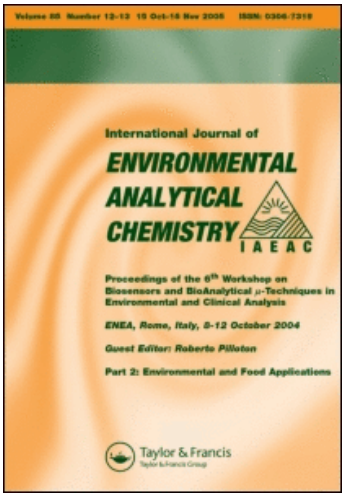

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DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE HERBICIDE PROPANIL

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Although the extensively used postemergence herbicide propanil itself is of low acute toxicity in mammals, it raises environmental concerns due to its effect on aquatic organisms and other adverse impacts. Therefore, in order to obtain a rapid analytical method for this pesticide, an indirect enzyme-linked immunosorbent assay (ELISA) has been developed. Antibodies obtained against a conjugate of 3,4-dichloroaniline coupled to succinylated proteins were tested in hapten-homologous and heterologous indirect ELISA formats using various *N*-(dichlorophenyl)-succinamic acid derivatives conjugated to carrier proteins as coating antigens. Titers in ELISA were found to be significantly affected by the type and quantity of coating antigen. One of the optimized systems using *N*-(2,4-dichlorophenyl)-succinamic acid and *N*-(3,5-dichlorophenyl)-succinamic acid conjugated to ovalbumin allowed serum dilution of 1 : 10,000 and IC_{50} values of 2.2 and 2.7 ng/mL for propanil, respectively. The limit of detection (LOD) of the immunoassays is 0.2 ng/mL. Other optimized ELISA systems based on different dichloroaniline-based coating antigens also offered similar sensitivities. The ELISA systems appeared to tolerate methanol and ethanol upto 5% concentration. For confirmatory purposes, the ELISA protocol was compared with a highly sensitive gas chromatographic method coupled with mass spectrometric detection (GC-MS). Spiked propanil content was detected both by ELISA and GC-MS in methanolic rice extract. Detection sensitivities of the two analytical systems appeared to closely correlate with each other in the range of 10–90 ng/mL (0.02–0.18 μg/g), indicating the utility of the immunoanalytical method in detecting propanil content in rice, the main commodity propanil is being applied on.

Keywords: Propanil; Enzyme immunoassay; Optimization; Sensitivity

INTRODUCTION

Propanil (*N*-(3,4-dichlorophenyl)propanamide) is a postemergence contact herbicide extensively used worldwide in rice. The compound is rapidly decomposed in the environment by microbial degradation [1] or by photolysis [2]. As a consequence, it exerts

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no residual activity; its half-life can be as short as 1.5 days in soil. Nonetheless, propanil appears to be mobile in soil and relatively stable to alkaline hydrolysis. Degradation pathways were extensively studied in distilled [3] and natural water [1], as well as in soil [4–8], and the major metabolite was found to be 3,4-dichloroaniline (3,4-DCA).

Although propanil itself is of low toxicity in mammals [9,10] and exerts no genotoxic effect in the *Salmonella* test [11], it raises environmental concerns due to its effect on aquatic organisms [12–19], as well as health related concerns due to its known adverse impacts including immunomodulatory effects [20–24], genotoxicity [25] and methemoglobinemia [26]. Moreover, its main metabolite, 3,4-DCA is mineralized only very slowly in soil, and appears to be bioaccumulated in fish [27], and some of its toxic metabolites and possible impurities (e.g., 3,3′,4,4′-tetrachloroazobenzene) [28] are considered to be structural analogs of certain dioxin compounds [9] and were shown to exert a comparable chloracnegenic potential [29].

As a result, maximal residue levels were specified in drinking water quality guidelines for Europe [30], and the compound was systematically monitored, e.g. in surface and ground waters in Greece [31] (where propanil was found a commonly encountered contaminant), in surface water and soil in Spain [32], and river waters in Portugal [33].

Propanil is most commonly analyzed by gas chromatography after chemical derivatization [34,35] preferably with electron-capture (EC) detection [36], or with mass spectrometry [37]. The parent compound can be detected through its aniline metabolite in matrices e.g., urine by GC using EC detector [38]. High-performance liquid chromatography [1,3,36,39–41] is also commonly used, mostly with UV detection, and recently with mass spectrometric detection [32]. In addition, both sorption chromatography on a two-step silica/aluminium oxide microcolumn [42] and special solid phase extraction with yeast cells used in an on-line silica gel precolumn prior to liquid chromatography [43] has been reported, offering detection limits within the range of 0.01–0.5 μg/L.

As for alternative detection methods, an amperometric sensor has been reported for propanil [44], but to date no immunoanalytical systems have been utilized for the detection of this compound. In order to allow rapid monitoring of propanil, we developed various immobilized antigen based enzyme-linked immunosorbent assay (ELISA) systems in hapten-homologous and hapten-heterologous formats for this analyte. The work reported in this paper includes immunoassay development and optimization, method development for chromatographic detection of propanil using GC-MS, and GC-MS verification of immunoanalytical results obtained in rice extract.

EXPERIMENTAL

Chemicals and Reagents

Propanil was received as a gift from Farmoplant (Milan, Italy). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL, USA) and goat anti-rabbit IgG–horseradish peroxidase conjugate from BioRad Laboratories (Hercules, CA, USA). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St. Louis, MO, USA). Pre-coated silica/aluminium sheets (250 μm silica gel F_{254} plates) for analytical thin-layer chromatography (TLC) were purchased from KGaA (Darmstadt, Germany). Solvents used were subjected to additional purification by fractional distillation.

The purity and structures of haptens and related compounds were confirmed by melting points (uncorrected), analytical TLC and [^{1}H]-NMR spectroscopy. Spectra/Por membrane from Spectrum (Houston, TX, USA) was used for dialysis (MWCO: 12–14 kD).

The following buffers and solutions were used: (1) carbonate (coating) buffer: 100 mM sodium hydrogencarbonate (pH 9.6) in distilled water; (2) phosphate buffer: saline (PBS) 150 mM sodium chloride and 15 mM potassium dihydrogenphosphate (pH 7.4) in distilled water; (3) assay buffer: PBS buffer containing 0.05% (v/v) Tween 20 (PBST 0.05); (4) washing buffer: PBS buffer containing 0.2% (v/v) Tween (PBST 0.2). Substrate, chromophore and other reagents for ELISA are listed in the detailed protocol description below.

Instruments

Gas chromatography was carried out on a Dani GC 1000 instrument coupled with a Hewlett Packard HP 5971A MSD quadrupole mass spectrometer or on a Varian Saturn 2000 workstation, a Varian 3800 gas chromatograph equipped with a Saturn ion-trap mass spectrometer (Varian, Walnut Creek, CA, USA). A fused silica capillary column Supelco MDN-5S, 0.25 μm film thickness, 30 m length × 0.25 mm internal diameter was employed. Three ions (*m*/*z* 161, 163, 217) were chosen for screening analysis in selected ion monitoring (SIM) mode on the quadrupol instrument or in selected ion storage (SIS) mode on the ion-trap instrument. Solvent delay was 4 min. Column temperature was programmed to rise from 80°C (held for 1 min) at a rate 20°C/min to a final temperature of 300°C (held for 3 min); injector temperature was 250°C in split/splitless mode. One μL of the corresponding propanil standards or spiked rice extracts were injected as methanolic solution in splitless mode for 1 min followed by 50% split mode afterwards. Helium was used as carrier gas, column head pressure was 0.07 MPa, held constant. The ion-trap was scanning in EI-mode (ionization current, 350 μA; electron energy, 70 eV) from 45 to 650 amu. The selected ions for quantitation of propanil were 161, 163 and 217.

ELISAs were carried out in high capacity 96-well microplates (Nunc, Roskilde, DK, #442404), and were read in an iEMS microplate reader (LabSystems, Helsinki, Finland).

Hapten Synthesis

Chemical structures of propanil (**1**) and the corresponding haptenic compounds are summarized in Fig. 1. Succinylated dichloroaniline haptens were synthesized by direct succinylation of the corresponding dichloroanilines (**2a–2c**). Thus, *N*-(3,4-dichlorophenyl)-succinamic acid (**3a**) was prepared from 3,4-DCA (**2a**): **2a** (0.32 g, 2 mmol) and succinic anhydride (0.20 g, 2 mmol) were dissolved in dry acetonitrile (10 mL), and the reaction mixture was stirred at 40°C for 8 h. The white crystalline product precipitated from the solution was filtered and washed with hexane to afford 0.12 mg (0.46 mmol) at a yield of 22.9%. Purity was checked by analytical TLC using a solvent system of hexane/acetone (6 : 4 v/v) with a few drops of methanol added (R_f 0.30–0.39). m.p. 158–160°C (Lit. 158–159°C) [45]. Anal. found: Cl, 25.07%. Calcd. for $C_{10}H_9Cl_2NO_3$: Cl, 27.05%; IR(KBr, cm^{-1}) 3300–2500 (ν COOH, br); 3290 (ν NH), 1698 (ν C=O); 1665 (ν CONH), 1588 (ν C–C aromatic), 852 (δ C–H, aromatic). [^{1}H]-NMR (ppm): δ 2.50–2.60 (m, 4H, CH_2), 7.46 (dd, 2H, $J_1 = 8.8$ Hz, $J_2 = 2.5$ Hz,

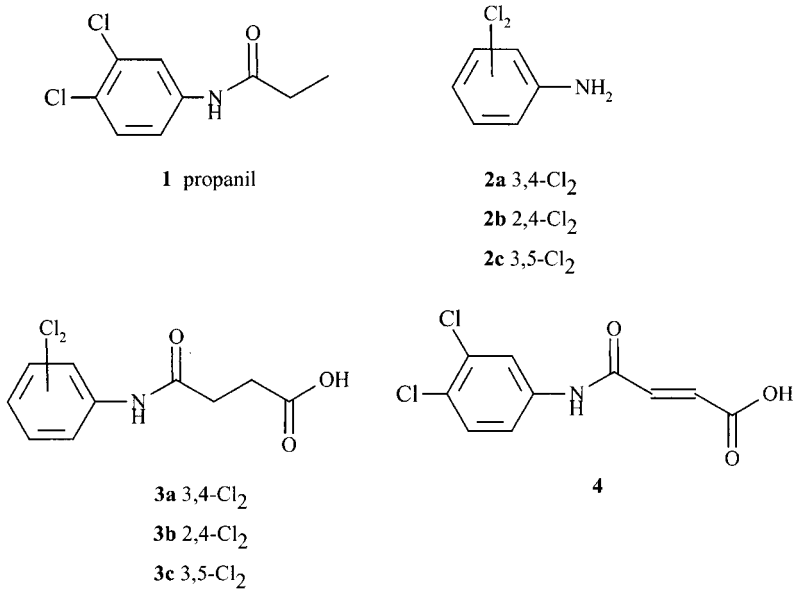

FIGURE 1 Chemical structure of propanil and the haptenic compounds used.

aromatic), 7.54 (d, 1H, $J = 8.8$ Hz, aromatic), 7.98 (d, 1H, $J = 2.5$ Hz, aromatic), 10.23 (br, 1H, CONH), 12.2 (br, 1H, COOH). (An additional 0.28 g (1.1 mmol) of the product could be isolated from the mother liquor by evaporation and subsequent purification by preparative TLC in the above solvent system.)

Succinylated derivatives of 2,4-DCA (**2b**) and 3,5-DCA (**2c**) were similarly prepared: **3b** m.p. 154°C (Lit. 150–151°C) [46], anal. found: Cl, 25.66% (calcd. for $C_{10}H_9Cl_2NO_3$: Cl, 27.05%); IR (KBr, cm^{-1}) 3300–2500 (ν COOH, br); 3282 (ν NH), 1698 (ν C=O); 1668 (ν CONH), 1581 (ν C–C aromatic), 848 (δ C–H, aromatic); [^{1}H]-NMR (ppm): δ 2.53 (t, 2H, $J = 6.9$ Hz, CH_2), 2.63 (t, 2H, $J = 6.9$ Hz, CH_2), 7.39 (dd, 2H, $J_1 = 8.8$ Hz, $J_2 = 2.5$ Hz, aromatic), 7.63 (d, 1H, $J = 8.8$ Hz, aromatic), 7.75 (d, 1H, $J = 2.5$ Hz, aromatic), 9.58 (br, 1H, CONH), 12.1 (br, 1H, COOH); **3c** m.p. 170–172°C, anal. found: Cl, 24.95% (calcd. for $C_{10}H_9Cl_2NO_3$: Cl, 27.05%); IR (KBr, cm^{-1}) 3300–2500 (ν COOH, br); 3290 (ν NH), 1700 (ν C=O); 1665 (ν CONH), 1587 (ν C–C aromatic), 851 (δ C–H, aromatic); [^{1}H]-NMR (ppm): δ 2.48–2.60 (m, 4H, CH_2), 7.23 (s, 1H, aromatic), 7.65 (s, 2H, aromatic), 10.29 (br, 1H, CONH), 12.2 (br, 1H, COOH). Moreover, *N*-(3,4-dichlorophenyl)-maleamic acid (**4**) was also synthesized from **2a** and maleic anhydride: m.p. 174–176°C (Lit. 175–177°C) [47], anal. found: Cl, 28.74% (calcd. for $C_{10}H_7Cl_2NO_3$: Cl, 27.26%); IR (KBr, cm^{-1}) 3300–2500 (ν COOH, br); 3288 (ν NH), 1699 (ν C=O); 1667 (ν CONH), 1621 (ν C=C), 1585 (ν C–C aromatic), 849 (δ C–H, aromatic); [^{1}H]-NMR (ppm): δ 5.40–5.90 (m, 2H, CH_2), 7.48 (dd, 2H, $J_1 = 8.8$ Hz, $J_2 = 2.5$ Hz, aromatic), 7.52 (d, 1H, $J = 8.8$ Hz, aromatic), 7.99 (d, 1H, $J = 2.5$ Hz, aromatic), 10.25 (br, 1 H, CONH), 12.2 (br, 1 H, COOH).

Preparation of Protein Conjugates

Protein conjugates were prepared either by coupling succinyl-DCAs or maleyl-DCA by the active ester method [48] using *N*-hydroxysuccinimide (NHS) and dicyclohexyl

carbodiimide (DCC) as a dehydrating agent, or by direct coupling of DCAs to succinylated proteins. In coupling succinyl-DCAs or maleyl-DCA to carrier proteins e.g. ovalbumin (OVA), the appropriate *N*-(dichlorophenyl)-succinamic acid hapten (49.8 mg, 0.19 mmol) and NHS (15 mg, 0.22 mmol) were dissolved in dry dimethyl formamide (DMF) (3.9 mL), DCC (45 mg, 0.22 mg) was added, and the reaction mixture was stirred for 2 h at room temperature, and was filtered. In the meantime, three solutions of OVA (150 mg) dissolved in the mixture of water (16 mL) and DMF (0.9 mL) were prepared in separate reaction vessels. Of the above activated hapten solution, 3.1 mL, 0.62 mL and 0.12 mL were given to the three vessels, respectively, resulting in three conjugates with different hapten/protein molar ratio (45.3, 9.06 and 1.75, respectively). The mixtures were stirred for 24 h at room temperature, and were dialyzed against distilled water for 2 days (water exchange every 4 h). The resultant conjugates were stored in a refrigerator until use. The same method was used to coupling hapten **3a** to gelatin and **4** to OVA.

In the direct coupling of DCA to proteins, the carrier protein was first succinylated. Thus, succinic anhydride (0.2 mL of a 0.1 M solution in dry DMF) was added dropwise at room temperature to a solution of bovine serum albumin (BSA) (7 mg, 0.1 μmol) in water (2 mL). The pH of the reaction mixture was kept at 9.0 by the addition of 1 M solution of sodium hydroxide. The reaction mixture was stirred for 2 h at room temperature, and was then dialyzed against water for 1 day and against 0.15 M phosphate buffer (pH 5.6) for two consecutive days. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (5 mg 26.01 μmol) was then added to the dialyzed solution of the activated protein, followed by the addition of 0.2 mL of a 50 mM solution of **2a**. The reaction mixture was stirred overnight at room temperature, and was then dialyzed against a 0.2% solution of sodium chloride. The dialyzed conjugate was diluted first with water and then 1 : 1 with glycerol to reach a final protein concentration of 1 mg/mL, and was kept at −20°C until use.

Immunization and Characterization of the Antisera

Two adult rabbits were immunized with **2a**-succinylated BSA conjugate. About 0.1 mg of conjugate was dissolved and emulsified with 1 mL Freund's complete adjuvant. Priming and booster injections were carried out by inoculating subcutaneously and intradermally at 20 different sites. Rabbits were boosted monthly and were bled through the ear vein within 1 week after each booster injection. For separation of cells, the blood was left at 4°C overnight, the serum was decanted and centrifuged. The serum was separated, freeze dried in 1 mL aliquots and stored in the refrigerator until use. The sera were used for ELISA either without further purification, or after collection of the IgG fraction by salt precipitation. The assessment of antiserum quality was undertaken after each immunization cycle, sera were lyophilized in aliquots of 0.5 mL and stored in a refrigerator until use. Sera were characterized by the titer (50% binding) observed in ELISA.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were carried out in 96-well microplates coated by incubating overnight at 4°C with 100 μL per well of the coating antigen in 0.1 M carbonate buffer (pH = 9.6). After washing, plates were blocked by incubation for 1 h at room temperature with 150 μL

per well of blocking agent (1% gelatin in PBS (pH 7.4)). After a washing step, standards or samples (50 μL per well) and diluted antiserum (50 μL per well) were added, and plates were incubated for 60 min at 37°C. After an additional wash, 100 μL per well of goat anti-rabbit IgG–horseradish peroxidase conjugate at a dilution of 1 : 12,000 were added, and incubated for 60 min as before. After a final washing step, 200 μL per well of the substrate solution (1.2 mM H_2O_2 with 3 mM 1,2-phenylenediamine in 0.5 M citrate buffer (pH 5.0)) were added, and after sufficient color development (after 10–60 min) the enzymatic reaction was stopped by the addition of 50 μL per well of 4 N H_2SO_4. Color intensities in the wells (endpoint mode) were read at 492 nm.

Detection of Propanil by GC-MS

Propanil was detected in methanolic solutions and in methanolic rice extracts using gas chromatography with a mass spectrometric detector (GC-MS). The injected volume was 1 μL. Propanil concentration was correlated with peak areas on the chromatogram using a standard calibration curve prepared with 6 standards and a blank (5, 3.5, 2, 1, 0.5, 0.1 and 0 μg/mL) in methanol. Thus, the injected amount of the analyte was 0–5 ng.

Sample Preparation

Because the main cultured plant propanil is being used on is rice, the developed ELISA systems were tested in spiked rice extracts for their utility to monitor propanil content. Rice samples were ground in a ball mill, and were solvent extracted for analysis. Extracts were prepared slightly differently for GC-MS or ELISA determinations. Briefly, 20 g of rice flour was extracted with 40 mL of methanol with vigorous shaking overnight. The filtered rice extract was then spiked with propanil at 8 different spiking concentrations i.e., at 0.78, 1.25, 1.56, 2.5, 3.13, 5, 6.5 and 10 μg/mL. Propanil content in spiked methanolic rice extracts was detected by GC-MS diluting the samples with methanol to reach theoretical amounts of injection of 1 ng or 0.1 ng. Concentrations of these samples were measured using the standard calibration curve in methanol (see above). For ELISA, the methanolic extract was diluted with methanol and with PBST (1 : 20) to reach optimal detection level (5–100 ng/mL in the diluted matrix), and the immunoassay protocol was carried out as seen before. In order to assess extraction recovery, rice samples were also spiked prior to extraction. Rice samples (20 g) were dried by heating at 60°C in a thermostat for 1 day, and propanil solutions in 0.5 mL of methanol were added to reach spike levels of 1–20 μg/g. Spiked rice samples were then extracted with methanol with vigorous shaking overnight, the filtered extracts were diluted 1 : 20 in PBST and were subjected to analysis as seen above. Alternatively, spiked rice samples were also subjected to extraction with PBST containing 5% of methanol.

RESULTS AND DISCUSSION

Hapten and Immunogen Syntheses

Conjugation of **2a** to succinylated proteins was sufficient for the synthesis of the immunogen. Thus, **2a** conjugated to succinylated BSA was used as the immunogen.

Nonetheless, for the purpose of coating antigen for ELISA, an excess of succinyl groups was to be avoided on the surface of the protein, therefore succinylated haptens were prepared based on methods from the literature [45,46], and were subsequently conjugated to carrier proteins OVA and gelatin. Moreover, a hapten with an unsaturated bridge, *N*-(3,4-dichlorophenyl)-maleamic acid (**4**) was also synthesized on the basis of a known procedure [47], and was conjugated to OVA. Chemical structures of haptenic compounds are depicted on Fig. 1.

Three conjugates of varying hapten/protein ratio were prepared to each succinylated DCA hapten, **3a–3c** and to the maleyl DCA hapten, **4** (conjugates of **4** were used as a quasi hapten-homologous coating antigens). In these conjugation reactions, OVA was used as a carrier protein in the coating antigens. In addition, a gelatin conjugate of **3a** was also prepared. The conjugates of each hapten corresponded to three different hapten/protein molar ratios (45.3, 9.06 and 1.75, respectively).

Serum Characteristics

Crude antisera were characterized by their titers (serum dilution causing 50% binding) in ELISA. For titration experiment, microtiter plates were coated with OVA and gelatin conjugates of the haptenic compounds, at various concentrations ranging from 0.02 to 1000 μg/mL in carbonate buffer. Optimal titers were found in the range of 1 : 1250 and of 1 : 60,000, and were found to be significantly affected by the type and quantity of coating antigen. Because three conjugates of varying hapten/protein molar ratios were available for haptens **3a–3c** and **4,** hapten coating on the microtiter plates in ELISA could be varied by either varying conjugate type or its concentration. Nonetheless, only OVA conjugates with the highest hapten/protein molar ratio allowed reasonable titers, and therefore only these conjugates were used in subsequent analyses.

As seen in Table I, C_{50} values in the optimized immunoassays appeared to be the highest (as expected) in the hapten-homologous systems using coating antigens **3a**-OVA or **3a-**gelatin. Hapten-heterologous ELISAs offered slightly improved IC_{50}

TABLE I Serum characteristics in ELISA using various coating antigens in indirect ELISA systems

Type	*Coating antigen*		*Titer*	IC_{50}[b] [ng/mL]
	Hapten/protein ratio[a]	*Concentration* [μg/mL]		
3a-OVA	h	1	1 : 4,600	11.5 ± 2.13 (0.49)
3a-gelatin	h	0.1	1 : 60,000	11.4 ± 1.89 (0.45)
3b-OVA	h	5	1 : 8,000	4.45 ± 1.69 (0.51)
3b-OVA	m	5	1 : 2,200	6.11 ± 2.14 (0.38)
3b-OVA	l	5	1 : 1,200	–[c]
3c-OVA	h	1	1 : 6,000	6.08 ± 1.70 (0.59)
4-OVA	h	5	1 : 3,000	3.77 ± 1.90 (0.60)
4-OVA	m	5	1 : 2,000	4.57 ± 1.51 (1.12)
4-OVA	l	5	1 : 1,000	3.35 ± 2.06 (1.35)

[a]Molar hapten/protein reagent ratio in the conjugate. h: high (45.3 mol/mol), m: medium (9.06 mol/mol), l: low (1.75 mol/mol).
[b]IC_{50} value defined as the analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal. Values in parentheses indicate the slope of the standard curve at the IC_{50} value.
[c]Low maximal signal level (0.2), not appropriate for analytical purposes.

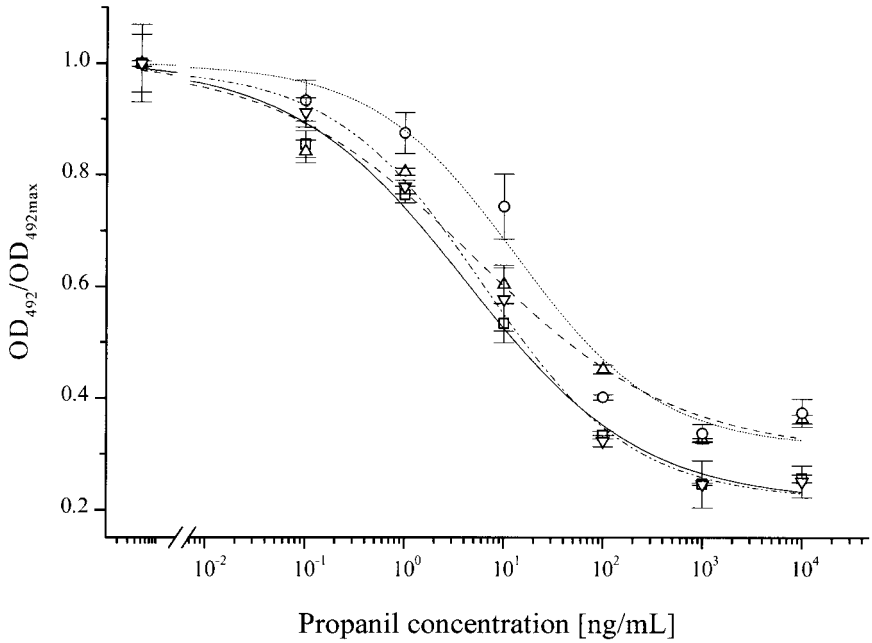

FIGURE 2 Standard inhibition curves by propanil in the optimized ELISA systems using different coating antigens. Assay conditions: coating antigen 0.1 μg/mL **3a**-gelatin, serum dilution 1 : 60,000 (◯···◯); coating antigen 5 μg/mL **3b**-OVA, serum dilution 1 : 10,000 (□—□); coating antigen 1 μg/mL **3c**-OVA, serum dilution 1 : 6,000 (▽ − · · −▽); (△− −△); coating antigen 5 μg/mL **4**-OVA, serum dilution 1 : 3000. Corresponding IC_{50} values were 11.4 ± 1.89, 4.45 ± 1.69, 6.08 ± 1.70 and 3.77 ± 1.90 ng/mL, respectively. Assays were carried out in a single microtiter plate using spiked concentrations of propanil of 10,000, 1000, 100, 10, 1 and 0.1 ng/mL and a blank.

values. In the optimized systems, antigens **3b**-OVA and **4**-OVA were both used at concentrations of 5 μg/mL for coating, while **3c**-OVA allowed a lower coating antigen concentration (1 μg/mL). With assay sensitivities, serum dilutions and standard curve shapes considered, the ELISA systems based on **3b**-OVA and **3c**-OVA appear to be optimal (with IC_{50} values of 4.45 and 6.08 ng/mL, respectively). Standard inhibition curves established for four coating antigens under optimized conditions are depicted in Fig. 2. Assay sensitivities were further increased by using the antiserum in the optimized immunoassays slightly below the corresponding titer values i.e. at a dilution 1 : 10,000. Thus coating antigen **3c**-OVA (applied at 1 μg/mL concentration in coating buffer) allowed an IC_{50} value of 2.7 ng/mL at a final serum dilution of 1 : 10,000. Alternatively, **3b**-OVA (applied at 5 μg/mL concentration in coating buffer) allowed an IC_{50} value of 2.2 ng/mL at a final serum dilution of 1 : 10,000.

The fact that hapten-homologous systems (using conjugates of **3a**) allowed poorer sensitivity is not surprising. Although the use of gelatin conjugate of **3a** allowed a better titer, it did not result in significant improvements in sensitivity relative to the corresponding OVA conjugate. The reason of the high titer seen for **3a**-gelatin relative to other titer values in the experiments is most likely the fact that this conjugate was prepared by the direct coupling of **2a** to succinylated gelatin (as opposed to coupling **3a** to gelatin) possibly resulting in either increased hapten density in the conjugate or in a large excess of free succinyl groups on the protein surface.

The effect of hapten density on assay performance is seen in Table I for conjugates in **3b**-OVA and **4**-OVA. Reducing the hapten density in the coating antigen severely affected serum binding: serum titers severely dropped when these conjugates were applied at decreasing molar hapten ratios. In the case of conjugate **3b**-OVA, the assay gradually ceased to produce a signal intensity suitable for analytical detection, while in the case of **4**-OVA, the IC_{50} value remained practically unaffected by molar hapten density in the coating antigen.

Good titers and assay sensitivities were seen with the OVA conjugate of haptens **2a** with a succinyl (**3a**) and a maleyl spacer (**4**). This indicates that a single difference of unsaturation in the spacer arm can improve assay sensitivity relative to the hapten-homologous system (**3a**-OVA).

Cross-reactivities of Related Compounds

To evaluate antibody specificity, a number of chloroanilines and derivatives, including certain urea herbicides (diuron, chlorobromuron, linuron, neburon, chlorotoluron, metoxuron), were tested for their cross-reactivities with the antibodies in two optimized ELISAs. Cross-reactivities were calculated as a percentage obtained by calculating the ratio of the IC_{50} value of the principal analyte, propanil to that of the given compound. Table II shows cross-reactivities obtained in the optimized ELISA systems. The assay showed low recognition of dichloroanilines **2a–2c** (0.56% and below), which also

TABLE II Cross-reactivities of various aniline derivatives, including propanil and certain urea herbicides in two optimized ELISA systems

Cross-reagent[a]	*%CR*[b]
Propanil	100.0
Diuron	55.2
Chlorobromuron	26.8
Linuron	20.0
3a	6.71
Neburon	5.68
3b	3.51
3c	2.78
Chlorotoluron	1.83
4	1.34
Metoxuron	0.39
2a	0.56
2b	0.31
2,4,6-Trichloroaniline	0.16
2c	< 0.16
2-Chloroaniline	< 0.16
3-Chloroaniline	< 0.16
4-Chloroaniline	< 0.16

[a]Chemical structures of the urea herbicides tested: diuron, *N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea; chlorobromuron, *N*′-(4-bromo-3-chlorophenyl)-*N*-methoxy-*N*-methylurea; linuron, *N*′-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea; neburon, *N*-butyl-*N*′-(3,4-dichlorophenyl)-*N*-methylurea; chlorotoluron, *N*′-(3-chloro-4-methylphenyl)-*N*,*N*-dimethylurea; metoxuron, *N*′-(3-chloro-4-methoxyphenyl)-*N*,*N*-dimethylurea.

[b]Relative cross-reactivity defined as a percentage obtained by calculating the ratio of the IC_{50} value of the reference compound (propanil) to that of the given compound in the ELISA system. Assay conditions: coating antigen 1 μg/mL **3c**-OVA, serum dilution 1 : 10,000.

means that the assay is of low sensitivity even for the main satellite of propanil, its metabolite 3,4-dichloroaniline. Recognition of the sucinylated or maleylated hapten (**3a–3c**, **4**) was also relatively low: below 7% even for the immunizing hapten (**3a**). (Note, however, that this hapten was present in the immunogen not as a semicarboxylic acid but as a diamide). This indicates that the serum also binds the conjugates of these haptens (as coating antigens) with low affinity, as also seen from the moderate titer values. On the other hand, the target analyte, propanil can easily compete the antibodies off the coating antigens, resulting in favorable selectivity. Similar cross-reactivities by haptens **3b**, **3c** and **4** correlate with the similar sensitivities of the ELISAs based on these conjugates as coating antigens. The assay appears to be rather sensitive to the number and position of the chlorine substituents on the aromatic ring: no monochloroaniline showed noticeable cross-reactivity, and the presence of an "extra" chlorine atom also decreased cross-reactivity, as seen for 2,4,6-trichloroaniline. Cross-reactivities by chloro-phenylurea herbicides followed a rather logical trend. The 3,4-dichloroaniline derivative diuron showed the highest cross-reactivity (above 50%), while the presence of an *N*-methoxy or a longer chain alkyl (butyl) group instead of the *N*-methyl group of diuron resulted in a sharp decline in the cross-reactivities of chlorobromuron, linuron and neburon. The loss or replacement of the chlorine atom in *para* position in diuron (chlorotolurom, methoxuron) caused an even stronger decline in cross-reactivity.

Although urea herbicides, especially diuron have significant influence on propanil ELISA, this need not necessarily present a limitation to the utility of the immunoassay as these herbicides are not used together: propanil is mostly applied for rice plantations, while diuron and other urea herbicides are used for cotton, corn, potatoes, vegetables and fruits. Therefore, urea herbicides hardly interfere with propanil determination in rice. Such interferences, however, have to be considered when these analytes are tested in river and ground water samples, if the pesticides are applied within the same region. In such cases, positive results on residues have to be verified by an alternative method (e.g. GC-MS).

Effect of Organic Solvents and Sample Matrix on Assay Performance

In order to test solvent effects of the organic solvent used for extraction from plant matrices, standard curves were established in the presence of methanol and ethanol at varying concentrations. The assay appeared to tolerate these solvents upto 5% (v/v) concentration in buffer, while 10% solvent content causes a drop in sensitivity and an apparent increase in standard error. In the case of methanol for example, the IC_{50} value of the assay increases from 4.7 at 5% methanol content to 7.9 at 10% methanol content. An apparent increase in the absolute signal was observed at methanol concentrations of 5% and 10%, while the standard error of the ELISA determinations seemed to significantly increase at methanol concentrations above 5%. The effect of methanol content on ELISA detection characteristics using conjugate **3b**-OVA is reflected in Fig. 3. The other optimized assays also displayed similar tolerance for methanol. The same tendency was observed for ethanol: IC_{50}s were found stable up to 5% ethanol content and increases over 5 times when ethanol was applied at 10% (v/v) concentration in the assay buffer.

The fact that the immunoassays tolerated methanol at least up to 5% (v/v) in the assay buffer allowed the use of solvent extraction with methanol in commodity samples.

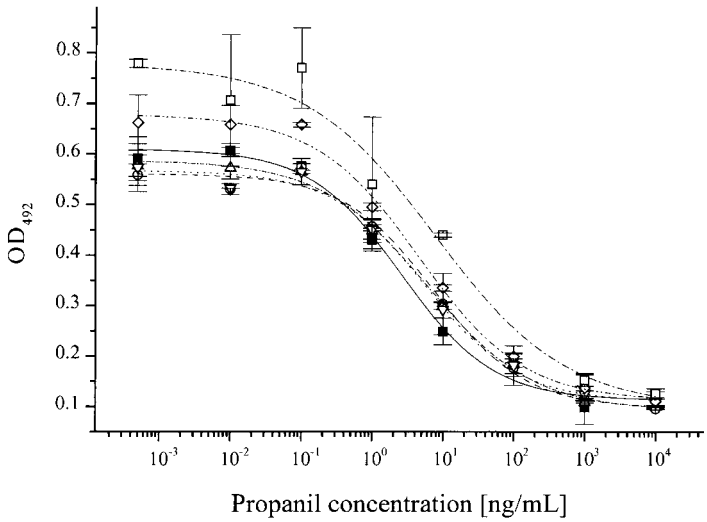

FIGURE 3 Standard curves for propanil in the optimized ELISA (**3b**-OVA as coating antigen at 5 μg/mL, serum dilution 1 : 10,000). Standard curves obtained in assay buffer (■—■) and in assay buffer containing 0.5% (◯—◯), 1% (△– · –△), 2% (▽ · · · ▽), 5% (◇– · ·–◇) and 10% (□– · –□) of methanol. Other assay conditions were as before.

For the analysis of propanil in produce, the main commodity this herbicide is applied on, i.e. rice was used. In order to assure representative sampling, sample sizes of 20 g (rice samples weighting at least 1000 grain weight) were applied. The sensitivity of the method allowed the herbicide to be readily detected in the rice matrix when methanolic rice extracts were diluted 1 : 20 with PBS; propanil content in methanolic extract samples spiked with propanil at concentrations between 5 and 250 ng/mL were detected correctly (within the range of experimental error) by ELISA. Detection at higher concentrations (e.g., 500 ng/mL or above) resulted in underestimation of propanil content. Alternatively, propanil content was also detected in methanolic extracts of spiked rice samples. For these experiments, rice samples were spiked with propanil at concentrations of 1–20 μg/g representing realistic levels with field application dosages and 1% residue levels considered.

GC-MS

Fragment ions chosen from propanil mass spectrum for selected ion monitoring (SIM) or selected ion storage (SIS) for quantification of propanil were 161 and 163. For further verification of the chemical composition, an additional fragment of m/z of 217 was also considered. A calibration curve for quantitative determination of propanil was established based on peak areas of standard solutions (5, 3.5, 2, 1, 0.5, 0.1 and 0 ng/μL) in methanol. The chosen GC column and temperature program allowed a peak retention time (R_t) of 7.76 min. The calibration between the peak area (m/z 161, 10^6) and the concentration of propanil (expressed in μg/mL) [$y = (0.744 \pm 0.026)\,x - 0.069 \pm 0.060$] offered excellent regression characteristics ($r^2 = 0.098$), and the fact that the intercept of the regression line was practically zero, indicated no systematic error in the determination. The regression line appeared to be suitable as standard curve for methanolic

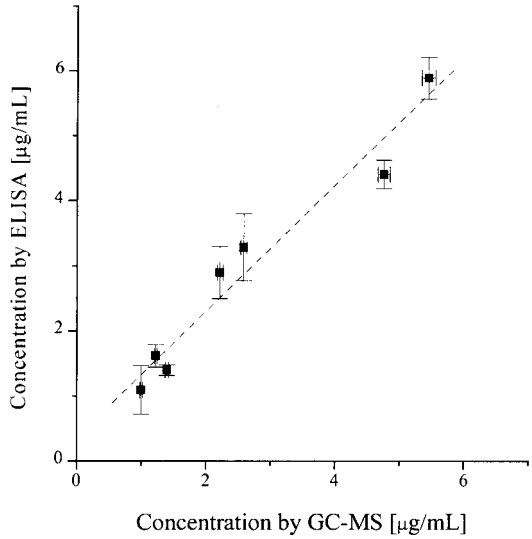

FIGURE 4 Correlation between propanil concentrations detected in spiked methanolic rice extract by ELISA and GC-MS ($n=7$, $r^2=0.953$, $c_{ELISA}=0.972\ c_{GC\text{-}MS}+0.363$). ELISA conditions: coating antigen 5 μg/mL **3b**-OVA, serum dilution 1 : 10,000.

matrix extracts. This analyte concentration range allowed the injection of 0.1–5 ng of propanil. It has to be noted, however, that the GC-MS detection would have allowed more sensitive detection as concentrations as low as 15 ng/mL (15 pg of injected amount) offered peak areas of 13,086 area units.

To test matrix effects in produce, rice extracts were spiked with propanil. These samples were measured with splitless injection using a quadrupole GC-MS instrument. Spike levels in the methanolic extract ranged between 0.78 and 10 μg/mL (corresponding to 0.39–5 μg/g in rice), where propanil was proven readily detectable by the calibration curve according to the sensitivity of the method. In GC-MS, no matrix effect was observed in the methanolic extracts, as compared to the standard calibration curve in methanol. The regression line between spiked and detected propanil concentrations indicated a slight underestimation by GC-MS (slope: 0.866 ± 0.027), in accordance with spike concentration uncertainties (data not shown). Methanolic extracts had to be analyzed by GC-MS shortly (within a few days) after being spiked: results indicated that the analyte was poorly detected in samples that have been stored longer (and transferred between laboratories). On the other hand, propanil appeared to be stable in pure methanol when stored at $-20°C$.

ELISA and GC-MS determination of propanil content in spiked methanolic rice extract, however, showed an excellent correlation (Fig. 4) with a regression line slope of 0.972 ± 0.096 indicating that the two methods detect the same concentration in the extract. Regression statistics were also good ($r^2=0.953$). The concentration range used in the correlation study corresponded to expected residue levels, and it was several orders of magnitude higher than the sensitivity of the GC-MS detection and approximately three orders of magnitude higher than the detection range of the ELISA systems allowing a sufficient dilution to eliminate possible solvent or matrix effects. These

results clearly demonstrated the utility of the optimized ELISA system for the detection of propanil in the matrix studied, methanolic rice extracts.

CONCLUSION

In the present study, an ELISA system for herbicide propanil was developed using a number of hapten-heterologous coating antigens with different chlorination pattern on the dichloroaniline moiety. The developed method allowed to detect propanil in rice grains at levels above 1 μg/g. Therefore the ELISA can successfully be used for propanil monitoring in rice under the regulatory maximum residue limit for this analyte in this commodity, 10 μg/g. Although the detection limit for the ELISA system exceeds that of one of the GS/MS and other recently developed chromatographic methods, but the great advantage of the ELISA process is rapidity and simplicity: it allows the analysis of up to 100 samples per day, while none of the chromatographic methods can offer such throughput. Thus the ELISA system can be used for screening a great number of samples and only in case of uncertainties, for instance in case of diuron influence, alternative verification is required.

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