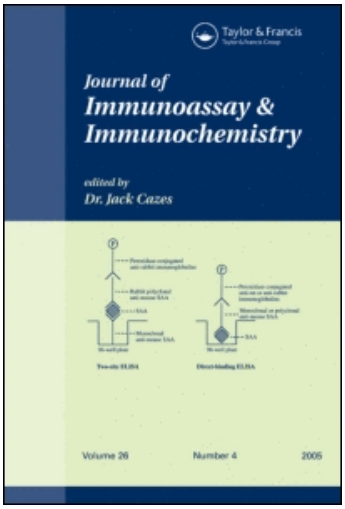

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Development of Fluorescence Polarization Immunoassay for the Detection of Organophosphorus Pesticides Parathion and Azinphos-Methyl

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Development of Fluorescence Polarization Immunoassay for the Detection of Organophosphorus Pesticides Parathion and Azinphos-Methyl

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Abstract: Organophosphorus Pesticides (OPPs) are a group of artificially synthesized substances used in farms to control pests and to enhance agricultural production. Although these compounds show preferential toxicity to insects, they are also toxic to humans and mammals by the same mode of action. ELISA now is an alternative method to detect OPPs. But, it must bear heterogenous properties, since several separation steps are needed during the ELISA method protocols. The FPIAs, which belong to homogenous assay, for determination of OPPs parathion and zainphos-methyl have been developed. The characteristics of Dep-EDF and PM-B-EDF tracers binding with antibodies A and D were investigated in the antibodies dilution experiments. The PM-B-EDF tracer combination with antibody D was selected to construct the standard curve for parathion detection. The IC_{50} value and the detection of limit were 1.96 mg/L and 0.179 mg/L, respectively, as shown in the standard curve. The tracers of PBM-EDF 2 and 3, which were chased from 4 PBM-EDF tracers, exhibited the good standard curves based on the MAb AZI-110. The FPIA constructed to analyze the azinphos-methyl showed the IC_{50} 1.003 mg/L and detection limit 0.955 mg/L when PBM-EDF 2 was employed and the IC_{50} 0.1487 mg/L and detection limit 0.150 mg/L were obtained when PBM-EDF 3 was used.

Keywords: Azinpjos-methyl, Fluorescence polarization immunoassay, Parathion

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INTRODUCTION

Organophosphorus pesticides (OPPs) have been extensively used for agricultural purposes for the last few decades. They provide perfect and cost-efficient treatments to prevent, repel, or mitigate the adverse effects caused by pests on a wide range of crops.

As a consequence, they are one of the major organic pollutants found in environmental matrices and foodstuff, making a human and animal health hazard. Therefore, it is necessary to monitor their residues regularly through some analytical methods.

Conventional methods for OPPs detection are generally based on chromatographic procedures such as gas chromatography (GC)[1] liquid chromatography (LC),[2] and electrophoretic methods,[3] which need sample pre-treatment and concentration, with a sensitive detector such as mass spectrometry (MS).[4,5] Presently, many methods are used in extraction, cleanup, and concentration steps within the sample pre-treatment in order to isolate OPPs and their degradation products from different environmental matrices effectively. The methods involve solid-phase extraction (SPE),[6] solid-phase micro extraction (SPME),[7] and microwave-assisted micellar extraction.[8]

Compared with these instrumental techniques, which are expensive, time-consuming, and complicated to perform, immunoassays provide a simple and economical alternative for OPs analysis. Immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), are gaining wide acceptance for the routine use for analysis because of the sensitivity, specificity, rapidity, simplicity, and cost effectiveness. It was reported that many pesticides such as isofenphos,[9] diazinon,[10] fenthion,[11] parathion-methyl,[12] acephate,[13] and bromophos-ethyl[14] have been analyzed successfully by ELISA.

However, ELISA is a heterogeneous method. It must separate free and antibody-bound analyte. In addition, it suffers from long reaction times (more than two hours), because this method involves multiple washing steps. A very promising way for the simplification of immunoassays for routine applications is a shift from heterogeneous methods (with separation) to homogeneous assays (without separation). Fluorescence polarization immunoassay (FPIA) is the most extensively used homogeneous technique, which meets the requirements of a simple, reliable, fast, and cost-effective analysis.[15]

In brief, the principles of FPIA are described as below. FPIA is a competitive immunoassay method based on the increase in the polarization of the fluorescence of a small fluorescent-labeled hapten (tracer) when it is bound by a specific antibody. If the sample contains unlabeled analyte, the tracer will compete for binding with the antibody and the

polarization signal will fall. The FPIA method is a direct assay without any sample pre-treatment or washing steps.[16]

The FPIA for pesticides was focused on herbicides such as atrazine, simazine,[17] 2,4-D,[18] 2,4,5-T,[19] chlorsulfuron,[16] acetochlor,[20] and paraquat.[21] In recent years, FPIAs have been developed scantily for detection OPs pesticides. A paper which was published in 2003 reported that parathion-methyl has been detected using FPIA by Eremin.[22] The aim of this work was to develop FPIA methods with simple and rapid characteristics to detect parathion and azinphos-methyl.

EXPERIMENTAL

Reagents and Equipment

Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), fluorescein isothiocyanate isomer I (FITC), ethylenediamine, N-hydroxysuccinimide (NHS), and N,N′-Dicyclohexyl carbodiimide (DCC) were obtained from Sigma (Poole, Dorset, UK). Pesticide parathion was purchased from the Institute of Environmental Protection, Ministry of Agriculture (Tianjin, China). Parathion tracer PM-B-EDF, azinophos-methyl tracer MBP-EDF $1 \sim 4$, and azinophos-methyl monoclonal antibody Azi-110 were kindly supplied by Professor S. A. Eremin. All chemicals and organic solvents were of analytical grade or higher. Water used in all experiments was purified with a NANOpure system (Barnstead International, Dubuque, IA, USA). Sodium borate buffer (BB, 0.05 m, pH 9.0) was used for all FPIA experiments. TLC plates (Silica gel 60, fluorescent, 1 mm, 20 cm × 20 cm) were obtained from Merck Co. (Darmstadt, Germany).

Fluoresceinthiocarbamyl ethylenediamine (EDF) was synthesized from FITC and ethylenediamine as described. Measurements of fluorescence polarization and intensity were performed using TDx/FLx Analyzer (Abbott Laboratories, Irving, TX, USA) in semiautomatic PhotoCheck mode. TDx/FLx glass cuvettes (up to ten in one run) were loaded into the special carousel, followed by the measurement of polarization (in mP units) and intensity (in conventional units) of fluorescence. The total time for measurement of ten samples was about 7 min.

Synthesis of Immunogen

The compound, diethyl (carboxymethyl) phosphonate (Dep), was selected as hapten of OPPs. Dep can be readily bought from reagent companies, so it is unnecessary to synthesize the OPPs hapten in the laboratory. It was conjugated to bovine serum albumin (BSA) to be used as the immunogen for production of polyclonal antibodies.

Hapten (0.32 mg, 1.63 mmol) was dissolved in 2 mL 1,4-dioxane to which N-hydroxysuccinimide (0.7 g, 6.08 mmol) dissolved in 1 mL 1,4-dioxane of followed by N,N-dicyclohexylcarbodiimide (0.41 g, 2 mmol) dissolved in 1 mL of 1,4-dioxane were added. After stirring for 5 to 6 hours, the mixture was filtered to remove dicyclohexylurea.

0.2 g carrier protein BSA was carried out in 5 mL borate buffer (0.2 M, pH 7.0) and a little DMF was added to give a homogeneous solution. Then, the active ester solution described above was added dropwise to the stirred protein solution and stirring was continued for 4 hours at room temperature and then at 4°C overnight. The hapten–protein conjugates were separated from the uncoupled haptens by gel filtration using Sephadex G-25 and PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The eluates were dialyzed in PBS for several days. The conjugates were measured by UV-spectrophotometer to acquire the molar ratio between protein and hapten molecule. The ratios were 22 and 15 of immunogen and coating antigen, respectively.

Production of Polyclonal Antibody

Male New Zealand white rabbits were immunized with Hapten-BSA. Routinely, 2 mg of the conjugated hapten dissolved in 0.9% NaCl solution emulsified with Freund's complete adjuvant (1:1 volume ratio) was injected intradermally at multiple sites on the back of each rabbit. After three weeks, each animal was boosted with an additional 2 mg of the conjugate emulsified with Freund's incomplete adjuvant. After these experiments, each booster was continued at two weeks intervals and each bleeding was 8 days after boosting. Serum was isolated by centrifugation at 3,000 rpm for 30 min, and sodium azide was added as a preservative at a final concentration of 0.02%. Serum was then aliquotted and stored at -70°C.

Synthesis of EDF

Fluoresceinthiocarbamyl ethylenediamine was synthesized from fluorescein isothiocyanate isomer I (FITC) and ethylenediamine as described previously by Pourzaneh et al.[23] with modifications (Nistor).[24]

49.8 mg ethylenediamine dihydrochloride was dissolved in 12 mL methanol and 120 μL triethylamine. 31.8 mg FITC was dissolved in 2.5 mL methanol and 25 μL triethylamine to form a FITC solution; then, add this solution dropwise with stirring to the solution of ethylenediamine over a 30 min period. Stir the reaction mixture in the dark at RT for 1 h, keep overnight at RT in the dark. After that, the orange precipitate was filtered via Whatman filter paper, then washed with 2.5 mL of methanol and dried in air at T in the dark.

Synthesis of Fluorescent Tracers

EDF-labeled Dep conjugates (tracer) were synthesized by the NHS ester method. Briefly, 2.7 mg (0.235 μmol) NHS and 4.8 mg (0.233 μmol) DCC was dissolved in 0.5 mL DMF, stirred and mixed. 3.7 mg Dep was added to the above solution, mixed, and kept overnight at RT.

Add 1.4 mg EDF into the solution of activated Dep. The reaction mixture was stirred at RT for 3 h, and left overnight at 4°C. This became the real mixture of tracer Dep-EDF.

The real mixture of tracer Dep-EDF was purified by TLC with a silica gel plate. The eluent solvent was chloroform/methanol (4:1, v/v). Bands of different R_f values were collected, eluted with 0.5 mL of methanol, and stored in the dark.

In our experiments, four fluorescence-labeled tracers were synthesized by duplicate experiments using small molecule Dep and EDF. Figure 1 shows the chemical structure of Dep, Dep-BSA and Dep-EDF. The tracers were synthesized by coupling the carboxyl group of the OP pesticide mimic molecule Dep to the amine group of fluorescein to form an amide bond between Dep and fluorescein. The tracers were separated by TLC using a solvent ($CHCl_3$:CH_3OH, 4:1). The fluorescent bands of proper R_f value, 0.4 and 0.5, were scraped, extracted, and purified using CH_3OH.

Polarization Fluoroimmunoassay

Dilution Curves

Assessment of tracer binding antibody dilution curves were obtained by incubating different antibody concentrations (dilution of antibody ranged from 1/100 to 1/51,200) with an appropriate amount of Dep-tracers

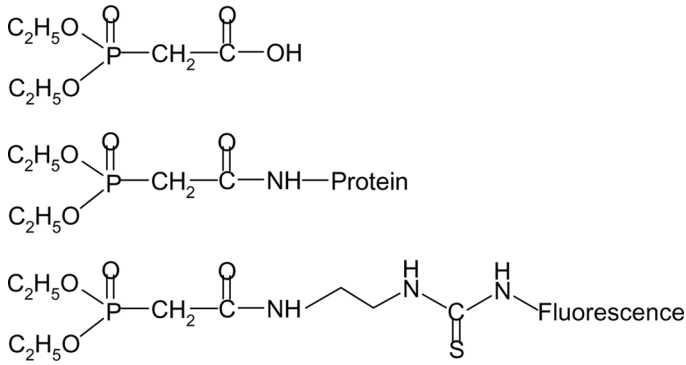

Figure 1. Structures of Dep, Dep-BSA, and Dep-EDF.

(Dep-EDF 0.4 F, Dep-EDF 0.4S, Dep-EDF 0.5 F, Dep-EDF 0.5S, and PM-B-EDF 0.6) or azinophos-methyl tracers (MBP-EDF 1 ~ 4) in a total volume of 0.5 mL for 1 min at RT. The fluorescence polarization (P) values, defined according to the formula: $P = (I_v - I_h)/(I_v + I_h)$, where I_v and I_h are the vertical and horizontal components of the emitted fluorescence intensities, were measured for each dilution of antibody. Fluorescence polarization expressed in "milli units" (mP) were used to construct antibody dilution curves.

Competitive FPIA Calibration Curves

A quantity of 50 μL pesticide standard solution (or sample), 0.5 mL tracer solution, and 0.5 mL antibody solution at optimal dilution were added sequentially to the cuvette and mixed, followed by measurement. Standard curves were plotted as mP vs. logarithm of analyte concentration fitted by the four-parameter logistic equation.

RESULTS AND DISCUSSION

Tracer Concentration

The tracer mass in the assay mixture system is a parameter influencing, most importantly, the sensitivity of the detection method. The concentration of tracer not only determines the intensities of emitted polarized light, but also contributes to the competition for antibody binding. In general, the tracer concentration is lower; the detection method constructed is more sensitive. Conversely, the tracer with low concentration will be not enough to compete for antibody binding with analyte. Thus, the lowest possible tracer concentration which permits the reliable detection of a label and produces the minimum effect on the competition, should be used to develop a sensitive assay. The lowest (optimal) concentration of tracers were approximately 5 nM in the final reaction. The tracer under this level concentration gave a total fluorescence intensity signal which could reach ~1000 fluorescence units, at least 20 times higher than the background signal from neat borate buffer.

Antibody Dilution

Antibodies produced by rabbits were antibody A, B, and D. The titer level of each antibody was assessed by IC-ELISA against parathion coating Dep-OVA. The result of the titer detection is the antibody D possesses the highest titer in 3 antibodies by IC-ELISA (data unpublished). Fluorescence-labeled tracer is an important component in FPIA, and the sensitivity and specificity depend on the characteristics

of the tracer. For this reason, four Dep-EDF tracers synthesized in duplicate experiments and PM-B-EDF tracer from professor Sergey's laboratory were used in the work. All tracers were checked for binding with antibody A and antibody D by FPIA.

Antibody dilution curves were constructed by plotting the fluorescence polarization in mP against the antibody dilution. Dilution curves were prepared by mixture of doubling dilutions of the antibody with the tracer at a concentration. Five tracers for both antibody A and antibody D were evaluated in terms of sensitivity, based on the dose–response curve of displacement. It's obvious that the result for the best tracer is shown in Fig. 2. The tracers synthesized by Dep, in

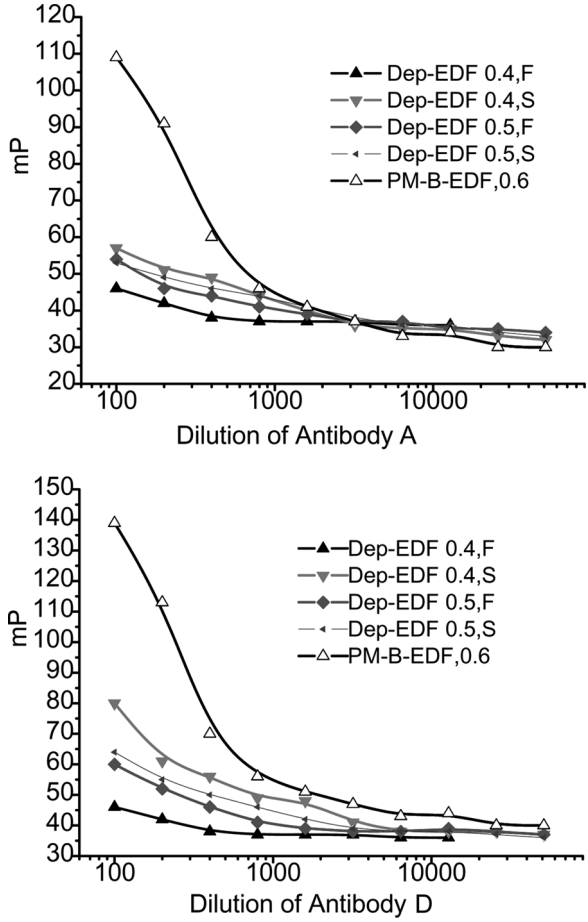

Figure 2. Dilution curves for antibodies A and D with four Dep-EDF tracers and PM-B-EDF tracer. The X-axis is the dilution of antibody, and the Y-axis is relative values (mP) of fluorescence polarization.

terms of the NHS ester method, do not bind with antibodies A and D because the results of reaction between the four tracers and two antibodies with the lowest dilution (highest concentration in reaction mixture) show Max mP values which are less than 60. As to the tracer of parathion PM-B-EDF, the Max mP values, not only for antibody A, but also for antibody D, are more than 110, though it is synthesized using parathion which belongs to the thiophosphonate esters pesticide category. This phenomenon could be explained by the frustrated experiments of binding between Dep and EDF.

Based on the results presented in Fig. 3, the antibody D offers the better four parameters fitted logistical trendline than antibody A. And, it also can be deduced from the figure that the tracer PM-B-EDF was apt to bind with the antibody D, because it made the Max mP value (the least dilution of antibody) which was more than the value produced by antibody A with the same concentration. For subsequent experiments, the optimum antibody concentrations for the construction of competitive calibration curves were chosen where approximately 70% of the tracer was bound to the antibody.

MBP-EDF $1 \sim 4$ tracers were checked for binding with MAb Azi-110 in order to find out the best tracer(s) which should be used in subsequent experiments to obtain a fair standard curve for azinophos-methyl. After MAb Azi-110 was diluted to 500 μL in 10 cuvettes, 500 μL tracer solution was added, followed the measurement of fluorescence polarization. Tracer MBP-EDF 2 and tracer MBP-EDF 3, which are characterized by $R_f = 0.9$ and $R_f = 0.6$, respectively, in silica gel plant eluted by solvent

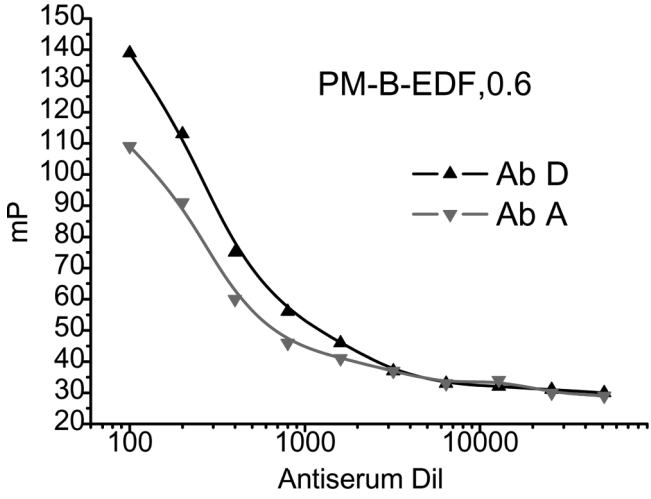

Figure 3. The specificity of two antibody binding with PM-B-EDF. The X-axis is the dilution of antibody, and the Y-axis is relative values (mP) of fluorescence polarization.

($CHCl_3$:CH_3OH, 4:1), exhibited strong binding with the MAb Azi-110. The same results were obtained with antibody A and antibody D. Tracer MBP-EDF 2 and tracer MBP-EDF 3, which are only different from tracers 1 and 4 in the aspect of R_f values under the same conditions of purification, showed about two times the signal produced by tracers 1 and 4. Virtually no binding was observed between tracer 4 and 3 type antibodies from Fig. 4. The concussion can be drawn that tracer 1 and

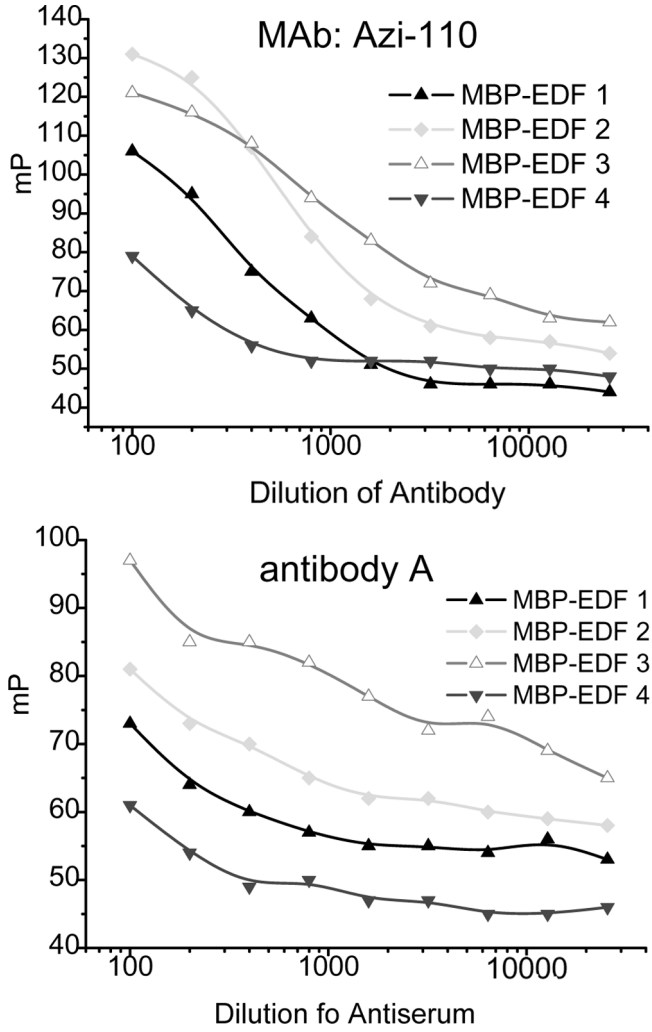

Figure 4. Dilution curves for antibodies Azi-110, A and D with four MBP-EDF $1\sim4$ tracers. The X-axis is the dilution of antibody, and the Y-axis is relative values (mP) of fluorescence polarization.

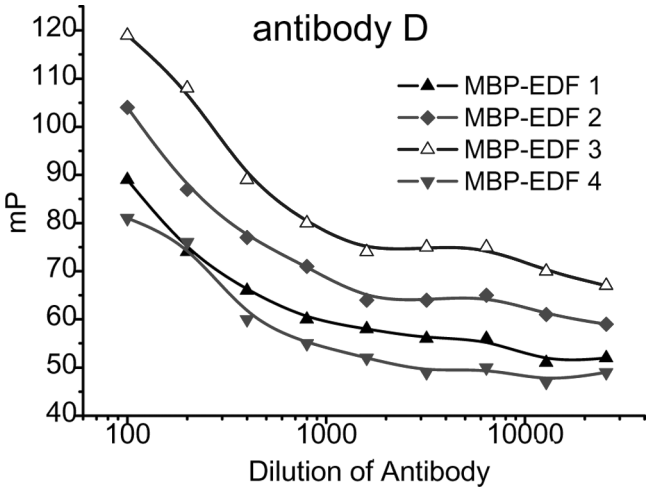

Figure 4. Continued.

4 likely were not the real compound, azinophos-methyl, bonding with EDF; only was the by-product created in the synthesis procedure. Comparing between the different kinds of antibodies binding with the four tracers, the MAb Azi-110 showed the greater affinity and specificity with the Tracer MBP-EDF 2 and tracer MBP-EDF 3 than the antibody A and the antibody D. Because it gave the higher Max mP values than the other two antibodies, and most importantly, it indicated the classical S-curve produced with the antibodies dilution in Fig. 4. On the basis of these results, Tracer MBP-EDF 2 and Tracer MBP-EDF 3 mating with the MAb Azi-110 was used for further investigation.

Analytical Performance of the Assay

The standard curves for parathion and azinphos-methyl detection by FIPA were obtained by means of the corresponding antibodies with the tracer PM-B-EDF and tracer MBP-EDF 2, 3, respectively. Assay sensitivity was defined as 50% inhibition (IC_{50}). IC_{50} values were extracted from the four-parameter logistic equation used to fit the standard curves. Assay dynamic range was defined by the analyte concentration inhibiting the maximum signal by between 20% and 80%. The detection limit in FPIA was determined in accordance with IUPAC recommendations. For this purpose, multiple measurements were performed in a reference sample with zero concentration of the analyte and the standard deviation S was calculated. The fluorescence polarization that corresponds to the limit of detection was calculated from the

equation: $mP_{min} = mP_0 - 3S$, where mP_{min} is the fluorescence polarization that corresponds to the limit of detection; mP_0 is the average value of fluorescence polarization in the 10 measurements of the zero reference samples; and S is the standard deviation. Next, the analyte concentration that corresponds to the limit of detection was found from a calibration graph. The calibration parameters were calculated with the use of Origin 7.5 or Windows.

Figure 5 shows parathion calibration curve which was obtained by mean of the antibody D with the tracer PM-B-EDF. The IC_{50} for parathion assay was found to be 1.96 mg/L, which corresponds to the detection limit of 0.179 mg/L. The assay dynamic concentrations ranged from 0.422 mg/L (corresponding to 20% inhibition) to 94.744 mg/L (corresponding to 80% inhibition).

Preliminary experiments showed that the optimal tracers for determination of azinophos-methyl by FPIA were Tracers MBP-EDF 2 and 3. Figure 6 presents the calibration curve for azinophos-methyl using these two tracers. The IC_{50} values were 1.003 mg/L and 0.955 mg/L with detecting limit of 0.1487 mg/L and 0.150 mg/L when the Tracers MBP-EDF 2, 3 were used, respectively. The assay had working ranges from 0.093 mg/L to 10.375 mg/L and from 0.102 mg/L L to 10.153 mg/L for Tracers MBP-EDF 2 and 3, respectively. It showed that there are slight differences between the results of FPIA using Tracer MBP-EDF 2 and 3.

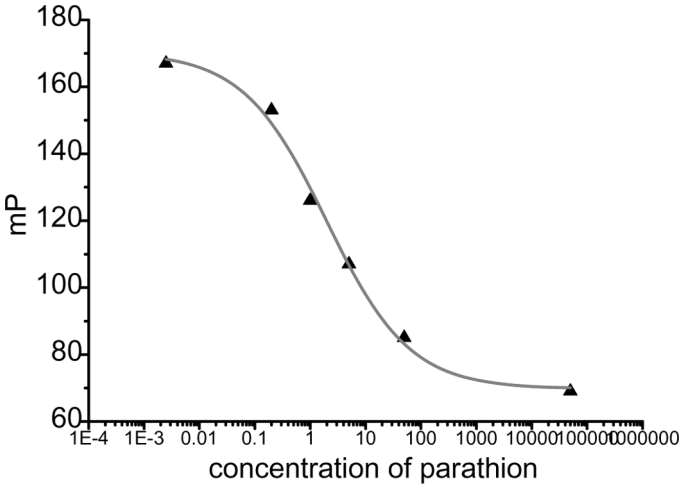

Figure 5. FPIA standard curve for parathion using antibody D with PM-B-EDF tracer. The X-axis is the concentration of parathion, and the Y-axis is relative values (mP) of fluorescence polarization.

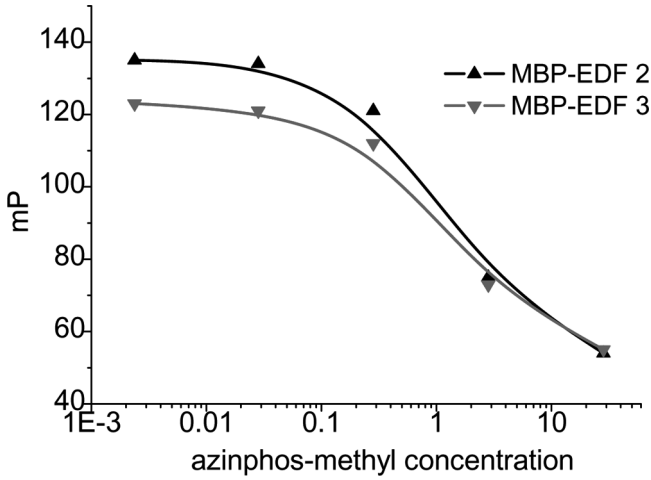

Figure 6. FPIA standard curve for azinphos-methyl using antibody Azi-110 with MBP-EDF 2 and 3 tracers. The X-axis is the concentration of azinphos-methyl, and the Y-axis is relative values (mP) of fluorescence polarization.

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