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A novel thermal biosensor based on enzyme reaction for pesticides measurement

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Abstract: A novel thermal biosensor based on enzyme reaction for pesticides detection has been developed. This biosensor is a flow injection analysis system and consists of two channels with enzyme reaction column and identical reference column, which is set for eliminating the unspecific heat. The enzyme reaction takes place in the enzyme reaction column at a constant temperature(40℃) realized by a thermoelectric thermostat. Thermosensor based on the thermoelectric module containing 127 serial BiTe-thermocouples is used to monitor the temperature difference between two effluents from enzyme reaction column and reference column. The ability of this biosensor to detect pesticides is demonstrated by the decreased degree of the hydrolytic heat in two types of thermosensor mode. The hydrolytic reaction is inhibited by 36% at 1 mg/L DDVP and 50 % at 10 mg/L DDVP when cell-typed thermosensor is used. The percent inhibition is 30% at 1 mg/L DDVP and 42% at 10 mg/L DDVP in tube-typed thermosensor mode. The detection for real sample shows that this biosensor can be used for detection of organophosphate pesticides residue.

Keywords: thermal biosensor; pesticides residue; thermoelectric module; flow injection analysis(FIA)

Introduction

Organophosphate pesticides (dichlorvos, dimethoate, parathion) have acute toxicity and can cause serious diseases and even death, which has increased public concerns (Donarski, 1989; Chepalamadugu, 1992). At present they are usually determined by laboratorial methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Leon-Gonzalez, 1991; Pylypiw, 1993). Although these methods have very high sensitivity and accuracy, they suffer from many disadvantages such as skillful technicians, high cost and time consuming, and their application for on-line monitoring is impractical. Clearly, the simple, rapid and inexpensive methods to detect pesticides are essential for on-site determining the source of pollutant and the magnitude of the threat(El-Yamani, 1998; Galgani, 1989).

Biosensors may provide the solutions of pesticides measurement (Marty, 1995). Several kinds of biosensors based on electrochemical, optical detection and other methods for this objective have been reported (Bernabei, 1991; Hoebei, 1992; Abad, 1998). Although such methods are sensitive and selective, their applications in real samples are limited because of the interferences from electroactive species, ions and turbidity of samples. In addition, some transducers, such as electrodes, require frequent recalibration. Therefore thermal biosensors are good alternatives to these devices. They are insensitive to the optical, electrochemical and other inherent properties of the real samples, and temperature measurements are very robust technique, which overall makes the system very easy to use.

Thermal biosensors are based on the measurement of heat produced by biochemical reaction(Ramanathan, 2001). Two approaches for measuring heat have been developed. In one approach, a thermistor is used to register temperature changes in the effluent flow from where an enzymatic reaction has taken place. The early studies using thermistors to determine heat were done around 1974 with the development of the so-called "enzyme thermistor"(ET)(Mosbach, 1974). Later miniaturized ETs in different versions have been reported(Danielsson, 1996). The other approach is to use a thermopile whose response is determined by the Seebeck effect. Compared with thermistor, the thermopile does not need amplification of electrical signals and has low noise, the great number of thermocouples in a thermopile is regarded to be a very valuable supposition to achieve a sufficient sensitivity. Thermopile was firstly used in conventional microcalorimeters whose high thermal inertia makes them unsuitable for rapid routine analysis. To overcome these problems some simple system were made in which enzyme was immobilized in a membrane on the surface of a thermopile, whereas the detection efficiency of enzyme membrane need to be enhanced(Xie, 1992). Other micro mechanical systems are complex and costly(Xie, 1995).

In this study, a novel thermal biosensor was designed and fabricated which employs thermoelectric (TE) module used for both thermosensor and thermostat of the thermal biosensor for its merits, including high sensitivity, low-cost and simple operating principle. An inherent disadvantage of calorimetry is the lack of specificity, which is mostly avoided by linking specific enzyme reaction. Other factors, such as pH, ionic strength and mixing of sample, may contribute unspecific effects. So, reference column is arranged for eliminating unspecific effects in system. The classical split-flow enzyme thermistor in which the solution flow is splited two identical channels by three-way pipe has been reported (Mattiasson, 1976). In our practice, the simple three-way pipe can not produce perfectly equal reagents to two channels even same flux. For optimizing the apparatus, two identical channels without three-way pipe are applied.

Enzyme is typically regarded as a biological receptor of biosensor for high selectivity and more species. Organophosphate pesticides detection by acetylcholinesterase (AChE) and organophosphrous hydrolase (OPH) hydrolization has been widely investigated(Boublik, 2002). However, their source is both hard to be gained and high in price. So the chicken liver-esterase is selected in our laboratory as the bio-recognition component for the attributes of easily got, low price and high sensitivity(Zhang, 2004).

The aim of this research is to develop a rapid, simple and sensitive calorimetric method for measurement of organophosphate pesticides. A thermal biosensor involving an

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immobilized enzyme and analysis performed by a flow injection technique are presented. Pesticides would inhibit the enzyme reaction, which produces a reduced level of hydrolysis heat. Therefore, the pesticides concentration can be measured from the thermal signals.

1 Description of the instrument

Fig.1 shows schematically the thermal biosensor based on FIA and the data acquisition unit (Hua, 2002; Zhao, 2004). A six-ports valve as an injector ensures the switch of reagents at same time into two channels. The peristaltic pump is used to push the solution through the two channels at constant flux rate. A thermoelectric thermostat can offer constant temperature for enzyme reaction. The reference column is set to eliminate unspecific heat inactivated immobilized, which are not produced by the enzymic reaction. The reference column and the enzyme reaction column are identical, but the reference column is full of enzyme inactivated immobilized, which is perished purposefully. The hydrolytic reaction takes places in the enzyme reaction column, but not in the reference column, when substrate is introduced to system. The thermosensor, which are used to monitor the temperature difference of effluents from enzyme reaction column and reference column, is connected with the outlets of the two columns and is coupled to A/D converter prior to the personal computer. The sampled data are analyzed in estimation of peak parameters by using an integration program written in Visual Basic 6.0.

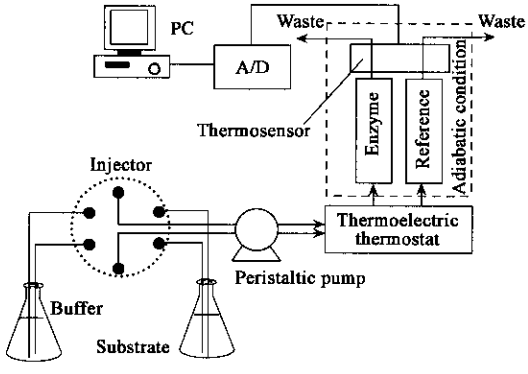

Fig.1 The schematic diagram of the thermal biosensor

Two types of thermosensor based on Thermoelectric (TE) module(CP1.4-127-06L), which have the sizes of 40 mm × 40 mm × 4 mm and consist of 127 serial Bi/Te thermocouples, were used in this research. Fig.2 gives the basic construction of a thermosensor which has two identical cells(35 mm × 35 mm × 1 mm) formed with a polymethyl methacrylate shell and a TE module. One of cells connects with the enzyme reaction column, and the other connects with the reference column. The tested fluxes are pushed through the two cells at upstream mode. The polymethyl methacrylate shell can eliminate the heat losses of the reaction solution. This cell-typed thermosensor has the sensitivity of 1.8 mV/K in real detection condition. The other thermosensor, which is consist of two identical copper tubes (ID = 2 mm, L = 10 mm) and a TE module, is showed at Fig.3. The copper tubes with elliptical shape have jointed with the copper plates, which contact with the surfaces of TE module. They are imbedded in foam for thermal shield. This thermosensor, called tube-typed thermosensor, has response of 2.6 mV/K in real detection condition. The thermal contacts of both the two thermosensor have been enhanced by heat-conduction grease.

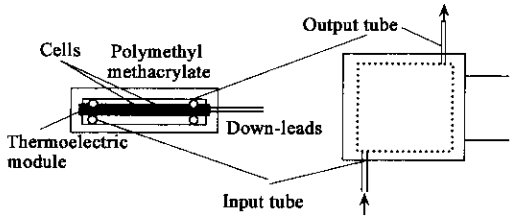

Fig.2 The basic construction of the cell-typed thermosensor

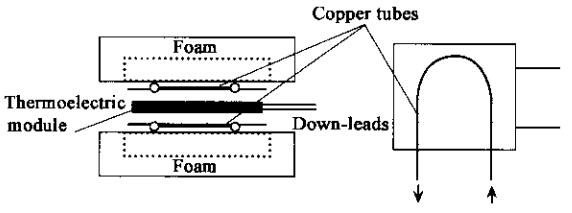

Fig.3 The basic construction of the tube-typed thermosensor

Temperature is a crucial factor in enzyme reaction. The reaction fluxes with constant temperature are essential in established biosensor. A thermoelectric thermostat, which has rapid response, simple operating and small volume, is used here for maintaining the two identical fluxs of 1 ml/min at set temperature from 0℃ to 70℃, and the temperature fluctuation is no more than ±0.1 K.

2 Principles of measurement

Thermometric measurement deals with the heat produced during a biochemical reaction (Bataillard, 1993). The total heat is proportional to the molar enthalpy change created in the biochemical reaction and to the total number of product moles (n_p). If the reaction is proceeded under the nearly adiabatic condition, the temperature change (ΔT) recorded by the thermosensor is directly proportional to the enthalpy change (ΔH) and inversely proportional to the heat capacity (C_p) of the system in which the reaction takes place.

$$\Delta T = \frac{-\Delta H n_p}{C_p}. \quad (1)$$

The TE module, as thermoelectric refrigerator and heater complex traditionally, has function of thermopile and is used as thermosensor of thermal biosensor in this research. The potential difference (ΔV) of TE module depends on the number of couples (n), the temperature difference (ΔT) and the Seebeck coefficient (ε). It is expressed by

$$\Delta V = n\varepsilon\Delta T, \quad (2)$$

$$\Delta V = n\varepsilon\frac{-\Delta H n_p}{C_p}. \quad (3)$$

Therefore, the enthalpy change (ΔH) created by hydrolysis and other reactions in thermal biosensor can lead to a direct (proportional) change in potential (ΔV) for the nearly adiabatic environment.

The flow injection analysis (FIA) technique is normally used in assay (Ruzicka, 1988). In most instances, the area under the peak and the ascending slope of the peak vary in the same way linearly with the substrate concentration (Kiba, 1984). The peak height of the thermometric recording is proportional to the enthalpy change corresponding to a specific hydrolysis heat. When the activity of the enzyme is completely or partly inhibited by the pesticide, the enzyme

reaction may be completely or partly restricted and the hydrolysis heat may correspondingly decrease. Therefore the inhibited degree of the enzyme can be measured by the decreased degree of the hydrolysis heat. So, pesticides concentration is measured after a certain incubation time based on the peak height difference.

3 Experimental

3.1 Reagents

Dichlorvos (DDVP), α-naphthyl acetate, acetone, sodium citrate, citric acid, fast blue B salt were analytic grade from Sigma Company. D113 resin was supplied by Shanghai Yadonghe Resin Company. Fresh chicken liver was from Shanghai Dajiang Food Company. Bidistilled water was used throughout for the preparation of solutions.

3.2 Chicken liver-esterase immobilization

Fresh chicken liver 30.0 g was placed in 100 ml of citric acid buffer(0.025 mol/L, pH 6.4). The mixture was homogenized with a tissue triturator. Then the extracts were centrifuged for 15 min at 5000 r/min with a centrifuge, and enzyme solution was obtained from the final supernatant. Enzyme solution was diluted up to 7 ± 0.5 U/ml. Resin with diameter of about 0.5 mm, which has been pretreated by acid and alkali, was mixed with diluted enzyme solution in conical flask with magnetic force stirrer for 2 h.

3.3 Assay preparation

The method from literature (Asperen, 1962) was adopted to detect activity of immobilized chicken liver-esterase by using spectrophotometer. The immobilized chicken liver-esterase was slurry-packed in enzyme reaction column. The reference column was full of the same batch of enzyme, but it was inhibited by abundant DDVP. The two columns should be replaced fro the next test.

4 Results and discussion

4.1 Performance of thermostat

When the buffer flow through the system at 1 ml/min, the experiment of thermostat has been processed. The refrigeration test and the heating test have been both performed. Fig. 4 gives the effects of the temperature constancy. during refrigeration, the system can cool the fluxes from 70℃ to 0℃ within 3 min; during heating, it is only 1.5 min for the fluxes from 0℃ to 70℃. When the temperature of the thermostat is stable, the temperature curve in the thermostat is almost a straight line and the temperature fluctuation has a scope of ± 0.1 K. Accordingly, the thermostat is fast response and good temperature constancy.

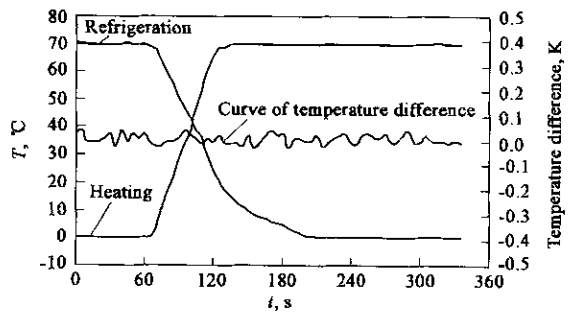

Fig.4 The performance test of the thermostat

After the temperature was stabilized at 40℃, the temperature difference between the two fluxes from the thermoelectric thermostat has been measured. As shown in Fig. 4, it is within 0.05 K. Which confirms the same temperature between the two fluxes and excludes the possibility of temperature noise effecting enzyme reaction.

4.2 Optimization of the system

The optimization of the system was performed by changing several parameters, such as the flux rate, size of columns and operational temperature, and so on. After stable base line was obtained, α-naphthylacetate acetate substrate solution was introduced in enzyme reaction column and reference column synchronously by switching the six-ports valve. The potential signals of thermosensor were recorded.

4.2.1 Operational temperature

Temperature is one of the most important operational parameter of biosensor and can affect the reaction rate acutely. The temperature of 30℃ or 37℃ is usually recommended for the assay of enzyme (Roth, 1972). The temperature of 40℃ is selected for chicken-liver esterase to achieve max reaction rate and min heat damage.

4.2.2 Diameter of columns

In general, the dispersion degree increase with the increasing diameter of enzyme reaction column and reference column, which result in low response signal. Simultaneously, the sufficient volumes ensure enough enzymes for reaction. The diameter of 8 mm is selected based on the experience (Satich, 1992).

4.2.3 Length of columns

Fig.5 shows that the response signal is maximum when the length of column is 80 mm. The result is brought by two trends, with increasing length of column, firstly, the response signal increase with more enzyme, secondly, the response signal decrease with increased dispersion degree.

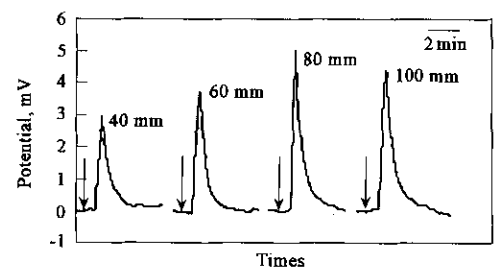

Fig.5 The response of different length of columns
With tube-typed thermosensor, 1 ml subsrate, enzyme acitivity is 0.81 U/g, flux is 1.0 ml/min, 40℃, arrow show the place of substrate introduced

4.2.4 Volume of substrate

Fig. 6 shows the response signal increase with the increasing volume of substrate, but the peak width of response signal is also improved, which is time consuming.

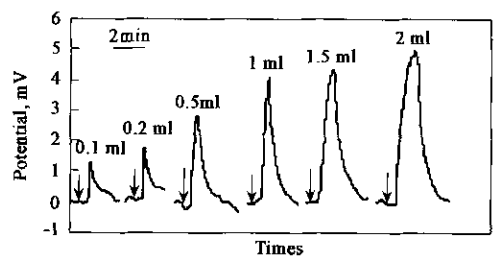

Fig.6 The response of different volume of substrate
With tube-typed thermosensor, φ8 mm × 80 mm columns, enzyme acitivity is 0.8 U/g, flux is 1.0 ml/min, 40℃, arrow show the place of substrate introduced

In addition, the peak shape deflects Gaussian distribution at high volume, which is not suitable for evaluation by integration program. So, the volume of 1 ml is optimum.

4.2.5 Flux rate

The relation between response signal and flux rate can be seen in Fig.7. The improving of flux rate can increase the response signal and decrease the width of peak, but a slight signal increase occurs at high flux. Considering reagent consuming at high flux, the optimum flux of is 1 ml/min.

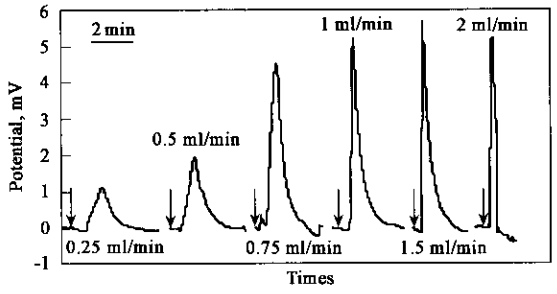

Fig.7 The response of different flux rate
With tube-typed thermosensor, φ8 mm × 80 mm columns, 1 ml subsrate, enzyme acitivity is 0.83 U/g, 40℃, arrow show the place of substrate introduced

4.3 Experiment of the system with reference column

Unspecific thermal signals may also occur when a continuous flux passing through the enzyme column. This problem is overcome by the use of the identical reference column containing inactive enzyme. Acetone, as solvent of substrate, can generate unspecific heat and is selected for this experiment.

The performance is seen from Fig.8 that buffer flow at 40℃ was pushed through two channels of system and the flux was tuned to 1.0 ml/min. After stable base line was obtained, acetone solution was introduced in a channel with enzyme reaction column at no reference mode and in two channels synchronously at reference mode for 1 min by switching the six-ports valve. The potential signals of thermosensor were collected in whole course.

As shown in Fig.8, almost no unspecific thermal heat was registered by the system in the reference mode. The system involving reference column can eliminate the unspecific effects availably. This result is valuable in analysis of crude solutions contained complex compound in particular in the on-site detection. It can also be concluded that reference column makes the system more stable toward noise of surrounding.

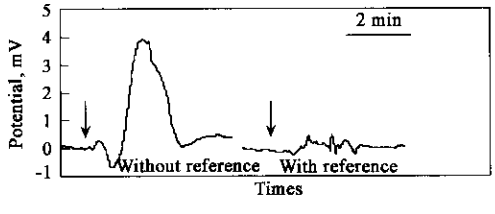

Fig.8 The response curves of acetone solution
With cell-typed thermosensor, 1 ml acetone, φ8 mm × 80 mm columns, enzyme activity is 0.8 U/g, flux is 1.0 ml/min, 40℃, arrow show the place of acetone introduced

4.4 Experiment of pesticide detection

The buffer contained concentration-known DDVP was used in the detection for stimulating the real sample. The thermostat temperature was stabilized at 40℃ and the flux was 1.0 ml/min. After stable base line was achieved, the pesticide detection was made in two steps: in the first step no pesticide used, the 1 ml α-naphthylacetate acetate substrate solution was introduced in system by switching six-ports valve for 1 min, the enzyme reaction signal was obtained. In the second step, the enzyme column was incubated with buffer containing concentration-known DDVP fro 10 min. Then, the enzyme reaction signal with inhibition was obtained by repeating first step. The degree of inhibition was calculated from the peak heights of signals with and without DDVP by using the Equation (4).

$$I = \frac{\Delta V_1 - \Delta V_2}{\Delta V_1} \times 100\% , \qquad (4)$$

where I is the degree of inhibition, ΔV_1 is the thermoelectric potential change in the case without inhibition and ΔV_2 is that in the case with inhibition.

4.4.1 Cell-typed thermosensor

Fig.9 gives the output curves of detection for DDVP by using cell-typed thermosensor. Enzyme inhibition can be evaluated by a visual observation of peak height of the none-pesticide-inhibited and pesticide-inhibited reaction.

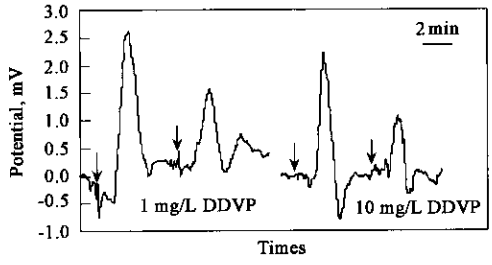

Fig. 9 The response curves of pesticide detection using cell-typed thermosensor
With 1 ml substrate, incubation time is 10 min, enzyme activity is 0.78 U/g, flux is 1.0 ml/min, arrow show the place of substrate introduced

The thermoelectric potential signals are about 2.5 mV without DDVP and 1.6 mV with DDVP at 1 mg/L from Fig. 9. Thus

$$I_{1\,mg/L} = \frac{\Delta V_1 - \Delta V_2}{\Delta V_1} \times 100\% = \frac{2.5 - 1.6}{2.5} \times 100\% = 36\% .$$

There are the total thermoelectric potential signals of 2.618 mV without DDVP and 1.31 mV with DDVP at 10 mg/L from Fig.9. Thus

$$I_{10\,mg/L} = \frac{\Delta V_1 - \Delta V_2}{\Delta V_1} \times 100\% = \frac{2.618 - 1.31}{2.618} \times 100\% = 50\% .$$

4.4.2 Tube-typed thermosensor

The output curves of detection for DDVP using tube-typed thermosensor are shown at Fig. 10. Similarly, the degrees of inhibition are obtained at 1 mg/L and 10mg/L DDVP. Thus

$$I'_{1\,mg/L} = \frac{\Delta V_1 - \Delta V_2}{\Delta V_1} \times 100\% = \frac{5.358 - 3.76}{5.358} \times 100\% = 30\% ,$$

$$I'_{10mg/L} = \frac{\Delta V_1 - \Delta V_2}{\Delta V_1} \times 100\% = \frac{5.183 - 3.014}{5.183} \times 100\% = 42\% .$$

When the enzyme is inhibited by DDVP, the reduced signal can be observed from Fig. 9 and Fig. 10 clearly. Hence, by comparing the signal differences produced by variously inhibited enzyme, the pesticide concentration can be checked. Although there are different values between cell-typed thermosensor and tube-typed thermosensor, the trend of reduced signal with inhibition is consistent. Which is likely due to some factors, such as manufacture, calculating method of peak height and so on.

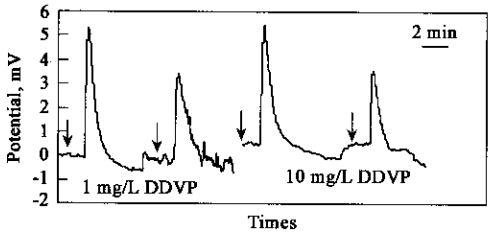

Fig. 10 The response curves of pesticide detection using tube-typed thermosensor
With 1 ml substrate, incubation time is 10 min, enzyme activity is 0.83 U/g, flux is 1.0 ml/min, arrow show the place of substrate introduced

The experiment of pesticide detection can be proceeded down to 0.5 mg/L DDVP by inhibition degree of 5%. So, 0.5 mg/L can be considered as detection limit of this biosensor for DDVP.

4.5 Pesticide detection for real samples

It is proved that this biosensor can be used to detect the pesticides residue in real samples by detection of DDVP for greengrocery.

The greengrocery, which has been detected by HPLC and has not pesticides residue firstly, is sprayed DDVP with concentration of 0.5 mg/kg. Then greengrocery with extracting reagent (acetone) is broken into slurry. So, the sample solution is obtained after filtration, and the DDVP detection in sample solution is performed as above mentioned. Finally, the result can be obtained at 0.43 mg/kg from calibration curve, which express the relations between inhibition degree and concentration of DDVP. The departure is resulted from the accuracy of detection and the recovery rate of extracting reagent.

It is universal in China now that food acute poisoning caused by mass dosage pesticides. Urgent needs for food safety focus on rapid on-site detection for pesticides. A portable system based on the developed thermal biosensor, which can achieve a detection-cycle within 30 min, has been developing for application in market.

5 Conclusions

A flow injection thermal biosensor for pesticide residue detection has been developed. Two types of thermosensor based on Thermoelectric Module have been performed which are cell-typed and tube-typed. For the cell-typed thermosensor, the degree of inhibition is 36% at 1 mg/L DDVP and 50% at 10 mg/L DDVP respectively. For the tube-typed thermosensor, the percent inhibition is 30% at 1 mg/L DDVP and 42% at 10 mg/L DDVP respectively. The DDVP detection for greengrocery has been also accomplished, and this biosensor shows the result of 0.43 mg/kg at 0.5 mg/kg DDVP in greengrocery.

The experiments show that the inhibition degree of pesticide on enzyme can be checked by the developed biosensor. It proves the feasibility of the use of this thermal biosensor in rapid detection of mass dosage pesticide residues. This biosensor is suitable for protection from acute toxicity.

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