JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Enzyme-Linked Immunosorbent Assay Based on a Polyclonal Antibody for the Detection of the Insecticide Fenitrothion. Evaluation of Antiserum and Application to the Analysis of Water Samples

EIKI WATANABE,[†] YUKIKO KANZAKI,[†] HIROSHI TOKUMOTO,[†] RYOKO HOSHINO,[†] HIROAKI KUBO,[‡] AND HIROYUKI NAKAZAWA*,[†]

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan, and Department of Analytical Chemistry, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

For development of an indirect competitive enzyme-linked immunosorbent assay (ELISA) for the organophosphorus insecticide fenitrothion, the specificity of the antiserum R-3 generated with the bifunctional hapten, LysMNPA (2-[[[(3-methyl-4-nitrophenyl)oxy]methylcarbonyl]amino]-6-(2,4-dinitrophenyl)aminohexanoic acid) and the application to the residual analysis of some water samples were evaluated. At optimized ELISA conditiond, the quantitative working range was from 1 to 39 ng/mL with a limit of detection of 0.3 ng/mL and an IC_{50} value of 6 ng/mL. Cross-reactivity to structurally similar organophosphorus compounds and related chemicals was determined. The antiserum R-3 showed significant cross-reactivity with fenitrooxon and 3-methyl-4-nitrophenol, which have a 3-methyl-4-nitrophenoxy group as common structures, but showed relatively low cross-reactivity with other compounds. Each water sample (river water, tap water, purified water, and bottled water) had a matrix effect and was investigated by adding Tween 20 in the assay buffer. These four kinds of water samples were fortified with fenitrothion at several concentration levels and were directly analyzed with only dilution with an equal volume of antiserum solution. The mean recovery was 105.9%, and the mean coefficient of variation was 10.9%. The results suggested that the developed ELISA would be very suitable for a preliminary screening for fenitrothion in water samples at such low levels.

KEYWORDS: ELISA; fenitrothion; fenitrooxon; 3-methyl-4-nitrophenol; bifunctional hapten; Tween 20; water analysis

INTRODUCTION

Fenitrothion [*O*,*O*-dimethyl *O*-(4-nitro-*m*-tolyl)phosphorothioate], also called Sumithion (Figure 1), is an organophosphorus insecticide. It has found widespread applications to control pests and has been mainly used as a grain protectant in many countries since the late 1970s. It is one of the broad spectrum insecticides, toxic to many malathion-resistant species (*1*−*3*), and is especially useful in combination with synthetic pyrethroids, by which the lesser grain borer can then be adequately controlled (*4*). Furthermore, fenitrothion also finds use in horticulture sprays, viticulture, forestry, control of locust plagues, household sprays, and public health applications. Because their extensive usage constitutes an important risk for nontarget species, including humans, and has already caused serious environmental problems, there is a growing concern about the toxicological and environmental risks associated with fenitrothion residues (*5*, *6*). This concern is creating a demand for more comprehensive monitoring programs.

Current fenitrothion analysis is carried out by multiresidue methods using gas chromatography (GC) (*7*−*13*). Chromatographic methods are laborious and time-consuming and require sophisticated equipment available in only well-equipped centralized laboratories. On the other hand, antigen−antibody reaction-based methods such as immunoassays (enzyme-linked immunosorbent assays, ELISAs) are being increasingly reported for residual pesticide analysis. ELISAs have an especially significant role to play in the analysis of environmental and food samples for the detection of various pesticides. Generally, immunochemical detection methods have proven to be simple, cost-effective, and rapid (*14*, *15*), although some ELISAs for several pesticides require a sample cleanup with solid phase extraction (SPE) (*16*−*18*). These features convert ELISAs into very

* Author to whom correspondence should be addressed (telephone +81-3-5498-5763; fax +81-3-5498-5765; e-mail nakazawa@hoshi.ac.jp).
[†] Hoshi University.
[‡] Kitasato University.

10.1021/jf0108359 CCC: $22.00 © 2002 American Chemical Society
Published on Web 12/06/2001

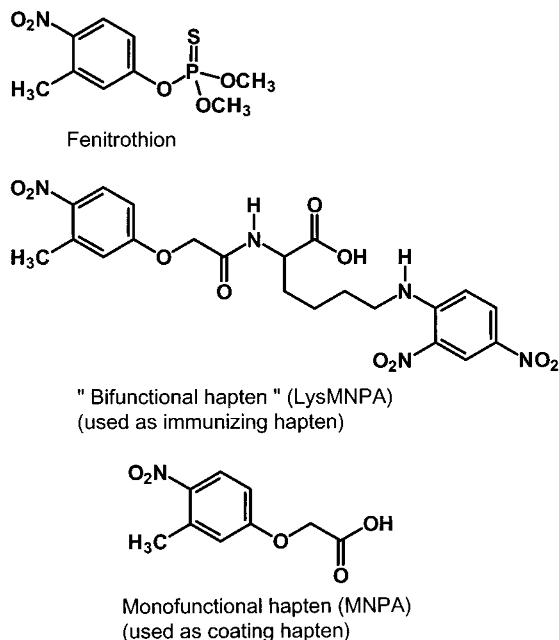

Figure 1. Chemical structures of fenitrothion and the haptens used to develop the fenitrothion ELISA.

powerful tools for pesticide residue analysis involved in large monitoring programs when high sample throughput and on-site screening analysis are required. For various organophosphorus compounds such as parathion (*19*), fenitrothion (*20*), chlorpyrifos (*21*), diazinon (*22*), azinphos-methyl (*23*), and their metabolites (*24*, *25*), there have been many reports concerned with developing sensitive methods.

Immunochemical detection methods require the production of antibodies to the targeted analytes and the optimization and validation of an assay system. To obtain desirable antibodies, it is very important to carefully design the structure of the hapten. Carlson advocated that assay specificity depends on the immunogenic hapten, whereas assay sensitivity depends on the competitive hapten (*26*). McAdam et al. have already reported the production of monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) to fenitrothion (*20*). They designed and synthesized some haptens with various approaches such as direct diazotization, linking through the phosphate group, or linking through the reduced nitro group using a six-carbon spacer arm.

We originated a unique concept to produce antibodies. We have been dealing with the synthesis of structurally unique haptens and the production of polyclonal antisera for fenitrothion and related compounds such as fenitrooxon and 3-methyl-4-nitrophenol (unpublished data). That is, to obtain antisera earlier than conventional technology for the production of antisera, we proposed the new and unique concept of a "bifunctional hapten". The bifunctional hapten has two functions: the conventional function of producing an antibody against an antigen and a unique function of promoting the production of the antibodies in the animal. On the basis of this unique concept, a group-specific antiserum was successfully obtained from rabbit immunized with the bifunctional hapten 2-[[[(3-methyl-4-nitrophenyl)oxy]methylcarbonyl]amino]-6-(2,4-dinitrophenyl)aminohexanoic acid (LysMNPA, Figure 1), which was synthesized by coupling between 3-methyl-4-nitrophenoxyacetic acid (MNPA, Figure 1) used as coating antigen in this study and commercial lysine derived with a 2,4-dinitrophenyl (DNP) group in the *N*ε-position in only 40 days (unpublished data). The purpose of this study was to develop, optimize, and characterize an ELISA based on the most sensitive antiserum (antiserum R-3) for the detection and quantification of fenitrothion in water samples.

MATERIALS AND METHODS

Chemicals and Immunoreagents. Analytical grade fenitrothion and other related pesticides were obtained from Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haën (Seelze, Germany), Kanto Chemical Co., Inc. (Tokyo, Japan), or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chicken egg ovalbumin (OVA), goat anti-rabbit immunoglobulin (IgG) conjugated to horseradish peroxidase (HRP), Tween 20, and *o*-phenylenediamine (OPD) tablets were obtained from Sigma Chemical Co. (St. Louis, MO). The production of the anti-fenitrothion pAb (antiserum R-3) generated with the bifunctional hapten LysMNPA-BSA was carried out by using rabbits (unpublished data), and the optimization of concentration of each immunoreagent also was carried out in our laboratory as described under Indirect Competitive ELISA. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccimide (NHS) were obtained from Wako Pure Industries, Ltd. Block Ace was obtained from Dainippon Chemical Industries (Osaka, Japan). The water used in ELISA tests was purified using a Milli-Q system (Millipore Corp., Milford, MA). The ELISA was carried out in 96-well polystyrene microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan).

Preparation of Coating Antigen. MNPA, selected as the most suitable coating hapten, contained a free carboxylic group that would react with amino groups of the carrier protein, OVA. MNPA−OVA conjugation was carried out by the NHS-active ester method according to the procedure of Karu et al. (*27*), with slight modifications. The MNPA (21.1 mg, 0.1 mmol) was dissolved in 500 μL of dry DMF, and then NHS (11.5 mg, 0.1 mmol) and DCC (20.6 mg, 0.1 mmol) were added. The reaction mixture was stirred for 4 h at room temperature. The precipitate was removed by centrifugation. Twenty-five milligrams of OVA was dissolved in 2.5 mL of 10 mM phosphate-buffered saline [PBS; 1.1 g/L Na_2HPO_4, 0.306 g/L KH_2PO_4, 0.9% (w/v) NaCl, pH 7.2] and 525 μL of dry DMF. Aliquots (125 μL) of the activated hapten solution were added dropwise to the stirred protein solution. The reaction mixture solution was stirred overnight at 4 °C, and then the content was dialyzed against PBS overnight at 4 °C. The purified conjugates were lyophilized and were stored at 4 °C.

Indirect Competitive ELISA. Microplates were coated overnight at 4 °C with 100 μL/well of 1.25 μg/mL solution of coating antigen (MNPA−OVA) in 0.1 M carbonate buffer (pH 9.6). After the plates had been washed with washing solution (PBS) by using a Nunc-Immuno Wash 8 microplate washer (Nalge Nunc International, Roskilde, Denmark), the surface of the wells was blocked with 300 μL/well of blocking solution [25% (v/v) Block Ace in distilled water containing 0.1% (w/v) sodium azide] by incubation for 2 h at room temperature to minimized nonspecific binding in the plate. The plates were washed and incubated with a mixture of diluted antiserum R-3 (1:8000, final dilution in wells) with various concentrations of analytes and further incubated with goat anti-rabbit IgG−HRP (1:8000 in PBS, 100 μL/well) for 1 h at room temperature after washing of the plates. The plates were washed again, and 100 μL/well of substrate solution (2.0 mg/mL OPD and 0.02% (v/v) H_2O_2 in phosphate−citrate buffer, pH 5.2) was added. Color development was stopped with sulfuric acid (1.0 M, 50 μL/well) after 20 min at room temperature. The absorbance at 490 nm was then read on a Bio-Rad model 550 microplate reader (Hercules, CA).

Determination of Cross-Reactivities. The ability of antiserum R-3 to recognize several related compounds was tested by performing competitive assays and determining their respective IC_{50} values (50% inhibition of control). Cross-reactivity was calculated as (IC_{50} of fenitrothion/IC_{50} of related compound) × 100.

Analysis of Water Samples. Water samples were fortified with fenitrothion to evaluate potential matrix effects on ELISA. The water samples tested were purified water, tap water (Tokyo, Japan), a commercial bottled water, and river water (Tama River, Tokyo, Japan). For ELISA analysis, 10 mL of water was spiked with known concentrations of fenitrothion covering the quantitative working range.

In the case of tap water and river water, the samples were filtered through a 0.45 μm nylon filter. After the solution had been adjusted to pH 7.2, a 500 μL aliquot of the sample was then mixed 1:1 with antiserum diluted with PBS, and concentrations were interpolated from a PBS standard curve.

RESULTS AND DISCUSSION

To perform the immunochemical detection of fenitrothion in four kinds of water samples, an indirect competitive ELISA format was optimized (unpublished data). The optimized configuration provided a highly sensitive immunoassay for fenitrothion. The combination of antiserum R-3 (1:8000 final dilution in wells) and the heterologous hapten, MNPA conjugated to OVA, as the coating antigen (125 ng/well) afforded the most sensitive ELISA to fenitrothion, and hence these immunoreagents were selected as specific components of the standard assay system.

Specificity of the Fenitrothion ELISA. Meulenberg et al. suggested that cross-reactivity between antibodies and compounds that are structurally similar to the target compound is an inherent problem with the ELISA (*28*). Cross-reactivity can affect analytical results by either indicating that the target compound is present when it is not (a false positive) or by elevating the predicted concentration of the target compound when both the target and one or more structurally similar compounds are present. Therefore, it is important to carefully evaluate the specificity of antibodies. The specificity of antiserum R-3, selected as best pAb with respect to the sensitivity of fenitrothion (IC_{50} = 6.1 ng/mL with the standard assay), had been evaluated with some compounds having molecular structures closely related to that of the competitor. That is, antiserum R-3 showed a cross-reactivity pattern almost identical to that of fenitrothion, its major metabolite, fenitrooxon (107%), and its degradation compound, 3-methyl-4-nitrophenol (113%). These results indicate that the chemical structure of the immunizing hapten, LysMNPA, greatly reflected the specificity of antiserum R-3. However, a simple change in the 3-methyl-4-nitrophenoxy compounds, the common structure of fenitrothion, fenitrooxon, and 3-methyl-4-nitrophenol, to the 4-nitrophenoxy compounds such as parathion, parathion-methyl, or 4-nitrophenol, reduced the recognition (<0.3% with these compounds), indicating the importance of the methyl group in the 3-position. As shown in Figure 1, LysMNPA contains the DNP group, which contributes to the acceleration of the antibody production as described by Eisen and Siskind (*29*). Various pesticides are present in the world, and some of these pesticides containing the DNP group, such as DNOC, dinoterb, and dinoseb, are also well-known. Thus, the specificity of antiserum R-3 generated with LysMNPA was further studied in detail with these three compounds. The cross-reactivities of these compounds were negligible (>0.01%) (Table 1). This is due to steric hindrance of the alkyl group, especially the bulky *tert*-butyl or *sec*-butyl groups.

However, the antigen−antibody interaction is not simple, and it is easily affected by slight differences in chemical structure, such as the difference between a hydroxyl group and a methoxy group. For instance, the interaction causes differences in the degree of electron donation and hydrophobicity of atoms or groups on target compounds. Although our focused compounds in this study have a common structure, the 3-methyl-4-nitrophenoxy group, the electrical contribution or the physicochemical property is slightly or greatly different among these compounds. Therefore, these factors may affect the interaction between the antiserum R-3 and each compound. However, from these findings it was clear that the antiserum R-3 had an ideal specificity, which recognizes the portion of LysMNPA, 3-methyl-4-nitrophenoxy group, as we expected.

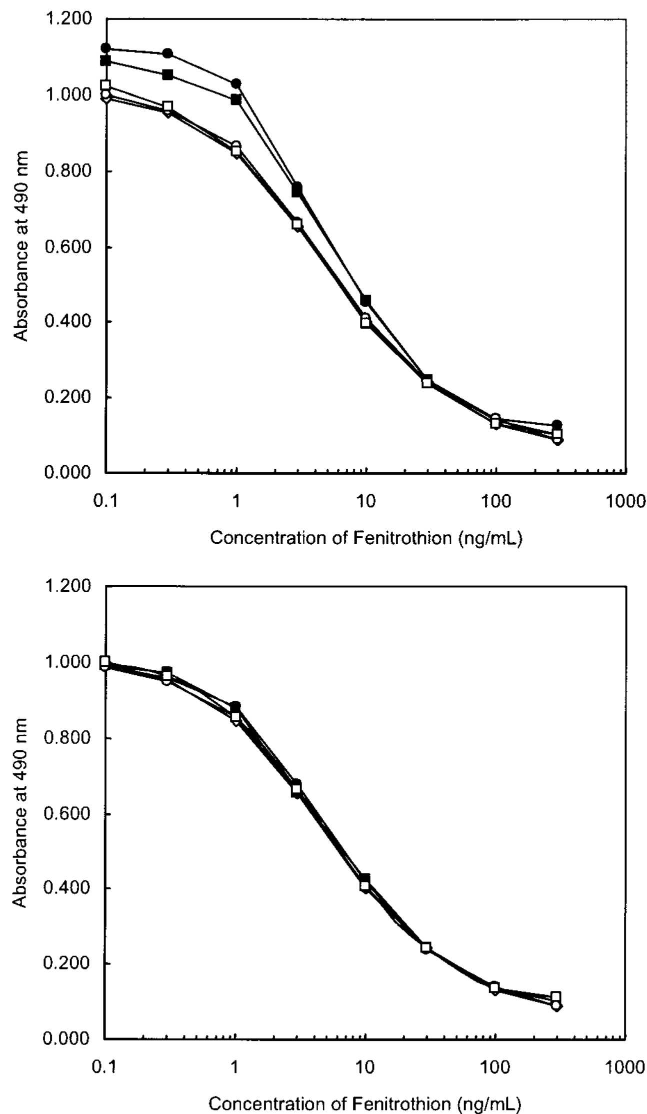

Figure 2. Comparison of fenitrothion competitive curves obtained from standards prepared in PBS not containing Tween 20 (top) and containing 0.05% (v/v) Tween 20 (PBST) (bottom): (◇) control; (●) river water; (■) tap water; (○) purified water; (□) bottled water. (Top) IC_{50} values were as follows: Std., 6 ng/mL; river water, 6.6 ng/mL; tap water, 8 ng/mL; purified water, 6.2 ng/mL; bottled water, 5.6 ng/mL. (Bottom) IC_{50} values were as follows: Std., 6 ng/mL; river water, 6.5 ng/mL; tap water, 6.3 ng/mL; purified water, 5.8 ng/mL; bottled water, 5.7 ng/mL.

Evaluation of Matrix Effects. Although it is generally known that immunoassays can simply and rapidly analyze pesticides, the antigen−antibody interaction can be affected by a variety of compounds (*30*). In fact, our research results (*31*) and those of others (*32*) show that there are matrix effects from food samples that affect the ELISA. To evaluate matrix effects caused by water samples, standards were prepared in several water samples. Because during the preparation of each water sample only a 2-fold dilution with antiserum solution was used as described under Analysis of Water Sample, in the river water samples and tap water samples, the resulting standard curves slightly shifted to higher maximum absorbances than the PBS standard curve and the other water samples curves (Figure 2). To correct the phenomena mentioned above, some additive such

Table 1. Cross-Reactivity of Antiserum R-3 to Fenitrothion and Related Compounds[a]

compound	chemical structure	CR (%) [b]	compound	chemical structure	CR (%) [b]
fenitrothion		100	diazinon		<0.01
fenitrooxon		107	pirimiphos-methyl		<0.01
parathion		0.2	malathion		<0.01
paraoxon		0.2	3-methyl-4-nitrophenol		113
methyl parathion		0.2	4-nitrophenol		0.1
methyl paraoxon		0.2	2-nitrotoluene		0.2
EPN		0.3	nitrobenzene		<0.01
fenthion		<0.01	DNOC		<0.01
dicapthon		<0.01	dinoterb		<0.01
chlorpyrifos		<0.01	dinoseb		<0.01

[a] ELISA conditions were 1.25 μg/mL coating antigen MNPA-OVA, 1:8000 dilution antiserum R-3. Preparatin of assay conditions were as described under Materials and Methods. [b] Percentage of cross-reactivity (CR) = (IC_{50} of fenitrothion/IC_{50} of related compound) $\times$ 100. IC_{50} is the analyte comcentration that reduces the assay signal to 50% of the maximum value.

as Tween 20 or BSA often is used (*33*−*35*). We also used the effect of Tween 20 to correct the rice matrix effect in the inabenfide ELISA (*31*). Tween 20, a nonionic surfactant, is the most commonly used additive in ELISA to reduce nonspecific interactions. In this study, to solve the phenomena in the river water and tap water samples, the dilution buffer contained 0.05% (v/v) Tween 20 (PBST) was used. The effect of Tween 20 apparently corrected the matrix effect. Although Watanabe et al. indicated that Tween 20 can greatly influence the sensitivity of the antibody (*31*), the addition of Tween 20 did not affect

Table 2. Recovery of Fenitrothion from Spiked Water Samples

sample	fenitrothion added (ng/mL)	fenitrothion recovered[a] (ng/mL)	SD[b] (ng/mL)	CV[c] (%)	recovery (%)
river water	0.5	0.53	0.088	16.6	105.5
	1	1.0	0.150	14.6	102.5
	5	5.0	0.658	13.2	100.0
	10	10.6	0.922	8.7	105.8
tap water	0.5	0.5	0.063	12.8	99.5
	1	1.2	0.126	10.3	122.5
	5	5.2	0.580	11.2	103.5
	10	10.2	0.592	5.8	101.5
purified water	0.5	0.54	0.060	11.1	107.5
	1	1.1	0.100	8.9	112.3
	5	5.0	0.670	13.5	99.5
	10	10.2	0.722	7.6	101.5
bottled water	0.5	0.5	0.067	13.4	99.5
	1	1.1	0.141	12.9	110.0
	5	5.8	0.483	8.3	116.0
	10	10.7	0.648	6.1	107.0
mean				**10.9**	**105.9**

[a] Each determination was run in quadruplicate, and the mean absorbance was interpolated in a standard curve performed in the same ELISA plate. Data are the average of four independent determinations. [b] Standard deviation. [c] Intra-assay coefficient of variation.

the sensitivity of the antiserum R-3 (Figure 2). The assay affinity for fenitrothion, represented by the IC_{50} value, was 6 ng/mL.

Analytical Parameters of the Optimized Fenitrothion ELISA. There is no general agreement for the calculation of assay sensitivity and working range of competitive immunoassays (*36*). Fleeker reported the limit of detection (LOD) of an assay to be 3 times the standard deviation of the A_0, negative control, from its mean absorbance (*37*), whereas Midgley et al. calculated the LOD as the concentration that corresponds to 90% of the A/A_0 (*38*). For the proposed assay, the LOD was calculated according to the Midgley et al. formula, and the LOD was estimated as 0.3 ng/mL. The quantitative working range was established between the concentrations producing 80 and 20% of the A/A_0, that is, 1−39 ng/mL.

Analysis of Spiked Water Samples. Spiking water samples with several amounts of fenitrothion is a common practice in the performance of a preliminary evaluation of analytical assay reliability. Some water samples from different sources were spiked at several concentrations of fenitrothion covering the optimized working range (0.5, 1, 5, and 10 ng/mL). Direct analysis of each spiked sample without dilution or SPE such as a C_{18} cartridge resulted in accurate determinations of fenitrothion concentrations. Determinations were made in quadruplicate, and the mean absorbance was used to estimate fenitrothion concentration by interpolation in the PBS standard curve performed in the same plate. Results of the analytical data, expressed as the percentage of recovery, are summarized in Table 2. Control samples without fenitrothion were also included in the analysis, and values lower than the assay detection limit were found in all cases, so no false positives were detected. Irrespective of the fortified concentration level and water type, very similar coefficients of variation (CV) were found, ranging from 5.8 to 16.6%. The ELISA data showed a slight tendency for overestimation compared to theoretical concentration level. However, accuracy was very excellent.

Conclusions. Antiserum R-3 generated with the bifunctional hapten, LysMNPA, was evaluated in detail. First, the ELISA conditions were optimized to achieve the best assay performance. Water sample interferences were observed for both river water and tap water samples. To correct the interferences, 0.05% (v/v) Tween 20 was added to dilution buffer, PBS. Tween 20 was investigated for assay interference and did not affect the sensitivity of the ELISA. Performance of the optimized fenitrothion ELISA for the determination of nanogram per milliliter fortified water samples was satisfactory: acceptable recoveries and good precision were obtained. Therefore, these analytical techniques are adequate for cost- and labor-effective environmental monitoring of fenitrothion.

LITERATURE CITED

(1) Kashi, K. P. An appraisal of fenitrothion as a promising grain protectant. *Int. Pest Control* **1972**, 20−22.

(2) Ardley, J. H.; Sticka, R. The effectiveness of fenitrothion and malathion as grain protectants under bulk storage conditions in New South Wales, Australia. *J. Stored Prod. Res.* **1977**, *13*, 159−168.

(3) Pearson, R. Effectiveness of liquid fenitrothion against pests in stored grain. *Milling Feed Fert.* **1979**, *162*, 30−32.

(4) Desmarchelier, J.; Bengston, M.; Connell, M.; Hennning, R.; Ridley, E.; Ripp, E.; Sierakowski, C.; Sticka, R.; Snelson, J.; Wilson, A. Extensive pilot use of the grain protectant combinations, fenitrothion plus bioresmethrin and pirimiphos-methyl plus bioresmethrin. *Pestic. Sci.* **1981**, *12*, 365−374.

(5) U.S. EPA. *Summary of the Risks and Uses of the Organophosphate Fenitrothion*; 2000; http://www.epa.gov/pesticides/op/fenitrothion/fensumm.htm.

(6) U.S. EPA. *Report of the Hazard Identification Assessment Review Committee, Hazard Assessment of the Organophosphates*, July 1998; http://www.epa.gov/pesticides/op/hazidrpt.pdf.

(7) Oliva, J.; Barba, A.; Vela, N.; Melendreras, F.; Navarro, S. Multiresidue method for the rapid determination of organophosphorus insecticides in grapes, must and wine. *J. Chromatogr. A* **2000**, *882*, 213−220.

(8) Gebreegzi, Y. T.; Foster, G. D.; Khan, S. U. Simultaneous determination of carbaryl, malathion, fenitrothion, and diazinon residues in sesame seeds (*Sesamum indicum* L.). *J. Agric. Food Chem.* **2000**, *48*, 5165−5168.

(9) Lambropoulou, D.; Sakellarides, T.; Albanis, T. Determination of organophosphorus insecticides in natural waters using SPE-disks and SPME followed by GC/FTD and GC/MS. *Fresenius' J. Anal. Chem.* **2000**, *368*, 616−623.

(10) Simplicio, A. L.; Boas, L. V. Validation of a solid-phase microextraction method for the determination of organophosphorus pesticides in fruits and fruit juice. *J. Chromatogr. A* **1999**, *833*, 35−42.

(11) Dagher, S. M.; Hawi, Z. K.; Kawar, N. S. Comparison of gas chromatography and immunoassay methods in quantifying fenitrothion residues in grape juice processed into alcoholic drinks. *J. Environ. Sci. Health B* **1999**, *34*, 849−858.

(12) Kim, D. H.; Heo, G. S.; Lee, D. W. Determination of organophosphorus pesticides in wheat flour by supercritical fluid extraction and gas chromatography with nitrogen−phosphorus detection. *J. Chromatogr. A* **1998**, *824*, 63−70.

(13) Oh-Shin, Y. S.; Ko, M.; Shin, H. S. Simultaneous quantification of insecticides including carbaryl in drinking water by gas chromatography using dual electron-capture and nitrogen−phosphorus detection. *J. Chromatogr. A* **1997**, *769*, 285−291.

(14) Harris, A. S.; Lucas, A. D.; Kramer, P. M.; Marco, M.-P.; Gee, S. J.; Hammock, B. D. Use of immunoassay for the detection of urinary biomarkers of exposure. In *New Frontier in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 217−235.

(15) Ellis, R. L. Rapid test methods for regulatory programs. In *Immunoassays for Residue Analysis. Food Safety*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 44−58.

(16) Lee, J. K.; Ahn, K. C.; Park, O. S.; Kang, S. Y.; Hammock, B. D. Development of an ELISA for the detection of the residues of the insecticide imidacloprid in agricultural and environmental samples. *J. Agric. Food Chem.* **2001**, *49*, 2159−2167.

(17) Aga, D. S.; Thurman, E. M. Application of solid-phase extraction for trace analysis of herbicides in water and soil by enzyme-linked immunosorbent assay. In *New Frontier in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 123−136.

(18) Karu, A. E.; Schmidt, D. J.; Richman, S. J.; Cooper, C.; Tran, D.; Hsu, J. Validation of a monoclonal immunoassay for diuron in groundwater. *J. Agric. Food Chem.* **1994**, *42*, 310−315.

(19) Morsy, M. H.; Ibrahim, A. A.; Hewedi, M. M. Detection of pesticides in human milk samples collected in Egypt by enzyme-linked immunosorbent assay. In *Immunoassays for Residue Analysis. Food Safety*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 167−175.

(20) McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphorus fenitrothion. 1. Approaches to hapten−protein conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466−1470.

(21) Manclús, J. J.; Primo, J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food Chem.* **1996**, *44*, 4052−4062.

(22) Beasely, H. L.; McAdam, D. P.; Larkin, K. A.; Ferfuson, B. S.; Bushway, R. J.; Skerritt, J. H. Laboratory and field enzyme-immunoassays for diazinon and their application to residue analysis in lanolin, water, and fruit juices. *Bull. Environ. Contam. Toxicol.* **1997**, *59*, 375−382.

(23) Mercader, J. V.; Montoya, A. Development of monoclonal ELISAs for azinphos-methyl. 1. Hapten synthesis and antibody production. *J. Agric. Food Chem.* **1999**, *47*, 1276−1284.

(24) Lober, M.; Krantz, S.; Herrmann, I. Erzeugung und charakterisierung von antiseren gegen *O*,*O*-diäthyl-*O*-(*p*-nitrophenyl)-phoshat (Paraoxon). *Acta Biol. Med. Germ.* **1982**, *41*, 487−496.

(25) Padki, M. M.; Bhide, M. B. Protective antibodies to fenitrooxon. *Indian J. Med. Res.* **1984**, *79*, 137−141.

(26) Carlson, R. E. Hapten versus competitor design strategies for immunoassay development. In *Immunoanalysis of Agrochemicals. Emerging Technologies*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 140−152.

(27) Karu, A. E.; Goodrow, M. H.; Schmidt, D. J.; Hammock, B. D.; Bigelow, M. W. Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron. *J. Agric. Food Chem.* **1994**, *42*, 301−309.

(28) Meulenberg, E. P.; Mulder, W. H.; Stoks, P. G. Immunoassays for pesticides. *Environ. Sci. Technol.* **1995**, *29*, 553−561.

(29) Eisen, H. N.; Siskind, G. W. Variations in affinities of antibodies during the immune response. *Biochemistry* **1964**, *3*, 996−1008.

(30) Skerritt, J. H.; Amita Rani, B. E. Detection and removal of sample matrix effects in agrochemical immunoassays. In *Immunoassays for Residue Analysis. Food Safety*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 29−43.

(31) Watanabe, E.; Tsuda, Y.; Watanabe, S.; Ito, S.; Hayashi, M.; Watanabe, T.; Yuasa, Y.; Nakazawa, H. Development of an enzyme immunoassay for the detection of plant growth regulator inabenfide in rice. *Anal. Chim. Acta* **2000**, *424*, 149−160.

(32) Watanabe, S.; Ito, S.; Omoda, N.; Munakata, H.; Hayashi, M.; Yuasa, Y. Development of a competitive enzyme-linked immunosorbent assay based on a monoclonal antibody for a fungicide flutolanil. *Anal. Chim. Acta* **1998**, *376*, 93−96.

(33) Mercader, J. V.; Montoya, A. Development of monoclonal ELISAs for azinphos-methyl. 2. Assay optimization and water sample analysis. *J. Agric. Food Chem.* **1999**, *47*, 1285−1293.

(34) Abad, A.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples. *J. Agric. Food Chem.* **1997**, *45*, 1495−1501.

(35) Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for 3,5,6-trichloro-2-pyridinol. 2. Assay optimization and application to environmental water samples. *J. Agric. Food Chem.* **1996**, *44*, 3710−3716.

(36) Sherry, J. P. Environmental chemistry: The immunoassay option. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217−300.

(37) Fleeker, J. Two enzyme immunoassays to screen for 2,4-dichlorophenoxyacetic acid in water. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 874−878.

(38) Midgley, A. R.; Nisender, G. D.; Rebar, R. W. Principles for the assessment of reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). *Acta Endocrinol.* **1969**, *63*, 163−179.

Received for review June 28, 2001. Revised manuscript received October 15, 2001. Accepted October 16, 2001.

JF0108359