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New Approach to Immunochemical Determinations for Triclopyr and 3,5,6-Trichloro-2-pyridinol by Using a Bifunctional Hapten, and Evaluation of Polyclonal Antiserum

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The present work describes the design and synthesis of the structurally unique hapten, "bifunctional hapten", to produce a group-specific polyclonal antiserum to triclopyr and 3,5,6-trichloro-2-pyridinol. A bifunctional hapten was designed and synthesized by conjugating commercially available $N\epsilon$-2,4-dinitrophenyl (DNP)-L-lysine to triclopyr, and then coupling this to carrier proteins such as bovine serum albumin (BSA). The synthesized bifunctional hapten greatly raised the antiserum titer in comparison with that of the conventional hapten, triclopyr. Antiserum with a sufficiently high titer to provide the determinations of targeted compounds was obtained only 63 days after the primary immunization. The obtained antiserum showed the highest affinity to triclopyr (IC_{50} = 3.5 nM) and 3,5,6-trichloro-2-pyridinol (IC_{50} = 5.1 nM) in homologous ELISA. The cross-reactivities to various agrochemicals and some chlorinated phenolic compounds were determined. Significant cross-reactivity was found to the herbicide 2,4,5-T. The antiserum reacted to both triclopyr and its metabolite. Assay sensitivity was evaluated for effects of various assay conditions, including pH value and concentrations of organic solvents and detergents. Under optimized assay conditions, the quantitative working range of triclopyr ELISA was from 0.1 to 5.2 ng/mL with a limit of detection (LOD) of 0.037 ng/mL, and an IC_{50} of 0.72 ng/mL. On the other hand, the quantitative working range of 3,5,6-trichloro-2-pyridinol ELISA was from 0.13 to 6.0 ng/mL with a LOD of 0.052 ng/mL, and an IC_{50} of 0.95 ng/mL. Water samples fortified with triclopyr or its metabolite at 1, 5, and 10 ng/mL were directly analyzed without extraction and cleanup by the proposed ELISA. The mean recovery was 101.6%, and the mean coefficient of variation (CV) was 7.1% in the case of the triclopyr ELISA. In the case of the 3,5,6-trichloro-2-pyridinol ELISA, the mean recovery was 99.8%, and the mean CV was 9.5%. The proposed ELISA turned out to be a powerful tool for monitoring of residual triclopyr or 3,5,6-trichloro-2-pyridinol in water samples at trace level.

KEYWORDS: Triclopyr; 3,5,6-trichloro-2-pyridinol; bifunctional hapten; ELISA; cross-reactivity; water analysis

INTRODUCTION

The herbicide triclopyr (3,5,6-trichloro-2-pyridyloxyacetic acid, **Figure 1**) has been manufactured and marketed by DowElanco since the mid 1970s. Triclopyr can be applied as either the triethylamine salt or the ethylene glycol butyl ether ester, and is often used in mixed formulation with other phenoxyalkanoic acids such as 2,4-D, mecoprop, and so on for control of woody plants and many broad-leaved weeds in grasslands, uncultivated lands, and rice fields. McCall and Gavit (*1*) and Woodburn et al. (*2*) reported that these compounds are quickly transformed to triclopyr though photolysis and hydrolysis. The environmental dissipation and transformation of triclopyr in river water and lake water was extensively studied by Woodburn et al. (*2*) and Solomon et al. (*3*). Norris et al. (*4*) reported that triclopyr undergoes biodegradation in aerobic soil environments. The major degradation product is 3,5,6-trichloro-2-pyridinol (**Figure 1**). Lickly and Murphy (*5*) reported that 3,5,6-trichloro-2-pyridinol is also the major metabolite produced by fish. 3,5,6-Trichloro-2-pyridinol is also the major degradation product of the organophosphorus insecticide chlorpyrifos and chlorpyrifos-methyl. Chlorpyrifos is a broad-spectrum insecticide that is widely used in agriculture and indoor disinfestation

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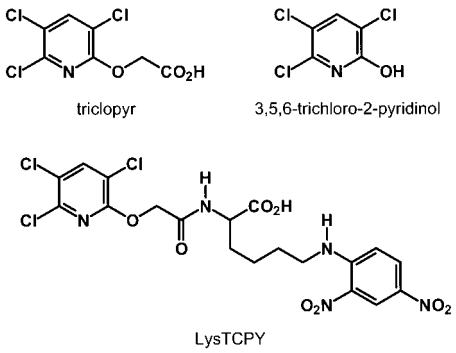


Figure 1. Chemical structures of triclopyr, 3,5,6-trichloro-2-pyridinol, and synthesized hapten (LysTCPY). Triclopyr (TCPY) was also used as coating hapten for indirect ELISA (checkerboard titration) and as immunogen for production of antiserum R-21. Bifunctional hapten, LysTCPY, was used as immunogen for production of antiserum R-20 and as optimal coating hapten (homologous hapten) for triclopyr and 3,5,6-trichloro-2-pyridinol ELISAs.

(*6*). Because of its widespread use in agriculture, a high chlorpyrifos residue occurrence in food has been reported (*7*), which poses potential health hazards (*8*). Furthermore, it was found not only in food but also in water − surface water, groundwater, or both (*9*, *10*). Therefore, there is actually a growing concern about toxicological and environmental risks associated with the remaining chlorpyrifos residues after its application. Because 3,5,6-trichloro-2-pyridinol is rapidly excreted in human urine after exposure to chlorpyrifos, it is used as a biomarker of exposure (*11*). On the basis of the above-mentioned background, in the United States active surveys of exposure to various pesticides were carried out (*12*, *13*). Kutz et al. (*12*) reported that 3,5,6-trichloro-2-pyridinol was found in 5.8% of urine samples provided from the U.S. population, and suggested that the results were consistent with the high occurrence of the parent pesticides. On the other hand, Hill et al. (*13*) also reported an interesting survey in which they analyzed 12 compounds containing 3,5,6-trichloro-2-pyridinol in urine samples of about 1,000 adults living in the U.S., and found 3,5,6-trichloro-2-pyridinol in 82% of those at observed maximum concentration of 77 ng/mL. They suggested that exposure to chlorpyrifos appears to be increasing.

Current triclopyr analysis is mainly carried out by using gas chromatography (GC) equipped with an electron capture detector (ECD) (*3*, *14*, *15*). These analytical methods involve methylation with diazomethane, which is an explosion hazard and requires handling of highly carcinogenic precursors. On the other hand, 3,5,6-trichloro-2-pyridinol analysis is also mainly carried out by instrumental analysis such as GC (*16*−*19*) or high-performance liquid chromatography (HPLC) (*20*−*23*). As these chromatographic methods require a number of clean-up procedures prior to determination, they are laborious and time-consuming, and they require sophisticated equipment available only in well-equipped centralized laboratories. Furthermore, using a large amount of organic solvents raises concern regarding health hazards to analysts and creation of environmental pollution, and there are limitations on the capacity of throughput of samples.

In the 1980s Hammock and Mumma (*24*) advocated the adaptability of immunoassays (enzyme-linked immunosorbent assays, ELISAs) for monitoring pesticides in various matrixes such as environmental media, foods, and so on. Immunoassays are simple, fast, cost-effective, and adaptable to on-site, high-sample-throughput analyses (*25*, *26*). They have been developed both as screening tools and as quantitative analytical methods for pesticide residues in environmental samples (*27*−*29*) and food samples (*30*−*32*). We developed a new type of hapten, "bifunctional hapten", with two functions: the conventional function of producing an antibody against an antigen, and a unique function of accelerating the production of the antibodies in the animal (unpublished data). Based on this unique concept, we succeeded in getting a group-specific polyclonal antiserum to the organophosphorus insecticide fenitrothion, its major metabolite product fenitrooxon, and its major degradation product 3-methyl-4-nitrophenol in 40 days only [unpublished data]. Although several immunoassays have been reported for the detection of triclopyr (*29*) and 3,5,6-trichloro-2-pyridinol (*33*), we designed the bifunctional hapten to obtain polyclonal antisera to these compounds based on the concept in the present study. The effect of synthesized bifunctional hapten was evaluated by comparison with conventional hapten, and the characterization of the obtained polyclonal antiserum for sensitivity and specificity was presented. Furthermore, the influence of the assay's performance in water matrix was evaluated.

MATERIALS AND METHODS

Chemicals and Instrumentation. Triclopyr and the structurally related pesticides used in cross-reaction studies were of analytical grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Dr. Ehrenstorfer (Augsburg, Germany), and Riedel-de Haën (Seelze, Germany). 3,5,6-Trichloro-2-pyridinol was produced from chlorpyrifos as mentioned in the Hapten Synthesis section below. All organic starting materials for hapten synthesis were purchased from Wako Pure Chemical Industries, Ltd., and were of reagent quality or better. 6-(2,4-Dinitrophenyl)aminohexanoic acid (*N*ϵ-2,4-DNP-L-lysine hydrochloride) was from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography (TLC) was performed using 0.2-mm precoated silica gel 60 F_{254} on glass plates from Merck (Darmstadt, Germany), and *Rf* values refer to TLC with visualization under exposure to either ultraviolet light or iodine vapor stain. Flash chromatographic separations were carried out on C_{18} (38−63 μm particle size) (Wako). Bovine serum albumin (BSA, fraction V), chicken egg ovalbumin (OVA), goat anti-rabbit immunoglobulin (IgG) conjugated to horseradish peroxidase (HRP), *o*-phenylenediamine (OPD) tablets, and complete and incomplete Freund's adjuvants were from Sigma Chemical Co. Block Ace was from Dainippon Chemical Industries (Osaka, Japan). Water used in ELISA tests was purified using a Milli-Q system (Millipore Corp., Milford, MA). The ELISAs were carried out in 96-well polystyrene microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectrum was obtained in deuteriochloroform ($CDCl_3$) or deuteriodimethyl sulfoxide (DMSO-*d6*) with tetramethylsilane (TMS) as an internal standard on a JEOL GSX270F instrument at 270 MHz and are described as multiplicity, coupling constant (*J*) in hertz (Hz), number of protons, and assignment. Chemical shift values (δ, ppm) are reported downfield from TMS. ELISAs were analyzed using a Bio-Rad model 550 microplate reader (Hercules, CA).

Hapten Synthesis. Synthesis of the hapten was carried out as outlined in **Figure 2**. A new concept of hapten, that is, bifunctional hapten, was synthesized by coupling between monofunctional hapten, triclopyr, and lysine with a 2,4-dinitrophenyl (DNP) group.

Methyl 2-Amino-6-(2,4-dinitrophenyl)aminohexanoate (**A**, **Figure 2**). In a 100-mL three-neck flask, 2 g of *N*ϵ-2,4-DNP-L-lysine hydrochloride (6.4 mmol) was dissolved in 40 mL of methanol, and the mixture was stirred in an ice bath. Furthermore, 4 mL of thionyl chloride (56.2 mmol) was added, and the reaction mixture was stirred overnight at room temperature. At the end of the reaction, the solvent was removed under reduced pressure, and the residual crystals were recrystallized from ether to give 2.06 g, a 99.1% yield of yellow crystalline. ^{1}H NMR (DMSO-*d6*) δ 1.36−1.93 (m, 7H, $NHCH_2CH_2CH_2$), 3.15−3.29 (m, 2H, CH_2CH), 3.51 (bs, 2H, NH_2), 3.75 (s, 3H, OCH_3), 4.01−4.17 (m, 1H, CHCO), 8.76−8.89 (m, 3H, Ar).

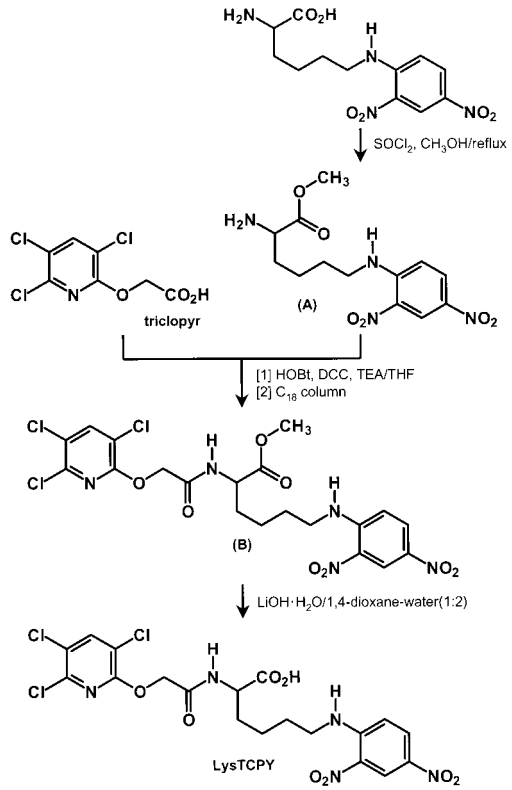


Figure 2. Synthetic pathway of bifunctional hapten, LysTCPY. HOBt, 1-hydroxy-1*H*-benzotriazole monohydrate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; TEA, triethylamine.

Methyl 2-[[[(3,5,6-Trichloro-2-pyridyl)oxy]methylcarbonyl]amino]-6-(2,4-dinitrophenyl)aminohexanoate (**B**, **Figure 2**). In a 100-mL three-neck flask, 500 mg of triclopyr (2.37 mmol), 0.73 g of 1-hydroxy-1*H*-benzotriazole monohydrate (HOBt), and 0.86 g of **A** (2.37 mmol) were dissolved in 10 mL of THF, and then 330 μL of triethylamine (2.37 mmol) and 0.51 mg of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (2.49 mmol) dissolved in 772 μL of THF were added to the mixture. The reaction mixture was stirred for 1 h in an ice bath and for 3 h at room temperature. At the end of the reaction, 50 mL of ethyl acetate was added, and the resultant mixture was filtered to remove dicyclohexylurea. After ethyl acetate was removed under reduced pressure, the resultant residue was reconstituted with 50 mL of ethyl acetate, and then the ethyl acetate phase was washed with saturated sodium bicarbonate solution, saturated ammonium chloride solution, saturated sodium bicarbonate solution, and water (50 mL of each). After the ethyl acetate phase was dried over anhydrous sodium sulfate, ethyl acetate was removed under reduced pressure. The resultant residue was purified with flash chromatography on C_{18} (6 g) (methanol/water, 1:1 v/v). After the eluted fraction was collected and lyophilized, the yellow crystalline was obtained in 57.1% yield (702 mg). ^{1}H NMR (DMSO-*d6*) δ 1.03−1.83 (m, 7H, $NHCH_2CH_2CH_2$), 3.63 (s, 3H, OCH_3), 4.30−4.39 (m, 1H, CHCO), 4.78 (s, 2H, $COCH_2$), 4.82−4.94 (q, J = 15.0 Hz, 2H, CH_2CH), 8.39 (s, 1H, pyridine-H), 8.55−8.88 (m, 3H, Ar).

2-[[[(3,5,6-Trichloro-2-pyridyl)oxy]methylcarbonyl]amino]-6-(2,4-dinitrophenyl)aminohexanoic acid (LysTCPY, **Figures 1** and **2**). The hydrolysis reaction of **B** (100 mg, 0.2 mmol) was conducted in 3 mL of 1,4-dioxane/water (1:2 v/v) with 5 equiv of lithium hydroxide monohydrate. After 2 h, the resultant reaction mixture was acidified to pH 4 with 2 M HCl. After the resultant mixture was lyophilized, the yellow crystalline was obtained in 90.9% yield (88.4 mg). ^{1}H NMR (DMSO-*d6*) δ 1.03−1.96 (m, 7H, $NHCH_2CH_2CH_2$), 3.92−4.09 (m, 1H, CHCO), 4.33 (s, 2H, $COCH_2$), 4.73−4.86 (q, J = 14.8 Hz, 2H, CH_2CH), 8.37 (s, 1H, pyridine-H), 8.59−8.98 (m, 3H, Ar).

3,5,6-Trichloro-2-pyridinol. This compound was produced as described by Beasely et al. (*34*) with slight modifications. Chlorpyrifos (742.5 mg, 2.13 mmol) was hydrolyzed by refluxing for 4 h in 9 mL of 70% (v/v) ethanol in water containing 394 mg of potassium hydroxide. The mixture was neutralized with 2 M HCl, and the product was extracted using chloroform (2 × 30 mL). After the chloroform phase was dried over anhydrous sodium sulfate, chloroform was removed under reduced pressure. The residual crystals were recrystallized from *n*-hexane to give 384.6 mg, a 91.7% yield of white needles.

Table 1. Selected Indirect Competitive ELISA Screening Data against Triclopyr and 3,5,6-Trichloro-2-pyridinol[a]

	antiserum	coating antigen	IC_{50} (nM)
triclopyr immunogen			
LysTCPY-BSA	R-20	LysTCPY-OVA	3.5
		TCPY-OVA	4.2
		245T-OVA	6.8
TCPY-BSA	R-21	TCPY-OVA	15.8
		245T-OVA	20.9
3,5,6-trichloro-2-pyridinol immunogen			
LysTCPY-BSA	R-20	LysTCPY-OVA	5.1
		TCPY-OVA	9.8
		256T-OVA	14.5
TCPY-BSA	R-21	TCPY-OVA	18.6
		245T-OVA	24.9

[a] Each standard was prepared in a 5% methanol/PBS solution.

Hapten Conjugation. The synthesized bifunctional hapten (LysTCPY, **Figure 1**), and the commercial triclopyr (TCPY, **Figure 1**), and 2,4,5-T (245T, **Table 2**) with a carboxylic acid functional group were coupled covalently to proteins by active ester method according to the procedure described by Karu et al. (*35*). Each hapten (0.1 mmol) was dissolved in 500 μL of dry DMF, and then 11.5 mg of *N*-hydroxysuccinimide (NHS) (0.1 mmol) and 20.6 mg of DCC (0.1 mmol) were added. The reaction mixture was stirred for 4 h at room temperature. The precipitate was removed by centrifugation. A 25-mg portion of BSA or 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 each) of the activated hapten solution were added dropwise to the two stirred protein solutions. The reaction mixture was stirred overnight at 4 °C, and then dialyzed against PBS (4×, 3 L each) overnight at 4 °C. The purified conjugates were lyophilized, and stored at 4 °C.

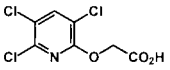
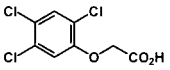
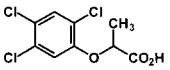
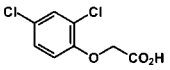
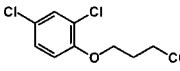
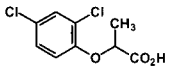
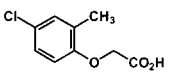
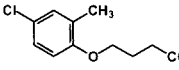
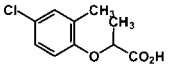
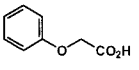
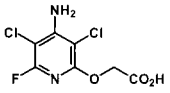
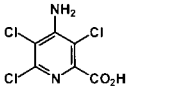
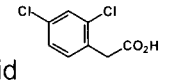
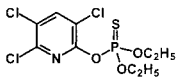
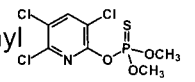
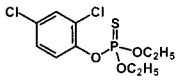
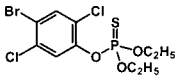
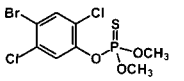
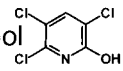
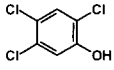
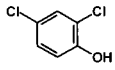
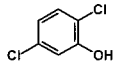
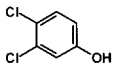
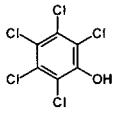
Polyclonal Antiserum Production. *System 1.* For production of group-specific polyclonal antiserum to triclopyr and 3,5,6-trichloro-2-pyridinol, a female Japan white rabbit (about 3 kg, Tokyo Animal Laboratory Inc., Tokyo, Japan) was immunized by intradermal injection in footpad sites with 1.0 mg of LysTCPY−BSA dissolved in 0.5 mL of PBS and emulsified with 0.5 mL of complete Freund's adjuvant. For the booster immunization, 0.5 mg of LysTCPY−BSA in 0.5 mL of PBS/0.5 mL of incomplete Freund's adjuvant was used. The booster immunization was made intradermally and subcutaneously at multiple sites on the back of the rabbit, and was performed at 2-week intervals after the initial immunization. The booster immunization was performed three times. The rabbit was bled from the marginal ear vein, and the hapten-specific titers of the obtained antisera were monitored. A week after the last injection, the rabbit was bled, and the antisera were collected and stored at −80 °C.

System 2. A female Japan white rabbit was immunized with 1.0 mg of TCPY−BSA. This immunization method was similar to that described above for system 1. The booster immunization (0.5 mg of TCPY−BSA) was repeated three times at 2-week intervals and then monthly.

Determination of Antiserum Titer. Triclopyr and 3,5,6-trichloro-2-pyridinol-specific polyclonal antiserum titer was determined by checkerboard titration based on indirect ELISA. Dilutions of coating antigens and antisera were chosen to produce an absorbance at 490 nm of approximately 0.5 after 30 min incubation at room temperature.

Indirect Competitive ELISA. Microplates were coated overnight at 4 °C with 100 μL of 0.1 M carbonate−bicarbonate buffer (pH 9.6) containing 62.5 ng of coating antigen. 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

Table 2. Cross-Reactivity (CR) to Triclopyr and Related Compounds with Antiserum 20[a]

compound	structure	IC_{50} (nM) [CR (%) [b]]
<phenoxyalkanoic acids>		
triclopyr		3.5 [100]
2,4,5-T		4.7 [75]
silvex		10.6 [33]
2,4-D		116.3 [3]
2,4-DB		4750 [0.07]
dichlorprop		>10000 [<0.01]
MCPA		>10000 [<0.01]
MCPB		>10000 [<0.01]
mecoprop		>10000 [<0.01]
phenoxyacetic acid		>10000 [<0.01]
fluroxypyr		>10000 [<0.01]
<other compounds>		
picloram		>10000 [<0.01]
2,4-dichloro phenylacetic acid		>10000 [<0.01]
<organophosphates>		
chlorpyrifos		42.2 [8.3]
chlorpyrifos methyl		49.3 [7.1]
dichlofenthion		3250 [0.1]
bromophos-ethyl		>10000 [<0.01]
bromophos		>10000 [<0.01]
<chlorinated pyridinol and phenols>		
3,5,6-trichloro-2-pyridinol		5.1 [69.3]
2,4,5-trichlorophenol		11.4 [30.8]
2,4-dichlorophenol		1750 [0.2]
2,5-dichlorophenol		>10000 [<0.01]
3,4-dichlorophenol		>10000 [<0.01]
pentachlorophenol		>10000 [<0.01]

[a] Concentrations for assay were as follows: immobilized concentration of coating antigen, LysTCPY-OVA (62.5 ng/well); antiserum R-20 (1:6000, final dilution in wells). Preparation of assay conditions were as described in the Materials and Method section. [b] CR = (IC_{50} of triclopyr/IC_{50} of related compound) x 100. IC_{50} is the analyte concentration that reduces the assay signal to 50% of the maximum value.

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(25% (v/v) Block Ace in distilled water containing 0.1% (w/v) sodium azide) by incubation for 2 h at room temperature to minimize nonspecific binding in the plate. The plates were washed and incubated with antiserum diluted with PBS for 1 h at room temperature. For this step of the competitive ELISA, the plates were incubated with a mixture of a constant concentration of antiserum with various concentrations of analytes. The plates were washed and further incubated with goat anti-rabbit IgG-HRP (1:8000 in PBS, 100 μL/well) for 1 h at room temperature. The plates were washed again, and 100 μL 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 to each well. After 30 min, the reaction was stopped with 0.5 M sulfuric acid, and the absorbance at 490 nm was read and recorded. The intensity of color was inversely proportional to the concentration of free triclopyr or 3,5,6-trichloro-2-pyridinol.

Effects of Organic Solvents. The effects of organic solvents were tested by dissolving the analyte in PBS containing various proportions of solvent (1, 5, 10, 20, and 30% (v/v) final concentration of each organic solvent) and incubating these with antiserum in PBS on the coating plate. Methanol, acetone, and acetonitrile were tested in this study.

Cross-Reactivity (CR). The ability of the obtained antiserum to recognize several structurally related compounds was tested by

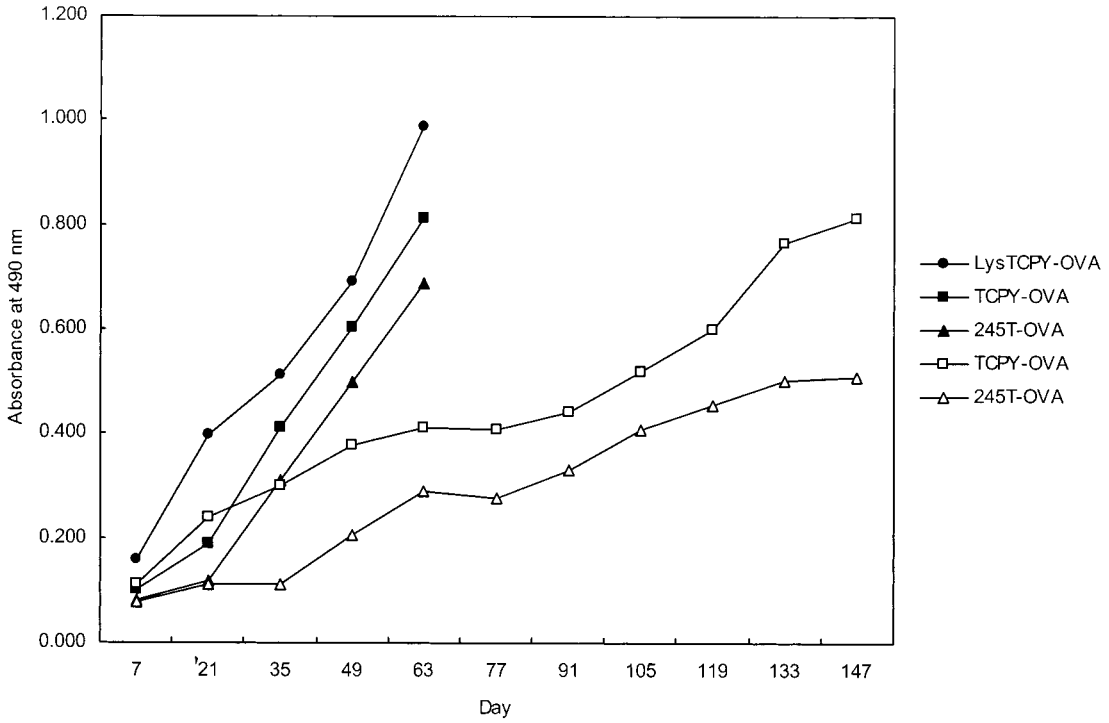

Figure 3. Antisera titers obtained with bifunctional hapten, LysTCPY-BSA and conventional hapten, TCPY-BSA. The antiserum R-20 titer obtained from rabbit immunized with LysTCPY-BSA is shown by solid symbols, and the antiserum R-21 titer obtained from rabbit immunized with TCPY-BSA is shown by open symbols. The antisera titers were estimated by indirect ELISA with each antiserum diluted at 1:6000 (final dilution in wells) and each coating antigen immobilized at 125 ng/well. The days (*x* axis) shown are the total number of days after first immunization. The booster injection was performed 3 or 5 times after first immunization.

performing competitive assays and determining their respective IC_{50} (nanomolar) values (analyte concentration that reduces the maximum signal of the competitive ELISA to 50%). CR value was calculated as [IC_{50}(triclopyr)/IC_{50}(related compound)] × 100.

Water Sample Analysis. The optimized ELISA was applied to triclopyr or 3,5,6-trichloro-2-pyridinol determinations in different water samples. Water samples were fortified with triclopyr or 3,5,6-trichloro-2-pyridinol to evaluate potential matrix effects in ELISAs. The waters tested were Milli-Q-purified water, tap water, a commercial bottled water, and samples from Tama River (Tokyo, Japan). The river water was collected in 1-L bottles and stored at 4 °C until required. For ELISA analysis, 10 mL of water was spiked with known concentrations of each targeted compound covering the quantitative working range. Tap water and river water samples were filtered through a 0.45-μm nylon filter and adjusted to pH 7.2 with PBS. Aliquots (500 μL) were then mixed with 500 μL of antiserum (1:6000 in PBS), and used in the ELISA. Analyte concentrations were interpolated from response curves for reference standards in PBS.

RESULTS AND DISCUSSIONS

Hapten Synthesis. Triclopyr and 3,5,6-trichloro-2-pyridinol have a common structure, that is, a 3,5,6-trichloro-2-pyridyloxy group in their molecules (**Figure 1**). In the present study, to obtain group-specific antiserum to triclopyr and 3,5,6-trichloro-2-pyridinol, we paid attention to this common structure, and a bifunctional hapten with a DNP group was designed and synthesized as shown in **Figures 1** and **2**. This hapten evoked an antiserum which group-specifically reacted to fenitrothion and its related compounds, fenitrooxon and 3-methyl-4-nitrophenol, only 40 days after the primary immunization. The function of the DNP group is the promotive effect of ability of antibody production in animals as reported by Eisen and Siskind (*36*). On the other hand, Goodman et al. (*37*) reported a unique bifunctional antigen introduced DNP group to activate T-lymphocyte responses. In the present study, we considered the interesting function of the DNP group, and applied it to antibody production for low molecular compound. The final goal in this study was to obtain antiserum in a shorter period by using bifunctional hapten. The previously described LysMNPA for fenitrothion and its metabolite consisted of lysine derivatized at its α-amino group with 3-methyl-4-nitrophenoxyacetic acid, and derivatized at its ϵ-amino group with DNP group. The LysTCPY described here has an analogue structure. The amide and carbonyl groups are thought to make the spacer arm stiffer and more planar, allowing more space for the lysine α-carboxyl group to be covalently coupled to the carrier protein.

Comparison of Immunoresponse to Conjugate of each Hapten. To evaluate the promotive effect of the DNP group introduced into LysTCPY in the immunized animal, the titer of the antiserum R-20 obtained from the rabbit immunized with LysTCPY−BSA (System 1) was compared with the titer of the antiserum R-21 from the rabbit immunized with the monofunctional hapten, TCPY-BSA (System 2). The titer of the antiserum R-20 was regularly monitored against three kinds of coating antigens, and the titer of the antiserum R-21 was regularly monitored against two kinds of coating antigens using checkerboard titration. As shown in **Figure 3**, the titer of the antiserum R-20 against the homologous hapten, LysTCPY-OVA, was remarkably increased after the first booster immunization, and the one against heterologous hapten, TCPY-OVA, was linearly increased after the second booster immunization. On the other hand, the increase of the titer of the antiserum R-21 was clearly later than the one of the antiserum R-20, and it took 147 days from the primary immunization to come to same titer level as that of the antiserum R-20 after 63 days. Although this result is the increase of the titer against homologous hapten, TCPY-OVA, the one against heterologous hapten, 245T-OVA, was still insufficient for triclopyr or 3,5,6-trichloro-2-pyridinol

measurement. From these findings, the DNP group introduced into LysTCPY had the promotive effect to the ability of antibody production in rabbit as might have been expected. It was successful in obtaining the antiserum with enough titer for fenitrothion or its related compounds measurement only 40 days after the primary immunization when the group-specific antiserum to these compounds was produced based on the concept in our previous report [unpublished data]. Thus, in the present results as well as the previous development of antiserum to fenitrothion and its metabolites, the bifunctional haptens with a DNP moiety made it possible to develop a strong antibody response in a shorter time, with fewer boosts.

Screening and Selection of Combination of Immunoreagents. In this section, the antiserum R-20 obtained from rabbit immunized with LysTCPY-BSA after 63 days from the primary immunization, and the antiserum R-21 obtained from rabbit immunized with TCPY-BSA after 147 days from the primary immunization were used to screen against each coating antigen. Furthermore, combinations of coating antigen and an antiserum having an optical density of >0.5 were selected, and then subjected to competitive inhibition experiments against triclopyr and 3,5,6-trichloro-2-pyridinol. When both the antisera R-20 and R-21 were diluted at 1:6000 (final dilution in wells), and the immobilized concentration of three kinds of coating antigens was 62.5 ng/well, the optimal absorbances were shown. On the basis of the conditions shown in **Table 1** competitive inhibition to triclopyr and 3,5,6-trichloro-2-pyridinol was performed. The homologous assay using LysTCPY-OVA as the coating antigen had a higher sensitivity to both compounds than the homologous assay using TCPY-OVA as the coating antigen. Furthermore, in the case of the heterologous assay using 245T-OVA changed from pyridyl nitrogen to carbon, the sensitivity to triclopyr and 3,5,6-trichloro-2-pyridinol was roughly 2−3 times more sensitive (lower IC_{50} value) than that of the homologous assay. The optimum ELISA was obtained using antiserum R-20 diluted 1:6000 and LysTCPY-OVA (62.5 ng/well) as the coating conjugate.

Cross-Reactivity (CR). The specificity of antiserum R-20 was evaluated with compounds of closely related molecular structure (such as phenoxyacetic acid derivatives, organophosphorus insecticides, and phenolic compounds): their respective IC_{50} values were obtained, and these data were compared with triclopyr IC_{50} value. CR values for each compound are given in **Table 2**. Antiserum R-20 showed 75% CR with 2,4,5-T, which has a carbon instead of the pyridyl nitrogen. Silvex, which also has the 2,4,5-trichlorination pattern, was 33% cross-reactive, indicating that the methyl group at the α-carbon caused relatively little hindrance of binding. By contrast, 2,4-D was only 3% cross-reactive, indicating that chlorine at the 5-position was very important for evoking the desired antibody. Dichlorprop, which lacks the 5-chlorine and has the methyl group at the α-carbon, had virtually no cross-reaction with antiserum R-20. Mecoprop, which has methyl groups on 2-position of the aromatic ring and on the α-carbon, and MCPA and MCPB, which are methylated at 2-position, all lack chlorination at 5-position, and their CR with antiserum R-20 is negligible. Chlorpyrifos and chlorpyrifos-methyl, with a 3,5,6-tichloro-2-pyridiyloxy group, had less than 9% CR, and no significant CR was found for the organophosphorus insecticides (dichlofenthion, bromophos-ethyl, and bromophos). The CR was about 69% for 3,5,6-trichloro-2-pyridinol. Together, these results are consistent with the hypothesis that LysTCPY evoked both types of antibodies in antiserum R-20. ELISAs with antiserum R-20 were sensitive enough for practical detection of triclopyr, 2,4,5-T, and 3,5,6-trichloro-2-pyridinol.

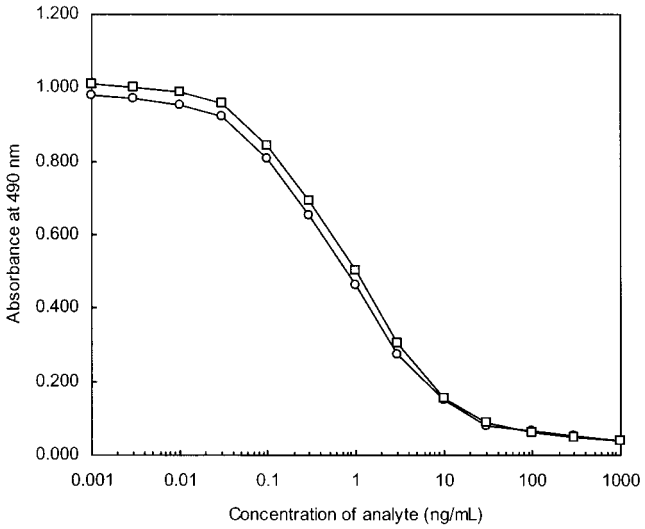

Figure 4. ELISA inhibition curve for triclopyr (○) and 3,5,6-trichloro-2-pyridinol (□). The data are corrected for background and are averages of three replicates with coefficient of variation below 13%. Concentrations for assay were as follows: immobilized concentration of coating antigen, LysTCPY-OVA, 62.5 ng/well; antiserum R-20, 1:6000, final dilution in wells; assay buffer, 10 mM PBS, pH 7.2; incubation time and temperature of each competition step, 1 h at room temperature.

Potential ELISA Interferences. Various matrixes may interfere with the antigen−antibody interaction, causing differences between expected and observed ELISA results, and poor correlation with instrumental analysis. Accordingly, we studied analyte solubility, pH, concentration of organic solvents, and the presence of Tween 20 surfactant as possible sources of interference in ELISAs with antiserum R-20.

Organic Solvent Effects. Methanol (*15*, *20*−*22*), acetone, and acetonitrile (*20*, *22*), which are commonly used to extract analytes from various matrixes or to elute analytes from solid-phase extraction (SPE) cartridges, were tested for their effects on the ELISA. The effects on hapten binding and assay sensitivity were measured as changes in the binding endpoint (maximum absorbance at 490 nm; A_{max}), and changes in IC_{50} value of a competition ELISA were both reduced (IC_{50} increased) when organic solvent concentration was increased (**Figure 5**). Methanol had the least effect and could be included at concentrations up to 10% (v/v).

Effect of pH. Many immunoassays are equally sensitive over a wide range of pH values. However, the carboxylic group on triclopyr and the hydroxyl group on 3,5,6-trichloro-2-pyridinol are potentially susceptible to ionization resulting from pH changes. To examine the influence of pH on the ELISA, competitive binding curves for each compound were obtained at pHs from 5.2 to 10.2. As shown in **Figure 6**, on the triclopyr ELISA, A_{max} did not change significantly in the tested pH change. On the other hand, IC_{50} value was remarkably increased at basic pH, that is, the sensitivity of the antiserum R-20 was about two times lower than at neutral or acidic pH. As shown in **Figure 6**, the 3,5,6-trichloro-2-pyridinol ELISA was very sensitive to pH, that is, A_{max} and IC_{50} values significantly changed at acidic and basic pH, and the sensitivity of the antiserum R-20 was about two times lower than at pH 7.2. On the basis of these results, pH 7.2 showing the highest affinity of the antiserum R-20 and the maximum absorbance, was selected as the optimum pH at which to carry out the ELISA for triclopyr and 3,5,6-trichloro-2-pyridinol.

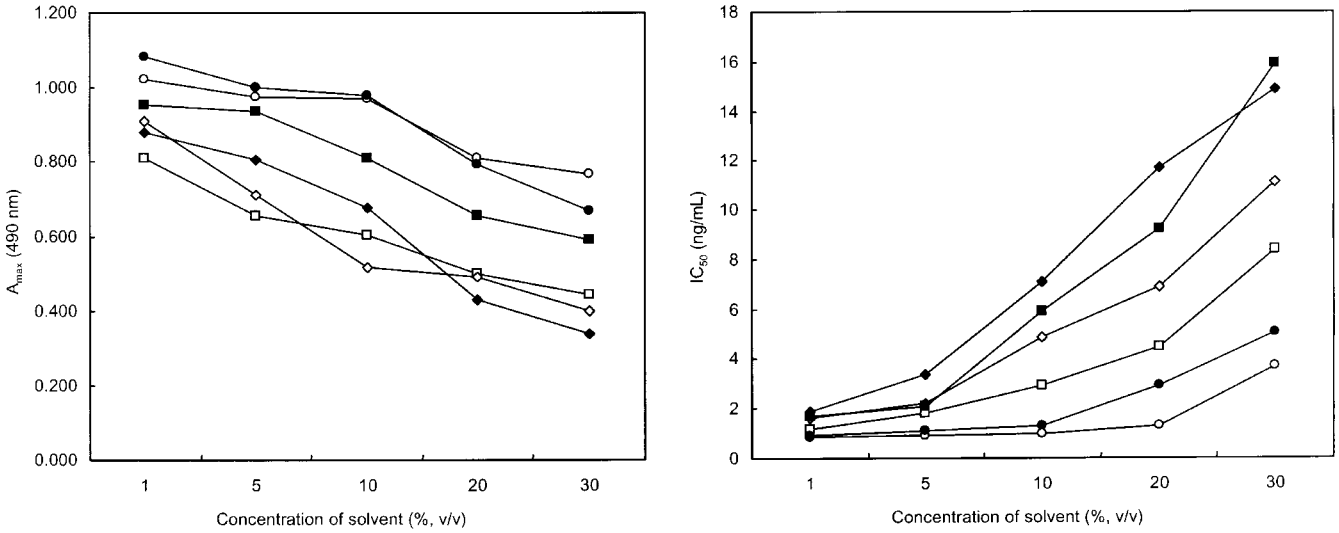

Figure 5. Influence of different organic solvents on the analytical parameters of the triclopyr (open symbols) and 3,5,6-trichloro-2-pyridinol (solid symbols) indirect competitive ELISAs. Data were obtained from standard curves performed in two replicates in buffers of different concentrations of each organic solvent (○ or ●, methanol; □ or ■, acetone; ◇ or ◆, acetonitrile). Results are the mean of three independent experiments.

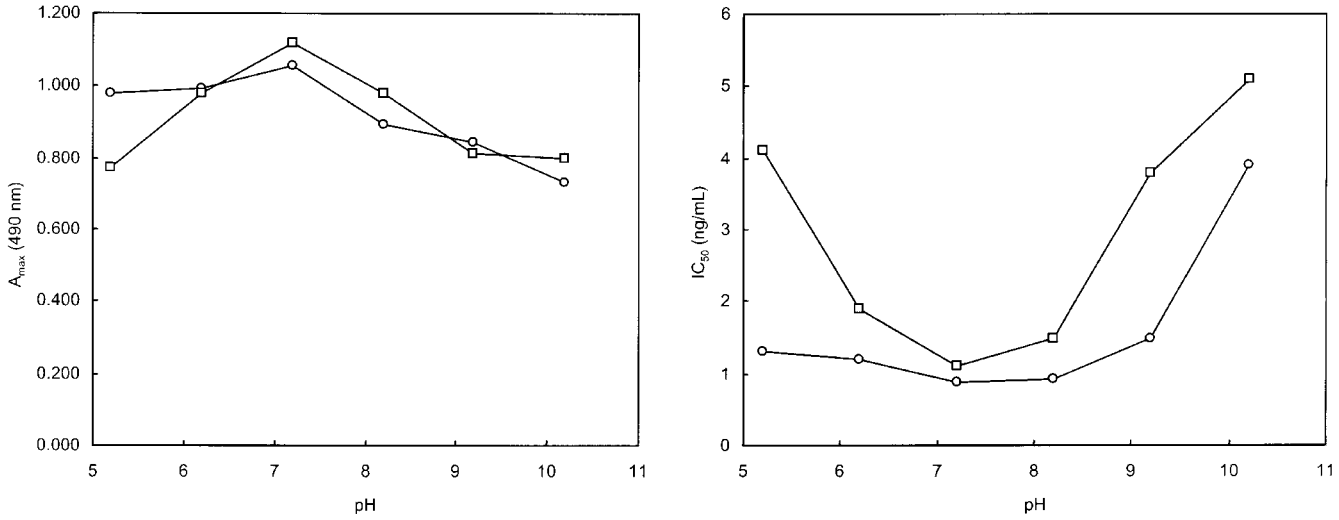

Figure 6. Influence of the assay buffer pH on the analytical characteristics of triclopyr (○) and 3,5,6-trichloro-2-pyridinol (□) competitive standard curves. The assay conditions were as follows: immobilized coating antigen, LysTCPY-OVA, 62.5 ng/well; antiserum R-20, 1:6000, final dilution in wells; incubation time and temperature of each competition step, 1 h at room temperature. The data are corrected for the background and are the average of two replicates.

Improvement of Matrix Effect and Effect of Tween 20. The influence of the matrix effects originated from four kinds of water samples was evaluated. As mentioned above, the maximum merits of ELISA are that it is possible to substantially reduce the labor of sample preparation such as concentration, SPE, or liquid−liquid partition only extraction procedure from samples. In the present study, each water sample was directly analyzed by very simple sample preparation in which the sample was diluted only with an equal volume of the antiserum R-20 solution. As shown in **Figures 7** and **8**, on both triclopyr and 3,5,6-trichloro-2-pyridinol ELISAs, a matrix effect was observed in river and tap water samples, that is, the competitive curves obtained from river water and tap water samples shifted to higher absorbance than the PBS control curve. To correct the matrix effect, it is well-known that the usage of an additive such as the nonionic detergent Tween 20 (*32*, *38*−*40*) or BSA (*41*) is very effective in suppressing nonspecific adsorption, so Tween 20 was selected as an additive. Tween 20 was investigated concerning the improvement of matrix effect and the influence on the sensitivity of the antiserum R-20. As shown in **Figures 7** and **8**, the matrix effects observed in river and tap water samples were improved by addition of 0.05% (v/v) Tween 20, and the addition of Tween 20 did not affect the sensitivity of the antiserum R-20. These results suggest that it is possible to analyze real water samples as the optimal condition in which the 0.05% (v/v) Tween 20 was added to dilution buffer.

Analytical Parameters of the Optimized Triclopyr and 3,5,6-Trichloro-2-pyridinol ELISAs. The optimized triclopyr and 3,5,6-trichloro-2-pyridinol ELISAs used coating antigen LysTCPY-OVA at 62.5 ng/well, the antiserum R-20 at a dilution of 1:6000 (final dilution in wells), and 0.05% (v/v) PBST as dilution buffer (**Table 3**). The assay affinity for triclopyr and 3,5,6-trichloro-2-pyridinol, represented by the IC_{50} values, were 0.72 and 0.95 ng/mL, respectively (**Table 3**).

There is not a general agreement for the calculation of assay sensitivity and working range of competitive immunoassays (*42*). Fleeker (*43*) reported the LOD of an assay to be three times the standard deviation of the A_0, negative control, from

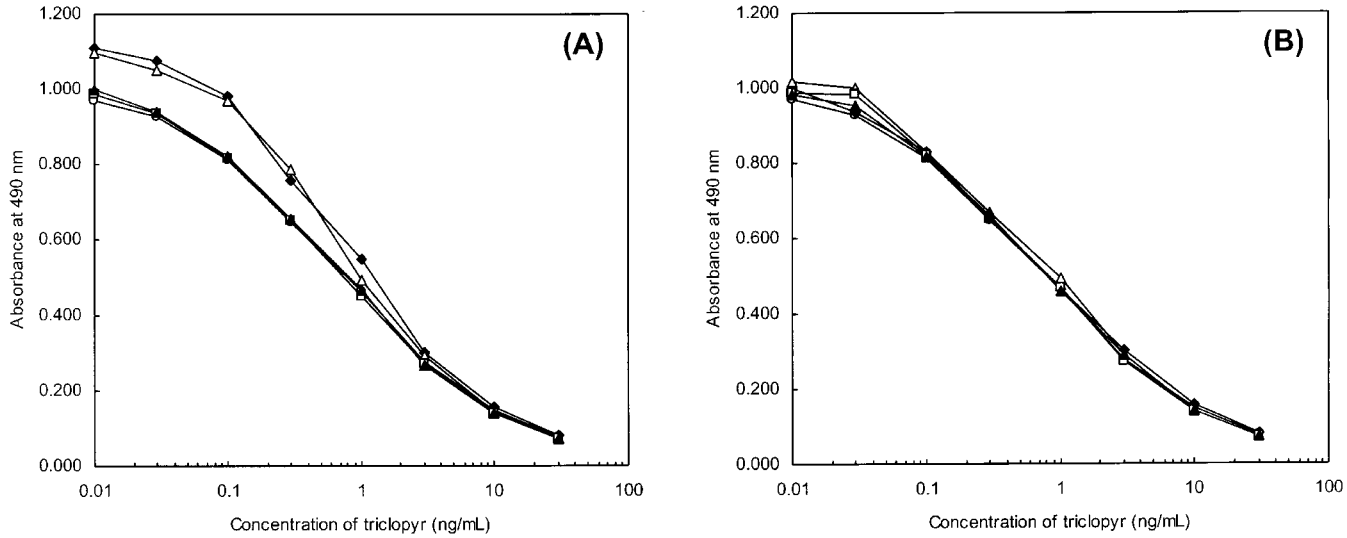

Figure 7. Comparison of triclopyr competitive curves obtained from standards prepared in PBS not containing Tween 20 (A) and containing 0.05% (v/v) Tween 20 (PBST) (B): (○) control; (◆) river water; (△) tap water; (□) purified water; and (▲) bottled water. The assay conditions were as follows: immobilized coating antigen, LysTCPY-OVA, 62.5 ng/well; antiserum R-20, 1:6000, final dilution in wells; assay buffer, PBS (A) or 0.05% (v/v) PBST (B); incubation time and temperature of each competition step, 1 h at room temperature; pH, 7.2. The data are corrected for the background and are the average of two replicates. (A) IC_{50} values were as follows: control, 0.84 ng/mL; river water, 0.84 ng/mL; tap water, 0.8 ng/mL; purified water, 0.7 ng/mL; bottled water, 0.75 ng/mL. (B) IC_{50} values were as follows: control, 0.87 ng/mL; river water, 0.75 ng/mL; tap water, 0.89 ng/mL; purified water, 0.8 ng/mL; bottled water, 0.82 ng/mL.

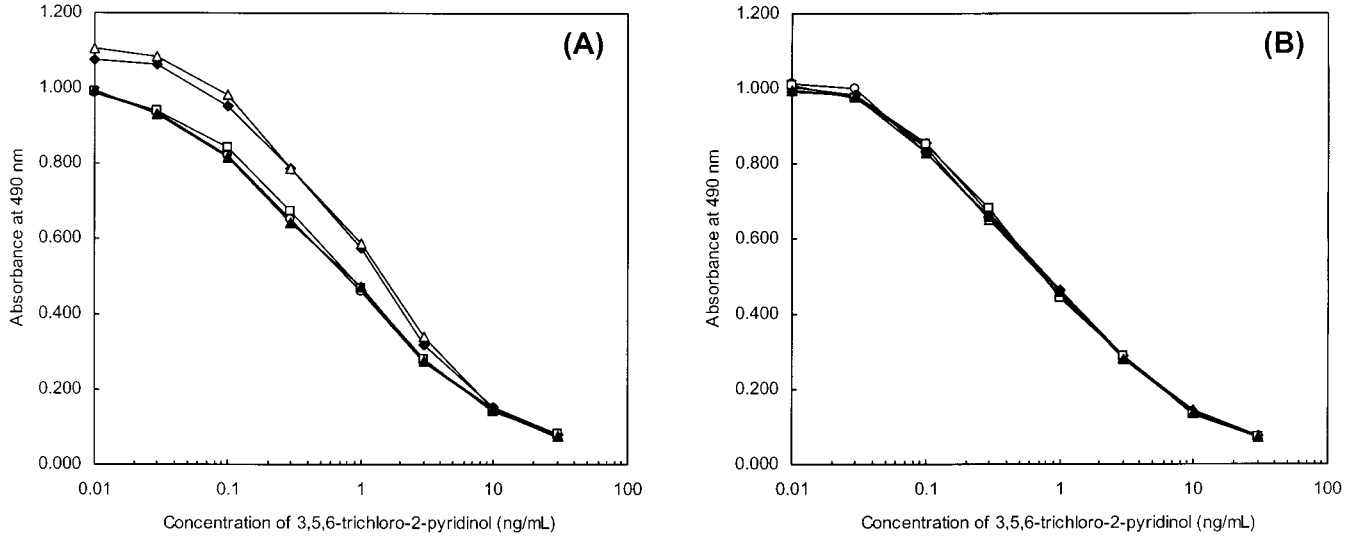

Figure 8. Comparison of 3,5,6-trichloro-2-pyridinol competitive curves obtained from standards prepared in PBS not containing Tween 20 (A) and containing 0.05% (v/v) Tween 20 (PNST) (B): (○) control; (◆) river water; (△) tap water; (□) purified water; and (▲) bottled water. The assay conditions were as follows: immobilized coating antigen, LysTCPY-OVA, 62.5 ng/mL; antiserum R-20, 1:6000, final dilution in wells; assay buffer, PBS (A) or 0.05% (v/v) PBST (B); incubation time and temperature of each competition step, 1 h at room temperature; pH, 7.2. The data are corrected for the background and are the average of two replicates. (A) IC_{50} values were as follows: control, 0.78 ng/mL; river water, 1.2 ng/mL; tap water, 1.25 ng/mL; purified water, 0.82 ng/mL; and bottled water, 0.8 ng/mL. (B) IC_{50} values were as follows: control, 0.76 ng/mL; river water, 0.75 ng/mL; tap water, 0.76 ng/mL; purified water, 0.71 ng/mL; and bottled water, 0.8 ng/mL.

its mean absorbance, whereas Midgley et al. (*44*) calculated the LOD as the concentration that corresponds to 90% of the A/A_0. For the optimized assay, the LOD was calculated according to the method reported by Midgley et al., and the quantitative working range was established between the concentrations producing 80% and 20% of the A/A_0. Using these criterions, the quantitative working range of the triclopyr ELISA was from 0.1 to 5.2 ng/mL with a LOD of 0.037 ng/mL. On the other hand, the quantitative working range of the 3,5,6-trichloro-2-pyridinol ELISA was from 0.13 to 6.0 ng/mL with a LOD of 0.052 ng/mL.

Applications to Water Samples. Spiking water samples with several amounts of triclopyr or 3,5,6-trichloro-2-pyridinol is a common practice to perform a preliminary evaluation of analytical assay reliability. Some water samples from different sources were spiked at several concentrations of triclopyr or 3,5,6-trichloro-2-pyridinol covering the optimized working range (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 triclopyr and 3,5,6-trichloro-2-pyridinol concentrations. Determinations were made in quadruplicate, and the mean absorbance was used to estimate triclopyr

Table 3. Recoveries of Triclopyr and 3,5,6-Trichloro-2-pyridinol from Spiked Water Samples Measured by the Optimized ELISAs

sample	added (ng/mL)	recovered[a] (ng/mL)	SD[b] (ng/mL)	CV[c] (%)	recovery (%)
		triclopyr			
river water	1	1.1	0.141	12.9	110.0
	5	4.9	0.250	5.1	97.5
	10	9.8	0.472	4.8	97.8
tap water	1	1.0	0.103	10.2	101.3
	5	5.1	0.311	6.2	101.0
	10	10.4	0.436	4.2	103.5
purified water	1	1.1	0.126	11.7	107.5
	5	4.9	0.340	6.9	98.5
	10	10.0	0.479	4.8	99.8
bottled water	1	1.0	0.086	8.8	97.8
	5	5.2	0.377	7.3	103.5
	10	10.1	0.275	2.7	100.8
mean				**7.1**	**101.6**
		3,5,6-trichloro-2-pyridinol			
river water	1	1.0	0.183	18.3	100.0
	5	4.9	0.497	10.1	98.0
	10	9.7	0.730	7.5	97.0
tap water	1	1.0	0.128	12.3	104.5
	5	5.0	0.350	7.0	99.5
	10	10.0	0.804	8.0	100.0
purified water	1	1.0	0.115	11.5	100.0
	5	4.8	0.332	7.0	95.0
	10	10.1	0.822	8.2	100.8
bottled water	1	0.9	0.128	13.9	92.0
	5	5.3	0.356	6.7	106.0
	10	10.4	0.330	3.2	104.3
mean				**9.5**	**99.8**

[a] Each determination was run in quadruplicate, and the mean absorbance was interpolated from a standard curve performed in the same ELISA plate. Data are the average of 3 independent determinations. [b] Standard deviation. [c] Intra-assay coefficient of variation.

or 3,5,6-trichloro-2-pyridinol 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 3**. Control samples without triclopyr or 3,5,6-trichloro-2-pyridinol 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 2.7−12.9% on the triclopyr ELISA, and from 3.2−18.3% on the 3,5,6-trichloro-2-pyridinol ELISA. The ELISA data showed a slight tendency for overestimation compared to theoretical concentration level. However, accuracy was excellent.

CONCLUSIONS.

Group-specific antiserum to triclopyr and 3,5,6-trichloro-2-pyridinol was raised to the novel hapten, "bifunctional hapten". Immunological response of the obtained antiserum to each coating antigen were satisfactory and finally rendered sensitive, group-specific antiserum to triclopyr and 3,5,6-trichloro-2-pyridinol at only 63 days after the primary immunization. The obtained antiserum affinity to these compounds was improved using structurally different hapten heterology. Significant cross-reactivity of the antiserum was found only to 2,4,5-T, while some (greater than 30%) cross-reactivity was found to silvex and 2,4,5-trichlorophenol. Next, to achieve the best assay performance, several external factors affecting the specific immunochemical interactions have been demonstrated, and the matrix effect observed in river water and tap water samples was improved by addition of Tween 20 into the assay buffer.

In particular, on the triclopyr ELISA, the IC_{50} value is about two times lower than that of the ELISA reported by Johnson and Hall (*29*). In the final optimum conditions, assay precision and accuracy in the presence of matrix effects from real water samples were investigated. The satisfactory recoveries suggested that the proposed ELISA for two compounds can be used for the preliminary screening method in water samples.

ABBREVIATIONS USED

A_{max}, maximum absorbance in the absence of competing analyte; BSA, bovine serum albumin; CR, cross-reactivity; CV, coefficients of variation; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, *N*,*N*-dimethyl sulfoxide; DNP, 2,4-dinitrophenyl; ECD, electron capture detector; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; ^{1}H NMR, proton nuclear magnetic resonance; HOBt, 1-hydroxy-1*H*-benzotriazole monohydrate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IC_{50}, concentration giving 50% inhibition of maximum response; IgG, immunoglobulin; LOD, limit of detection; LysMNPA, 2-[[[(3-methyl-4-nitrophenyl)oxy]methylcarbonyl]-amino]-6-(2,4-dinitrophenyl)aminohexanoic acid; LysTCPY, 2-[[[(3,5,6-trichloro-2-pyridinyl)oxy]methylcarbonyl]amino]-6-(2,4-dinitrophenyl)aminohexanoic acid; MCPA, 4-chloro-*o*-tolyloxyacetic acid; MCPB, 4-(4-chloro-*o*-tolyloxy)butyric acid; NHS, *N*-hydroxysuccinimide; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, 10 mM phosphate buffer, 0.9% (w/v) NaCl, pH 7.2; PBST, PBS containing 0.05% (v/v) Tween 20; SD, standard deviation; SPE, solid-phase extraction; 245T, 2,4,5-T; TCPY, triclopyr; TEA, triethylamine; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMS, tetramethylsilane.

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