sigma.

2.2.1. Design of Electrode Biosensor of Layered Wire

The design of biosensor based on acetylcholinesterase for analysis of diazinon is based on electrochemical analysis by using Potentiometer. Electrode biosensor consists of two electrodes, i.e. standard and working electrodes. Electrode of Ag/AgCl as the standard electrode is made through electrolysis of Ag wire as anode connected to positive pole of battery and platinum (Pt) wire as cathode connected to negative pole of the battery where silver (Ag) wire was sunk in a solution of KCl 0.1 M for 30 minutes.
The enzymatic biosensor or working electrode bodies were made from a 7 cm bronze wire of 0.5 cm in diameter which was connected with a 2.0 cm Gold (Au) wire of 0.4 mm in diameter, which was then soldered with tin (Sn) wire. It was then put into a blue tape with the Gold wire (Au) sticking out at 1.5 cm which was used as the working electrode bodies. At the bodies of all electrode were the parafilm coiled around as Cu and Au wire. The edge of the electrode, which was Au wire, was immersed in homogeneous cellulose acetate (CA). Working electrode of diazinon pesticide biosensor is made of layering membrane (CA) with a concentration of 15% in the surface of the gold wire. After the membrane electrode was formed, the electrodes were washed using a distilled water at 3 times. Next, the part of Au wire layered with membrane CA was immersed in glutaraldehyde 25% (GA) for 8 hours. After that, the electrodes were washed with phosphate buffer pH 8.0. This formed membrane electrode which was then immersed in phosphate buffer pH 8.0 containing acetylcholinesterase enzyme for 48 hours (enzymatic biosensor). The enzymatic biosensor or working electrode which had not been used remained immersed in the phosphate buffer solution pH 8.0 at the temperature of 4 °C [9].

2.2.2. Measurement of Range of Working Concentration, Sensitivity, Detection Limit and Response Time of the Diazinon Pesticide Biosensor.
The prepared biosensor was immersed into acetylthiocholine chloride (ATCh) substrate solution with the concentration of 10-3 M, and the initial potential was recorded. The potential of ATCh substrate with diazinon pesticide inhibitor was measured by way of immersing into varying concentration between 10-6 - 1 ppm of the diazinon pesticide solution for 30 minutes, then immersed into ATCh solution, and the potential response values were finally measured under the optimal measurement conditions. Determining the response time is done in the substrate concentration of 10-3 M, i.e. when the measured potential is already constant. To determine response time potential of pesticides, analyte solutions are measured every interval of 1 minute until the constant value is gained.

3. Results And Discussion
The Working principle of the biosensor to analyze the diazinon pesticide is based on the enzyme of AChE. The biological component (enzyme) was immobilized in a matrix of polymer membrane of CA which was connected to the gold wire integrated with transducer signal of Potentiometer. Component of AChE enzyme in the membrane of working electrode of biosensor functions as an electroactive sensor. The potentiometric method is based on electricity of a solution substance analyzed in an electrochemical cell. The potentiometric method used in working electrode in the type of layered wire. The use of working electrode of layered wire to detect diazinon pesticide is because of having some advantages, i.e. quick analysis process, simple, usable for routine analysis, and having a small size so it is easier to carry. Work of biosensor is very influenced from the thickness of membrane as supporting material in working electrode of the biosensor. The process of making membrane is conducted by using phase inversion method of instantaneous liquid-liquid demixing, i.e. change of polymer form from liquid to solid by removing solvent using a solvent having a different character. In this research, the solvent of working electrode of biosensor that has been layered by a membrane of cellulose acetate is then removed by using a distilled water and enzyme of AChE with the cross-linking method using 25% of glutaraldehyde is immobilized. Glutaraldehyde can form a bound connecting enzyme and cellulose acetate 15% as supporting material. The success of work of pesticide biosensor depends not only on the potentiometric transducer and membrane as supporting material but also on the existence of enzyme immobilized in electrode membrane of biosensor functioning as substrate catalysator being a product measurable in potentiometer transducer in the form of potential signal.
3.1. Performance of Diazinon Pesticide Biosensor

3.1.1. Range of Working Concentration, Sensitivity and Detection Limit.

Work test of pesticide biosensor is based on the mechanism of inhibiting diazinon pesticide towards enzyme of AChE in the hydrolyzing substrate of acetylthiocholine into thiocholine and acetic acid as shown in Figure 1.

$$2(CH_3)_3N^+CH_2CH_2SCCH_3 + H_2O \xrightarrow{AChE} 2(CH_3)_3N^+CH_2CH_2SH + CH_3COOH$$

Acetylthiocholine $\xrightarrow{\text{O}}$ Thiocholine $\xrightarrow{-2e^-, -2H^+}$ Acetic acid

$$(CH_3)_3N^+CH_2CH_2S-SCH_2CH_2N^+(CH_3)_3$$

dithiobischoline

Figure 1. Hydrolysis reaction of acetylthiocholine substrate with AChE enzyme.

Interaction happening between acetylthiocholine substrate and AChE enzyme in the reaction of hydrolysis produces thiocholine and acetic acid (Figure 1), with inhibition from diazinon pesticide so it causes phosphorylation i.e. insertion of the phosphate group in serine amino acid inactive side of enzyme which can decrease the working activity of AChE enzyme in hydrolyzing substrate [10]. AChE catalyzes the cleavage of acetylthiocholine chloride substrate to thiocholine, which was oxidized to give a dithiobischoline compound. All the experiments were carried out in phosphate buffer solution at pH 8.0 at room temperature. The response of the biosensor showed a good linearity range with an incubation time of 30 min for diazinon (Table 1). Work test of diazinon pesticide biosensor conducted covers: range of working concentration, sensitivity, and detection limit.

Table 1. The range of working concentration and Nernst factor (sensitivity) and detection limit of the biosensor of diazinon pesticide for the composition of electrode membrane CA 15%, GA 25%.

| Substrate concentration [ATCh] M | Inhibitor concentration [diazinon] ppm | Potential (mV) |
|---------------------------------|---------------------------------------|---------------|
| $1 \times 10^{-3}$              | 1                                     | 30.7          |
| $10^{-3}$                       | $10^1$                                 | 42.1          |
| $10^{-3}$                       | $10^2$                                 | 58.1          |
| $10^{-3}$                       | $10^3$                                 | 78.6          |
| $10^{-3}$                       | $10^4$                                 | 109.1         |
| $10^{-3}$                       | $10^5$                                 | 137.4         |
| $10^{-3}$                       | $10^6$                                 | 139.4         |

Nernst factor (sensitivity) 20.275 mV
$R^2$ (coefficient of correlation) 0.9722
Range of working concentration 10$^{-6}$ - 1 ppm
The value of sensitivity can be seen from Nernst factor value of 29.6 mV/decade in temperature of 24 °C ± 1 °C. It happened because oxidation of acetylthiocholine involved two electrons. Figure 2 shows the curve of potential towards –log [diazinon] ppm for membrane composition of CA 15% towards GA 25% in substrate concentration of 10-3 M and value of Nernst factor as well as measurement range. Electrode of the biosensor with the membrane of CA 15% and GA 25% has measurement range in diazinon pesticide concentration of 10-6 ppm to 1 ppm. Based on the obtained data, it shows that value of regression approaches 1, i.e. 0.9722 and value of Nernst factor (sensitivity) is 20.275 mV/decade.

The detection limit is the smallest concentration of an analyte that is still able to be determined or detected by a biosensor after inhibition by the pesticide. Detection limit shows that the smallest concentration that still gives linear in the range of working concentration in this research is 10-6 ppm.

3.1.2. Response Time.
Pesticide biosensor is an enzyme-containing biosensor immobilized on the surface of the electrode that is able to give a specific response to the substrate [11]. The working principal of enzyme-based biosensor that was produced from immobilization of biological component (enzyme, bacteria, etc) on matrix membrane polymer integrated with transducer signal in the analyte. The biological components on the membrane of working electrode of biosensor function as an electroactive sensor. Response time is the time needed by a biosensor electrode to reach the constant potential. Determining the response time is done in the substrate concentration of 10-3 M, i.e. when the measured potential is already constant. To determine response time potential of pesticides, analyte solutions are measured every interval of 1 minute until the constant value is gained. The faster the time needed to reach the constant value, the better the quality of biosensor electrode.
Table 2. The response time of biosensor of diazinon pesticide for the composition of electrode membrane CA 15%, GA 25%.

| Minute | 1 ppm | $10^1$ ppm | $10^2$ ppm | $10^3$ ppm | $10^4$ ppm | $10^5$ ppm | $10^6$ ppm |
|--------|-------|------------|------------|------------|------------|------------|------------|
| 1      | 39.7  | 48.7       | 64.5       | 83.4       | 109.3      | 144.6      | 146        |
| 2      | 38.4  | 46.7       | 63.2       | 81         | 108.9      | 143.4      | 144.9      |
| 3      | 37.2  | 45.5       | 61.9       | 80.3       | 107.9      | 142.5      | 143.6      |
| 4      | 36.5  | 44.5       | 60.7       | 79.1       | 107.6      | 141.7      | 142.2      |
| 5      | 34.9  | 43.9       | 59.6       | 78.1       | 107.9      | 140.2      | 141.4      |
| 6      | 33.1  | 43.3       | 58.7       | 77.9       | 108.1      | 139.6      | 140.4      |
| 7      | 32    | 42.8       | 58.2       | 78.2       | 108.3      | 138.1      | 139.7      |
| 8      | 31.2  | 42.5       | 58.1       | 78.5       | 108.7      | 137.7      | 139.5      |
| 9      | 30.9  | 42.1       | 58.3       | 78.7       | 109.1      | 137.4      | 139.4      |
| 10     | 30.7  | 42.2       | 58.2       | 78.6       | 109.1      | 137.4      | 139.4      |

Figure 3. The curve of the response time of biosensor in membrane composition of CA 15% and GA 25%.

The value of response time is determined from the duration of every electrode to reach a constant potential in analyzing pesticide in a variation of diazinon pesticide concentration, each has $10^{-6}$ ppm, $10^{-5}$ ppm, $10^{-4}$ ppm, $10^{-3}$ ppm, $10^{-2}$ ppm, $10^{-1}$ ppm dan 1 ppm. The faster the time needed to reach the constant value, the better the quality of biosensor electrode. The analysis result of response time value for biosensor electrode membrane composition is CA 15% and GA 25% is shown in table 2 and figure 3. It shows about 5 to 10 minutes to produce constant electrode potential value in analyzing diazinon pesticide.

4. Conclusion

The biosensor of diazinon pesticide was developed by using gold electrode in the type of layered wire with immobilization of acetylcholinesterase (AChE) enzyme in the membrane of
cellulose acetate (CA) 15% and glutaraldehyde (GA) 25% used as working electrode and Ag/AgCl as a standard electrode. The research result shows that biosensor of diazinon pesticide gives optimal work in the analysis of diazinon pesticide using acetylthiocholine chloride substrate 10-3 M and potentiometer transducer with a range of working concentration of 10-6 ppm to 1 ppm and sensitivity of 20.275 mV/decade. The limit of detection value was found to be 10-6 ppm. The response time is the time needed by a biosensor electrode to reach the constant potential. The response time of electrode is on the measurement of pesticide diazinon with concentration variation of 10-6 ppm to 1 ppm is about 5 minutes.

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