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Fluorescence Polarization Immunoassay Based on a Monoclonal Antibody for the Detection of the Organophosphorus Pesticide Parathion-methyl

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A fluorescence polarization immunoassay (FPIA) based on a monoclonal antibody for the detection of parathion-methyl (PM) was developed and optimized. Fluorescein-labeled PM derivatives (tracers) with different structures were synthesized and purified by thin-layer chromatography. The influence of immunogen and tracer structures on the assay characteristics was investigated. PM concentration determinable by the FPIA ranged from 25 to 10000 ppb. The detection limit was 15 ppb. Methanol extracts of vegetable, fruit, and soil samples were diluted 1/10 for the analysis. Recovery in spiked samples averaged between 85 and 110%. The method developed is characterized by high specificity and reproducibility (CV ranged from 1.5 to 9.1% for interassay and from 1.8 to 14.1% for intra-assay). The FPIA method can be applied to the screening of food and environmental samples for PM residues without complicated cleanup.

KEYWORDS: Fluorescence polarization immunoassay; parathion-methyl; pesticides; monoclonal antibodies

INTRODUCTION

Due to the widespread use of pesticides, serious problems in the environment are emerging, and they are an important risk to human health. Organophosphorus pesticides (OPs) have been used in agricultural and domestic applications for more than 40 years. The utilization of this class of pesticides is favored over their more persistent organochlorine counterparts because of their ability to degrade more readily in the environment. Contaminant residues have been reported in food (*1*) and environmental samples (*2*).

Organophosphorus compounds are among the most toxic substances known. They are used not only as pesticides but as chemical warfare agents, too (for example, sarin and soman). This creates a need for fast and simple assay methods of OPs. Such assays could also be helpful for scientific and practical purposes in the emerging research areas that involve bioremediation of OPs: genomic toxicology, specifically the identification of genetic polymorphisms (differences) in human paraoxonase, the product of the PON1 gene, that affect susceptibility to the effects of OPs; and development of a PON-1 gene "knockout" mouse (*3*−*5*).

Parathion-methyl (*O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate, PM) is widely used as an organophosphorus insecticide and acaricide to control many insect pests of agricultural crops, primarily on cotton. It is also used widely on fruits and vegetables (*6*). PM is a highly toxic insecticide in EPA (U.S. Environmental Protection Agency) toxicity class I. It is highly toxic to birds, aquatic invertebrates, and bees. PM primarily affects the nervous system. It can cause cholinesterase inhibition in humans; that is, it can overstimulate the nervous system, causing nausea, dizziness, confusion, and, at high exposures, respiratory paralysis and death (*6*). Because the extensive usage of OPs, among them PM, constitutes an important risk to nontarget species, including humans, there is a serious concern about the toxicological and environmental risks associated with PM residues (*7*, *8*). This creates a demand for more comprehensive monitoring programs.

Current PM analysis is basically carried out by multiresidue methods using gas chromatography (GC) (*9*−*15*) and liquid chromatography (LC) (*16*−*19*). However, these methods are laborious and time-consuming and require complicated cleanup procedures. The ability of PM, along with other OPs, to inhibit cholinesterases has found application in some analytical techniques such as flow injection (*20*) and biosensor (*21*) analyses. The main limitation for using biosensors is the regeneration of the receptor surface, which should be at least washed or even

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entirely replaced between tests of different samples. Recently, a simple potentiometric enzyme electrode was developed for the direct, sensitive, selective, and rapid determination of organophosphates, PM included, using organophosphorus hydrolase immobilized via cross-linking on the surface of a pH electrode (*22*).

Pesticide analysis using immunochemical methods is gaining acceptability as a simple, cost-effective screening method for many samples (*23*, *24*). Over the past 15 years the importance and application of immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), has grown significantly. In general, ELISA has many advantages over the other techniques and allows direct analysis of a large number of samples. The detection limits of ELISA can be comparable to, or even lower than, that obtained with instrumental methods. However, ELISA is a heterogeneous method, and separation of free and antibody-bound analyte as well as a long reaction time (1−2 h) is needed; besides, this method involves multiple washing steps.

Homogeneous methods of immunoassay that are simpler and faster and do not require separation or washing steps can be a good alternative to ELISA, especially for screening purposes. Fluorescence polarization immunoassay (FPIA) is one such technique. It is reliable, simple, fast, and cost-effective. Theory and applications of FPIA for the determination of pesticides and biologically active compounds have been previously described (*25*, *26*). In brief, homogeneous FPIA is based on differences in polarization of the fluorescent labeled species in the free and bound fractions. In this technique free and labeled analyte (tracer) compete for binding to a specific antibody. Free tracer rotates quickly, and light emitted is polarized to a very small extent. An antibody−tracer complex has a high molecular weight of >150 kDa, so it rotates slowly, resulting in a high degree of polarization of the emitted fluorescence. Free analyte added to the system competes with the tracer for binding to the antibody, resulting in a polarization decrease and a monotonically decreasing standard curve. Recently, the use of FPIA for the determination of fumonisins and aflatoxins in grains has been reported (*27*, *28*).

For parathion, the diethyl ester analogue of PM, several immunoassay methods have been developed (*29*−*33*). Polyclonal, monoclonal, and recombinant antibodies that cross-reacted with PM, and their immunoassay applications, have also been reported (*32*, *34*−*36*). We did not find any publication concerning specific immunoassays of PM, except one in which ELISA was mentioned in connection with measuring birds' dietary exposure to pesticides, PM included (*37*). This paper focuses on the development, optimization, and characterization of FPIA using monoclonal antibodies for PM detection and application of this technique for the analysis of different samples.

MATERIALS AND METHODS

Materials. Analytical grade PM and other related pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Supelco Inc. (Bellefonte, PA). Complete and incomplete Freund's adjuvants (FCA), *N*,*N*-dicyclohexylcarbodiimide (DCC), common solvents, and salts were supplied by Aldrich Chemical Co. (Milwaukee, WI). Bovine serum albumin (BSA, fatty acid free fraction V), keyhole limpet hemocyanin (KLH), *N*-hydroxysuccinimide (NHS), and fluorescein isothiocyanate (FITC, isomer I) were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol [PEG 1500 in 75 mM Hepes (PEG 50%, w/v), sterile, fusion tested] was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Dialysis membrane (Spectra/Por; MW cutoff 6000−8000) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA). P3-X63-Ag8.653 mouse plasmacytoma line was obtained from Microbiology Laboratory, Medical College, Gyeongsang National University (Chinju, Republic of Korea). Cell culture media (DMEM), fetal calf serum, and supplements were obtained from Gibco BRL (Paisley, Scotland). Microculture plates (96 and 24 wells) were obtained from Falcon Co. (Lincoln Park, NJ). Thin-layer chromatography (TLC) plates (silica gel 60, fluorescent, 1 mm, 20 × 20 cm) were obtained from Merck Co. (Darmstadt, Germany). All chemicals and organic solvents were of reagent grade or higher. The water used in all experiments was purified with a NANOpure system (Barnstead, Dubuque, IA).

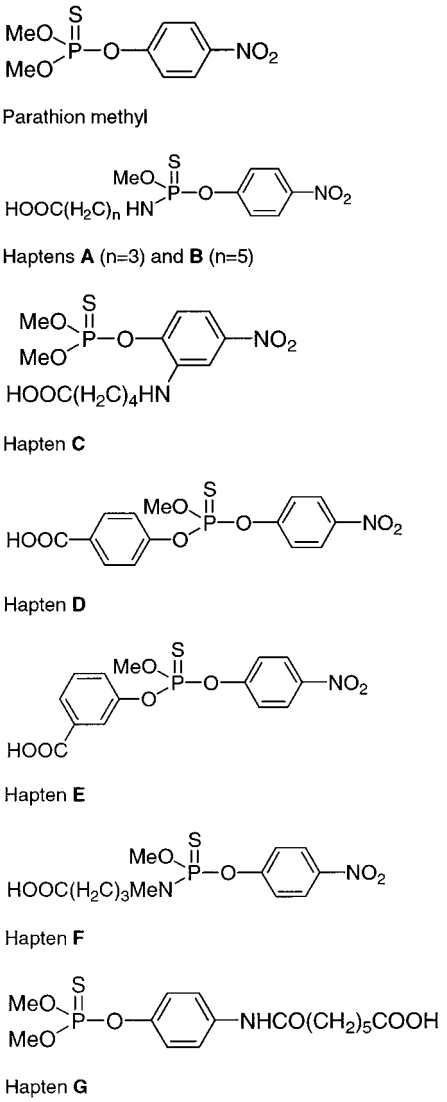


Figure 1. Chemical structures of PM and haptens used for the preparation of immunogens and fluorescein-labeled tracers.

Sodium borate buffer (BB) (0.05 M, pH 9.0) was used for all FPIA experiments.

Standard solutions of PM and cross-reactants were prepared by dilution of stock solutions of these compounds (1 mg/mL, in methanol).

Fluoresceinthiocarbamyl ethylenediamine (EDF) was synthesized from FITC and ethylenediamine as described previously (*38*) with modifications (*39*).

Haptens **A**−**G** (**Figure 1**) were kindly provided by Professor Yong Tae Lee from Yeungnam University (Kyongsan, Korea). The structures of all haptens and intermediate compounds were verified by NMR spectra obtained with a Bruker ARX spectrometer (General Electric, 300 MHz for ^{1}H and ^{13}C nuclei).

Apparatus. Measurements of fluorescence polarization and intensity were performed using a TDx/FLx analyzer (Abbott Laboratories, North Chicago, IL) in semiautomatic PhotoCheck mode. TDx/FLx glass cuvettes (up to 10 in one run) were loaded into the special carousel

Table 1. Results of PM Tracer Purification and Binding with MAb

tracer	first purifn, R_f	mP (bound tracer)[a]	second purifn, R_f	mP (bound tracer)	mP (free tracer)
A	0.2	171	0.4	258	65
	0.7	178			
B	0.4	49			29
	0.5	65			36
	0.6	213	0.4	253	77
C	0.4		0.4	97	53
D	0.2		0.2	43	29
	0.5		0.4	55	32
E	0.5		0.4	64	32
F	0.5		0.4	170	45
G	0.2	141			
	0.7	160	0.4	213	63

[a] After purification, fluorescence polarization was measured for free tracer solution in BB (without MAb), and then 10 μL of MAb 1A11 was added to each tracer solution followed by the measurement of fluorescence polarization.

followed by the measurement of polarization (in mP units) and intensity (in conventional units) of fluorescence. The total time for measurement of 10 samples was ~7 min.

Sample Preparation. The pesticide-free samples of different fruits, vegetables (bean sprouts, lettuce, apple, banana, and red bean), and soil were taken from a field in Chinju (Gyeongnam Province, Republic of Korea). The fruits and vegetables were washed with distilled water and chopped. Five grams of each sample was spiked to 0.5 or 1 ppm with analytical grade PM diluted in methanol and then extracted with 25 mL of methanol/water (80:20, v/v) for 1 h at room temperature with shaking at intervals. Soil samples (5 g) were dried, spiked to 1 ppm with PM, and extracted with absolute methanol. Extracts were filtered through cotton, and after dilution analyzed in triplicate by FPIA. Blank samples were prepared as described above but not spiked with PM.

Synthesis of Fluorescein-Labeled Tracers. Twenty-three milligrams (200 μmol) of NHS and 41 mg (200 μmol) of DCC were dissolved in 5 mL of absolute DMF and mixed for 30 min; 0.5 mL of this solution (containing 20 μmol of NHS and 20 μmol of DCC) was added to 10 μmol (~3 mg) of each hapten (**A**−**G**, **Figure 1**). The reaction mixture was kept overnight at room temperature (RT). Then, 2.5 mg (5 μmol) of EDF was added to the mixture and mixed well, followed by 1 h of incubation at RT. Red EDF was dissolved in 5 min, and the solution color changed to yellow. Small portions of the reaction mixture (~20−50 μL) were separated by TLC using chloroform as eluent. Several yellow bands were observed at the starting line of the TLC plates. The plates were dried and eluted again using chloroform/methanol (4:1, v/v). The major yellow bands at different R_f values (**Table 1**) were collected, eluted with 0.5 mL of methanol, and stored at −20 °C in the dark.

Synthesis of Immunogens. Conjugates of haptens **A** and **B** with BSA and KLH were synthesized by activated ester method (*40*). One hundred micromoles of each hapten was dissolved in 1 mL of absolute DMF with equimolar NHS and 10% molar excess of DCC. The reaction mixture was kept at RT for 3.5 h, and then the precipitated dicyclohexylurea was removed by centrifugation. Afterward, the solution of activated compound was added drop by drop to the protein (BSA or KLH) solution (50 mg of protein in 5 mL of 0.2 M borate buffer, pH 9.0, and 1 mL of DMF). The reaction mixture was stirred gently overnight at 4 °C and then centrifuged at 3000 rpm for 10 min. Subsequently, dialysis against 2 L of 0.05 M phosphate-buffered saline (pH 7.4), which was changed three times a day, was carried out for 3 days. The immunogens obtained, **A**−BSA, **A**−KLH, **B**−BSA, and **B**−KLH, were lyophilized and stored at −20 °C. Conjugation of haptens to proteins was confirmed spectrophotometrically. The hapten/protein ratio (moles of hapten/mole of protein) was 39 for the hapten−BSA conjugates and 200 for the hapten−KLH conjugates. Protein concentration was determined by using the method of Bradford (*41*) and hapten content by HPLC, after hydrolysis of 1 mg of each conjugate in 25% ammonium hydroxide.

Production of Monoclonal Antibodies. *Immunization.* One hundred micrograms of each immunogen (**A**−BSA, **A**−KLH, **B**−BSA, and **B**−KLH) in 0.1 mL of sterilized PBS (pH 7.4) was emulsified with an equal volume of Freund's complete adjuvant and given as an intraperitoneal injection to each of eight female BALB/c mice, 7−8 weeks of age (two mice were immunized with each immunogen). Boost injections were given 2, 4, and 6 weeks later. One week after the third injection, serum was collected from the caudal vein of each mouse, and titers of antisera were determined by the indirect ELISA. Three days before cell fusion, the mice that produced antisera with high titers were given another intraperitoneal boost injection without adjuvant.

Cell Fusion and Culture. Antisera were obtained before spleen extraction. Immunized mouse spleen cells (1.5×10^8) were fused with myeloma cells (1×10^7 ; P3-X63-Ag8.653) using 1 mL of 50% PEG 1500. After fusion, the cells were selected with HAT medium (DMEM medium with 20% fetal calf serum, 100 units/mL gentamicin, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Aliquots of the cell suspension (50 μL) were added to the wells of four 96-well plates (4×10^5 cells/well) and incubated at 37 °C in 7% CO_2 atmosphere. After 12 h, 50 μL more of the selection medium was added to the wells. After 2 or 3 days, the medium was replaced with fresh selection medium. After day 12, aminopterin was omitted from the medium. Between the 12th and 14th days supernatants from the 96-well plates were screened for antibody activity by the indirect ELISA in the absence and presence of PM (1 ppm). ELISA-positive hybridoma cells were cloned by the limiting dilution method (*42*) in aminopterin-free selection medium (HT medium), and 10 stable clones were obtained.

Two cell lines from the **A**−KLH-immunized mouse (**A**−KLH-1D12, **A**−KLH-2F5), six from the **B**−KLH mouse (**B**−KLH-1H2, **B**−KLH-1G7, **B**−KLH-1B6, **B**−KLH-1D1, **B**−KLH-1A11, **B**−KLH-1D9), and two from the **B**−BSA-immunized mouse (**B**−BSA-2C8 and **B**−BSA-2D9) were established. All 10 were of the IgG1 k isotype. No hybridomas from the **A**−BSA-immunized mice were found to be suitable. To obtain higher concentrations of antibody, each cell line was cultured in six-well plates, in which the culture space was separated from the larger medium-containing space by a membrane permeable to medium and metabolites, but not antibody (CelLine CL-6; Integra Biosciences Inc., Wallisellen, Switzerland). The cells were cultured, and antibody-enriched medium was harvested according to the manufacturer's instructions.

Fluorescence Polarization Immunoassay. *Dilution Curves.* To 0.5 mL of tracer solution (10 nmol/L) in the TDx/FLx glass cuvettes was added 0.5 mL of supernatant in various dilutions, mixed, and analyzed using fluorescence polarization.

Competitive FPIA Procedure. One hundred microliters of PM standard solution (or sample), 0.5 mL of tracer solution, and 0.5 mL of supernatant in optimal dilution were added sequentially to the cuvette and mixed followed by measurement. Standard curves were plotted as mP versus logarithm of analyte concentration. Cross-reactivity (CR) for different compounds structurally related to PM was determined by performing competitive assays and comparing the analyte concentration giving half-maximal inhibition (IC_{50}, ng/mL), and calculated as

$$\% \text{ CR} = (IC_{50} \text{ for PM}/IC_{50} \text{ for analyte}) \times 100$$

PM concentration in spiked samples was calculated after fitting of the standard curve using the four-parameter logistic model.

RESULTS AND DISCUSSION

Production and Characterization of Immunoreagents. Immunochemical detection methods require the production of immunoreagents (immunogens, antibodies, and competitors) as well as the optimization and validation of an analytical system. To obtain desirable immunoreagents, it is very important to design the hapten structure carefully. It was shown that assay specificity generally depends on the immunogenic hapten,

whereas assay sensitivity is usually affected by the competitive hapten (*43*). The functional groups chosen for attachment of the hapten, the length of the spacer between hapten and protein, and the conjugation method have all been shown to be crucial in determining properties of the resultant antibodies (*44*, *45*). Several PM derivatives (**Figure 1**) were synthesized and used in our study. Haptens **A** and **B**, which differed in the length of the spacer linked to the thiophosphate group of PM molecule, were conjugated to the carrier proteins (BSA and/or KLH) via the carboxyl group using attachment to the phosphate ester backbone for use as immunogens. Haptens **A** and **B** as well as five other haptens were conjugated to EDF using attachment to the phosphate ester backbone (haptens **A**, **B**, and **D**−**F**) or to the benzene ring of PM structure, the phosphate ester group being left exposed (haptens **C** and **G**), and used as the competitors (fluorescein tracers) in the FPIA method. Conjugation was carried out via the carboxyl group except for hapten **G**, for which conjugation was accomplished via the nitro group of PM. Thus, the tracers **A**, **B**, and **D**−**F** differed in the length and structure of the spacer between hapten and fluorophore, and tracers **C** and **G** differed in the conjugation position.

All of the tracers were twice purified by TLC, and reaction products with different R_f values were obtained. Results are summarized in **Table 1**. After the second purification, only one band ($R_f = 0.4$) for each tracer (except tracer **D**) was observed. It is worth mentioning that the secondary purification of tracers **A** and **G** yielded the same reaction product ($R_f = 0.4$) for both forms $R_f = 0.2$ and $R_f = 0.7$ obtained after the initial purification. Probably, after the first (preliminary) purification, the purity of the tracers was not good enough, because the TLC plate could be overloaded, and the elluent polarity was not correct, because the reaction mixture obviously comprised some amount of DMF, which increased the polarity significantly, and moreover, induced the gradient of polarity. As a result of it, after the first run, TLC could give several bands that were identical after the additional purification.

The 10 monoclonal antibodies (MAbs) obtained against immunogens **A** and **B** were tested by the FPIA using homologous tracer **A** (data not shown). For all MAbs except 1D12 and 2F5 (against immunogen **A**) good binding with the tracer was observed; titers ranged from 1/1280 (MAb 1A11) to 1/160 (MAb 1D9). It is worth noting that MAbs obtained against immunogen **B** (five methylene groups in the spacer, **Figure 1**) appeared to be of greater affinity and specificity than the MAbs against immunogen **A** (three methylene groups in the spacer). In theory, immunogens with longer spacers between hapten and carrier protein (five to seven methylene fragments) are preferable to those with shorter spacers (*45*, *46*). Longer spacers presumably give the hapten more flexibility and separation from the carrier, resulting in improved hapten recognition and antibodies of greater affinity and specificity. Conversely, shorter spacers may allow bonding interactions between hapten and carrier, increase steric hindrance, and/or promote inclusion of carrier protein moieties in hapten recognition. MAbs 1A11 and 2C8, produced from immunogens **B**−KLH and **B**−BSA, respectively, had the best titers for binding PM and were used for further investigations.

All tracers were checked for binding with MAb by adding 10 μL of MAb to the tracer solution followed by the measurement of fluorescence polarization (**Table 1**). Tracers **D** and **E**, which differed from the immunogens by spacer heterology, were not bound detectably by the MAbs. In this case also, steric hindrance probably occurs; no antibody−tracer complex was formed, possibly because of the large size of the substituent

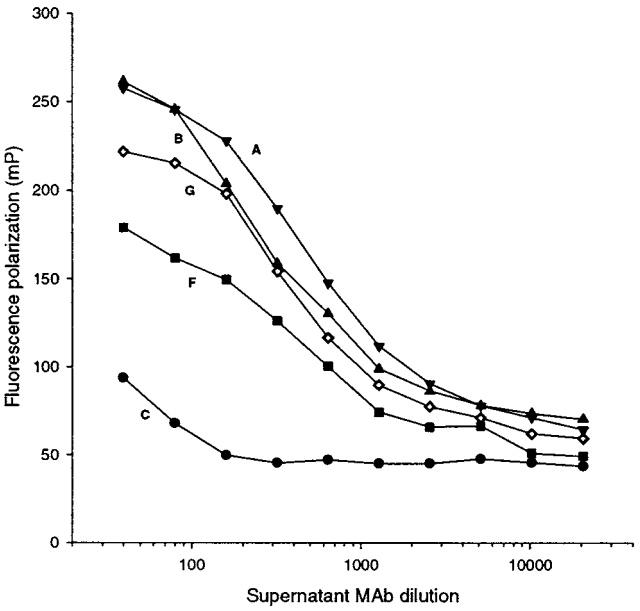


Figure 2. Dilution curves for MAb 2C8 with different tracers (**A**–**C**, **F**, and **G**).

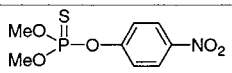
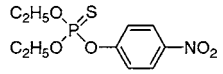
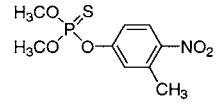
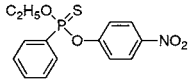
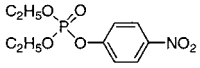
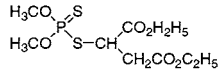
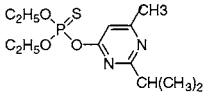
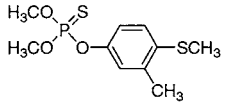
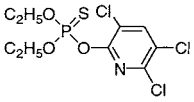
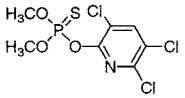
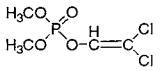
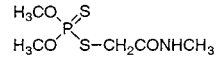
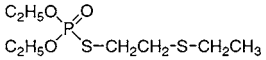
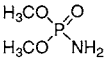
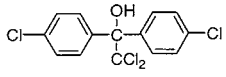
incorporated in the spacer structure of these tracers. **Figure 2** shows FPIA dilution curves for MAb 2C8 in culture fluid harvested from CL-6 plates and tracers **A**−**C**, **F**, and **G**. Tracers **A** and **B**, which are most homologous to the immunogen, exhibited strongest binding. Similar results were obtained with tracers **G** and **F**. Tracer **F**, which differed from **A** only by the *N*-methyl group in the spacer structure (**Figure 1**), gave about two-thirds the signal obtained with **A**. Virtually no binding was observed with tracer **C**. Tracers **G** and **C** were heterologous to the immunogen in spacer arm position. Tracer **G** was conjugated through the nitro group of PM, presumably exposing the rest of the PM molecule. As predicted, this tracer was bound well (titer 1/320; **Figure 2**). Tracer **C**, which incorporated one additional *N*-alkyl substituent into the benzene ring of PM and was conjugated through this position, was bound very weakly. Tracers **A**, **B**, **F**, and **G** were used for further investigation.

Cross-reactivity of the compounds structurally related to PM was investigated using MAb 1A11 and tracer **A** (**Table 2**). Parathion-ethyl, the diethyl ester analogue of PM, was characterized by the highest percent of cross-reactivity (36%). For fenitrothion, differing from PM in structure only by one methyl group in the benzene ring, and for EPN, some cross-reactivity was also observed (14 and 15%, respectively). For other organophosphorus pesticides, cross-reactivity was negligible (<0.1%).

The antigen−antibody complex formation is a complicated interaction, and it is easily affected by slight differences in chemical structure. Although compounds used in this study are structurally related, the electrical contribution and physicochemical properties are slightly or greatly different among these compounds. Therefore, these factors may affect the interaction between the antibodies and each compound, but it can be concluded from this study that MAb were probably produced mainly against thiophosphate and nitrophenoxy groups of PM structure because antibodies recognized only the compounds possessing these groups.

Assay Optimization. Competition between free and fluorescein-labeled PM for binding with antibodies was investigated, and assay conditions were optimized using different combinations of tracers and MAbs. The tracer determines the intensity of emitted polarized light and contributes to the competition

Table 2. Cross-Reactivity of MAb to PM and Related Compounds[a]

Compound	Structure	Cross-reactivity, %
Parathion methyl		100
Parathion ethyl		36
Fenitrothion		14
EPN		15
Paraoxon		2
Malathion		<0.1
Diazinon		<0.1
Fenthion		<0.1
Chlorpyrifos		<0.1
Chlorpyrifos methyl		<0.1
Dichlorvos		<0.1
Dimethoate		<0.1
Demeton S		<0.1
Methamidophos		<0.1
Dicopol		<0.1

[a] Tracer **A** and MAb 1A11 (dilution 1/900) were used.

for antibody binding, so tracer concentration influences assay sensitivity markedly. The lowest possible tracer concentration that allows the reliable detection of the label and does not affect the competition should be used to achieve the most sensitive assay. All of the tracers were adjusted to a concentration of ~10 nM in the final reaction, corresponding to ~1000 fluorescence units. Under these conditions, the best standard curve for PM detection by FPIA was obtained with MAb 1A11 at 1:900 dilution (data not shown).

Tracers **A** and **G** gave the best assay sensitivity (IC_{50} = 380 and 392 ng/mL, respectively; **Table 3**), and dose−response curves with these tracers had nearly identical slopes (**Figure 3**). FPIA standard curves with tracers **B** and **F** were less sensitive: the differences between maximal and background binding decreased, and more MAb had to be used **(Table 3)**. The IC_{50} values obtained with tracers **A** and **B** indicated that a spacer of three, instead of five, methylene groups gave the more sensitive assay, which is in agreement with some data described previously (*47*−*50*). Compared to tracer **A**, tracer **F** gave a weaker signal, as well as a less sensitive assay (higher IC_{50}). The standard curve with tracer **G** was similar to that for tracer **A**, and the assay had a high signal and sensitive (low) IC_{50} (**Table 3**). In summary, the optimal FPIA for PM was obtained using tracer **A** or **G** and MAb 1A11, in assay buffer with 10% (v/v) methanol. **Figure 4** shows a typical standard curve. The detection limit for PM was 15 ng/mL, IC_{50} = 607 ng/mL, and

Table 3. Analytical Parameters of Standard Curves for PM Detection by FPIA Method Using Different Tracers and MAb 1A11

tracer	dilution of supernatant in assay system	IC_{50}, ng/mL	δmP range[a]
A	1/900	380	123
B	1/450	563	93
F	1/150	427	75
G	1/900	391	124

[a] Difference between fluorescence polarization values (in mP) for standard solution with maximum PM concentration (10 μg/mL) and that without PM.

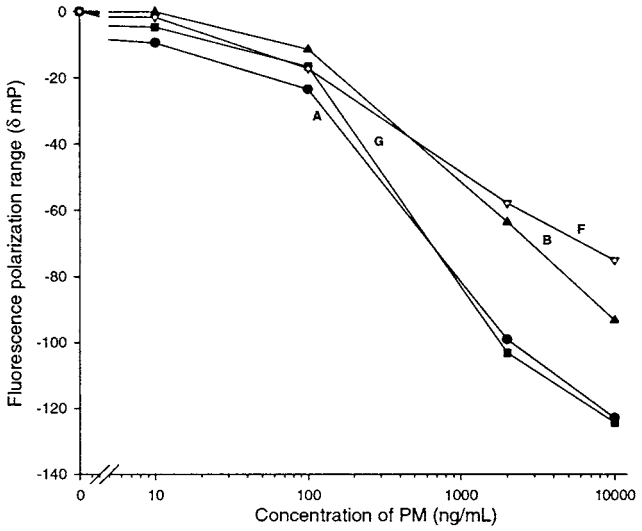


Figure 3. FPIA standard curves for PM using MAb 1A11 and different tracers (**A**, **B**, **F**, and **G**).

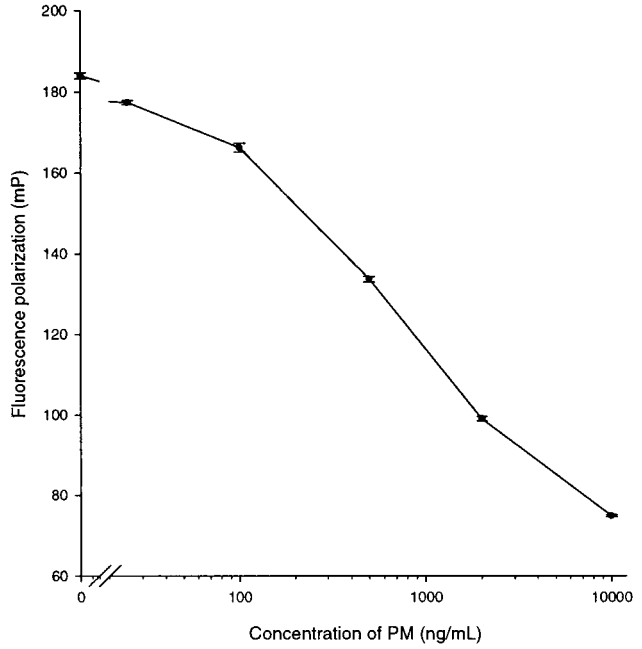


Figure 4. FPIA standard curve for PM detection in buffer with 10% methanol. Each point of the curve represents the mean ± SD (standard deviation) of $n = 5$ assays.

with appropriate dilutions, PM could be determined from 25 to 10000 ng/mL. The FPIA was characterized by good reproducibility; coefficients of variance (CVs) ranged from 1.8 to 14.1% within assay and from 1.5 to 9.1% between assays.

Matrix Effects. There was an optimum volume of sample above which sensitivity decreased due to interfering substances in the sample matrix. Near-optimal standard curves could be obtained with buffer containing up to 10% (v/v) methanol, and little or no matrix effect was observed when samples, extracted in 80−100% methanol, comprised 5−10% of the final assay volume. No matrix effects were observed with water washes from pesticide mixing tanks or from river and field water samples. In most cases these could be assayed without any cleanup or dilution.

Sample Analysis. We applied the FPIA to analyze extracts of fruits, vegetables, and soils. Although it is generally known that immunoassays are used to simply and rapidly analyze pesticides, the antigen−antibody interaction can be affected by a variety of compounds (*51*). FPIA is susceptible to interference with different components existing in some matrices such as plant extracts and serum. As described above, the most common ways to reduce matrix effects are (a) selective extraction ("cleanup") and (b) dilution to bring the interfering substances below a concentration that would interfere with the assay. Cleanup is relatively time-consuming and may affect reproducibility and recovery of the analyte. To keep the analysis as simple as possible, we extracted PM from fruits and vegetables with 80% methanol and from soil with 100% methanol. Samples that had received pre-extraction spikes of 500 and 1000 ppb of PM were subsequently diluted 10-fold and analyzed in triplicate by FPIA. Spike recoveries averaged between 85 and 110%, as shown in **Table 4**. No false positive results for blank (unspiked) samples were observed.

Table 4. Amounts of PM Recovered from Different Fruit, Vegetable, and Soil Samples

	parathion-methyl concn, ppb		
sample	spiked	exptl	recovery, %
bean	500	523 ± 6	104.6 ± 1.2
sprouts	1000	1093 ± 44	109 ± 4
lettuce	500	499 ± 24	100 ± 5
	1000	943 ± 6	94.3 ± 0.6
apple	500	504 ± 5	100.8 ± 1.0
	1000	863 ± 169	86 ± 17
banana	500	788 ± 28	158 ± 6
	1000	1018 ± 38	102 ± 4
red	500	516 ± 18	103 ± 4
bean	1000	877 ± 156	88 ± 16
soil	1000	993 ± 25	99.3 ± 2.5

The maximum residue level (MRL) for PM on most fruits and vegetables is 1 ppm, so the FPIA can be applied for PM determination in these, as well as in soil and river and field water, in the concentration range mentioned above. Although PM is degraded rather quickly in water and soil [with reported half-lives of 1−30 days, (*52*, *53*)], it can accumulate on fruits and vegetables. Thus, monitoring of these is very important. PM can be detected in water and soil samples just after it is applied. However, a concentration step would be necessary before FPIA could be used to monitor drinking water, for which European Community regulations specify an action level of 0.1 ppb for PM and other pesticides (*54*).

In conclusion, the FPIA method based on a monoclonal antibody for the detection of parathion-methyl was developed and optimized. Tracers with different structures were synthesized and purified. One tracer homologous to the immunogen with three methylene groups in the spacer and a tracer heterologous to the immunogen were shown to provide the best analytical characteristics. The FPIA had an unusually wide determinative range, from 25 to 10000 ng/mL. PM spikes in vegetable, fruit, and soil extracts were determinable by the FPIA with excellent recovery and CV. The method was highly specific and reproducible. On the basis of these initial results, this FPIA appears to meet the performance criteria for PM residue testing of food and environmental samples without complicated cleanup.

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