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14. ABSTRACT

Impregnating uniforms with the insecticide permethrin may result in dermal exposure to mili-tary personnel. We identified key metabolite(s) from a low dose exposure using accelerator mass spectrometry and developed immunoassays for them for future monitoring. Metabolite stan-dards were synthesized and an HPLC system to separate individual metabolites in urine was developed. Six subjects were exposed dermally for 8h. Blood, saliva and urine samples were taken for 7d. Absorption/elimination rates and metabolite concentrations varied by individu-al. Average absorption was 0.2% of dose. Serum concentrations rose until 12-24h then rapidly declined reaching predose levels by 72h. Maximum saliva excretion occurred 6h post dosing. Maximum urinary excretion rate was 12-24h; average elimination half life was 56h. 3-Phenoxy-benzyl alcohol glucuronide (PBAG) was the most abundant metabolite identified, but most of the radioactivity was in more polar fractions for which there were no standards. The PBAG immunoassay LOQ was 1 ng/mL urine. Assays were also developed for 3-phenoxybenzoic acid, its glycine conjugate and cis/trans-dichlorovinylcyclopropane carboxylic acid glycine.

15. SUBJECT TERMS
Permethrin, immunoassay, LC/MS, accelerator mass spectrometry, human exposure, metabolism

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INTRODUCTION

The current practice of impregnating uniforms with the insecticide permethrin produces significant potential for dermal exposure to personnel such that rapid monitoring methods for exposure risk assessment would be desirable tools for troop commanders and field leaders. In addition, ensuring a safe as well as efficacious amount of repellent on uniforms is very important to soldiers to protect themselves from insecticide toxicity and yet effectively prevent insect-borne infectious diseases. The repellency of pyrethroids on uniforms is certain to decrease with aging as affected by many variables including exposure to sunlight and moisture. A portable, rapid and on-site monitoring technique for the detection of the repellent parent compound and thus repellent efficiency as well as human metabolites as an indication of exposure will be valuable to troop personnel. It is the purpose of this work to develop such monitoring tools. The scope of this project encompasses three key technologies (liquid chromatography/mass spectrometric detection; accelerator mass spectrometry and immunoassay) that are being used to conduct a human exposure study to identify key metabolite(s) of permethrin and to develop an immunoassay to the identified key metabolite(s) as a biomarker of human exposure to permethrin.

RESEARCH REPORT

A. Statement of Work

The original objectives of the research, as shown below, are unchanged from the original Statement of Work with the following exceptions: In IIA the work has been expanded to include collecting saliva samples during the same times that blood samples are collected. In IIB the work includes the analysis of saliva samples for total radioactivity. In IIIE, Dr. Alex Lu, formerly of the University of Washington, Seattle, WA, currently of Emory University (Atlanta, GA) was identified as a potential source of urine samples from people with an exposure to permethrin. This modification of the statement of work was approved in September of 2003.

I. Develop an LC/MS method for separation of permethrin and its putative human metabolites.
   - A. Synthesize metabolite standards.
   - B. Synthesize standards of conjugated metabolites.
   - C. Develop a separation method for pyrethroid metabolites in a human urine matrix using HPLC.
   - D. Evaluate the potential of LC/MS as an analytical method for permethrin and metabolites.

II. Determine the human metabolite profile of permethrin using accelerator mass spectrometry (AMS).
   - A. Expose 6 human subjects dermally, to low doses of ^{14}C-labeled permethrin.
   - B. Analyze blood for total radioactivity.
   - C. Analyze urine using the methods developed in Objective I.

III. Develop an immunoassay to the key metabolite identified in Objective II as a biomarker of human exposure to permethrin.
   - A. Synthesize haptens based on the structure of the identified metabolite.
   - B. Synthesize antigens from the haptens synthesized in IIIA.
   - C. Immunize rabbits with the antigens made in IIIB.
   - D. Develop and optimize immunoassays using reagents from IIIB and IIIC.
   - E. Conduct a laboratory validation of the optimized immunoassay using spiked urine samples.
   - F. Conduct a field sample validation of the optimized immunoassay.

B. Research Progress

Figure 1 shows the known metabolic pathways for permethrin in mammals. In mammals, the major 'detoxification' reaction for carboxylic acid-containing xenobiotics is conjugation, either with an amino acid to form a peptide or with glucuronic acid to form a glucuronide. In general, larger and more complicated cyclic acids tend to be excreted as glucuronides, and simpler acids such as benzoic acid are more commonly eliminated in the form of amino acid conjugates (Killenberg and Webster, 1980; Sipes and Gandolfi, 1991). In the few studies that have been conducted on metabolism of permethrin in humans, the urine collected from pest control workers following occupational exposure (Angerer and Ritter, 1997) or healthy volunteers using a shampoo for head lice (Tomalik-Scharte et al., 2005) was subjected to hydrolysis and then analysis. Results from these studies indicated 3-phenoxybenzoic acid (3PBA) and the *cis/trans*-dichlorovinylchrysanthemic acids (*cis/trans*-DCVA) were the major metabolites found in human urine. However, no study has reported the relative concentrations of free metabolites vs. conjugated metabolites in human urine or identified conjugated metabolites. Thus, both glucuronic acid and amino acid conjugates are possible. Because we do not know which of these conjugated

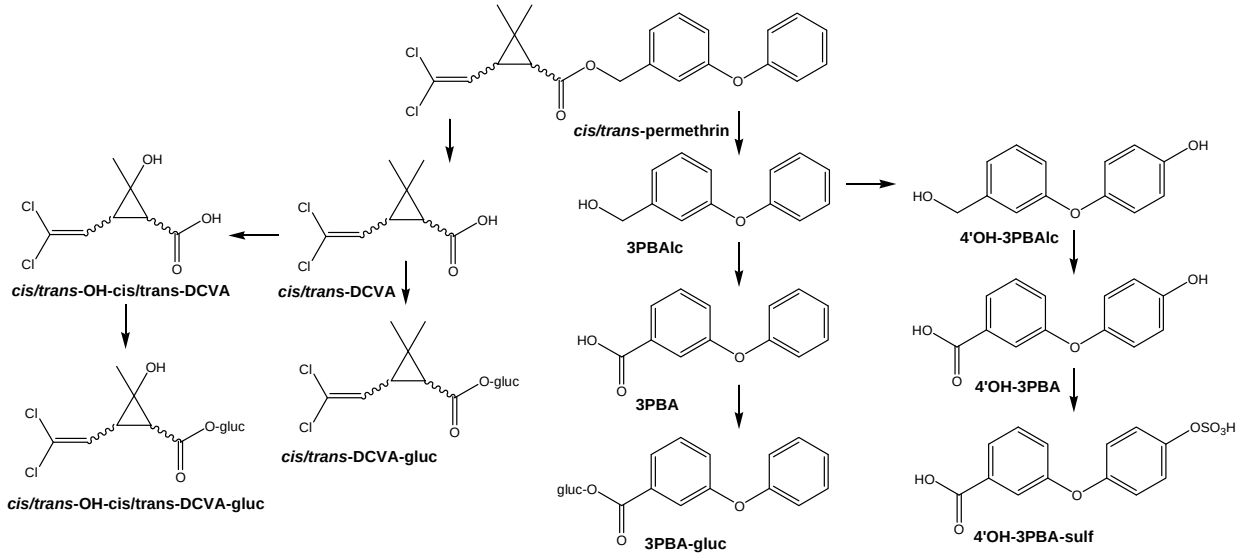


Figure 1. Metabolism of permethrin. Subjects were administered either a mixture of permethrin containing a ^{14}C label in each position or permethrin labeled in one position or the other. Carboxylesterase readily hydrolyzes permethrin to the dichlorovinylcyclopropane carboxylic acid (DCVA) and phenoxybenzyl alcohol (3PBAlc). The 3PBAlc is conjugated resulting in a glucuronide (3PBAlc-gluc, not shown) or further hydrolyzed to 3-phenoxybenzoic acid (3PBA) which is then glucuronidated. Hydroxylated 3PBA (4'OH-3PBA) is conjugated forming either a glucuronide (gluc) or a sulfate (sulf). DCVA is glucuronidated (DCVA-gluc) or hydroxylated on the gem-dimethyl groups (OH-DCVA) then glucuronidated (OH-DCVA-gluc). Glycine conjugates of DCVA and 3PBA are also postulated as well as hydroxylated parent.

metabolites will be the most abundant in humans, all were synthesized as standards for the analysis of human urine.

I. Develop an LC/MS method for separation of permethrin and its putative human metabolites.

Synthesis of metabolite standards and conjugates. We have published the synthesis of several metabolites/conjugates whose structures shown in Table 1. Structures were confirmed by melting point, thin layer chromatography (TLC), nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS). The synthesis of the glycine conjugate of DCVA and the

glucuronide conjugate of 3-PBAlc are detailed below.

N-(cis/trans-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine (cis/trans-DCVA-glycine (Ahn et al., 2004). Thionyl chloride ($SOCl_2$, 3 mL) was added to *cis*-DCVA (1500 mg, 7.17 mmol) in a 10 mL round bottomed flask. The mixture was stirred under N_2 at 65 °C for 1.5 h. The solution was concentrated under reduced pressure to remove excess $SOCl_2$. Hexane was added, and then the solution was concentrated again. The crude acid chloride was obtained as a pale yellow liquid. It was added drop wise to a vigorously stirred ice-cooled solution of glycine in 10 mL of 2 N KOH. A 1 N solution of KOH was added to keep the mixture slightly basic. After 3 h, the odor of the acid chloride was no longer detectable. The mixture was acidified with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was recrystallized from a mixture of ethyl acetate and *tert*-butyl chloride to give 1084 mg (56%) of *cis*-DCVA-glycine as a white solid; mp 141-143 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] *Rf*, 0.85. ^{1}H NMR (DMSO-*d*6): δ 1.15 (s, 1H, CH_3), 1.32 (s, 3H, CH_3), 1.85 (d, *J* = 8.5 Hz, 1H, CHCO), 2.03 (dd, *J* = 8.7, 8.7 Hz, C=C-CH), 3.78 (t, *J* = 5.8 Hz, 2H, NCH_2), 6.06 (d, *J* = 8.9 Hz, 1H, C=CH), 8.40 (t, *J* = 5.8 Hz, 1H, NH). MS-ESI$^-$ *m*/*z* calcd for [M - H]-) $C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 264.09. The *trans*-isomer (1023 mg, 53%) was prepared as a white solid from *trans*-DCVA by the same method as described above for the *cis*-isomer; mp 178-179 °C. TLC (ethyl acetate/hexane/acetic acid [1:1:0.1, v/v/v)] *Rf*, 0.75. ^{1}H NMR (DMSO-*d*6): δ 1.14 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.83 (d, *J*= 5.3 Hz, 1H, CHCO), 2.04 (dd, *J* = 5.3 Hz, 8.2 Hz, 1H, C=C-CH), 3.78 (t, *J* = 5.6 Hz, 2H, NCH_2), 6.04 (d, *J* = 8.3 Hz, 1H, C=CH), 8.36 (t, *J* = 5.8 Hz, 1H, NH). MS-ESI- *m*/*z* calcd for [M - H]-) $C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 263.95.

3-Phenoxybenzyl β-D-glucuronide (Kim et al., 2007). The synthesis requires the use of a protected glucopyranoside to prevent unwanted mixtures. The protected glucopyranoside was brominated and the brominated compound reacted with 3-phenoxybenzyl alcohol in the presence of a catalyst. The resulting compound is then de-protected (Figure 2). Methyl 1,2,3,4-

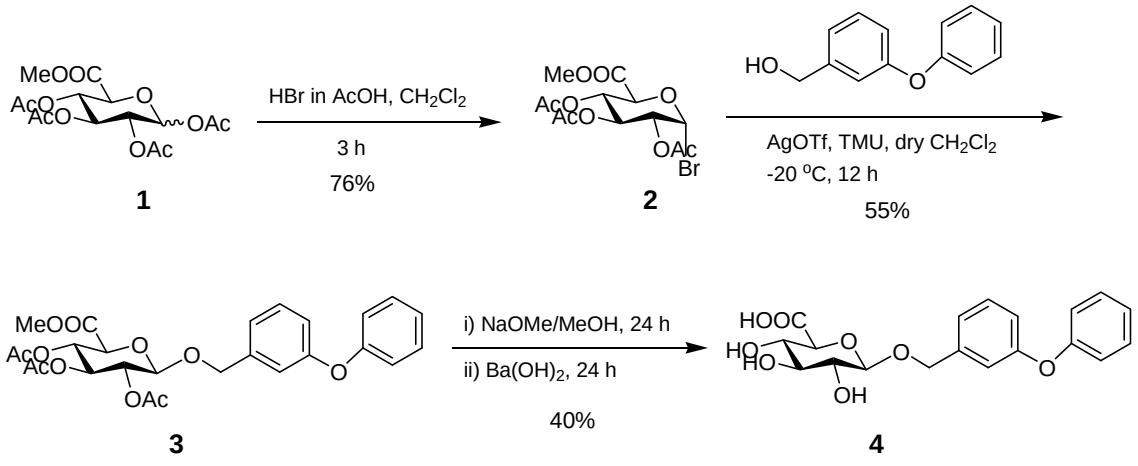


Figure 2. Scheme for the synthesis of the glucuronide conjugate of 3-phenoxybenzyl alcohol.

tetra-*O*-acetyl-D-glucopyranouronate (**1**; 7 g, 18.6 mmol) prepared from the glucuronolactone by methylation with sodium methoxide and acid-catalyzed acetylation with acetic anhydride was dissolved in dry CH_2Cl_2 (100 mL), and 45% hydrobromic acid in acetic acid (20 ml, 111.6 mmol) was added in an ice bath. After stirring at room temperature for 3 h, the mixture was diluted

with $CHCl_3$ and successively washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl. To this organic layer activated charcoal was added and then filtered to remove the color. After evaporation, the residue was recrystallized with ethyl ether to afford methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl bromide)uronate (**2**; 5.6 g, 76%). To a solution of **2** (500 mg, 1.3 mmol) in dry CH_2Cl_2 (10 mL), were added 3-phenoxybenzyl alcohol (350 mg, 1.8 mmol), dry silver (I) trifluoromethane sulfonate (400 mg, 1.6 mmol) and tetramethylurea (180 mg, 1.6 mmol), and the mixture was stirred under an argon atmosphere at -20 ˚C for 12 h. The reaction was filtered and diluted with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$ and applied to flash chromatography (stepwise elution with hexane-ethyl acetate; 20, 30 and 40% ethyl acetate) to afford 3-phenoxybenzyl methyl 2,3,4-tri-*O*-acetyl-D-glucopyranouronate (**3**; 350 mg, 55%). Compound **3** (250 mg, 0.48 mmol) was dissolved in methanol (5 mL), and a 1 M sodium methoxide solution (10 mL) was added. The mixture was stirred at room temperature for 24 h, an excess of $Ba(OH)_2$ added, and the reaction mixture was stirred at room temperature for an additional 24 h. After neutralizing with Dowex-50 (H^+-form), the mixture was filtered and evaporated *in vacuo*. The residue was purified by flash chromatography (stepwise elution with $CHCl_3$-methanol; 10, 20 and 30% methanol) and by C_{18} column (10 g; stepwise elution with acetonitrile-H_2O; 0, 10 and 20% acetonitrile). Freeze-drying gave analytically pure 3-phenoxybenzyl β-D-glucuronide (**4**) as a white powder (70 mg, 40%; total yield from **1**, 17%). Compound **4**: ^{1}H-NMR δ (CD_3OD): 3.26-3.63 (2H, Gluc H-4 and 5), 3.64 (1H, dd, *J* = 7.8 and 6.3 Hz, Gluc H-3), 4.41 (1H, dd, *J* = 7.8 and 6.3 Hz, Gluc H-2), 4.67 (1H, dd, *J* = 11.7 and 5.1 Hz, $OCH\underline{H}_aPh$), 4.87 (1H, d, *J* = 7.8, Gluc H-1), 5.01 (1H, dd, *J* = 11.7 and 5.1 Hz, $OC\underline{H}_bHPh$), 6.92-7.40 (9H, -<u>Ph</u>-O-<u>Ph</u>). HRMS $[M-H]^+$: calcd. for $C_{19}H_{19}O_8$, 375.1080 found, 375.1052 (Figure 3).

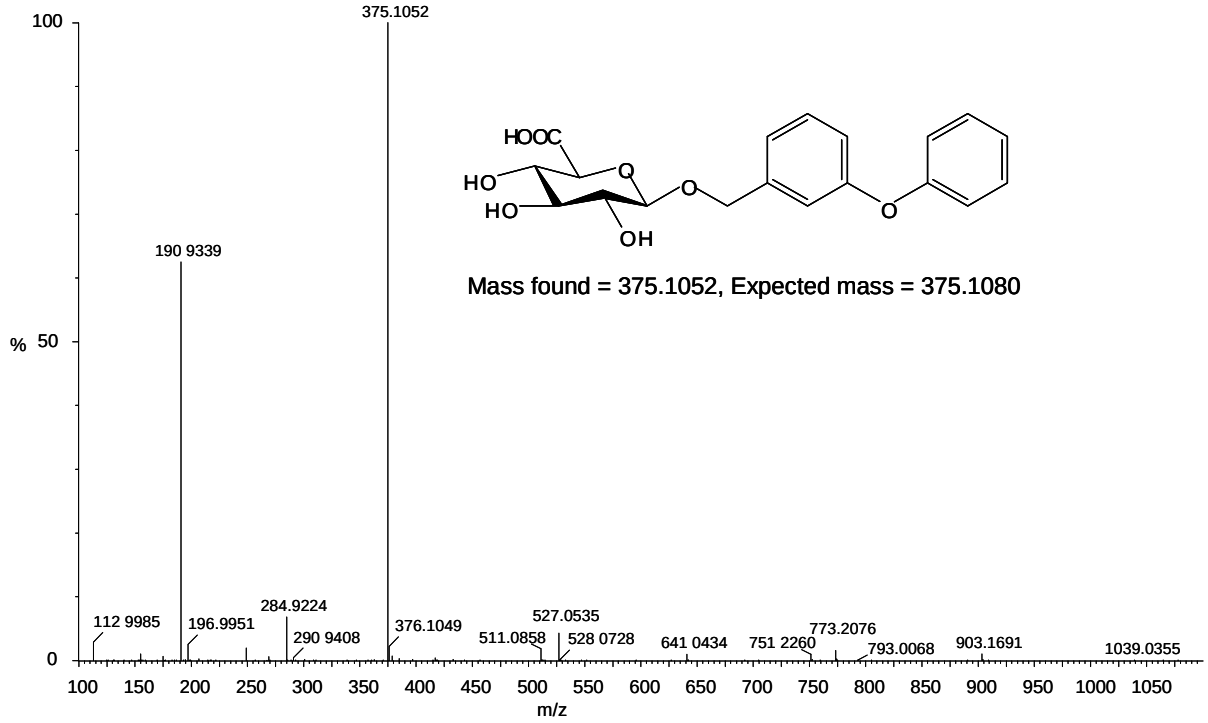
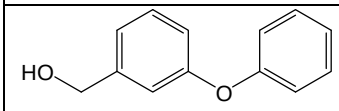
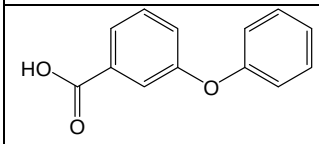
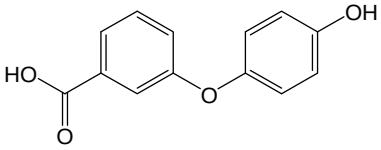
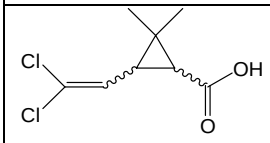
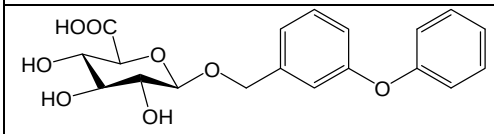
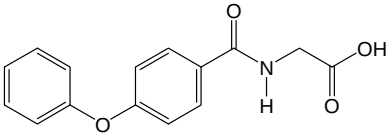
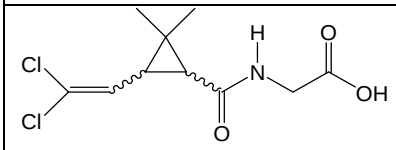
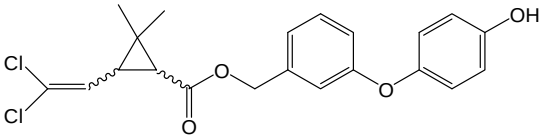


Figure 3. Exact mass determination of the glucuronide conjugate of 3-phenoxybenzyl alcohol.

Table 1. Permethrin metabolites synthesized or acquired for this project.

Metabolite	Reference
 3-Phenoxybenzyl alcohol (3-PBAlc)	Purchased
 3-Phenoxybenzoic acid (3PBA)	Purchased
 4'-Hydroxy-3-phenoxybenzoic acid	Shan et al., 2004
 *cis/trans*-Dichlorovinylcyclopropane carboxylic acid (DCVA)	Ahn et al., 2004
 3-Phenoxybenzyl alcohol glucuronide (3-PBAlc-gluc)	Kim et al., 2007
 3-PBA glycine	Shan et al., 1999 esfenvalerate metabolites
 *cis/trans*-DCVA glycine	Ahn et al., 2004
 4'-Hydroxypermethrin	Synthesized according to Zhang & Scott 1994

Development of an HPLC method to separate permethrin metabolites. An HPLC separation method was needed for both the separation of metabolites prior to analysis of samples by accelerator mass spectrometry (AMS) and for the validation of the immunoassay by LC/MS. Since the LC/MS requires a microbore LC to keep the volumes very low, we chose to use this method for the urine analysis by AMS as well. The optimized system used a Magic 2002 microbore HPLC (Michrom BioResources, Inc., Folsom, CA) equipped with an Onyx monolithic C18 (5 x 4.6 mm) precolumn and an Onyx monolithic C18 (100 X 3.0 mm) column in tandem with a Magic C_{18} (1.0 x 150 mm, 3 µ particle size, 100 Å pore size) column. The mobile phase was a gradient mixture of double distilled water adjusted with trifluoroacetic acid to pH 2.2 and 90% acetonitrile in water (v/v) adjusted to pH 2.2 with trifluoroacetic acid at a flow rate of 50 µL/min at room temperature. The gradient is detailed in Figure 4. Standards were prepared in 4% acetonitrile in 0.1M sodium phosphate, pH 7.5 and were detected with a UV/visible detector at either 230 or 210 nm. The urine samples were prepared by mixing 960 µL of filtered urine and adding 40 µL of the standard mixture and 50 µL were injected onto the column. Figure 5 is an example UV chromatogram for a urine sample collected between 12 and 24 hours post-dosing that was spiked with standards. The standards are baseline resolved and there is good resolution between *cis* and *trans* isomers of DCVA (peaks 5 and 7), DCVA-glycine (peaks 2 and 3) and permethrin (peaks 9 and 10). There is adequate time resolution between peaks to assure that fractions collected for AMS will be clearly separated. We have found previously that although UV resolution of the peaks is at baseline, resolution by AMS shows that the peaks are much broader (Buchholz et al., 1999). For LC/MS analysis, this separation system requires flow

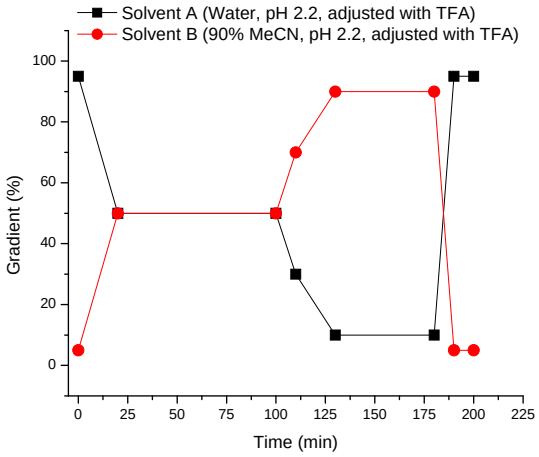


Figure 4. Details of the gradient used to separate metabolites of permethrin.

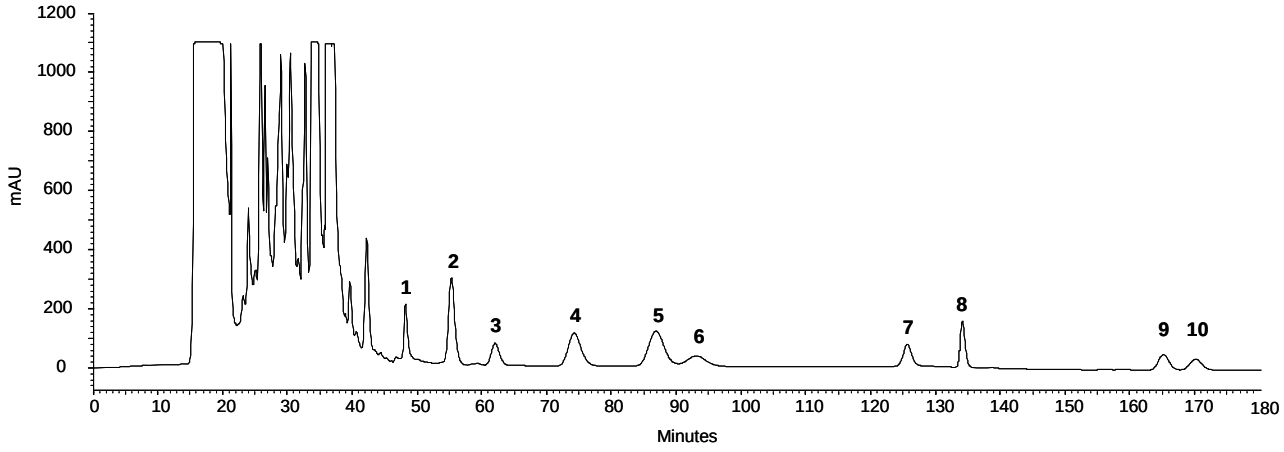


Figure 5. LC separation of the metabolites of permethrin in a spiked urine sample. Peaks for each standard were (1). Glucuronide conjugate of 3-PBAlc; (2). trans-DCVA-glycine/3PBA-glycine; (3). cis-DCVA-glycine; (4). 3-PBAlc; (5). trans-DCVA; (6). 3PBA; (7). cis-DCVA; (8). 3-phenoxybenzaldehyde; (9). trans-permethrin; (10). cis-permethrin and were detected by UV absorbance.

splitting in order to reduce the volume to the MS. Alternatively, it would be a more efficient use

of the LC/MS to develop a faster LC separation since the wider separation is not required for MS analysis or if fewer analytes will be measured.

II. Determine the human metabolite profile of permethrin using accelerator mass spectrometry (AMS).

Recruiting. Subjects recruited for this study were healthy (self-report) adult men and premenopausal women between the ages of 18-50 years. Individuals reported that they did not consume alcohol beyond a rare drink during a social event, use cigarettes, or drugs, or maintained any unusual exercise or dietary habits for the last 3 years. They were not under the care of a physician for a disease and had not been subjects in a radioactive drug research study. Women reported that they were not pregnant or breastfeeding and a urine pregnancy test was conducted just prior to beginning the study. No one reported any use of permethrin in the previous six months from common home pesticide products or to their knowledge, commercial application to their residence, nor did any of the subjects report that they were pest control operators. Six subjects were recruited to participate. Some generalized demographics are shown in Table 2.

Table 2. Subject demographics.

Gender	2 Male (Subjects 10, 14), 4 Female (Subjects 12, 16, 18, 20)
Race	3 White (2 male, 1 female), 2 Hispanic (2 female), 1 African American/Asian (female)
Age (average)	35 years (range 22 – 48 year)
Weight (average)	87 kg (range 69.5 – 118.2 kg)
Height (average)	172 cm (range 163 – 180 cm)
Body mass index (BMI)	29.2[a] (range 23.4 – 39.5)

[a] BMIs of 18.5 – 24.9 are classed as normal; BMIs of 25-29.9 are classes as overweight (BMI Calculator: http://www.nhlbisupport.com/bmi/bmicalc.htm)

Exposure. Prior to exposure, the treatment area on the forearm was cleaned with an alcohol wipe to remove any lotions or surface skin oils. Each subject was administered dermally, 25 mg of permethrin containing one µCi of radioactivity. Subjects 10, 12, 14, and 20 received a dosing solution consisting of an equal mixture of permethrin that was labeled in cyclopropane ring or the phenoxybenzyl ring. Subject 16 received permethrin labeled only in the phenoxybenzyl ring only and Subject 18 received permethrin labeled only in the cyclopropane ring. The dosing solution was prepared in isopropyl alcohol and applied onto an approximately 50-cm^2 area of one forearm. The treated area was monitored with a pancake probe Geiger counter then covered with gauze and remained in place for 8 hrs. At the end of 8 hrs, the area was swabbed several times with soap- and then water-saturated gauzes to remove surface material. Between washes, the area was monitored with a pancake probe Geiger counter until the count rate did not change. The Geiger counter provided a crude estimate of the counts applied and remaining as the probe was not large enough to cover the entire treated area at once. The probe was moved over the surface and count rates recorded at 3 different parts of the treatment area. Because the count method was relatively crude, we could not determine the amount absorbed from this method. The results averaged for the six subjects are shown in Table 3. The amount on the skin and found in the gauze cover combined accounted for about one-half of the activity, implying that one-half of the activity was absorbed; however, activity determined by accelerator mass spectrometry indicates that the absorbed

dose was closer to 0.2% of the applied dose (described below). This discrepancy is likely due to the inaccuracy and inefficiency of the survey meter. However, the survey meter did provide useful information during the individual treatments. No adverse effects were reported during the one-week period of the study.

Table 3. Dosing procedure monitoring by survey meter.

Measurement point	Average cpm ± SD	n
Immediately after applying dose	15648 ± 3323	6
Just before decontamination	4397 ± 1799	6
Retained on the gauze cover	2760 ± 701	6
Following decontamination	176 ± 116	6
7 Days post dosing	19 ± 19	5*

*One subject withdrew from the study just prior to 24 hours.

Sample collection. A 24-hour urine sample was collected the day before treatment. On treatment day, a pre-dose blood and saliva sample were taken, and then the radiolabeled permethrin was applied to the forearm and covered with a gauze patch. Blood (sampled from the untreated arm), and saliva samples were taken at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 hours post application. Urine samples were collected from 0-6 hr, 6-12 hr, 12-24 hr, 24-48, 48-72, 72-96, 96-120, 120-144 and 144-168 hrs post application. Blood samples were centrifuged to separate the serum. The serum was removed to a separate tube and both fractions were stored at -80 ^{o}C until analysis. The saliva collection device was centrifuged to extract the saliva from the cotton collection swab. The separated saliva was then stored at -80 ^{o}C until analysis. The volume of the saliva and urine samples at each time point was recorded. Five 50-mL aliquots of urine were taken and stored at -80 ^{o}C until analysis. The remainder of the urine was discarded.

Analyze blood, saliva and urine: Liquid scintillation counting of whole tissues. Saliva, urine and serum samples were assessed by liquid scintillation counting. Because of the low amount of radioactivity administered and the even lower amount absorbed, as expected, there were no counts detected above background.

Analyze blood, saliva and urine: Carbon analysis. The accelerator mass spectrometer (AMS) process is most efficient when measuring about 1 mg of total carbon. Thus, aliquots of the samples were submitted for carbon analysis. For measurement, a small volume of sample was added to a silica support and analyzed using an elemental analyzer (Gilman et al., 1998). Saliva and urine %C contents were similar among time points for each subject (Table 4). Extensive experience has shown that the carbon content of serum samples is consistent and averages 4.2% so serum samples were not analyzed for carbon content. The concentration of permethrin equivalents in each saliva and urine sample was corrected for carbon content according to the measured amount in that sample. Serum samples were corrected using the 4.2 %C figure.

Analyze blood, saliva and urine: AMS measurement. Based on the carbon content, aliquots of each sample were submitted for AMS measurement. For sample preparation (Buchholz et al., 2001; Getachew et al., 2006) the tissue sample (approximately 1 mg carbon) was placed into a small quartz vial nested inside two borosilicate tubes and dried in a vacuum centrifuge. A small glass fiber filter was placed into the top of each set of nested tubes during

centrifugation to minimize inter-sample contamination by aerosols. The inner quartz vials were transferred to quartz combustion tubes, which were evacuated and sealed. The samples were combusted to carbon dioxide and reduced to carbon using this method. Graphite samples were packed into aluminum sample holders, and carbon isotope ratios were measured on the spectrometer. Typical AMS measurement times were 3 min/sample, with a counting precision of 1.4-2.0% and a standard deviation among 3-7 measurements of 1-3%.

Table 4. Percent carbon content in saliva and urine[a].

Subject #	***010***	***012***	***014***	***016***	***018***	***020***
			Average ± SD %C			
SALIVA						
Time						
Predose	0.19 ± 0.05	0.14 ± 0.01	0.37 ± 0.06		0.09 ± 0.01	0.19 ± 0.02
1 hr	0.16 ± 0.03	0.14 ± 0.02	0.99 ± 0.04		0.15 ± 0.05	0.16 ± 0.10
3 hr	0.18 ± 0.01	0.12 ± 0.01	0.58 ± 0.07	0.20 ± 0.03	0.30 ± 0.06	0.17 ± 0.07
6 hr	0.43 ± 0.11	0.12 ± 0.01	0.36 ± 0.02	0.53 ± 0.11	0.19 ± 0.01	0.13 ± 0.04
9 hr	0.12 ± 0.02	0.16 ± 0.01	0.29 ± 0.03		0.06 ± 0.05	0.25 ± 0.08
12 hr	0.16 ± 0.04	0.12 ± 0.01	0.52 ± 0.03		0.09 ± 0.03	0.31 ± 0.19
24 hr	0.17 ± 0.02		0.47 ± 0.07		0.18 ± 0.03	0.38 ± 0.06
48 hr	0.19 ± 0.04		0.35 ± 0.03	0.17 ± 0.02		0.18 ± 0.05
72 hr	0.22 ± 0.04			0.13 ± 0.03	0.17 ± 0.04	0.25 ± 0.06
96 hr	0.19 ± 0.05		0.35 ± 0.03		0.10 ± 0.02	0.31 ± 0.15
120 hr	0.15 ± 0.01				0.20 ± 0.03	0.20 ± 0.03
144 hr	0.24 ± 0.03		0.54 ± 0.06		0.24 ± 0.01	0.28 ± 0.06
168 hr	0.19 ± 0.03		0.22 ± 0.07		0.12 ± 0.03	0.35 ± 0.07
URINE						
Time						
Predose	0.78 ± 0.02	0.80 ± 0.04	0.33 ± 0.02	0.70 ± 0.05	0.35 ± 0.05	1.58 ± 0.09
0-6 hr	0.31 ± 0.02	0.65 ± 0.03	0.14 ± 0.03	0.46 ± 0.05	0.56 ± 0.06	0.52 ± 0.02
6-12 hr	0.35 ± 0.01	0.52 ± 0.04	1.09 ± 0.04	0.35 ± 0.03	0.53 ± 0.03	0.32 ± 0.02
12-24 hr	0.67 ± 0.03		1.29 ± 0.04	0.51 ± 0.07	1.05 ± 0.17	0.47 ± 0.04
24-48 hr	0.68 ± 0.08		0.67 ± 0.03	0.45 ± 0.03	1.33 ± 0.05	0.84 ± 0.40
48-72 hr	0.75 ± 0.02		0.41 ± 0.05	0.59 ± 0.03	1.09± 0.16	1.01 ± 0.05
72-96 hr	0.68 ± 0.06		0.21 ± 0.05		1.10 ± 0.04	1.14 ± 0.07
96-120 hr	0.76 ± 0.03		1.08 ± 0.05		1.42 ± 0.05	0.95 ± 0.21
120-144 hr	0.73 ± 0.02		1.03 ± 0.05	0.67 ± 0.06	1.38 ± 0.29	1.00 ± 0.04
144-168 hr	0.79 ± 0.13		0.86 ± 0.03	0.30 ± 0.03	1.19 ± 0.09	1.31 ± 0.09

[a] n=5

The absorption from the skin and the elimination from the blood expressed as permethrin equivalents are represented in Figure 6A. The amount of permethrin absorbed from the skin varied widely by individual. Permethrin is a lipophilic compound and one might anticipate that absorption and distribution may be related to body fat stores. However, there is no apparent trend to the amount absorbed and the body mass index (a measure of obesity, Table 5). The highest absorption was seen for Subjects 16 and 18 (both female), whereas absorption was more similar for Subjects 10, 12, 14 and 20 (10 and 14 were males and 12 and

20 were females). The serum concentration of ^{14}C permethrin equivalents climbed steadily over 12 hours (the treatment was removed at 8 hours), leveling between 12 and 24 hours (Figure 6B), and then showing a rapid elimination phase. By 72 hours, the blood concentrations of ^{14}C permethrin equivalents had returned to pre-dose levels. Interestingly, one would anticipate that the pre-dose levels of ^{14}-C would be zero, but each subject showed some 'basal' level of ^{14}-C above anticipated background for an unexposed subject. One cannot presume that these pre-dose sample values were spurious because the 7-day values for each subject were similar to the respective pre-dose values.

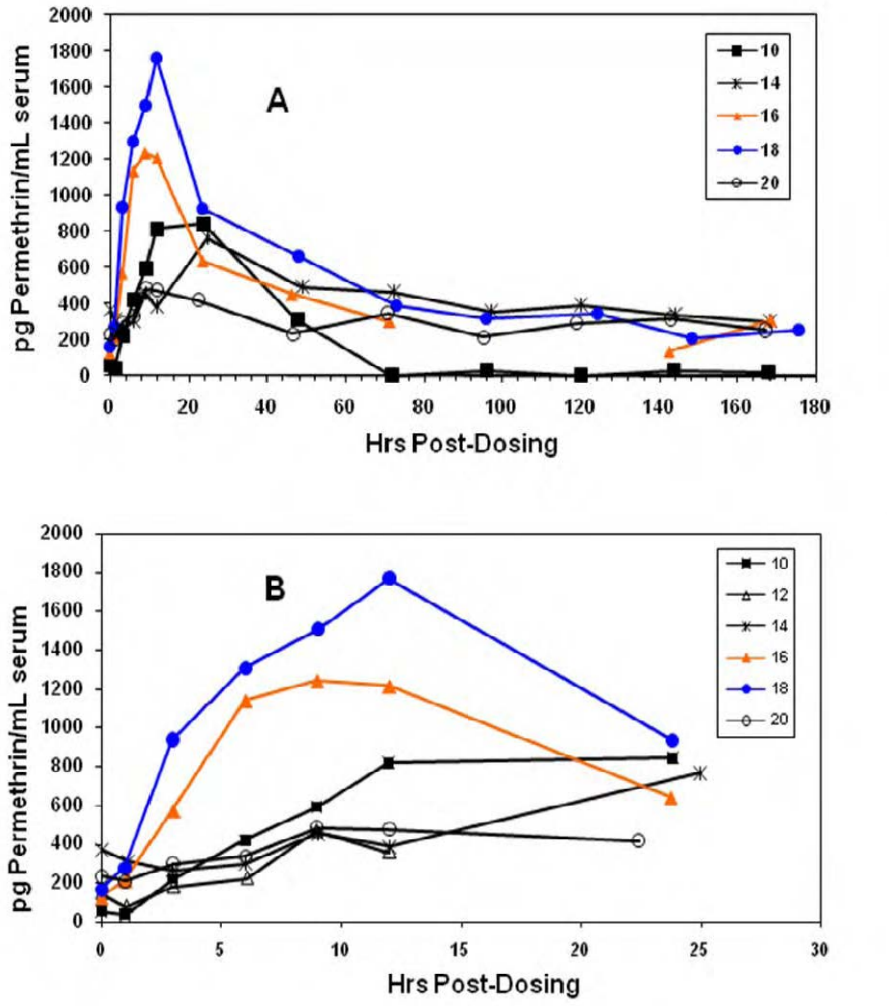


Figure 6. Time course of the absorption and elimination of permethrin in the serum. (A) full time course; (B) 0-24 hr time period.

Table 5. Relationship of body mass index (BMI) to peak serum levels of permethrin

Subject	***BMI***	***[Permethrin] at peak serum level (pg/mL serum)***
010	27.9	814.9
012	29.8	464.8
014	39.5	765.2
016	23.4	1239.2
018	28.5	1765.4
020	26.3	483.9

Not much is known about excretion of xenobiotics in saliva. To reliably estimate internal dosimetry from a spot saliva samples requires good understanding of the pharmacokinetics of the chemical and the relationship between chemical concentration in the saliva and blood (Timchalk et al., 2004).This has been demonstrated for the herbicide atrazine (Denovan et al., 2000). In this study, saliva samples were taken to determine if permethrin is excreted in the saliva, and if so, to assess saliva as a potential biomonitoring fluid for permethrin. ^{14}C-Permethrin equivalents do appear to be excreted in the saliva (Figure 7). Although the trends are not as obvious as with serum or urine, there is a distinct increase following dosing. The peak excretion time varies by individual, but occurs between 6 and 24 hours. Subjects 16 and 18 show the highest absorbed concentrations (Figure 6). Subject 18 also shows the highest saliva elimination. The correlation cannot be made with Subject 16 as critical samples are missing between 9 and 48 hr (trend line is indicated only for the collected samples). Subjects were allowed to eat and drink at their discretion during the study. The amount of ^{14}C label found in saliva may be more related to the abundance of saliva (i.e. those subjects that ate or drank more, excreted more saliva, and consequently, more ^{14}C label may appear in the samples), relative to the time that the samples were taken, but this cannot be verified. For subjects 14 and 18, we know that the non-occlusive cover came loose sometime in the 8 hour dosing period. Therefore, it is also possible that the high levels seen for these two subjects is related to hand-to-mouth activity, where they attempted to adjust the cover, contaminating a hand that was later used for eating. Saliva may be a viable biomonitoring media, but further studies are needed.

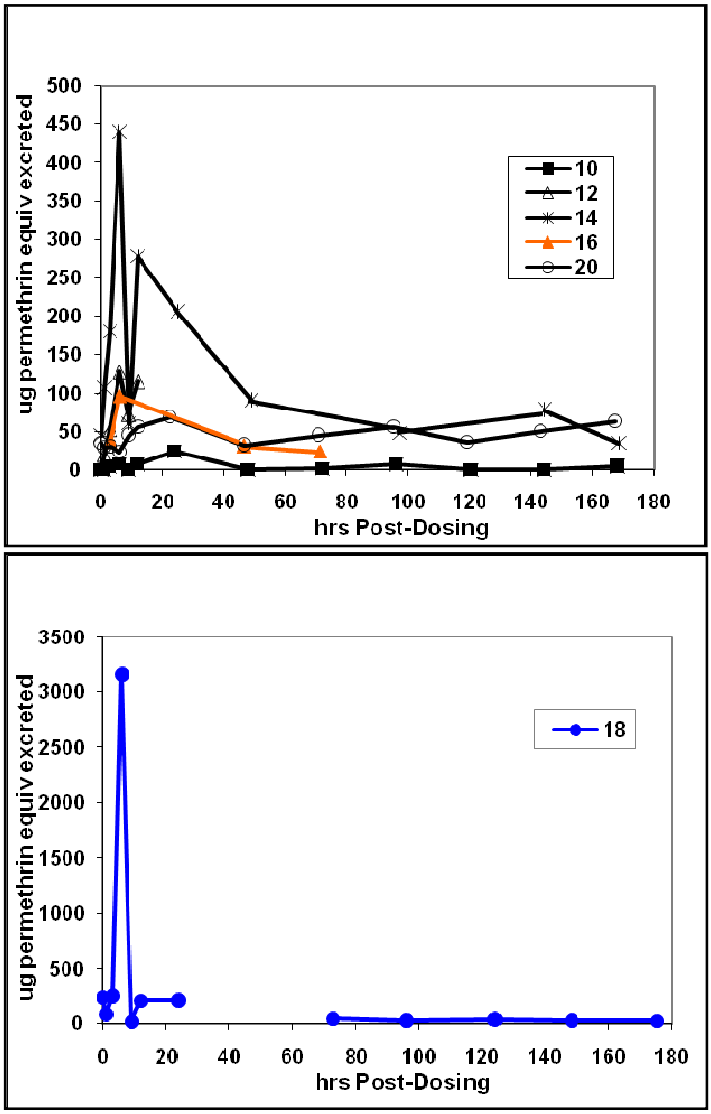


Figure 7. Excretion of 14-C permethrin equivalents in saliva.

The total amount of ^{14}C-permethrin equivalents excreted in urine at each time point is shown in Figure 8 for the 6 subjects. The higher levels of permethrin that was excreted correlates loosely to the levels absorbed for each individual, with the exception of Subject 16 who had relatively high absorption, but excretion in urine was low. It is possible that this subject

retained the labeled permethrin/permethrin metabolites, or they were excreted by alternate routes (feces, sweat) or urine collections were incomplete. Assuming all absorbed permethrin is excreted only in urine, is not sequestered in other compartments and is complete by the end of 7 days, the estimated % of the dose absorbed was 0.25, 0.12, 0.27 and 0.06 for subjects 10, 14, 18 and 20 who completed all urine collections. Similar levels (0.35-0.52%) were found by

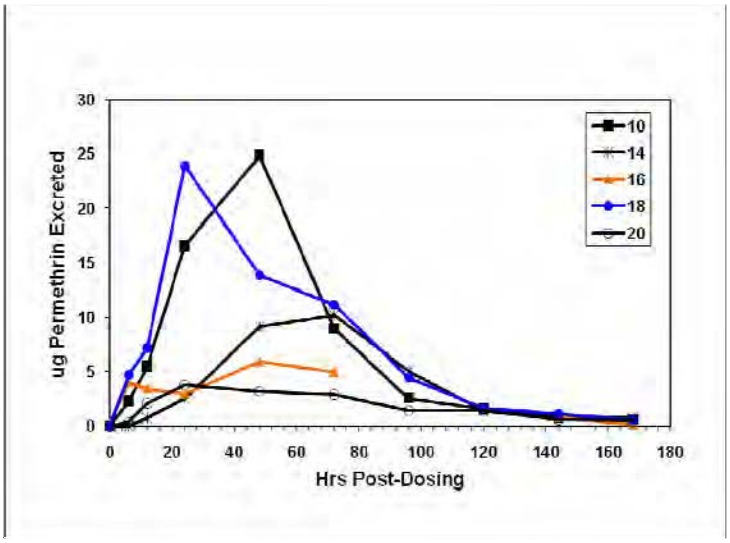


Figure 8. Total 14-C permethrin equivalents excreted in the urine at each time.

Tomalik-Scharte et al., (2005) where subjects were exposed to permethrin in a head shampoo and by Woollen et al. (1992; 0.85-1.2%) where subjects were exposed to 31 mg cypermethrin to an 800 cm^2 area of skin for 8 hr.

The elimination rate of ^{14}C permethrin equivalents in the urine increased through the first 24 hours, being maximal in the 12-24 hour period and then declined rapidly, correlating to the availability of permethrin in the serum (Figure 9). Woollen et al., (1992) described maximum urinary excretion rates to occur between 12 and 36 hr post dosing for cypermethrin. The elimination half-life was 33.8, 38.9, 72.9, 67.9 and 68.6 hours for Subjects 10, 14, 16, 18 and 20, respectively. The shorter half-lives were found in male subjects, while the longer ones were found in female subjects. These values are in close agreement with Tomalik-Scharte et al. (2005) who determined maximal urinary excretion at 13.9 hr and elimination half-life of 32.7 hr from male subjects dosed with 215 mg of permethrin in alcohol to the hair and head. However, are much longer than determined by Woollen et al. (1992) for cypermethrin (13 hr ± 5.1 hr) following a dermal exposure or Leng et al. (1997) for cyfluthrin (6.44 ± 0.64 hr) following an oral exposure.

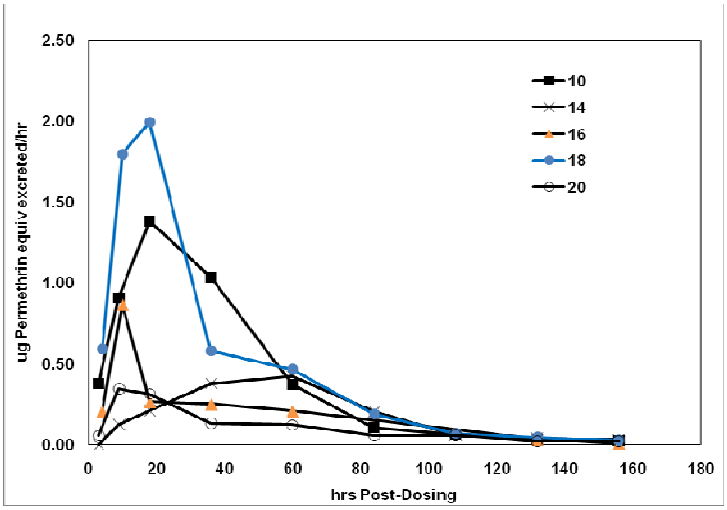


Figure 9. Permethrin elimination rate in urine.

The permethrin equivalents eliminated in the first 24 hours averaged 7.6 µg/L for the 5 subjects with complete 24 hour urine collections (range (2.8 – 17.1 µg/L). These values were within the range reported by Leng et al. (1997) for pest control operators in Europe with daily occupational exposure for one week (<0.5 µg/L and 277 µg/L) and demonstrates that our low dose exposure is similar to typical occupational exposure scenarios.

Analyze urine samples for metabolite profile. The LC method developed in Objective IA was used to separate metabolites in the urine samples. As mentioned above, urine samples were spiked with standards so that peaks could be identified for collection of fractions. We

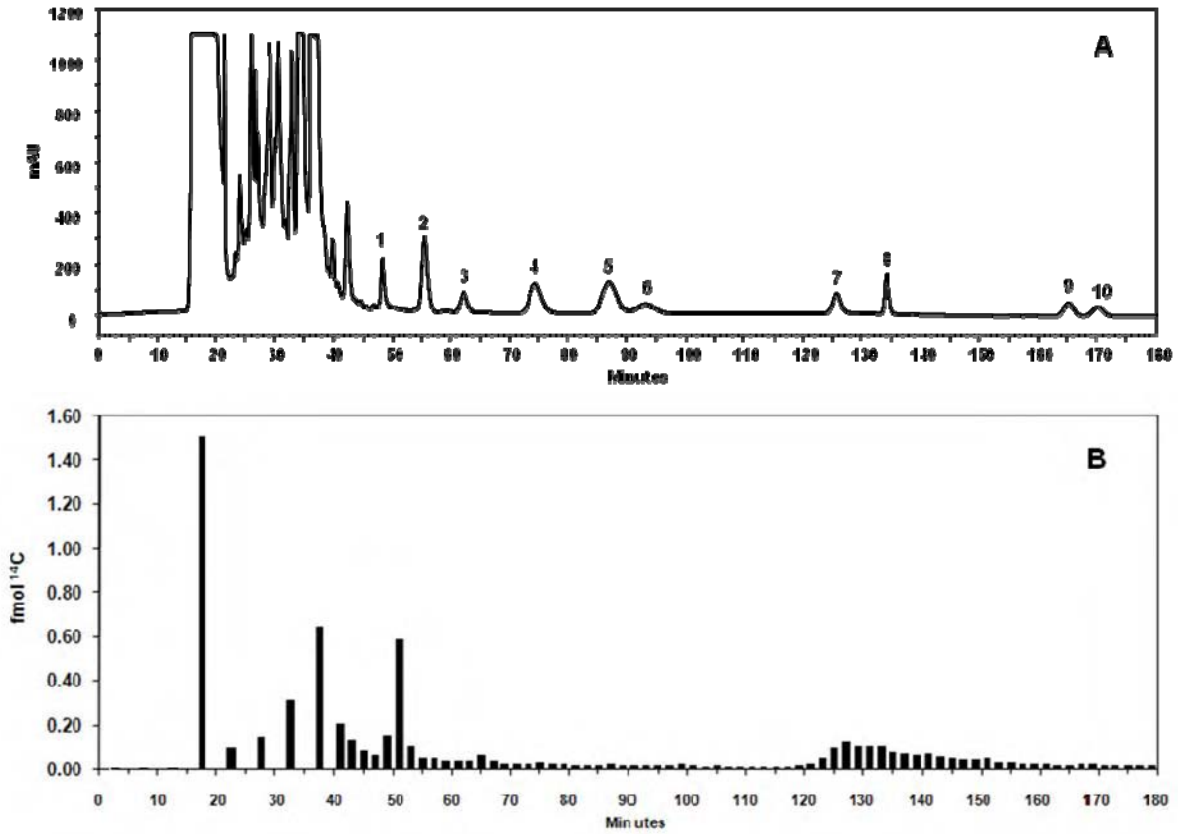


Figure 10. (A). HPLC chromatogram of metabolites detected by UV. 1 – glucuronide of 3-phenoxybenzyl alcohol; 2 – trans-DCVA-glycine + 3PBA-glycine; 3 – cis-DCVA-glycine; 4 – 3-phenoxybenzyl alcohol; 5 – trans-DCVA; 6 – 3PBA; 7 – cis-DCVA; 8 – 3-phenoxybenzaldehyde; 9 – trans-permethrin; 10 – cis-permethrin. (B). Corresponding AMS measurement of collected fractions from the same HPLC run. The values are plotted at the center point of the collection time period.

collected 2 min fractions throughout the chromatographic run, but collected 5 min fractions in the first 40 min as none of our standards eluted in that period of time. Figure 10A is the UV chromatogram for the 24-hour urine sample of Subject 10 while Figure 10B is the AMS measurements of the corresponding fractions.

Some radioactivity is associated with peaks 2, 7 and 8 (Figure 10A) that correspond to the glucuronide of 3-phenoxybenzyl alcohol, *cis*-DCVA and 3-phenoxybenzaldehyde, respectively. No parent compound was detected. Surprisingly, a large percentage of the radioactivity chromatographed was found in the first 40 min of the run and did not correspond to the known standards. The peak at about 18 minutes corresponds to about 45.4% of the radioactivity chromatographed. The broad peak between 22 and 45 min constitutes an

additional 31.7%, while the peak centered around 52 min (putatively the glucuronide of 3-phenoxybenzyl alcohol) made up 10.2%. The broad peak between 122 and 150 min was made up of about 11.2%.

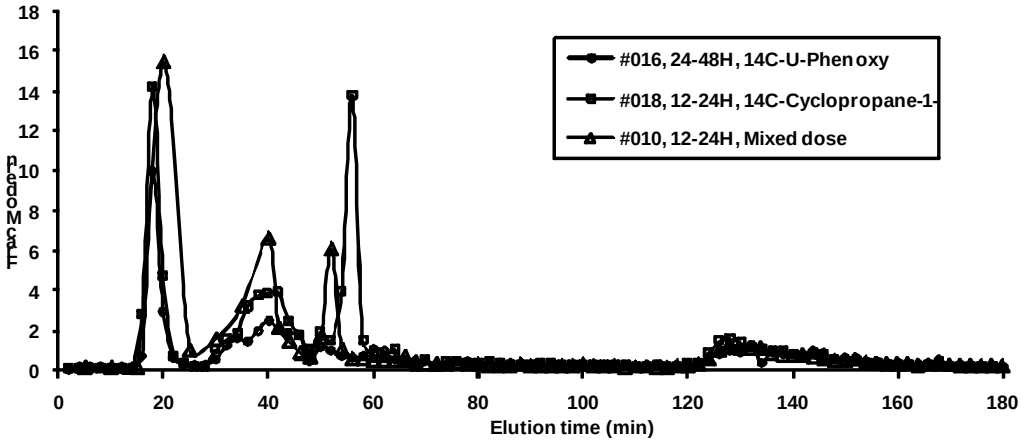


Figure 11. Representation of AMS analysis of HPLC fractions for subjects treated with a mixed label (triangle), the cyclopropane only label (square) and the phenoxy label only (circle).

One subject each was administered permethrin containing a label in either the phenoxy group or the cyclopropane group. At about 20 min and 40 min, peaks appear for all three subjects suggesting that the compounds eluting at those times are mixtures of very polar metabolites (Figure 11). At about 55 min, there is a peak for Subject 18 administered the cyclopropane label and this corresponds to the *trans*-DCVA as the co-eluting 3PBA-glycine is not labeled. However, one would expect Subject 10 who was administered a mixed label to also have a peak at that elution time, which does not appear to be the case. Subject 10 shows a distinct peak around 48-50 min that is tentatively identified as the glucuronide of 3PBA. Subject 16, who received permethrin labeled in the phenoxy group also shows at peak at that time, although much smaller. All three subjects show a broad band around 128 min that could be associated with *cis*-DCVA and the 3-phenoxybenzaldehyde. Generally, we did not expect the presence of 3-phenoxybenzaldehyde as it is rapidly converted to 3PBA. Alternatively then, the broad peak that elutes just before the parent compound could be the hydroxylated parent compounds.

Further confirmation of the metabolite identities is needed. One approach would be to hydrolyze the urine prior the HPLC/AMS analysis to determine if the distribution of radioactivity changes to less polar peaks. Further work on the HPLC separation system could be conducted to determine how many compounds may be present in the early eluting peaks. Mass spectrometry can be used for specific identification of the peaks following concentration and purification of the samples to provide adequate mass.

Preliminary analysis of urine samples using the immunoassays for 3PBA (Table 6) shows that acid hydrolysis results in the appearance of 3PBA suggesting that conjugates of 3PBA are present. The AMS data suggest that 3PBA-glycine may be one conjugate, but another may be 3PBA-glucuronide for which we did not have a standard. The 3PBA found comprised only a portion of the total permethrin equivalents found by AMS.

Table 6. Determination of 3-PBA in urine of Subject 10 before and after hydrolysis.

Sample	*ng 3PBA/mL by ELISA before acid hydrolysis*	*ng 3PBA/mL by ELISA following acid hydrolysis*	*ng permethrin equiv/mL by AMS*
0-6 hr	<LOQ of 2 ng/mL	<LOQ of 2 ng/mL	1.56
12-24 hr	<LOQ of 2 ng/mL	4.78 ± 0.70	19.28
24-48 hr	<LOQ of 2 ng/mL	6.53 ± 1.31	16.47
48-72 hr	<LOQ of 2 ng/mL	20.89 ± 1.40	5.82
72-96 hr	<LOQ of 2 ng/mL	3.70 ± 0.40	1.65

We will continue to work with these samples to identify the metabolites. An examination of Figure 1 suggests that the very polar metabolites might be hydroxylated DCVA or 3PBA or glucuronide or sulfate conjugates of the hydroxylated products. The later eluting peaks around 128 min may in fact be hydroxylated parent compound. The uptake and clearance of permethrin was clearly different among individuals, thus it may not be surprising that the metabolic profiles are not exact matches. Metabolism of permethrin occurs primarily by carboxylesterases and cytochrome P450s. The metabolite pattern may depend upon genetic differences in the expression of these important enzymes as well as the possibility of interactions among permethrin and other substrates such as phthalate esters, ester pro-drugs and endogenous lipid esters for these enzymes (Ross and Crow, 2007).

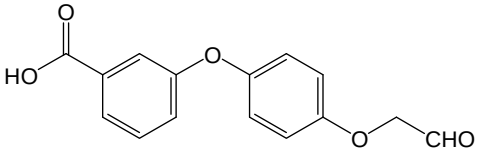
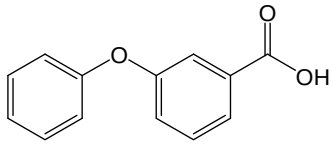
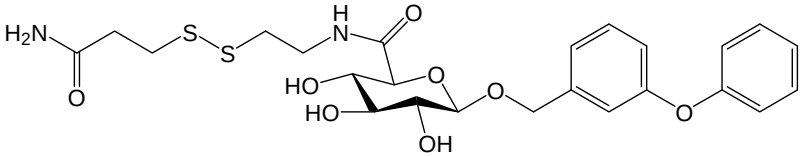
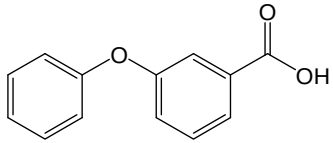
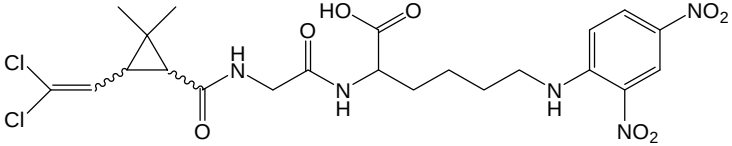
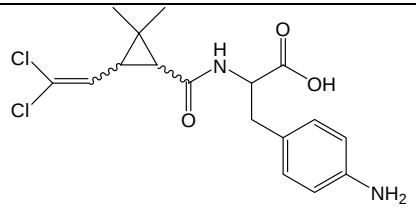
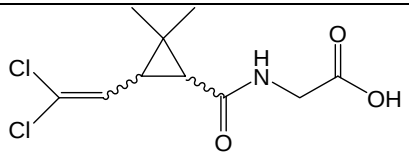
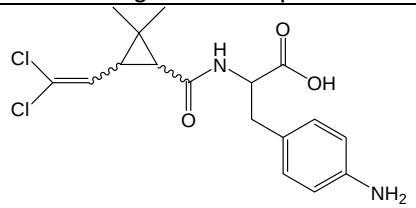
Difficulties:

- Need to instruct subjects and nursing staff better in the taking of saliva samples. Instructions were followed, but subjects did not report and nursing staff did not ask if the cotton plug was soaked with saliva. Consequently, several time points did not contain enough saliva for analysis.
- The non-occlusive gauze that covered the treatment sight was not always securely fastened. It shifted and sometimes did not remain on the treatment sight particularly while subjects were napping. Since a significant amount of radioactivity was associated with the gauze cover, these problems with the cover will reflect in the inability to recover the total amount of radioactivity applied.
- Saliva and urine samples may have been contaminated if handled by subject after touching the loose cover.
- Signal for individual metabolites would be improved by injecting a larger volume of urine onto the HPLC column, or concentrating the sample before injection.

III. Develop an immunoassay to the key metabolite identified in Objective II as a biomarker of human exposure to permethrin.

Immunoassays were developed for 4 putative metabolites: 3PBA, 3-PBAlc-glucuronide, DCVA, and DCVA-glycine. The development of the 3PBA assay is detailed below as an example of the method development and validation. The assay development details for DCVA are also reported below as this assay has yet not been published. Other assays developed under this project are summarized in Table 7 with reference to publications.

Table 7. Properties of the immunoassays developed for this project

Target analyte	Immunizing hapten	Coating hapten	IC_{50} ng/ml	LOD/LOQ
3PBA	 coupled to THY (Rabbit #294)	 coupled to BSA	1.6	0.1/2[a]
3-PBAlc-glucuronide	 coupled to THY (Rabbit #1891)	 coupled to BSA	0.5	0.1/20[b]
DCVA	 *cis* configuration coupled to THY (Rabbit #3709)	 *trans*-configuration coupled to BSA	18	ND[c]
DCVA-glycine	 *trans* configuration coupled to THY (Rabbit #3703)	 *cis* configuration coupled to BSA	1.2	0.1/1.0[a]

[a] LOQ determined on urine samples extracted by solid phase extraction
[b] 1 ng could be detected in a urine sample diluted 1:20. Extraction or cleanup will improve the LOQ.
[c] Not determined.

Development of immunoassay for 3PBA: Design and synthesis of haptens for 3PBA. In order to generate a specific antibody for an analyte, it is important to maintain the structure of target compound with as little change as possible when it is modified and coupled to a carrier

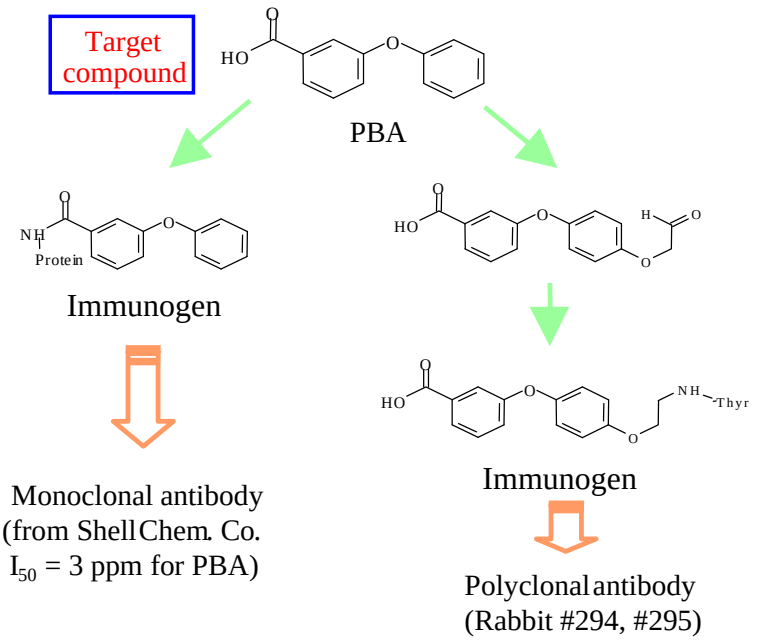


Figure 12. Hapten design for 3-PBA immunogen

protein. In this respect, it is prudent to attach the handle as distally as possible from the partial structure that defines the target (including the carboxylic acid group of 3PBA). This approach was demonstrated in the previous study for developing an immunoassay for the 3PBA-glycine conjugate by attachment of a handle at the α-position of the glycine and leaving the carboxylic acid group unchanged. Similarly, in this study, haptens for 3PBA were synthesized with the handle attached to the 4-position of distal phenyl group (Figure 12).

The structure and purity of the synthesized haptens were verified by TLC, melting point, NMR and HRMS. Stereo configuration was determined using polarimetry. Once the haptens were synthesized, they were covalently coupled to a variety of proteins. Typical peptide chemistry methods were used. 3-((2-Oxethoxy)ethoxy)phenoxybenzoic acid was coupled to thyroglobulin (Thyr) by Schiff base formation to yield the immunogen shown in Figure 12 (right;**6**-Thyr). The coating antigens were synthesized using an activated ester method (Hermanson, 1996). Compounds 3-(4'-methoxyphenoxy)benzoic acid, 4-phenylbenzoic acid, or phenoxyacetic acid (0.025 mmol) were dissolved in 1.6 mL of dry N,N-dimethylformamide, and then, 6 mg (0.05 mmol) of N-hydroxysuccinimide and 5.8 mg (0.03 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added. The reaction mixture was stirred overnight at room temperature. Forty milligrams of bovine serum albumin (BSA) or ovalbumin (OVA) was dissolved in 12 mL of phosphate buffered saline (PBS; 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KCl, pH 7.4) and 4 mL of carbonate buffer (pH 9). The activated hapten was added drop wise to the protein solution. The mixture was stirred for 30 min at room temperature and 7 h at 4 °C. The solution was then dialyzed against PBS over 72 h at 4 °C and stored at -20 °C.

Development of an immunoassay for 3PBA: *Immunization.* The production of antibody was made according to Shan et al. (1999). Briefly, two New Zealand white rabbits were immunized (rabbit nos. 294 and 295) with **6**-Thyr. The antigen solutions (100 µg in PBS) were emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. After 1

month, the animals were boosted with an additional 100 µg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1 v/v). Booster injections were given at 4-week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters. These terminal bleeds were used for enzyme linked immunosorbent assay (ELISA) development.

Development of immunoassay for 3PBA: ELISA method. The competitive inhibition ELISA format in this study was based on methods described by Voller et al. (1975). Microplates were coated overnight at 4 °C with 100 µL/well of the appropriate coating antigen concentration in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After the plates were washed with PBS containing Tween-20 (PBST: PBS + 0.05% Tween-20), the plate was incubated with 200 µL per well of a 0.5% BSA solution in PBS for 30 min at room temperature. After another washing step, 100 µL per well of antiserum diluted in PBS per well (for titration experiment) or 50 µL /well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 µL/well of standard analyte or sample solution were added and incubated for 1 h. The standard analyte concentrations ranged from 0.05 to 5 µg/L. Following a washing step, the secondary antibody goat anti-rabbit-horse radish peroxidase conjugate (GAR-HRP; diluted 1:3000 in PBS with 0.05% Tween 20, 100 µL/well) was added and incubated for 1 h at room temperature. The plates were washed again, and 100 µL/well of substrate solution (3.3 µL of 30%H_2O_2, 400 µL of 0.6% 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl sulfoxide (DMSO) per 25 mL of acetate buffer, pH 5.5) was added. The color development was stopped after 15 min with 50 µL/well of 2 M H_2SO_4. The absorbance was measured using the dual wavelength mode at 450 nm minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four parameter logistic equation: y) {(A - D)/[1 + (x/C)B]} + D where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is IC_{50}, and D is the minimum absorbance at infinite concentration.

Initial screening. A checkerboard titration system was used for screening of antibody and antigen combinations (Gee et al., 1994). The antisera of 4 rabbits were tested against 5 coating antigens. All antisera showed higher titers in a homologous system than in the heterologous systems. In a homologous system, the hapten in coating antigen and immunogen is the same and is the main reason for the higher titer. Because haptens used in coating for heterologous systems are different from the immunizing hapten, the binding to these coating antigens is less. Since no significant difference was observed in antibody titers between coating antigens with different carrier proteins (BSA or OVA), only BSA conjugates of 3PBA were used for further screening with antibody 294, and 295.

All combinations tested showed a very low IC_{50} (<6.0 µg/L) for 3PBA, ranging from 1.3 to 6.0 µg/L. The homologous systems had higher IC_{50}s than the heterologous systems. For example, with antibody 294, the IC_{50} in a homologous system was about 50 times higher than in heterologous systems. In this study, only the combination of antibody 294 and coating antigen 3PBA-BSA was used for further assay development.

Assay Optimization. The assay conditions were optimized in such a way that the IC_{50} values were minimized. This goal was achieved by screening antibodies and antigens in a two dimensional titration for best dilution of coating antigen and antibody. Then, competitive inhibition curves were measured for different antibody and antigen combinations, and the one with the lowest IC_{50} was selected for further assay development.

Cross-Reactivity (CR). The optimized assay was submitted to cross-reactivity studies by using the standard solution of the analytes and other structurally related compounds (listed in Table 8). The CR was obtained from the IC_{50} values of metabolite standard and the related compounds from the same plate where esfenvalerate, cypermethrin, permethrin, deltamethrin, cyfluthrin, permethrin metabolites and other structurally related compounds were tested for cross-reactivities (Table 8). Antibody 294 was highly specific for the target analyte 3PBA, and

the related cyfluthrin metabolite, 4-fluoro-3-phenoxybenzoic acid (FPBA; 72%). In all cases, the inhibition by parent compound, other metabolites and other tested compounds was negligible. Although 3PBA-glycine, phenoxybenzyl alcohol, and other tested pyrethroids contain the phenoxybenzyl group, which is present in the immunogen hapten, they did not interfere in the assay.

Table 8. Summary of Cross-Reactivities

Compound	I_{50} (µg/mL)	CR (%)
3-PBA	1.98	100
FPBA	2.75	72
3-PBA-glycine	>10,000	0
3-Phenoxybenzyl alcohol	>10,000	0
Permethric acid	>10,000	0
Permethrin	>10,000	0
Esfenvalerate	>10,000	0
Cypermethrin	>10,000	0
Deltamethrin	>10,000	0
Cyfluthrin	>10,000	0

Matrix Effects. Since this assay was intended to analyze urine samples, the influence of pH and ionic strength in urine samples was examined.

1. pH. The pH effect was tested by preparing analytes in phosphate buffers at pH 4, 6, and 8. This assay showed good tolerance to the pH of the solution (Figure 13). Compared with PBS buffer (0.15 M, pH 7.5), no significant influences were observed at pH 5-8 indicating that a slight difference in the pH of sample or buffer (in this range) would not affect the accuracy of **3**PBA quantitation.

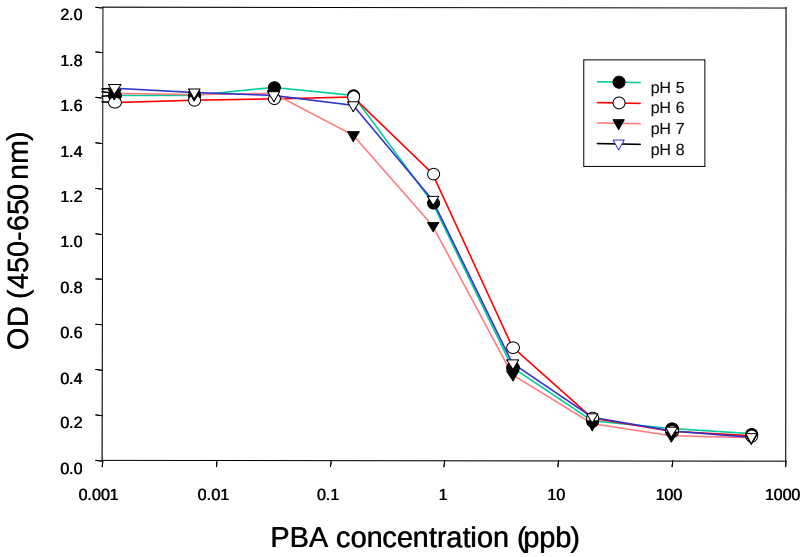


Figure 13. ELISA competition curves of 3PBA prepared at various pHs. Reagent concentrations: coating antigen (PBA-BSA) (0.4 µg/mL); antiserum (antibody 294, 1/6000, final concentration in wells); goat anti-rabbit IgG-HRP (1/3000).

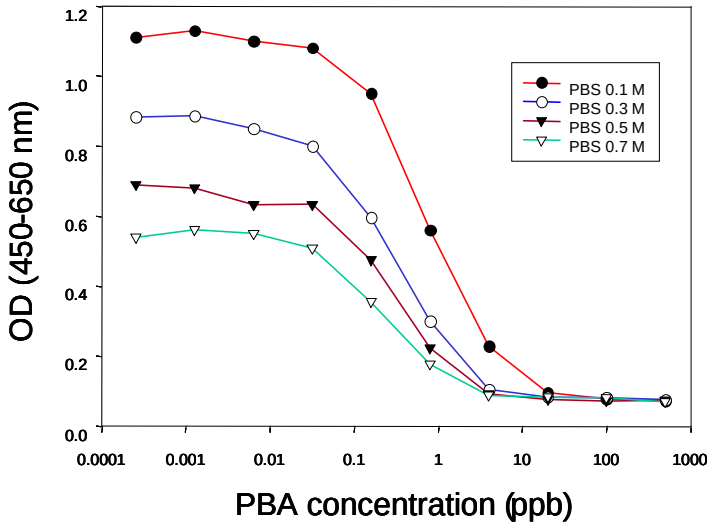


Figure 14. ELISA competition curves of PBA prepared at various ionic strengths.

2. Ionic Strength. The effect of ionic strength on the quantitation of PBA was studied by preparing analyte standard solutions in 0.1, 0.3, 0.5, and 0.7 M PBS all at pH 7.5. The assay is very sensitive to higher concentrations of salt in solution (Figure 14). At ionic strength of 3x PBS (0.45 M), the binding between antibody and antigen was suppressed about 30%. These results are consistent with the previous report about atrazine mercapturic acid, which was assumed to disrupt the antigen-antibody interaction (Jaeger et al., 1998).

3. Organic solvent. Since analytes are usually extracted with organic solvent, the effect of methanol on the assay was tested. Methanol was chosen because it can successfully solubilize the analyte and is miscible in the mostly aqueous-based immunoassay. Table 9 shows the effect of methanol on the assay. The IC_{50} increased when the solvent concentration exceeded 10% with a corresponding decrease in slope value. Ideally, the assay performs best without methanol. However, if a co-solvent is needed, 10% methanol can be used. In this study, no methanol was used during further optimization studies.

Table 9. Effects of Methanol Concentration

MeOH (%)	A_{max} (A)	Slope (B)	IC_{50} (µg/L) (C)	A_{min} (D)	R^2
0	1.46	1.14	2.29	0.182	0.99
10	1.46	1.06	4.03	0.174	0.99
20	1.51	0.97	5.41	0.164	0.99
40	1.57	1.00	8.49	0.17	0.99
60	1.57	0.741	21.7	0.17	0.99
80	-	-	-	-	-

4. Urine. The effects of urine matrix on the quantitation of PBA were evaluated by preparing analyte standard solutions in a buffer with different concentrations of urine (0, 1, 2, 4, or 10% of urine). Urine samples tested in this study were from healthy individuals without known exposure to pyrethroids. In tests with 4 different urine samples, this assay could tolerate 2% of urine (Figure 15) before calibration curves began to deteriorate. Therefore, dilution prior to ELISA would be necessary for the assay. Considering the variation among the different urine samples,

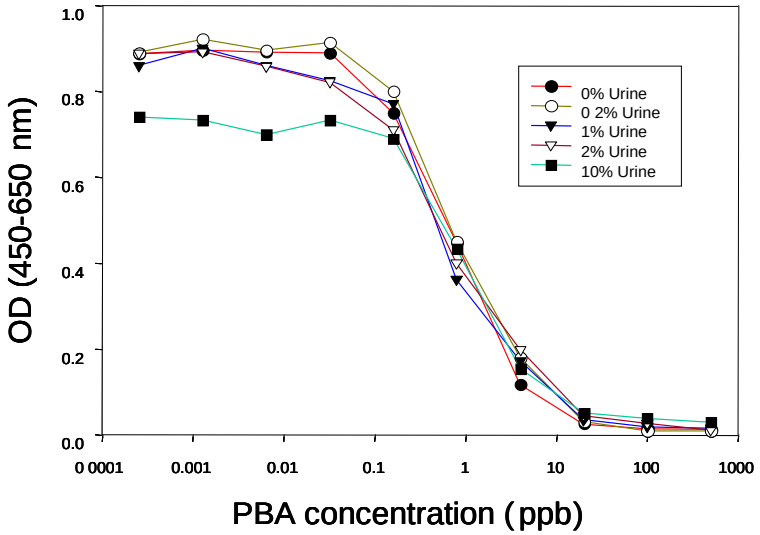


Figure 15. ELISA competition curves of 3-PBA prepared at various urine concentrations.

a 100x dilution of the urine before the assay was chosen. In recovery experiments for the direct dilution of urine samples, urine matrix had a little effect on accurate quantitation for urines spiked with PBA $\geq$ 20.0 µg/L. The recovery rate for PBA at 25.0 µg/L was 110.0 ± 10.5 %. According to the recommended LOQ determination guideline (Brady, 1995), the approximate limit of quantitation was 20.0 µg/L for the assay. This is consistent with the estimated concentration that corresponded to the absorbance of the control (zero analyte) minus 3 times the standard deviation of control, when standard analytes were prepared in a buffer system with 1% unspiked urine.

The assay validation was performed in a blind fashion by direct dilution of the urine samples, which were spiked with PBA concentration ranging from 0 to 160 µg/L (Figure 16). The linear regression analysis of ELISA results showed good correlation (R^2 = 0.900). All recoveries were over 86% of the spiked values. These results demonstrated that these assays are

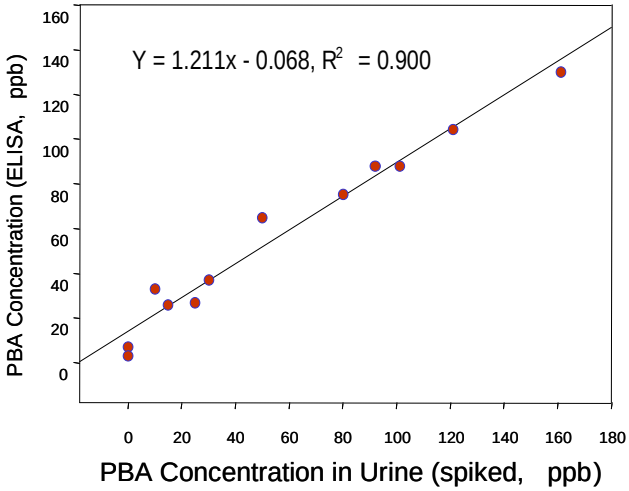


Figure 16. Relationship between spiked PBA in urine and measured by ELISA.

suitable for the quantitative detection of pyrethroid metabolite (as total 3PBA) at trace levels in urine samples using direct dilution.

Urine sample analysis with cleanup. To further validate the 3PBA immunoassay for urine samples and improve the limit of quantitation, a method was developed in which urine was subjected to acid hydrolysis to free 3PBA that might be conjugated. After thawing, the urine was thoroughly mixed and then left to stand for about 2 hours. A 0.5 mL aliquot of the supernatant was added to a tube followed by 0.1 mL of 6 N HCl. The samples were hydrolyzed at 100 ^{o}C for 1 hr. After cooling, the sample was neutralized with 6N NaOH (about 93-98 µL), then diluted with 1 mL of 0.2M sodium acetate buffer, pH 4.5. The neutralized hydrolysate was subjected to solid phase extraction on a Strata Screen-A 100 mg/1 mL sorbent (Phenomenex). The column was conditioned with 1 mL methanol followed by 1 mL water, then 1 mL sodium acetate buffer (pH 4.5) at a flow rate of 3-6 mL/min. The hydrolyzed sample (1.5 mL) was loaded at a flow rate of about 1-2 mL/min. The column was then washed sequentially with 1 mL water and 1 mL methanol and vacuum dried for about 5 min. The analyte was eluted with 1.5 mL of 1% acetic acid in a mixture of ethyl acetate:hexane (30:70, v/v). The eluate was completely evaporated in a centrifugal vacuum concentrator and the residue re-dissolved in 2.5 mL of 10% methanol-PBS buffer (pH 7.5) for immunoassay analysis. Each urine sample was analyzed in triplicate.

Laboratory-generated urine samples with no known exposures to pyrethroids were spiked with 3PBA and subjected to hydrolysis and SPE. The SPE eluate was diluted 1:5, then analyzed. 3PBA is known to be present at detectable levels in most of the population. Thus, these laboratory-generated samples were also subjected to hydrolysis, SPE and analysis without spiking in order to subtract any background 3PBA present. Acceptable recoveries were set at 70-135% with a %CV of less than 25%. Table 10 shows that 3PBA levels between 1 and 50 µg/L are recovered within acceptable levels each day. The intra-assay (intra-plate) precision of the ELISA, expressed as a coefficient of variation (%CV) between three replicates was <25%, and in most cases <10%. The inter-assay (inter-plate) precision measured for the same sample solutions during 4 different days did not exceed 16%. For quality assessment, pooled urine samples were spiked at four different concentrations (1–50 ng/mL), and the samples were analyzed on multiple days. The average recovery efficiency and %CV for all tested concentrations was 109.86% and 9.8%, respectively.

Table 10. Recovery (%) of 3-PBA spiked into urine samples and the precision of ELISA results using an SPE clean-up with Strata Screen-A.

3-PBA spiked (µg/L)	No. of samples	Recovery (%)					CV (%)
		Day 1	Day 2	Day 3	Day 4	Mean	
1	4	101.0±13.4	95.0±3.4	99.0±24.7	133.0±14.3	107.0±17.5	16.4
3	4	109.7±12.0	93.7±3.3	106.7±18.6	100.7±20.7	102.7±7.1	6.9
5	4	97.4±6.7	112.6±0.6	100.0±6.4	117.4±17.3	106.8±9.7	9.1
10	4	112.9±1.8	103.0±2.3	116.2±7.0	136.5±6.6	117.2±14.1	12.0
50	4	116.0±1.9	115.1±4.2	109.4±2.3	121.9±1.4	115.6±5.1	4.4

Number of different urine samples used for each concentration and the variability among urine samples is indicated in the table.

An estimate of the method detection limit was made by identifying the concentration of 3PBA that resulted in a 20% inhibition (IC_{20}) and multiplying by the dilution factor of the original

sample. The estimated value was 2 µg/L. The recovery data in Table 10 demonstrated empirically that a method detection limit of 1 µg/L is acceptable.

Field sample validation: Urine sample analysis without cleanup. Two urine samples collected from persons exposed to cyfluthrin were analyzed for the total FPBA after hydrolysis via GCMS. Although the hydrolytic product of cyfluthrin is FPBA rather than 3PBA, because FPBA cross reacts to a large extent (72%) in the 3PBA assay, we used this assay to test which form of conjugate is predominant in the urine, these samples were tested by different immunoassays including the PBA-glycine ELISA (Shan et al., 1999). Each urine sample was split into three aliquots. One aliquot was analyzed directly by 3PBA and 3PBA-glycine ELISAs to determine the free FPBA and FPBA-glycine conjugate in urine. The second aliquot was incubated with glucuronidase and then measured with the 3PBA ELISA to determine the FPBA-glucuronide conjugate in urine (the difference in FPBA concentration measured before and after enzyme treatment). The third aliquot was hydrolyzed as described by Leng et al. (1997b) followed by the 3PBA ELISA to measure total free FPBA and FPBA conjugates in urine. As shown in Table 11, no FPBA-glycine was detected in either urine sample. A moderate amount of FPBA-glucuronide was detected representing about 7.2 and 6.2% of the total FPBA in the samples, respectively. Free FPBA appeared to be the major metabolite.

Table 11. Summary of cyfluthrin metabolites in human urine as measured by the 3PBA immunoassay using FPBA as the standard.

	Prehydrolysis				Posthydrolysis	
	FPBAG (ng/mL)	Glucuronide (ng/mL)	FPBA ng/mL	Others ng/mL	FPBA (ng/mL)	
					ELISA	GC-MS
Urine I	ND[a]	8.4	68.7	19.4	117	97.6
Urine II	ND	2.4	28.2	8.4	39	34.6

[a] ND: not detectable at the detection limit of the PBA-glycine immunoassay (5 ng/mL)

Field sample validation: Urine sample analysis with cleanup. Field-collected samples for subjects occupationally exposed to esfenvalerate and laboratory controls were assessed by immunoassay and LC/MS following acid hydrolysis and SPE cleanup. Acid hydrolysis of esfenvalerate results in the formation of 3PBA similar to hydrolysis of permethrin. For LC/MS, samples were analyzed using a Shimadzu AP-10 HPLC coupled to a Quattro Ultima MS/MS in negative electrospray ionization mode at a cone voltage of 40 and a collision energy of 25. The MRM trace for 3PBA was 213 > 99 and for ^{13}C-3PBA was 219 > 99. Separation on the LC utilized a 2.1 x 150 mm Atlantis dC_{18} 3 µm, column (Waters, Milford, MA). The mobile phase consisted of a 50% mixture of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid run isocratically at a flow rate of 0.3 mL/min. The retention time was about 4.9 min. For analysis of urine samples, ^{13}C-3PBA (100 µL of a 50 ng/mL solution in acetonitrile) was added to glass tubes and mixed using a vortex for 30 sec. Water (100 µL) was then added and the tube mixed again followed by the addition of 150 µL sample. The mixture was transferred into inserts of HPLC vials and 25 µL injected onto the column (2.1 x 150 mm Atlantis dC_{18} 3 µm, Waters, Milford, MA). The immunoassay analysis was conducted as described above. Figure 17 and Table 12 show close agreement between the two methods over the range of 1-140 ng/mL of 3PBA.

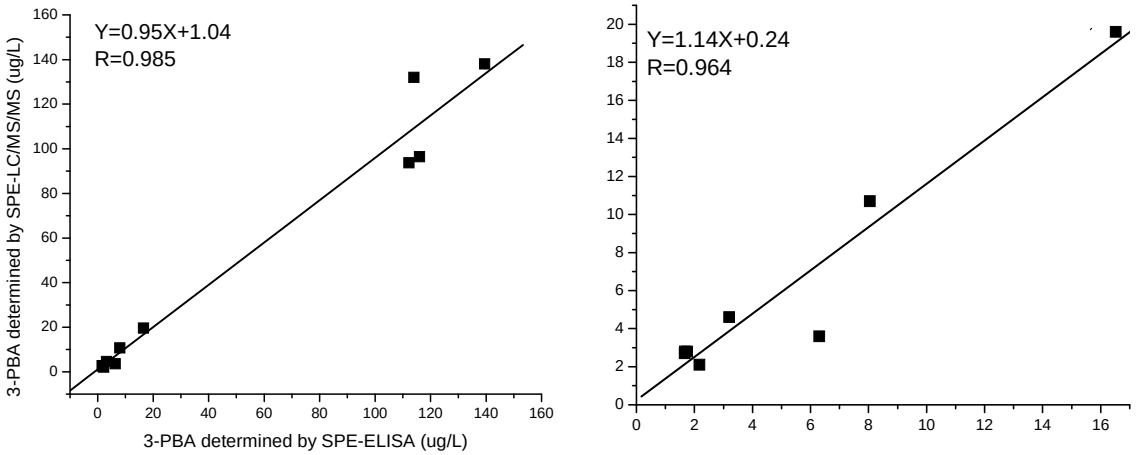


Figure 17. Correlations between the spiked and measured concentrations of 3-PBA for ELISA and LC/MS/MS. The ELISA and LC/MS/MS analysis was performed after the mixed-mode SPE for sample preparation. A) The full concentration range tested. B). The scale expanded between 0-20 ug/L. The limit of detection of the LC/MS/MS was 2 ng/mL.

Table 12. Comparison of 3-PBA concentrations determined with a LC/MS/MS and ELISA, urine extract being prepared with a mixed mode SPE.

Sample	3-PBA (ng/mL)	
	Instrument (LC/MS/MS)	ELISA
CTL-1 (PBS buffer)	1.4	0.14
CTL-2 (PBS buffer)	1.3	0.20
LS-2 3.1	138	139.5
LS-2 3.2	132	114
LS-7 1.1	96.4	116
LS-7 1.2	93.7	112.2
MWV-4 5.1	2.7	1.66
MWV-4 5.2	2.8	1.75
MWV-4 7.1	2.8	1.67
MWV-4 7.2	2.1	2.17
001	4.6	3.2
Composite 1B	3.6	6.3
SJM	19.6	16.522
KCA IV	10.7	8.044

Development of immunoassay for DCVA. Synthesis of haptens for DCVA. In order the elicit antibodies, the molecular weight of the target compound (also known as the antigen) must generally be larger than 10,000 kilodaltons. Since the pyrethroid metabolites are all much smaller than this, they must be covalently linked to a protein. Proteins contain carboxylic acid, amine, sulfhydryl or hydroxyl groups that can be used for the covalent linkage. DCVA contains a carboxylic acid thus linkage could be made by direct coupling. However, the most selective antibodies are those to haptens that are coupled to proteins through a linker that places the

hapten at some distance from the surface of the protein. Figure 18 shows the structure of haptens used to elicit antibodies to DCVA; note that compared to DCVA, two of these haptens have 'linkers' such that the protein would be attached at one or three carbons away from the protein coupling. *trans*-DCVA haptens 1 and 2 were synthesized as follows: A mixture of the *trans*-DCVA, 2-bromo-acetic acid *tert*-butyl ester (or 4-bromo-butyric acid *tert*-butyl ester), and potassium carbonate in 1 mL of anhydrous N,N-dimethylformamide was reacted at 100 °C for 3 h. The resulting mixture was filtered to remove excess K_2CO_3 and HBr produced in the reaction. The filtrate diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to obtain the ester intermediate as a transparent oil. Trifluoroacetic acid (TFA) (0.5 mL) was added to the ester intermediate, and the mixture was allowed to stand at ambient temperature for 15 min. The TFA was stripped off under vacuum, and ethyl acetate was added twice and stripped to remove residual TFA. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:1, v/v). Fractions containing pure product by TLC were stripped under high vacuum, and the residue was recrystallized from ethyl acetate and *tert*-butyl chloride to give *trans*-haptens 1 and 2 as a white solid. t*rans*-Hapten 1 and 2 were designated as DCVA-acetyl and DCVA-butyl.

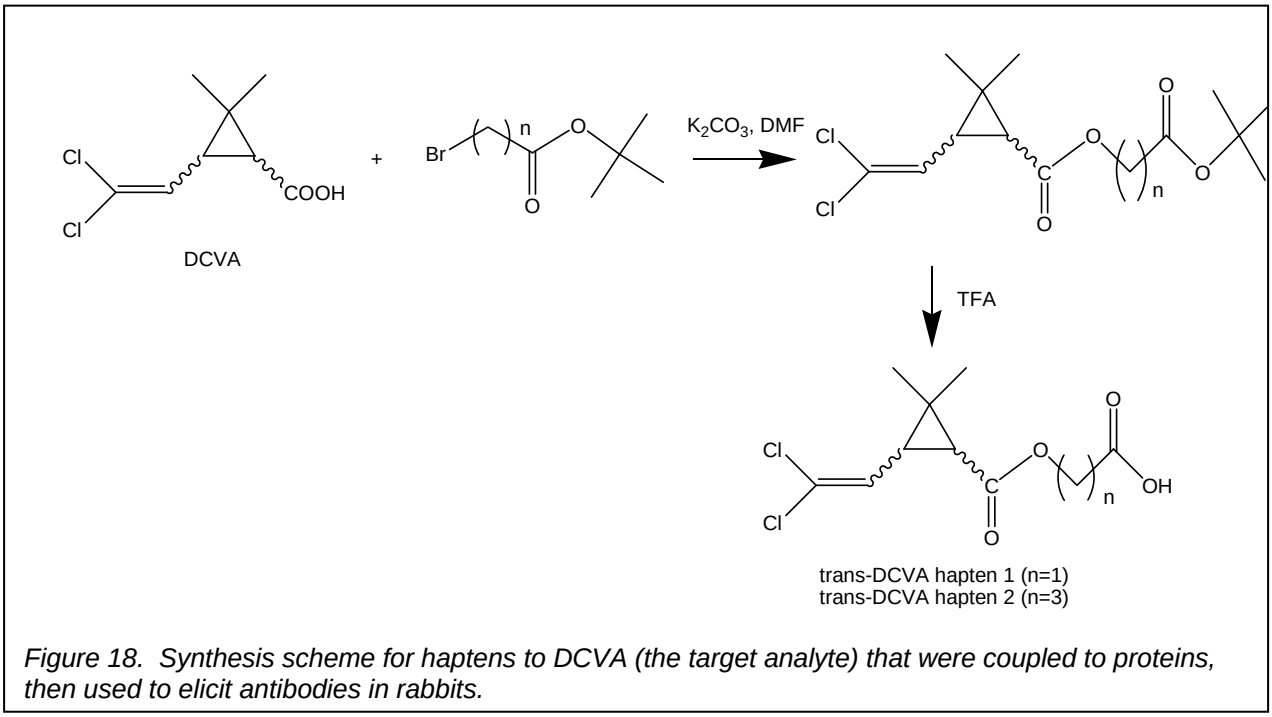


Figure 18. Synthesis scheme for haptens to DCVA (the target analyte) that were coupled to proteins, then used to elicit antibodies in rabbits.

<u>*Development of immunoassay for DCVA: Immunization*</u> *trans*-Hapten 1 (DCVA-butyl) and *trans*-hapten 2 (DCVA-acetyl) were used as an immunizing hapten. The haptens *trans*-DCVA-acetyl and *trans*-DCVA-butyl were conjugated by the active ester method using N-hydroxysuccinimide and dicyclohexylcarbodiimide and then conjugated to thyroglobulin. Two rabbits were used to produce antiserum for each immunogen. Antisera #1886 and #1887 for *trans*-hapten 2 (DCVA-butyl) and antisera #1888 and 1889 for *trans*-hapten (DCVA-acetyl).

<u>*Development of immunoassay for DCVA. Initial screening.*</u> The antisera were screened against various coating antigens. None of the antibody/coating antigen combinations were

inhibited by DCVA, so no assay could be developed using these reagents. Reagents developed for the detection of DCVA-glycine were then screened for sensitivity to DCVA alone. We found that the combination of antiserum (#3709) produced against *cis*-hapten 9-Thyr (Ahn et al., 2004) and *trans*-hapten 5-BSA as a coating antigen showed a good sensitivity at an IC_{50} value of 18 µg/L to determine a mixture of *cis/trans*-DCVA (Figure 19). Further optimization and characterization are necessary. If DCVA-glycine is a major metabolite, then it may not be important to use an assay for DCVA. However, if DCVA-glycine is not found, this assay for DCVA may prove useful.

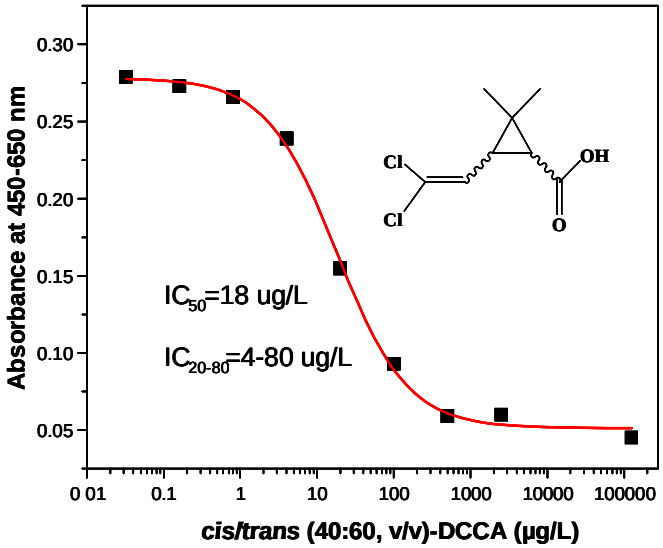


Figure 19. Calibration curve for the detection of cis/trans-DCVA.

KEY RESEARCH ACCOMPLISHMENTS

- Synthesis of dichlorovinylchrysanthemic acid-glycine conjugate standard
- Synthesis of glucuronide conjugate of 3-phenoxybenzyl alcohol standard
- Synthesis of 4'-hydroxy-3-phenoxybenzoic acid
- Development of an HPLC method for separating putative metabolites of permethrin
- Determination of the time course of serum and urinary elimination of permethrin
- Development of an immunoassay for 3-phenoxybenzoic acid
- Development of an immunoassay for the glucuronide conjugate of 3-phenxoybenzyl alcohol
- Development of an immunoassay for dichlorovinylcyclopropane carboxylic acid
- Development of an immunoassay for the glycine conjugate of dichlorovinylcyclopropane carboxylic acid

REPORTABLE OUTCOMES

- 7 Peer reviewed publications.(Appendix I)
- 6 Presentations at national scientific conferences (Appendix II)
- One postdoctoral researcher applied for and received employment at Dow AgroSciences, LLC, Indianapolis, IN in their regulatory affairs section
- One postdoctoral researcher applied for and received employment at Marrone Organic Innovations, LLC, Davis, CA as an analytical chemist

- The PI has applied for and received funding, as part of a larger program project, from the National Institute of Occupational Safety and Health, to utilize the assays developed in this project to assess farmworker and farmworker family exposure to pesticides.
- The research coordinator along with the PI have applied for and received funding, as part of a larger program project, from the National Institute of Environmental Health Science to utilize the assays developed to evaluate exposure and effect of environmental chemicals.

CONCLUSION

The goal of this project was to identify the most abundant human metabolite of permethrin found in urine and to develop a rapid immunoassay to be used in biomonitoring of exposure. The project utilized three complementary state-of-the-art techniques to carry out the objectives, accelerator mass spectrometry (AMS), liquid chromatography tandem mass spectrometry (LCMS) and immunoassay. A very low dose of permethrin, containing only 1 microcurie of carbon-14 label, was used for the dermal exposure. AMS was used to measure total carbon-14 in urine, serum and saliva samples taken over a 7 day period. The average absorbed dose was about 0.2% of the applied dose. Analysis of saliva samples shows a peak excretion at about 6 hr, while peak serum concentrations were reached in 12-24 hrs. Permethrin was eliminated in the urine with an average half-life of 56 h. An LCMS method was developed to separate metabolites using urine spiked with metabolite standards that were synthesized for the project. The AMS allowed measurement of individual metabolites in urine even though only 0.2% of the dose was absorbed. The glucuronide conjugate of 3-phenoxybenzyl alcohol was the major identified metabolite. However, about 55% of the radioactivity that was chromatographed was unidentified, eluting in the most polar fractions. An immunoassay for the glucuronide of 3-phenoxybenzyl alcohol (3PBAlc-gluc) was developed with an limit of quantitation in urine estimated at 1-2 ng/mL. The only sample preparation required is dilution in buffer. The accelerator mass spectrometer proved to be a highly sensitive tool for determining metabolite patterns in this low dose study where the amount of radioactivity excreted could not be measured by standard liquid scintillation counting. Using the initial development work with the LCMS, further research should be conducted to characterize the unidentified metabolites. The assay for 3PBAlc-gluc can be used in biomonitoring studies to develop a further understanding of the relationship between exposure from permethrin-treated uniforms and potential toxicity.

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APPENDIX I - Publications pdfs

Immunoassay, biosensors and other nonchromatographic methods

Guomin Shan, Cynthia Lipton,
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pp. 623–679

in

Handbook of Residue Analytical Methods for Agrochemicals
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Edited by

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Immunoassay, biosensors and other nonchromatographic methods

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1 Introduction

Nonchromatographic methods for residue detection consist of a wide variety of techniques. For illustrative purposes these may be divided into 'biological'- and 'physical'-based methods, based on whether or not biological reagents are involved. Biological techniques include immunoassays, biosensors, bioassays, enzyme assays and polymerase chain reaction (PCR). Among the physical techniques that fit this category are spectrophotometry and voltammetry. The focuses of this article are the 'biological' techniques, in particular immunoassays and PCR, with a brief introduction to biosensors.

2 Immunoassay for pesticides

The concept of immunoassay was first described in 1945 when Landsteiner suggested that antibodies could bind selectively to small molecules (haptens) when they were conjugated to a larger carrier molecule.[1] This hapten-specific concept was explored by Yalow and Berson in the late 1950s, and resulted in an immunoassay that was applied to insulin monitoring in humans.[2,3] This pioneering work set the stage for the rapid advancement of immunochemical methods for clinical use.

The first application of immunologically based technology to pesticides was not reported until 1970, when Centeno and Johnson developed antibodies that selectively bound malathion.[4] A few years later, radioimmunoassays were developed for aldrin and dieldrin[5] and for parathion.[6] In 1972, Engvall and Perlman introduced the use of enzymes as labels for immunoassay and launched the term enzyme-linked

Handbook of Residue Analytical Methods for Agrochemicals.

immunosorbent assay (ELISA).[7] In 1980, Hammock and Mumma[8] described the potential for ELISA for agrochemicals and environmental pollutants. Since then, the use of immunoassay for pesticide analysis has increased dramatically. Immunoassay technology has become a primary analytical method for the detection of products containing genetically modified organisms (GMOs).

The advantages of immunoassay technology relative to other analytical techniques have been discussed in several reviews,[8–12] and include the following:

- low detection limits
- high analyte selectivity
- high throughput of samples
- reduced sample preparation
- versatility for target analytes
- cost effectiveness for large numbers of samples
- adaptability to field use.

As is the case with every analytical method, immunoassay technology has limitations, including:

- interferences from sample matrices
- cross reactivity to structural analogs of the target analyte
- poor suitability for some multi-analyte applications
- low availability of reagents
- longer assay development time than some classical analytical methods
- a large number of anticipated samples required to justify the development of a new assay for an analyte of interest.

The immunoassay is clearly not the best analytical method for all analytes in all situations. For example, gas–liquid chromatography (GLC) remains the method of choice for the analysis of volatile compounds. However, immunoassay technology is important for the analyst because it complements the classical methods, thus providing a confirmatory method for many compounds and the only reasonable analytical choice for others.[13] Most immunoassays can be used to obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods. They are generally applicable to the analysis of small molecules, including pharmaceuticals and pesticides, identification of pest and beneficial species, characterization of crop quality, detection of GMOs, product stewardship, detection of disease and even monitoring for bioterrorism.

2.1 Principles of immunoassays

Immunoassays are based on the reaction of an analyte or antigen (Ag) with a selective antibody (Ab) to give a product (Ag–Ab) that can be measured. The reactants are in a state of equilibrium that is characterized by the law of mass action (Figure 1).

Several types of labels have been used in immunoassays, including radioactivity, enzymes, fluorescence, luminescence and phosphorescence. Each of these labels has advantages, but the most common label for clinical and environmental analysis is the use of enzymes and colorimetric substrates.

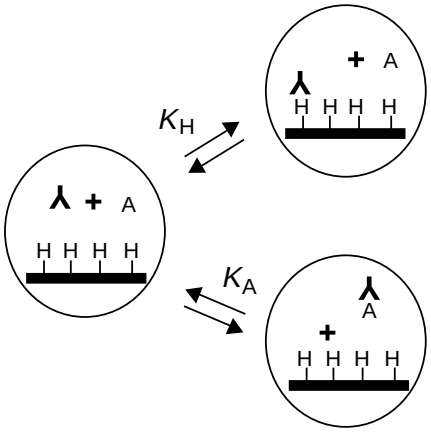


Figure 1 Schematic of the quasi-equilibria using heterologous haptens in coating antigen immunoassay formats. K_A represents the equilibrium constant for binding of antibody (**Y**) to target analyte (**A**). K_H is the equilibrium constant for the binding of antibody to hapten–protein conjugate (**H**–) immobilized on a solid phase

Enzyme immunoassays can be divided into two general categories: homogeneous and heterogeneous immunoassays. Heterogeneous immunoassays require the separation of bound and unbound reagents (antibody or antigen) during the assay. This separation is readily accomplished by washing the solid phase (such as test-tubes or microtiter plate wells) with a buffer system. Homogeneous immunoassays do not require a separation and washing step, but the enzyme label must function within the sample matrix. As a result, assay interference caused by the matrix may be problematic for samples of environmental origins (i.e., soil, water, etc.). For samples of clinical origin (human or veterinary applications), high target analyte concentrations and relatively consistent matrices are often present. Thus for clinical or field applications, the homogeneous immunoassay format is popular, whereas the heterogeneous format predominates for environmental matrices.

2.2 Immunoassay formats

The microplate ELISA test is conducted in standard 96-well microplates. A microplate consists of a 12×8 grid of wells for test solutions. The three most widely used ELISA formats are immobilized antigen competitive immunoassay, immobilized antibody competitive immunoassay and sandwich immunoassay.[14,15]

The following is a generic description of the immobilized antigen ELISA (Figure 2), commonly termed indirect competitive immunoassay, on a microtiter plate.

Preparation of microtiter plates. A constant amount of the coating antigen is bound to the surface of polystyrene microtiter plate wells by passive adsorption. After a predetermined incubation time, the plate is washed to remove unbound coating antigen.

Competitive inhibition. A constant amount of anti-analyte antibody (primary antibody) and a series of solutions containing increasing amounts of analyte are added to the prepared microtiter plate wells. During incubation, the free analyte and bound

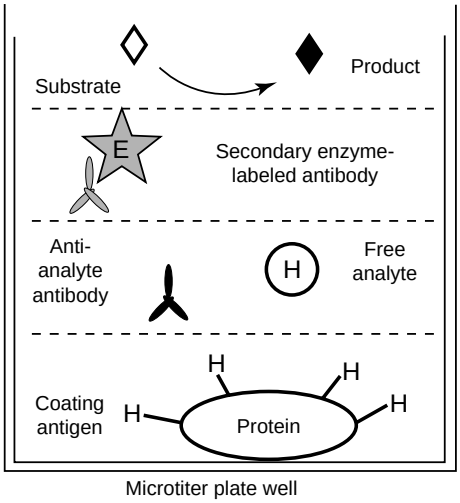


Figure 2 Immobilized antigen ELISA format. Antigen is immobilized to a solid phase by passive adsorption. Following removal of unbound antigen, analyte (free **H**) and antigen (**H**–protein) compete for a fixed number of primary antibody (**Y**) binding sites. Unbound materials are removed (dotted line). Secondary antibody–enzyme conjugate (**Y**–**E**) is added to bind to primary antibody followed by another wash step. Substrate (◇) for the enzyme is added to detect the bound enzyme. The amount of colored product (◆) detected is inversely proportional to the amount of analyte present

coating antigen compete for binding to antibodies in the mixture. Unbound reagents are washed out.

Secondary antibody and determination. A secondary antibody labeled with an enzyme is added which binds to the primary antibody that is bound to the coating antigen. If the primary antibody were produced in a rabbit, an appropriate secondary antibody would be goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (or another enzyme label). Excess secondary antibody is washed away. An appropriate substrate solution is added that will produce a colored or fluorescent product after enzymatic conversion. The amount of enzyme product formed is directly proportional to the amount of first antibody bound to the coating antigen on the plate and is inversely proportional to the amount of analyte in the standards.

Another commonly used ELISA format is the immobilized antibody assay or direct competitive assay (Figure 3). The primary anti-analyte antibody is immobilized on the solid phase and the analyte competes with a known amount of enzyme-labeled hapten for binding sites on the immobilized antibody. First, the anti-analyte antibody is adsorbed on the microtiter plate wells. In the competition step, the analyte and enzyme-labeled hapten are added to microtiter plate wells and unbound materials are subsequently washed out. The enzyme substrate is then added for color production. Similarly to indirect competitive immunoassay, absorption is inversely proportional to the concentration of analyte. The direct competitive ELISA format is commonly used in commercial immunoassay test kits.

Sandwich ELISAs (Figure 4) are the most common type of immunoassay used for the detection of proteins. A capture antibody is immobilized on the wells of a microplate. The solution containing the analyte is introduced and antibody–analyte

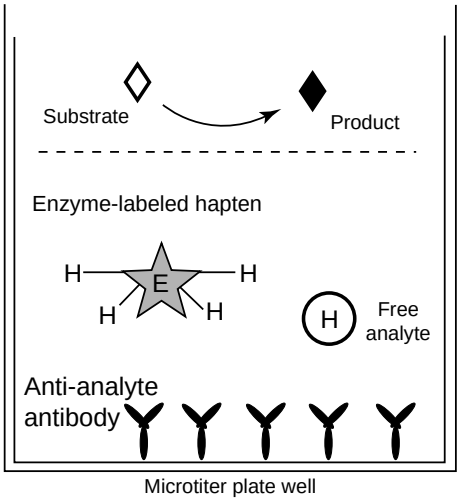


Figure 3 Immobilized antibody ELISA. Primary antibody (**Y**) is passively adsorbed to the surface of a polystyrene microtiter plate. Analyte (free **H**) and an enzyme-labeled hapten (**H**–**E**) compete for the fixed number of primary antibody binding sites. Following a wash step (dotted line), the substrate for the enzyme is added (◇) and a colored product formed (◆). The amount of product is inversely proportional to the amount of analyte present

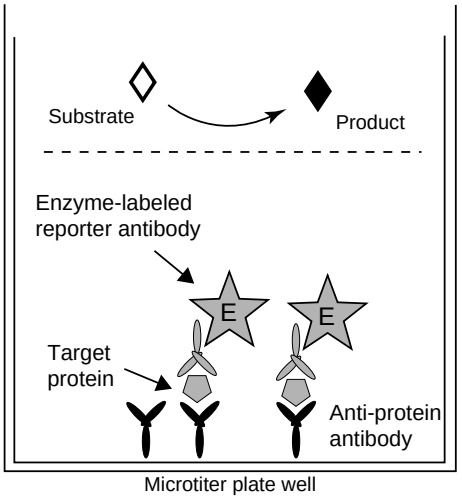


Figure 4 Sandwich immunoassay. A capture antibody (**Y**) is passively adsorbed on a solid phase. The target protein contained in the sample and the enzyme-labeled reporter antibody (**Y**–**E**) are added. Both the capture antibody and enzyme-labeled reporter antibody bind to the target protein at different sites, 'sandwiching' it between the antibodies. Following a wash step, the substrate (◇) is added and colored product (◆) formed. The amount of colored product is directly proportional to the amount of target protein captured

binding occurs. A second, analyte-specific, enzyme-labeled antibody is added and it also binds to the analyte, forming a sandwich. A substrate is added, producing a colored product. Unlike the competitive immunoassays described in Figures 2 and 3, the absorbance in the sandwich immunoassay is directly proportional to the concentration of the analyte in the sample solution.

A commonly used field-portable immunoassay format is the lateral flow device. Lateral flow devices are designed for threshold or qualitative testing. Advantages of this format are that the cost per test is low, it is field portable, it can be done at ambient temperature, it requires no specialized equipment and only minimal user training is required. Each immunoassay strip test (lateral flow device) is a single unit allowing for manual testing of an individual sample. The device contains a reporter antibody labeled with a colored particle such as colloidal gold or latex, which is deposited in a reservoir pad. An analyte-specific capture antibody is immobilized on the membrane. When the strip is placed into the test solution, the solution enters the reservoir pad and solubilizes the labeled reporter antibody, which binds to the target analyte. This analyte–antibody complex flows with the liquid sample laterally along the surface of the strip. When the complex passes over the zone where the capture antibody has been immobilized, the complex binds to the capture antibody and is trapped, accumulating and producing the appearance of a colored band at the capture zone on the strip. If the result is negative and no analyte is present in the test solution, only the control band appears in the result window. This band indicates that the liquid flowed properly up the strip. If the result is positive, two bands appear in the result window. A lateral flow strip test can provide a yes/no determination of the presence of the target analyte or a threshold (semi-quantitative) result, typically in 5–10 min.

Commercial test kits that use 96-well microtiter plates or test tubes have been available for some pesticides since the 1980s.[16] Several vendors have assays for analytes such as herbicides that appear in groundwater or runoff water, e.g., triazines, alachlor, diazinon and chlorpyrifos. More recent emphasis has been the production of kits for compounds of concern in developing countries (such as DDT) and for GMOs. When selecting a test kit, the user should determine the intended use, (i.e., as a screening method or a quantitative method) and whether the method will be used in the laboratory or the field. The cost per assay, assay sensitivity, cross-reactivity, availability of published validation by independent groups and the availability of technical support are important considerations in selecting a test kit. It is critical that the assay has been validated in the matrix of interest. If a kit or method intended for water is used for another aqueous media such as urine, inaccurate results may be obtained. Because the test kit must be validated in the matrix of concern, the sponsoring company will usually actively collaborate or assist with the validation. Several papers on test kit validations or comparisons of test kits from different manufacturers have been published.[16–19]

2.3 Data reduction

The absorbance values obtained are plotted on the ordinate (linear scale) against the concentration of the standards on the abscissa (logarithmic scale), which produces a sigmoidal dose–response curve (Figure 5). The sigmoidal curve is constructed by

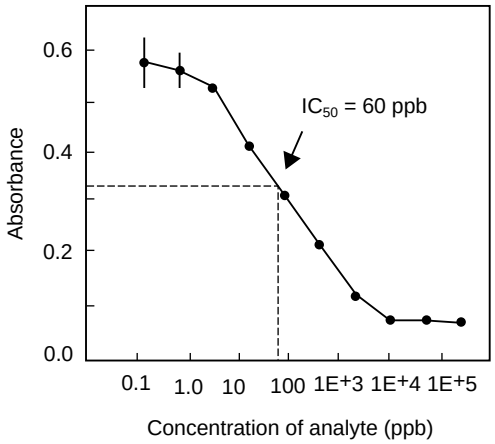


Figure 5 An example calibration curve. Absorbance is plotted against log (concentration of analyte). The competitive equilibrium binding process results in a sigmoidal curve that is fitted using a four-parameter fit. [20] The IC_{50} is defined as the concentration of analyte that results in a 50% inhibition of the absorbance

using the four-parameter logistic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance.[20]

Assay sensitivity is defined here as the concentration of analyte that inhibits the observed absorbance by 50% or the IC_{50}. The lower limit of detection (LLD) is the lowest analyte concentration that elicits a detector response significantly different from the detector response in the absence of analyte. In some cases, the LLD is defined as three standard deviations from the mean of the zero analyte control. In other cases, the LLD is defined empirically by determining the lowest concentration of analyte that can be measured with a given degree of accuracy. Readers are referred to Grotjan and Keel[21] for a simplified explanation and to Rodbard[22] for the complete mathematics on the determination of LLD.

The concentration of analyte in the unknown sample is extrapolated from the calibration curve. To obtain an accurate and precise quantitative value, the optical density (OD) for the sample solutions must fall on the linear portion of the calibration curve. If the sample OD is too high, the sample solution must be diluted until the OD falls within the quantitative range of the assay. The concentration of the analyte in the original sample is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the microplate.

2.4 Sample collection and preparation

Once the immunoassay that meets the study objectives has been identified, sample collection begins. Proper sampling is critical in order to obtain meaningful results from any type of analytical assay. An appropriate sampling scheme will support the objective of the test. For example, a plant breeder may take a single leaf punch to determine quickly whether a specific protein has been expressed in an experimental plant. A more complex sampling regime would be used to determine the expression

profile of a specific protein in corn grain, leaves and stalks for a regulatory study. These regulatory field studies are often modeled after crop residue studies for chemical pesticides. The protocol typically describes sampling from representative plants, tissues, growth stages and geographical sites.

Sampling has the potential to introduce significant uncertainty and error into a measurement; therefore, a proper plan should be devised with the assistance of a qualified statistician. Grain sampling is a routine practice and standard methods for taking samples from static lots – such as trucks, barges and railcars – and for taking samples from grain streams can be found in the United States Department of Agriculture Grain Inspection Protection Service (USDA GIPSA) 'Grain Inspection Handbook, Book 1, Grain Sampling'.[23] Ultimately, the optimum sampling strategy is a balance between sensitivity, cost and confidence.

Sample preparation techniques vary depending on the analyte and the matrix. An advantage of immunoassays is that less sample preparation is often needed prior to analysis. Because the ELISA is conducted in an aqueous system, aqueous samples such as groundwater may be analyzed directly in the immunoassay or following dilution in a buffer solution. For soil, plant material or complex water samples (e.g., sewage effluent), the analyte must be extracted from the matrix. The extraction method must meet performance criteria such as recovery, reproducibility and ruggedness, and ultimately the analyte must be in a solution that is aqueous or in a water-miscible solvent. For chemical analytes such as pesticides, a simple extraction with methanol may be suitable. At the other extreme, multiple extractions, column cleanup and finally solvent exchange may be necessary to extract the analyte into a solution that is free of matrix interference.

The protein analyte is extracted from the plant material by adding a solvent and blending, agitating or applying shearing or sonic forces. Typical solvents used are water or buffered salt solutions. Sometimes detergents or surfactants are added. As with chemical pesticide extraction methods, the protein extraction procedure must be optimized for the specific sample matrix. Processed samples may have been subjected to processes resulting in protein precipitation and/or denaturation. These factors can influence protein extraction efficiency. The problem can often be overcome by changing the buffer composition and the extraction procedure.

Because the protein analyte is endogenous to the plant, it can be difficult to demonstrate the efficiency of the extraction procedure. Ideally, an alternative detection method (e.g., Western blotting) is used for comparison with the immunoassay results. Another approach to addressing extraction efficiency is to demonstrate the recovery of each type of protein analyte from each type of food fraction by exhaustive extraction, i.e., repeatedly extracting the sample until no more of the protein is detected.[24]

Some examples are given below to illustrate extraction procedures for proteins that have been optimized for different matrices and testing strategies.

Neomycin phosphotransferase II (NPTII) extraction from cotton leaves and cottonseed. The extraction buffer consists of 100 mM Tris, 10 mM sodium borate, 5 mM magnesium chloride, 0.2% ascorbate and 0.05% Tween 20 at pH 7.8. The frozen leaf sample is homogenized in cold (4 °C) buffer. An aliquot of the homogenate is transferred to a microfuge tube and centrifuged at 12 000 *g* for 15 min. The supernatant is diluted and assayed directly by ELISA.

The extraction procedure for cottonseed samples is the same, except that the cottonseed samples are crushed before the buffer is added for homogenization.[25]

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) extraction from processed soybean fractions. The extraction buffer consists of 0.138 M NaCl, 0.081 M Na_2HPO_4, 0.015 M KH_2PO_4, 0.027 M KCl and 2% sodium dodecyl sulfate (SDS) at pH 7.4. Aqueous buffers are inadequate to extract EPSPS efficiently from processed soybean fractions owing to protein precipitation and the denaturation that occurs throughout soybean processing. Efficient extraction is achieved through the use of detergent in an aqueous buffer, mechanical tissue disruption and heating.[25]

Bt11 endotoxin extraction from corn grain. The following example is a description of a commercial kit procedure for extraction of the Cry1A (b) and Cry1A (c) from corn grain for analysis with an immunoassay strip test (lateral flow device). It is important to note that for the Bt11 event the endotoxin is expressed in seed (grain) and plant tissue. However, corn plants from the Bt176 event do not express detectable quantities of the *Bacillus thuringiensis* (Bt) endotoxin in grain, and therefore a negative result in a corn grain sample does not necessarily mean the sample does not contain genetically modified material.

Reagents A and B are supplied with the kit, but the composition of these solutions is not described. A sample (25 g) of corn grain is weighed into a 4-oz glass Mason jar. Using a Waring blender, the sample is ground for 10 s on the low-speed setting. Buffered water (40 mL), consisting of 200 mL of Reagent A in 1 gal of distilled water, is added to the ground corn. The jar is capped and shaken vigorously for at least 30 s. The solids are allowed to settle and the supernatant is withdrawn with a transfer pipet. Six drops of the supernatant are dispensed into the reaction tube and three drops of Reagent B are added. The reaction tube is capped and mixed by inverting it three times. The sample is analyzed with the lateral flow device.[26]

2.5 Development of pesticide immunoassays

The development of sensitive and inexpensive immunoassays for low molecular weight pesticides has been an important trend in environmental and analytical sciences during the past two decades.[8,10,27–29] To design an immunoassay for a pesticide, one can rely on the immunoassay literature for agrochemicals,[30–32] but many of the innovations in clinical immunoanalysis are also directly applicable to environmental analysis.[11,33,34] Conversely, the exquisite sensitivity required and difficult matrices present for many environmental immunoassay applications have forced the development of technologies that are also useful in clinical immunoassay applications. In the following discussion we will describe widely accepted procedures for the development of pesticide immunoassays.

The major steps in the development of an immunoassay are as follows:

- design and synthesis of haptens
- conjugation of haptens to antigenic macromolecular carriers
- immunization of host animals and subsequent generation of antibodies

- characterization of antibodies
- assay optimization
- assay validation.

2.5.1 Basic analysis of the target analyte structure

In general, immunoassays are more readily developed when the target analyte is large, hydrophilic, chemically stable and foreign to the host animal.[8] In theory, the sensitivity and selectivity of an immunoassay are determined by the affinity of the antibody to the analyte, and hence immunogen design and antibody production are of fundamental importance to assay development. For a molecule to be immunogenic it must have a molecular mass of at least 2000 Da and possess a complex and stable tertiary structure. Low molecular weight antigens (less than 2000 Da), a size that includes most pesticides, are not directly immunogenic. Such nonimmunogenic molecules are termed 'haptens'. Haptens possess no, or very few, epitopes that are recognizable by immune systems of host animals. As a consequence, they must be linked to larger molecules in order to become immunogenic to host animals.

Factors an analyst should consider when designing a hapten–immunogen system are outlined in Table 1. The immunizing hapten should be designed to mimic closely the target analyte. Ideal haptens have close chemical similarity to the target analyte and possess a functional group to allow coupling to carrier molecules; coupling to carrier antigens usually occurs through a 'linker,' 'spacer' or 'handle' molecule (discussed below). Retention of the unique functional groups of the analyte, especially ionizable groups or groups that form hydrogen bonds, are critical for the production of high-affinity antibodies. Also important are the ease of hapten synthesis, hapten solubility, and the nature of the method to be used for conjugation to proteins.

2.5.2 Design of the immunizing hapten

(1) Position of spacer arm. The position of the linker group on the target analyte that connects it to the immunogen has a profound influence on the selectivity and sensitivity of the subsequent assays. The handle should be attached as far as possible from the unique determinant groups, allowing maximum exposure of the important

Table 1 Guidelines for the design and synthesis of an immunogen hapten

1. Position of handle on target molecule
Distal to hapten determinant groups
Avoid attachment to functional groups
2. Handle selection
Length of handle
Avoid functional groups in handle (unless used to increase exposure or improve solubility)
3. Coupling of haptens
Type of coupling reaction
Compatibility of reaction with target molecule functional groups
4. Stability of hapten under coupling conditions and subsequent use
5. Ease of synthesis
6. Characterization of conjugates and determination of hapten/protein ratio

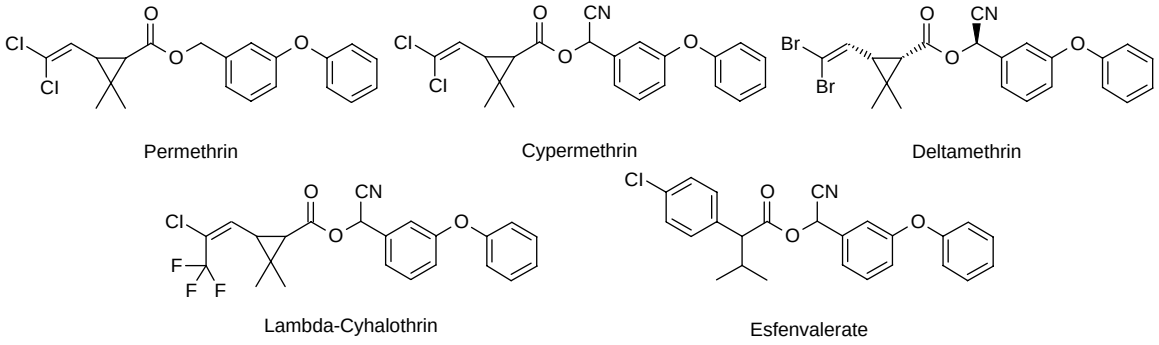


Figure 6 Structures of some major use pyrethroids

structural features of the analyte to the immune system. Presentation of unique features of the target analyte is particularly important for ensuring selectivity to a single chemical structure within a chemical class. For example, we attempted to develop compound-specific immunoassays for the major pyrethroids esfenvalerate, permethrin and cypermethrin. As shown in Figure 6, these pyrethroids have similar or identical alcohol moieties, while containing relatively unique acyl substituents. If a carrier protein was linked through the acid portion, leaving the common phenoxybenzyl group unchanged, the resulting antibodies generated from such an immunogen would be expected to recognize many pyrethroids. In order to develop a compound-specific assay, we retained the relatively unique acid substituents, and attached the linkers to the aromatic phenoxy benzyl groups (Figure 7). Using this strategy, sensitive and selective assays for permethrin and esfenvalerate were developed.[35,36] Another design option was to modify the α-cyano group to support a linker for protein conjugation (Figure 8). In this case, nearly the whole pyrethroid is unchanged; antibodies developed based on this strategy were specific for the target compounds.[37,38]

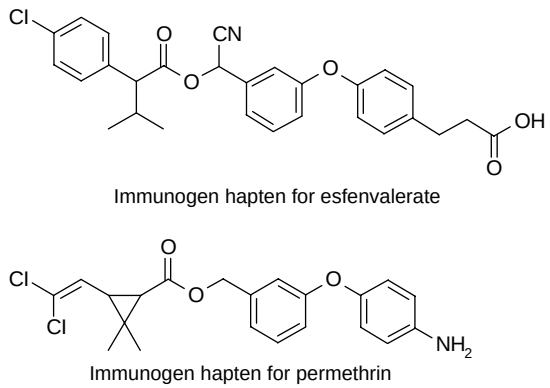


Figure 7 Structure of the haptens used in the immunogen for the development of antibodies that recognize pyrethroid insecticides, esfenvalerate and permethrin. The esfenvalerate hapten was coupled to proteins through the carboxylic acid group and the permethrin hapten was coupled to proteins through the amine group. Because antibody recognition of the structure is greatest most distal to the point of attachment to the protein, the antibodies were selective for the acid portions of the pyrethroid molecules resulting in highly selective assays for esfenvalerate and permethrin, respectively

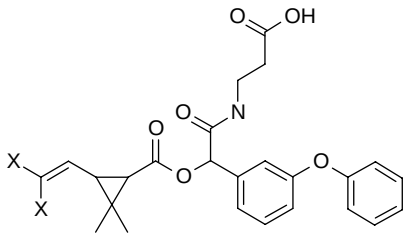


Figure 8 Structure of immunogen haptens for pyrethroids with spacer arm attachment at the α-position of the alcohol moiety. Since the whole pyrethroid molecule is available for recognition by the antibody, assays resulting from these immunogens were selective for the parent pyrethroids

However, if a class-selective assay is desirable (for multi-analyte assays), the handle should be located at or near a position that differentiates members of the class and exposes features common to the class. Using the pyrethroid example, an ideal immunogen should retain the phenoxybenzyl moiety and link the protein from the distal acid end (Figure 9). Using such an immunogen hapten, a class-specific immunoassay was developed that was highly cross-reactive with the type I pyrethroids permethrin, phenothrin, resmethrin and bioresmethrin.[39]

For small molecules, the retention of each determinant group identity is very important. Attaching the handle to a determinant group should be avoided because this alters the target molecule's structure, geometry and electronic properties relative to the parent compound. Some target analytes may contain acid, amino, phenol or alcohol groups that can be directly conjugated. Because hydrogen bonding is often the major force for interaction between an antigen and an antibody, such groups are very important determinants for antibody affinity and specificity. A good example of functional group importance is the immunoassay for phenoxybenzoic acid (PBA), a major metabolite of some pyrethroids. To develop an antibody against PBA, two options were used to design and conjugate haptens to the carrier protein. Phenoxybenzoic acid was directly conjugated with the antigenic protein using its $-$COOH group (Figure 10, site 2). This reaction could be accomplished using relatively simple chemistry for conjugation, but would likely result in poor antibody specificity because the phenoxybenzyl moiety is present in many parent pyrethroids. In addition,

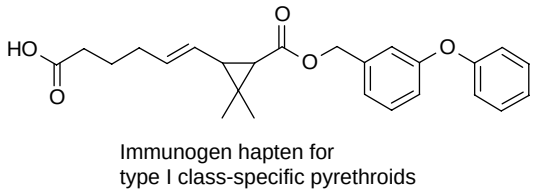
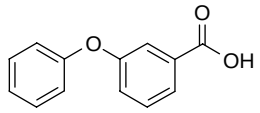


Figure 9 Structure of the immunogen hapten used to generate antibodies for a type I pyrethroid class-selective assay. Pyrethroids lacking an α-cyano group are generally termed type I. This hapten exposed the features most common to type I pyrethroids, the phenoxybenzyl group, the cyclopropyl group and the lack of a cyano group, resulting in antibodies that recognized permethrin, phenothrin, resmethrin and bioresmethrin, but not cypermethrin

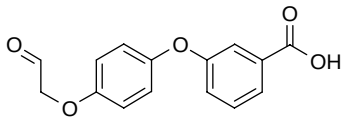


Phenoxybenzoic acid (PBA)

Figure 10 Structure of the target analyte phenoxybenzoic acid (PBA). The arrows point to the ideal sites for conjugation of the molecule to proteins for optimum recognition. Use of site 2 for conjugation to protein resulted in antibodies that recognized free PBA poorly

the lack of hydrogen bonding elements and reduced solubility of conjugates would likely significantly influence subsequent antibody affinity and specificity. Alternatively, we designed a hapten that left the −COOH group unchanged by attaching to the distal aromatic benzene, site 1, a linker containing a terminal aldehyde group that was used to conjugate to protein (Figure 11). The resulting antibodies had a high binding affinity and resulted in the development of a highly sensitive and selective assay [($IC_{50} = 1\ \mu g\ L^{-1}$ (ppb)] that was about 1000 times more sensitive than the assay developed from an antibody raised against an immunogen conjugated at site 2. No cross-reactivity to any other parent pyrethroid or their metabolites was measured for the antibody resulting from site 1 conjugation. Although some structural change in the target molecule is usually unavoidable, when selecting a handle for the immunogen hapten the original steric and electronic characteristics of the target molecule should be preserved as much as practical. Especially electronic features including electron density around important atoms, net charge at important atoms and hybridization of electronic orbitals of characteristic groups should be preserved.

(2) Handle selection. For small molecules (including most pesticides), the selection of a spacer or linker arm is important. Omitting the spacer arm from the structure of immunogen may result in assays with poor sensitivity and/or weak recognition of the portion of the target molecule near the attachment to the carrier protein. Generally, the optimal linking group has a chain length of about four to six atoms.[40–42] For hydrophobic haptens such as pyrethroids and dioxins, the role of the spacer may be of critical importance because the hapten may fold back on the protein surface or within the protein core after conjugation. The antibody resulting from such an immunogen will have low affinity and poor selectivity. A hapten with a rigid spacer can overcome such hydrophobic interactions. A double bond-containing spacer for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) immunogen hapten (Figure 12) resulted in a highly sensitive immunoassay with an IC_{50} of 240 ng L^{-1}.[43,44] In contrast, when a flexible hexanoic acid spacer was used for development of an ELISA



Immunogen hapten for PBA

Figure 11 Structure of the phenoxybenzoic acid (PBA) immunogen hapten. Conjugation to the protein through the aldehyde resulted in an immunogen that generated antibodies selective and sensitive for PBA

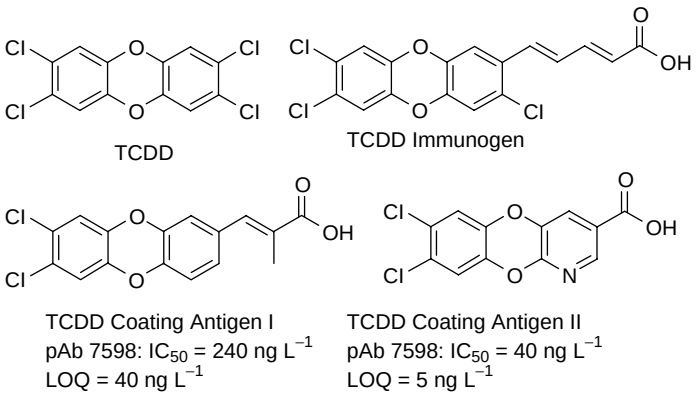


Figure 12 Structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), immunogen and coating haptens. The immunogen was synthesized with a rigid spacer so the lipophilic hapten would not fold back into the hydrophobic core of the protein preventing recognition by the immune system. The affinity of the antibody for coating antigen II is less than for coating antigen I owing to structural changes, hence the assay using coating antigen II is more sensitive for TCDD

for polychlorinated biphenyls (PCBs), a modestly successful assay with an IC_{50} of 100 μg L^{-1} resulted.[45]

In concept, a lipophilic hapten can be attached to glycoprotein linkers to prevent the hapten from folding into the protein. However, the use of glycoprotein linkers may lead to the recognition of the handle. In general, the spacer arm should not include polar, aromatic or bulky groups; at a minimum, these moieties should not be linked directly to the target structure. An aliphatic straight-chain linker is preferred.[46]

2.5.3 Haptens for coating antigens and tracers

Careful design of coating haptens should take into consideration the reversible antibody/analyte equilibrium competition with an antibody/hapten–protein conjugate that is illustrated in Figure 1. Assuming that no analyte (**A**) is present, only the K_H, which is variable by changing hapten structure, for coating hapten–protein (**H**) is in operation between antibody (**Y**) and coating antigen (**H**), and a maximum signal from the **Y–H** is observed. On the addition of analyte (**A**), this equilibrium is shifted towards the formation of antibody–analyte (**Y–A**), described by K_A. Formation of **Y–A** dramatically reduces the amount of **Y–H** and hence the tracer signal decreases. Thus, for a fixed quantity of antibody; the lowest IC_{50} (or sensitivity) is observed when the affinity of the antibody for the analyte is greater than the affinity of the antibody for the coating-hapten ($K_A \gg K_H$). Therefore, with a fixed K_A for **Y–A**, one can shift the equilibrium by selecting a coating hapten with decreased relative affinity for the antibody; lower analyte concentrations may compete with these reagents under equilibrium conditions, resulting in assays with greater sensitivities. This competition is the rationale for improving assay sensitivity through use of heterologous haptens[47] and is employed extensively in our laboratory for triazine herbicides,[41,48] arylurea herbicides,[46,49] pyrethroid insecticides[35,36,39] and dioxins.[44,50] Guidelines for obtaining this heterology are outlined in Table 2.

Table 2 Guidelines for design of coating/tracer haptens

1. Heterology of hapten structure
Position of handle
Composition of handle
Conjugation chemistry
2. Alterations in target molecule structure
Use of partial structure
Change of key determinants
3. Cross-reactivity data of hapten structures (or derivatives)
4. Determination of hapten/protein ratio

Hapten heterology, site heterology, linker heterology, geometric heterology and the use of different conjugation techniques (discussed later) are useful tools to improve assay performance for both coating-antigen and enzyme tracer formats. In the development of TCDD immunoassays, our first assay employed a heterologous hapten **I** containing a short linker that lacked chlorine at position 2; a sensitive immunoassay resulted.[44] To improve the sensitivity, a new coating antigen (hapten **II**) was designed by replacing the benzene ring proximal to the linker with a pyridine ring (Figure 12). The resulting assay was five times more sensitive than the original assay having an IC_{50} of 40 ng L^{-1} and a limit of quantitation (LOQ) of 5 ng L^{-1}.[50]

Immunoassays for diuron (Figure 13) are another example of improved assay performance using heterologous assay conditions. One antibody was derived from a hapten that extended the dimethylamine side chain of diuron with methylene groups.

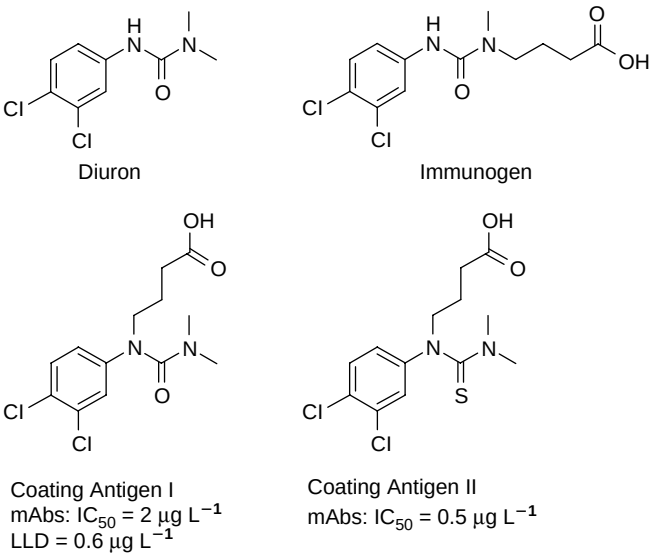
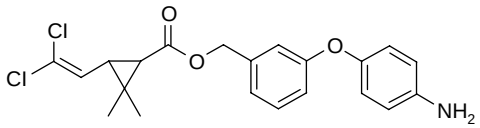


Figure 13 Structures of haptens used for immunizing and coating antigens in a monoclonal antibody-based immunoassay for diuron. A sensitive assay was developed using coating hapten I that had the handle in a position different from the immunogen hapten. When the oxygen in the urea moiety of hapten I was replaced with a sulfur (hapten II), increasing the heterology, even greater sensitivity was achieved

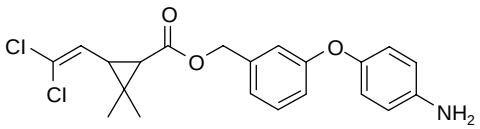
The best coating antigen of three evaluated consisted of an isomer in which the butyric acid handle was attached to the dichloroaniline nitrogen. The IC_{50} was 2 μg L^{-1} with an LOQ of 0.6 μg L^{-1}.[49] Using the rationale that a coating hapten with a lower affinity for the antibody was desirable, we replaced the oxygen of the diuron immunogen hapten with a sulfur to make a thiourea coating antigen. The resulting assay had an IC_{50} of 0.5 μg L^{-1} for diuron.[46] Sulfur, being larger than oxygen, probably did not fit well in the anti-diuron antibody pocket and there would be a substantially lower affinity owing to the loss of hydrogen bonding between the thiocarbonyl and antibody.

For chiral haptens, the use of enantiomers or diastereoisomers as the coating hapten may significantly improve the assay sensitivity. This was the case in the development of the permethrin immunoassay. The antibody was raised against a *trans*-permethrin hapten (Figure 14). Use of the corresponding *cis*-permethrin hapten as a coating antigen resulted in a sensitive and selective assay with an IC_{50} of 2.5 μg L^{-1} and an LOQ of 0.4 μg L^{-1}, which is about 200 times more sensitive than the homologous system in which the *trans*-permethrin hapten was the coating antigen.[35]

There are tradeoffs with developing assays based on assay heterology. For example, the highest titer of antibody is normally identified with a coating hapten that is very similar to the immunizing hapten. Rabbit antisera raised against acylurea insecticide haptens had high titers for the acylurea haptens that were similar to the immunizing structure. However, the target acylurea insecticide could not inhibit these assays because the antibodies bound to the coating hapten with greater affinity than to the acylurea insecticide. Changing the coating hapten to one containing a different handle than used for the immunizing hapten resulted in a decrease in antibody titer, demonstrating that the antibody bound with less affinity to the new coating antigen. However, the affinity for the target analyte was improved and a very sensitive assay for the acylurea insecticides resulted.[47] The benefit of careful design of a heterologous assay normally is greater with small haptens and spacers (primary or secondary amines compared with tertiary amines and amides) that are readily distinguished by the immune system than it is with large haptens.



trans-Permethrin hapten



cis-Permethrin hapten

Figure 14 Permethrin immunogen and coating antigen haptens. Using enantiomers or diastereoisomers is a strategy to provide hapten heterology. Assays using antibodies raised to the *trans*-permethrin hapten were more sensitive when the *cis*-permethrin hapten was used instead of the *trans*-permethrin hapten for the coating antigen

2.5.4 Hapten conjugation

In order to elicit a satisfactory immune response, haptens must first be covalently attached to a carrier protein, which is usually foreign to the animal being immunized. In addition, the hapten used for immunization and other similar haptens are conjugated to enzymes and (or) other proteins for use in the assay. For hapten–protein conjugates, protein solubility, the presence of functional groups and stability under reaction conditions are important variables to consider during immunoassay development. Many conjugation methods are available[14,51–53] and the selection of an appropriate method is ultimately dependent on the functional group available in the hapten.

(1) Carrier protein. A wide variety of proteins are available for the synthesis of immunogens or antigens including bovine serum albumin (BSA) and human serum albumin (HSA), ovalbumin, thyroglobulin, keyhole limpet hemocyanin (KLH) or horseshoe crab hemocyanin (LPH), and the synthetic polypeptides poly-L-lysine and polyglutamic acid. Among these, KLH is often the first choice as an immunogen carrier protein because it is large (approximately 10^6 Da) and is highly immunogenic. In addition, KLH contains an abundance of functional groups available for conjugation, including over 2000 lysine amines, over 700 cysteine sulfhydryls and over 1900 tyrosine residues. It should be noted that KLH requires a high-salt buffer (at least 0.9 M NaCl) to maintain its stability and solubility. In solutions with NaCl, concentrations lower than 0.6 M KLH will precipitate and denature, and maintaining solubility after hapten conjugation can be difficult. Hence conjugation reactions using KLH should be carried out under high-salt conditions to preserve the solubility of the hapten–carrier complex.

Thyroglobulin has been increasingly used as an immunogenic carrier protein owing to its excellent water solubility. Another frequently used protein in immunoassay is BSA. Although BSA is immunogenic, it is mostly used as a coating antigen carrier. Advantages of BSA include its wide availability in relatively pure form, its low cost and the fact that it is well characterized. BSA has a molecular weight of 64 000 and it contains 59 primary amino groups, one free cysteine sulfhydryl, 19 tyrosine phenolate residues and 17 histidine imidazolides. It is also relatively resistant to denaturation and is suitable for some conjugation procedures that involve organic solvents. Moreover, BSA conjugates are usually readily soluble, which makes their isolation and characterization easier. Although a general rule states that large and phylogenetically foreign proteins make the best antigenic proteins, we have obtained antibodies when smaller proteins such as fetuin were used as carriers.[54]

(2) Conjugation methods. The selection of conjugation method is dependent on the functional group on the hapten (e.g., carboxylic acid, amine, aldehyde). A hapten with a carboxylic acid group can conjugate with a primary amino group of a protein using the carbodiimide, activated *N*-hydroxysuccinimide (NHS) ester or mixed anhydride methods. Haptens with free amines can be coupled to proteins using glutaraldehyde condensation or diazotization. Haptens that have been designed to contain spacers may be linked directly to the protein with methods such as the mixed anhydride, whereas haptens lacking a spacer should be coupled using methods that insert a linker between the hapten and the protein such as with glutaraldehyde. Typical procedures

Table 3 Conjugation of a carboxyl-containing hapten to a protein using a carbodiimide method

Materials
BSA (Sigma, Fraction V or similar)
Hapten
EDC[a]
Phosphate buffer (0.1 M, pH 6): prepared from KH_2PO_4 (3.025 g), Na_2HPO_4 (0.39 g) and water (250 mL)
Method
1. Dissolve the hapten (0.04 mmol) in phosphate buffer containing 50 mg of BSA
2. Add 150 mg (0.78 mmol) of EDC to the buffer solution. Stir the mixture at room temperature to allow all the reagents to dissolve
3. React at room temperature for 24 h
4. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl.

are provided below for methods that have been successfully used in this laboratory or for which extensive literature is available.

(3) Haptens with free carboxylic acids. Methods for linking hapten carboxyl groups to amine groups of antigenic proteins include activation by carbodiimides, isobutyl chloroformate or carbonyldiimidazole. In the widely used carbodiimide method, the carbodiimide activates the carboxylic acid to speed up its reaction with the amine. Acidic conditions catalyze the formation of the active *O*-acylurea intermediate while the protein is more reactive at higher pH, when the lysine amino groups are unprotonated. Therefore, as a compromise, a pH near 6 is used. The choice of carbodiimide is dependent on the reaction conditions. For example, dicyclohexylcarbodiimide (DCC) is used in nonaqueous media with nonpolar, water-insoluble haptens where the carrier protein, in aqueous solution, is added to the activated hapten in a two-step reaction. For more water-soluble haptens, water-soluble derivatives of DCC such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC or Morpho CDI) are used in one-step reactions (Table 3, Figure 15). However, EDC will react directly with protein, and some antibodies are certain to be

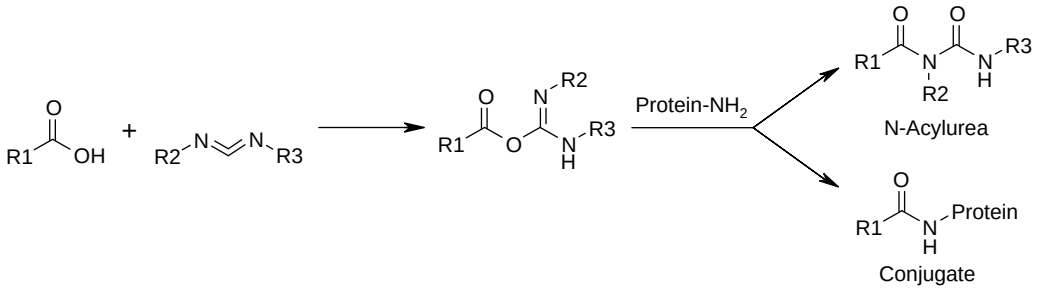


Figure 15 Conjugation of a carboxylic acid and an amine using the carbodiimide method. The carbodiimide activates the carboxylic acid to speed up the reaction to the amine. Carbodiimides can be used with nonpolar or polar solvents, including water. Undesirable urea complexes may form as by-products. Details of the reaction are given in Table 3

Table 4 Conjugation of a carboxyl-containing hapten to a protein using *N*-hydroxysuccinimide

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DCC
- NHS
- DMF[a]
- Phosphate buffer (0.1 M, pH 7.4): prepared from KH_2PO_4 (0.67 g), Na_2HPO_4 (0.285 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.04 mmol) in DMF (0.5 mL)
2. Add DCC (15 mg, 0.15 mmol) followed by NHS (20 mg, 0.17 mmol)
3. React at room temperature for 3.5 h
4. Remove the precipitate, dicyclohexylurea, by centrifugation
5. Add the supernatant to phosphate buffer (∼5 mL) containing 50 mg of BSA
6. React at room temperature for 2 h
7. Purify conjugate by gel filtration, dialysis or ethanol precipitation

[a] DMF = dimethylformamide (>99%, from Aldrich).

generated to the resulting highly immunogenic protein–urea complex. Formation of these antibodies is not a drawback as long as a different coupling chemistry is used to prepare coating antigens.

Activated NHS esters of carboxylic acids are prepared by reacting the acid with NHS in the presence of DCC (Table 4, Figure 16). *N*-Hydroxysuccinimide esters are stable when kept under anhydrous and slightly acidic conditions, and they react rapidly with amino groups to form an amide in high yield.

Like the carbodiimide method, the mixed anhydride method[55,56] results in an amide complex (Table 5, Figure 17). The acid-containing hapten is dissolved in a dry, inert, dipolar, aprotic solvent such as *p*-dioxane, and isobutyl chloroformate is added with an amine catalyst. The activated mixed anhydride is chemically stable and can be isolated and characterized. The aqueous protein solution is added to the activated acid and the pH is maintained at around 8.5. A low temperature (around 10 °C) is necessary during the reaction to minimize side reactions.

(4) Haptens with an amino group. Amine groups in haptens, carrier proteins or both can be modified for conjugation through homo- or heterobifunctional cross-linkers such as acid anhydrides (e.g., succinic anhydride), diacid chlorides (e.g.,

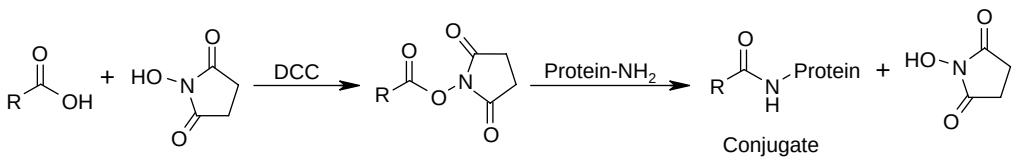


Figure 16 Conjugation of an amine and a carboxylic acid via the *N*-hydroxysuccinimide (NHS)-activated ester method. NHS esters may be isolated and characterized and are stable to long term storage as the powder. Alternatively, the NHS esters may be used immediately upon formation without isolation. Details of the reaction are given in Table 4

Table 5 Conjugation of a carboxyl-containing hapten to a protein using the mixed anhydride procedure

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- Isobutyl chloroformate
- 1,4-Dioxane (>99%, from Aldrich)
- Tributylamine

Method
1. Dissolve the hapten (0.04 mmol) in dioxane (5 mL) in a small tube and cool to 10 °C
2. Add tributylamine (11 μL, 0.044 mmol) to the solution followed by isobutyl chloroformate (6 μL, 0.044 mmol)
3. React at 10 °C for 60 min to activate the carboxylic acid
4. Add BSA solution (50 mg of BSA dissolved in 5 mL of distilled water and adjusted to pH 9 with NaOH) and stir for 4 h
5. Monitor the solution pH over the period and maintain it at 8.5 by the addition of dilute NaOH
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

succinyl chloride) or dialdehydes (e.g., glutaraldehyde). Glutaraldehyde condensation (Table 6) has been used widely to produce protein–protein and hapten–protein conjugates. The glutaraldehyde reagent should not have undergone polymerization. To check for polymerization, add a few drops of water to an aliquot of stock glutaraldehyde solution; a white precipitate is indicative of polymerization whereas unpolymerized reagent will not precipitate.

A disadvantage of the glutaraldehyde condensation method is that dimers of the hapten and polymers of carrier protein may also form. To overcome this problem, the reaction time is limited to 2–3 h, or an excess of an amine-containing compound, e.g., lysine or cysteamine hydrochloride, is added. A two-step approach also minimizes dimerization.[57]

Aromatic amine-containing haptens are converted to diazonium salts with ice-cold nitrous acid. Diazonium salts can then react with a protein at alkaline pH (around 9) through electrophilic attack of the diazonium salt at histidine, tyrosine and(or) tryptophan residues of the carrier protein (Table 7).

(5) Other reactions. Other reactions can also be used to couple haptens to proteins. The periodate oxidation is suitable for compounds possessing vicinal hydroxyl groups such as some sugars. Schiff's base method has been used for conjugating aldehyde-containing haptens to primary amino groups of carrier proteins. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional reagent that will cross-link a free amine at one end and a free thiol at the other. Heterobifunctional

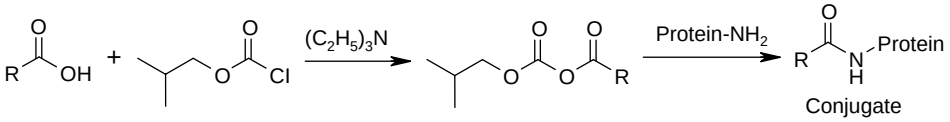


Figure 17 Conjugation of an amine and a carboxylic acid via the mixed anhydride method. Although the activated mixed anhydride is stable, it is usually used without purification. Use of low-temperature reactions will limit undesirable side products. Details of the reaction are given in Table 5

Table 6 Conjugation of an amino-containing hapten to a protein using the glutaraldehyde method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- Glutaraldehyde solution (0.2%, 0.02 M) in buffer
- Lysine monohydrochloride (1 M) in water
- Phosphate buffer (0.1 M, pH 7): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.03 mmol) and BSA (40 mg) in phosphate buffer
2. Add the glutaraldehyde solution (2 mL) dropwise over a period of 30 min
3. React at room temperature for 90 min. During this period the reaction mixture should turn yellow
4. Add the lysine solution to quench the reaction and stir for 60 min
5. Purify conjugate by gel filtration, dialysis or ethanol precipitation

reagents are commercially available but their use for immunizing antigens may lead to extensive handle recognition. A more complete discussion of other cross-linking and conjugation reagents can be found in Hermanson.[51]

2.5.5 Characterization of conjugates

Hapten density is important for both immunization and assay performance, and hence the extent of conjugation or hapten density should be confirmed by established methods. A characteristic ultraviolet (UV) or visible absorbance spectrum that distinguishes the hapten from the carrier protein or use of a radiolabeled hapten can be used to determine the degree of conjugation. If the hapten has a similar λ_{max} to the protein, the extent of incorporation can still be estimated when the concentration of the protein and the spectral characteristics of the hapten and protein are known. The difference in absorbance between the conjugate and the starting protein is proportional to

Table 7 Conjugation of an amino-containing hapten to protein using the diazotization method

Materials
- BSA (Sigma, Fraction V or similar)
- Hapten
- DMF (>99%, from Aldrich)
- Sodium nitrite (0.2 M) in water
- Phosphate buffer (0.1 M, pH 8.8): prepared from KH_2PO_4 (1.40 g), Na_2HPO_4 (2.04 g) and distilled water (250 mL)

Method
1. Dissolve the hapten (0.10 mmol) in 4 drops of ethanol and treat with 1 mL of 1 N HCl
2. Stir the solution in an ice-bath while adding 0.5 mL of 0.20 M sodium nitrite
3. Add 0.4 mL of DMF dropwise to give a homogeneous solution
4. Dissolve 45 mg of BSA in 5 mL of 0.2 M borate buffer (pH 8.8) and 1.5 mL of DMF
5. Add the activated hapten solution dropwise to the stirred protein solution. Stir in an ice-bath for 45 min
6. Purify conjugate by gel filtration, dialysis or ethanol precipitation

the amount of hapten conjugated.[41] Hapten density can also be determined indirectly by measuring the difference in free amino groups between conjugated and unconjugated protein using trinitrobenzenesulfonic acid.[58] These methods are at best rough estimates because the process of conjugation usually alters the apparent number of amine or sulfhydryl groups on the protein. Careful titration of reactive groups on very large proteins is particularly difficult.

Alternatively, competitive ELISA can be used to estimate the hapten density if an antibody that specifically recognizes the hapten is available.[59] At first observation this approach seems circular because the immunoassay developed is used to determine hapten density on proteins used for immunization. However, if a small molecule mimic of the protein conjugate is used as a standard, the method can be accurate. For example, a hapten containing a carboxylic acid can be coupled to phenethylamine or tyramine, its structure confirmed and the material used to generate a calibratron curve to estimate hapten density.

Advanced mass spectrometry (MS) techniques offer a new way of determining the hapten density of protein conjugates. For example, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) detects covalently bound haptens.[60] Increasingly powerful instruments allow higher resolution of conjugates. However, large proteins cannot be analyzed by MS. Protein heterogeneity and some post-translational modifications, particularly glycosylation, will obscure the results and lower resolution instruments cannot distinguish among desired conjugates and unwanted reaction by-products. It is possible, however, to measure hapten density on small peptides unequivocally by MS techniques and extrapolate to proteins such as KLH and thyroglobulin that are too large and/or heterologous for MS analysis.

Hapten density, and also the common positions where haptens are bound, can also be estimated by cyanogen bromide or enzymatic cleavage of the protein and either MALDI-MS or separation of the components by reversed-phase ion-pair chromatography and electrospray or electrospray time-of-flight (TOF) analysis.

Conjugates with a broad range of hapten/protein or hapten/enzyme ratios of about 1–30 have been used successfully to elicit antibody production or as enzyme tracers.[29,61,62] The optimum hapten ratio may depend on the study objectives, the nature of the antigen, immunization protocol, etc. A general rule of thumb is to target high hapten ratios for immunogens and low hapten ratios for coating antigens or enzyme tracers. For immunogens, a high hapten ratio implies greater exposure of the immune system to the hapten; for coating antigens or enzyme tracers, a lower hapten density implies fewer haptens to compete with the analyte in the assay. Optimum hapten density is often determined empirically with checkerboard titration procedures. Such procedures are very rapid and are normally adequate to optimize ELISAs without knowing the exact hapten density. With the development of more sophisticated biosensors, the determination of exact hapten densities may become increasingly important.

2.5.6 Antibody production

Essentially any vertebrate can be used as a source of antibodies. Rabbits are easy to care for, and produce a moderate amount of serum, often with high antibody titers.

Goats or sheep also produce high-quality antiserum in larger amounts. Antibodies derived from serum consist of a population of antibodies that recognize a variety of antigenic determinants with varying degrees of specificity and affinity and are thus termed polyclonal. Although two antisera are rarely identical, even if they come from the same rabbit at different times, it is simple to evaluate each antiserum for specificity and affinity.

In contrast, monoclonal antibodies are obtained from a murine cell line ultimately traceable to a single cloned cell. If carefully screened and selected, the monoclonal antibody will recognize a single antigenic determinant with constant affinity and specificity. The hybrid cell line comes ultimately from spleen lymphocytes (from a previously immunized animal) that have been fused to an immortal myeloma cell line. This fusion ensures that the cell line will continue to produce the selected antibody while it grows and replicates. Although it is attractive to have a permanent supply of antibody with constant specificity and affinity, these cell lines may contain an unstable chromosome complement and their immortality depends upon proper storage and maintenance. The advantages, disadvantages, and production of monoclonal antibodies have been discussed.[63–65]

Immunization procedures and schedules vary depending on the laboratory.[66,67] Usually an initial series of injections is followed by booster injections some weeks later. Animals are generally bled 7–14 days after each booster injection and the characteristics of the serum determined. Serum may be collected or pooled following numerous booster injections and(or) the animal may be exsanguinated.

For long-term storage, antibodies are best stored frozen either in solution or as a lyophilized powder. Similarly to most biological materials, repeated freeze–thaw cycles are detrimental to antibodies, and hence antibodies should be stored in clearly labeled aliquots. A single vial may be used for a set of experiments extending over several months. Antibodies can be kept in solution containing 0.1% sodium azide (to prevent growth of microorganisms) in a refrigerator for up to a year. Solutions can also go through freeze–thaw cycles several times without alarming loss of activity. Although antibodies are relatively hardy proteins, the concentration should be kept above 1 mg mL^{-1} during storage, solutions should be frozen quickly in liquid nitrogen before placing in a standard freezer, and for long-term storage antibodies should be lyophilized and the container sealed under dry nitrogen.

Building on the monoclonal antibody technology and the advent of molecular biology techniques, it is now possible to isolate antibodies from combinatorial libraries and express them in a variety of expression systems. Efficient systems for the cloning and expression of antibody genes in bacteria were developed in the late 1980s.[68] The discovery of PCR simplified the cloning of monoclonal antibody genes from mouse monoclonal cell lines. These functional recombinant antibody fragments could be expressed in bacteria for use.[69] To take advantage of recombinant technology, efficient, large-scale screening techniques must be used. A variety of techniques have been reviewed by Maynard and Georgiou.[70] The ability to engineer antibodies for therapeutic uses, such as neutralizing toxins (antivenoms), cancer therapy and imaging of tumors, is attractive. For environmental residue analysis, the most likely use of recombinant antibodies is as detector molecules in biosensors, where engineering could provide useful surface linkage chemistry, unique labels or improved robustness of the sensor. A few recombinant

antibodies for pesticides have been developed and at least one applied to a sensor format.[71–75]

2.5.7 Assay optimization

Assay optimization involves determining the optimum coating antigen/hapten–enzyme conjugate and anti-pesticide antiserum concentrations using a checkerboard titration. Using a 96-well plate, the coating antigen concentration is varied by row and the antibody concentration is varied by column so that each well has a different combination of antigen and antibody concentrations. By plotting the resulting absorbance values versus either reagent concentration an estimate can be made of the concentrations that will yield a reasonable signal and at which the system is not saturated.[76]

Using the optimum reagent concentrations, the assay is tested for inhibition by the target analyte. If a useable IC_{50} is obtained, then further optimization is conducted. This second stage of optimization includes determining the optimum assay temperature and incubation times and the effect of potential interferences (e.g., solvent, salt, pH, matrix). When evaluating immunoassays, it is important to remember that the law of mass action applies and interferences affect the equilibrium condition. For example, assays are conducted with reagents that have been equilibrated to room temperature. If room temperature is not constant (within 3–5 °C), then assays should be conducted using a forced-air incubator. Shaking the plate periodically during incubation may improve precision because reactions occur at the surface of the microtiter plate, causing a localized concentration of reactants. For immunoassays utilizing 30-min or longer incubation periods, the reactants have likely come nearly to equilibrium, and precise timing of the incubation period is less critical than for nonequilibrium immunoassays. Each of these variables should be evaluated and controlled if necessary in order to improve the precision of the measurements.

2.5.8 Validation

Consistent with other analytical methods, immunoassays must be validated to ensure that assay results are accurate. Initial validation involves an evaluation of the sensitivity and specificity of the immunoassay, while later validation includes comparison with a reference method. Because a goal of immunoassays is to minimize sample preparation, validation also includes testing the effects of sample matrices and(or) sample cleanup methods on results. The final steps in validation involve testing a limited number of samples containing incurred residues to determine if the method provides reliable data.

Structurally related compounds may cross-react with the antibody, yielding inaccurate results. In screening for the herbicide alachlor in well water by immunoassay, a number of false positives were reported when compared with gas chromatography (GC) analysis. A metabolite of alachlor was found to be present in the samples and it was subsequently determined that the cross-reactivity by this metabolite accounted for the false-positive results.[77] On the other hand, cross-reactivity by certain structural analogs may not be an issue. For example, in an assay for the herbicide atrazine, cross-reactivity by propazine is 196%;[78] because of atrazine and propazine field use

patterns, they are not usually found together. Conversely, this assay also cross-reacts with simazine by 30% and simazine is expected to be present. Hence, if the sample is positive and the presence of simazine is expected, another method of analysis would be necessary to determine the relative contribution of each triazine.

The second phase of validation involves comparing the immunoassay with an established method with a known accuracy using an identical same sample set. For most pesticides, reference methods are based on gas chromatography/mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC). When comparing two methods, it is important to be aware of the strengths and weaknesses of each. For example, many pesticide immunoassays require minimum sample cleanup before analysis, relative to the corresponding GC/MS or HPLC methods. Thus, immunoassay data may reflect higher values if there are losses occurring during further sample workup for GC/MS. On the other hand, the immunoassay data may be higher because a cross-reacting species is present that the GC/MS differentiates by chromatography. Comparison of immunoassay results with results obtained from a validated method will determine if the immunoassay is accurate.

For pesticide residue immunoassays, matrices may include surface or groundwater, soil, sediment and plant or animal tissue or fluids. Aqueous samples may not require preparation prior to analysis, other than concentration. For other matrices, extractions or other cleanup steps are needed and these steps require the integration of the extracting solvent with the immunoassay.[79] When solvent extraction is required, solvent effects on the assay are determined during assay optimization. Another option is to extract in the desired solvent, then conduct a solvent exchange into a more miscible solvent. Immunoassays perform best with water-miscible solvents when solvent concentrations are below 20%. Our experience has been that nearly every matrix requires a complete validation. Various soil types and even urine samples from different animals within a species may cause enough variation that validation in only a few samples is not sufficient.

Matrix effects are determined by running calibration curves in various dilutions of matrix and comparing the results with those for corresponding calibration curves run in buffer. Overlapping curves indicate no effect of matrix. Parallel curves are an indication that a matrix interference is binding the antibody in the same manner as the analyte. Nonparallel curves are indicative of nonspecific matrix interferences. Grotjan and Keel[21] described parallelism tests, similarity of curves and the corresponding statistics. A second test for matrix effects is to analyze a sample before and after a known amount of analyte has been added (test of additivity). If the values for the 'before' and 'after' samples are not additive, a matrix effect is presumed. If matrix effects are present, then adjustment of the immunoassay method, such as running the calibration curve in the matrix or further sample preparation, is necessary.

2.5.9 Quality control (QC) and troubleshooting

Unlike GC/MS methods, internal standards are not appropriate for immunoassays. Internal standards that would react with the antibody but would not interfere with the assay are nonexistent. In the place of internal standards, external QC must be maintained.

One strategy is to use appropriately stored batch QC samples that are analyzed with each assay because intra- and interassay variability are easily tracked. Various types of QC samples can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can indicate any background response that can be subtracted from the sample and standard responses. A negative control sample (i.e., matrix extract solution known to contain no analyte) can reveal whether a nonspecific response or matrix effect is occurring. A positive control or matrix extract fortified with a known amount of the analyte can determine accuracy. Precision can be determined using standards and samples run in replicate. Blanks, negative controls, positive controls, fortified sample extracts standardized reference material extracts and replicates are typically run on each microplate to control for plate-to-plate variation.[80] Recording assay accuracy and precision and maximum (no analyte present) and minimum (completely inhibited) absorbances over time will provide a warning of deteriorating assays.[81,82]

If an assay does not meet performance criteria, there are a variety of corrective measures (Table 8). The most frequent immunoassay performance problem is a high coefficient of variation for replicates or spurious color development. Plate washing and pipetting techniques are the greatest sources of this error.[76,83] A decrease in the maximum absorbance can be attributed to loss of enzyme activity or hapten conjugate degradation. To check enzyme activity, dilute the enzyme–conjugate about 2–5 times greater than normal for the assay. For example, if the method calls for a 1:2500 dilution of the enzyme label, then make dilutions of 1:5000 to 1:10 000, or greater. Add the substrate solution to the enzyme dilution and incubate for the time indicated in the method. Color development should be similar to that obtained in the assay when it is performing according to specifications. If the color development is lower, the enzyme label reagent should be replaced. Hapten–conjugate degradation can only be remedied by replacing the reagent.

Another important factor for QC is temperature. Reagents should be used at room temperature and plates should be protected from wide fluctuations in temperature while conducting the immunoassay. If an incubator is used or the ambient temperature is high, uneven heating of the wells may occur. Variations in final absorbances may be manifested in what is called an ‘edge effect’, in which greater variation occurs among the wells on the edges of the plate. Use of a forced-air incubator can reduce this problem. Detailed immunoassay troubleshooting information has been presented by Schneider *et al*.[84]

2.6 Applications

Pesticide immunoassays have been developed for a variety of pesticides and, more recently, GMOs, and have been used for matrices such as surface water, groundwater, runoff water, soil, sediment, crops, milk, meat, eggs, grain, urine and blood.[85–90] Table 9 is a partial list of immunoassays for chemical pesticides developed since 1995 and includes notations on the matrices studied. A fairly comprehensive list of pesticide immunoassays developed prior to 1994 was provided by Gee *et al*.[91]

Table 8 Troubleshooting the optimized immunoassay

Symptom	Cause	Remedy
Poor well to well replication	Poor pipetting technique	Check instrument, practice pipetting, calibrate pipet
	Poor binding plates	Check new lot, change manufacturer
	Coating antigen or antibody is degrading	Use new lot of coating reagent or antibody
	Coated plates stored too long	Discard plates, coat a new set, decrease storage time
	Poor washing	Wash plates more, or more carefully, remake buffer
	Uneven temperature in the wells	Deliver reagents at room temperature, avoid large temperature fluctuations in the room
	Sample carryover	Watch for potential carryover in pipetting and washing steps
Low or no color development	Loss of reagent integrity	Systematically replace or check reagents, including buffers and beginning with the enzyme label
	Incubation temperature too cold	Lengthen incubation time or increase temperature by using a circulating air-temperature controlled incubator
	Sample matrix effect	Dilute matrix if possible, check pH of matrix, increase the ionic strength of the buffer, re-evaluate matrix
Color development too high	Incubation too long or temperature too high	Decrease incubation time or temperature
	Matrix effect	Dilute matrix or re-evaluate matrix effects
Change in calibration curve parameters	Degradation of reagents	Systematically check or replace reagents, including buffers

2.6.1 Human exposure monitoring

The immunoassay is one of the most promising methods for the rapid monitoring and assessment of human exposure. The great specificity and sensitivity of immunoassays allow their use for monitoring pesticide exposure levels by determining parent compound, key metabolites[92] or their conjugates in human urine, blood,[93] and(or) saliva.[94] Recently, several immunoassays have been developed to assess human exposure to alachlor,[95,96] atrazine,[97,98] metolachlor,[99] and pyrethroids.[100] In the case of the herbicide atrazine, the mercapturic acid conjugate excreted in human urine[101] is a specific biomarker for exposure. A sensitive immunoassay has been developed for this metabolite[97] that can be detected at 0.1 $\mu g\ L^{-1}$ in urine. The great advantage of the immunoassay over chromatographic methods is high throughput, which is

Table 9 Immunoassays developed since 1995

Class	Name, matrix	Reference
Herbicide	Chlorpropham, food	139
	Isoproturon, water	140
	Metsulfuron-methyl, water	141, 142
	Bensulfuron-methyl, water	143
	Chlorsulfuron	144
	Fluometuron, soil	145
	Trifluralin, soil, water, food	146, 147
	Cyclohexanedione	148, 149
	Triazines, water, food	19, 150, 151
	Dichlobenil	152
	Propanil, water	153
	Dichlorprop methyl ester	154
	Hexazinone, water	155
	Fluroxypyr, triclopyr, soil	156
Insect growth regulator	Fenoxycarb	157, 158
	Flufenoxuron, soil, water	159
Insecticide	Hexachlorocyclohexane, water, soil	160
	Azinphos-methyl, water	161
	Carbofuran, food	162–164
	Chlorpyrifos, water	165, 166
	Chlorpyrifos-ethyl	74
	Pymetrozine, plants	167
	Azinophos-methyl, water	161, 168
	Pyrethroids	37, 39, 169
	Allethrin	170
	Esfenvalerate, water	36
	Flucythrinate, soil, water, food	171
	Permethrin, air, water	35, 172
	Organophosphates	112, 173, 174
	Fenitrothion, food, water	175, 176
	DDT, soil, food	177–179
	Etofenprox	180
	Phosalone	181
	Spinosyn A, water	182
	Spinosad, food, water, sediment	89, 183
	Imidacloprid, water, food	13, 175, 184
	Acetamiprid, water, food	175
	Azadirachtin, food, formulations	185
	Oxamyl, food	186
	Propoxur	187
Fungicide	Myclobutanil, soil, water, food	188
	Procymidone, food	189
	Benalaxyl, food, water	190
	Thiram, food	191, 192
	Chlorothalonil, water, plant residues, food	193–195
	Tebuconazole, food	196, 197
	Thiabendazole, food	198–200
	Imazalil, food	201
	Tetraconazole	197, 202
	Myclobutanil, water, soil, food	188, 202
	Hexaconazole, formulations	203
	Didecyldimethylammonium chloride	204
	Methyl 2-benzimidazolecarbamate, soil, food	205, 206
	Captan, food, water	207

particularly suitable for screening large numbers of samples generated during human exposure studies.

2.6.2 Immunoassay in agricultural biotechnology

Agricultural biotechnology providers include agricultural biotechnology companies, seed companies, food companies and other research organizations. Technology providers use qualitative, quantitative and threshold immunoassays during all stages of the research and development of biotech crops, the choice depending on the specific application. Immunoassays are used for gene discovery, event selection, screening, transformant identification, line selection, plant breeding and seed quality control. Agricultural biotechnology companies also use immunoassays for product support, product stewardship and intellectual property protection.

Technology providers use quantitative immunoassays to determine expression data of field material for regulatory submissions. Regulatory authorities require that expression levels of introduced proteins in various plant parts be determined by quantitative, validated methods. Immunoassays are also used to generate product characterization data, to assess food, feed and environmental characteristics, to calculate concentrations for toxicology studies and to obtain tolerance exemption or establish tolerances for pesticidal proteins.

Immunoassays are also useful in the food handling and distribution system. Threshold assays are most commonly used to test agricultural commodities entering the food distribution channel to ensure compliance with relevant labeling regulations.[102] Immunoassays can be applied to raw, fresh and or lightly processed foods. The protein analyte can be denatured during processes such as heating. This creates potential difficulties in the analysis of heavily processed finished food products.

2.6.3 Flow injection immunoassay (FIIA)

In FIIA, antibodies are immobilized to form an affinity column and analyte is pumped over the column. The loading of the antibodies with analyte is followed by pumping over the column enzyme tracers that compete with the pesticide for the limited binding sites of the antibodies. Generally, the indirect format produces a result inversely proportional to the pesticide concentration. FIIA can be used with electrochemical, spectrophotometric, fluorimetric and chemiluminescence detection methods. Conventional UV visible spectrophotometry is also suitable for the FIIA detection of bioligand interactions.[103] FIIA has been used for the detection of diuron and atrazine in water.[104] The method was developed as a cost-effective screen for determining compliance with the European drinking water directive. One analysis for either atrazine or diuron, including column regeneration, took about 50 min using the system that is shown schematically in Figure 18. The column material was regenerated up to 1600 times over a 2.5 month period. FIIA is a powerful analytical tool for semi-continuous, high sample throughput applications and may serve as an alternative or complementary technique to solid-phase immunoassay by providing real-time monitoring data.[105] In addition, the continuous flow system is easier to automate than assays using tubes or microplates. More rapid results and sensitive detection will be possible by miniaturizing the column and fluid handling and

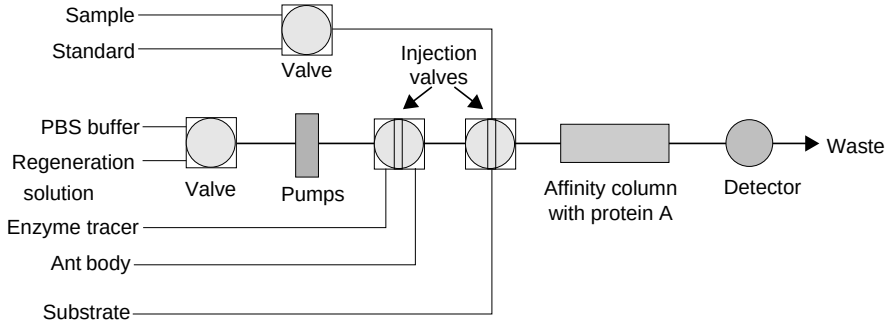


Figure 18 Flow chart of the automated on-line flow injection immunoassay (FIIA). Six steps are involved in each cycle: (1) addition of antibody and incubation; (2) addition of analyte (or standard) and incubation; (3) addition of enzyme–tracer and incubation; (4) addition of substrate and incubation; (5) downstream measurement of fluorescence; (6) regeneration of affinity column

with the development of sensors that can detect antibody–antigen binding events directly.

2.6.4 Multi-analyte analysis

Immunoassays traditionally have been used as a single-analyte method, and this is often a limitation of the technology. However, several approaches are possible to overcome this limitation. A simple approach is to have highly selective assays in different wells of a single microtiter plate, as was demonstrated for the sulfonylureas.[106] A more elegant approach than using a microtiter plate is to use a compact disk (CD)-based microarray system.[107] A microdot system was developed that utilized inkjet technology to 'print' microdots on a CD. The CD was the solid phase for immunoassay, and laser optics were used to detect the near-infrared fluorescent label. The advantage of the CD system is the ability both to conduct assays and to record and/or read data from the same CD. Since the surface of a single CD can hold thousands of dots, thousands of analyses can be made on a single sample simultaneously. Such high-density analyses could lead to environmental tasters where arrays of immunosensors are placed on chips[108,109] or high-density plates. Because the CD format has the potential for high-density analyses, there will be the opportunity for easily generating multiple replicates of the same sample, including more calibration standards, thus improving data quality.

The development of class-selective antibodies is another approach to multi-analyte analysis. The analyst may design haptens that will generate antibodies that recognize an epitope common to several compounds, as explained above for the analysis of pyrethroids by measuring PBA. Other examples of class-selective immunoassays that have been developed are mercapturates,[110] glucuronides,[111] pyrethroids,[37,39] organophosphate insecticides,[112] and benzoylphenylurea insecticides.[113]

Rather than have one antibody that can detect a class, a third approach is to analyze a sample using multiple immunoassays, each with a known cross-reactivity spectrum, and determine the concentration of the analytes and confidence limits mathematically.[114–116] A drawback to using class-selective assays or assays with known cross-reactivity is that for a given antibody, the sensitivity for each analyte

will vary, and the sensitivity for some analytes may not be sufficient, hence selection of well-characterized antibodies will be a critical step.

2.6.5 Future prospects

Immunoassays designed for environmental applications are mostly sold as some variation of the ELISA format. ELISA-like formats dominate the field because they are inexpensive and because they provide high sensitivity and precision without requiring complex instrumentation. The basic ELISA format supports both field and laboratory-based applications but is limited by multiple steps and inadequate sensitivity for some applications, excessive variability and sometimes long analysis times. Some of the other formats discussed in this article may replace the ELISA for selected applications; however, because many laboratories are familiar with the ELISA technology, there will be a significant delay before alternative formats are widely accepted.

In the near term, to improve throughput, the 96-well ELISA is likely to be replaced by higher density arrays. For example, plates, readers and robotic systems are being developed for high-throughput screening in the pharmaceutical industry in 384-, 768-, and 1536-well formats. Other high-throughput formats will utilize inkjet printing technology on CD surfaces or FIIA-like systems, which offer advantages for sequential analysis as discussed above. Biosensor technology will also likely be integrated with ELISAs to generate improved formats.

It is critical to keep in mind that existing reagents can be used for multiple formats. For example, polyclonal antibodies dominate the environmental field because they generally provide greater sensitivity and specificity for small molecules at a much lower cost than do monoclonal or recombinant antibodies. With some biosensors monoclonal or engineered antibodies or recombinant binding proteins may offer advantages.

3 PCR for products of agricultural biotechnology

The recent introduction of genetically modified crops has changed both the agriculture and food industries. United States Department of Agriculture (USDA) surveys report that 25% of corn, 61% of cotton and 54% of soybean acreage grown in the USA in 2000 were genetically modified.[117]

Agricultural biotechnology involves inserting a novel gene [deoxyribonucleic acid (DNA) sequence] into plants or animals using recombinant DNA techniques. These techniques even allow the transfer of DNA from a donor organism to a recipient organism that is not genetically related, a feat not possible using conventional breeding techniques. The novel DNA codes for the expression of a specific protein that confers a new trait or characteristic to the plant or animal. Most traits are described as either input or output traits. Input traits are useful for crop production and include commercial biotech crops that contain herbicide tolerance or resistance to insect pests or diseases. Output traits offer valuable quality enhancements such as improved nutritional value or improved handling or processing characteristics.

Since the commercial introduction of biotech crops, a need has emerged for analytical methods capable of detecting the novel DNA sequences introduced into the plant genome and also methods for detecting the protein products expressed by the

plant. PCR is a powerful tool for the amplification and detection of defined DNA sequences. This section describes the basic principles of agricultural biotechnology and covers principles of both conventional and real-time PCR for DNA analysis. Examples of how these techniques are currently used for analytical testing of raw agricultural commodities and finished food are presented.

3.1 Basic principles of agricultural biotechnology

Within the nuclei of plant cells, chromosomal DNA provides instructions for the cells to replicate themselves and to carry out vital functions. Individual, unique DNA sequences (genes) code for the production of individual, unique proteins. With the tools of modern biotechnology, it is possible to introduce novel DNA sequences that instruct plant cells to synthesize or over-express proteins that confer new traits to the plant. It is also possible to 'down-regulate' or turn off a native gene, thereby suppressing or eliminating the synthesis of a native protein, which can also produce a new trait. Plants that have been transformed in these ways have been called transgenic, genetically modified (GM), genetically engineered (GE), biotech plants and(or) genetically modified organisms (GMOs).

There are several methods that can be used to introduce foreign genes into plant cells, a process called, in general, transformation. Among the most common plant transformation methods are biolistics and exposure to *Agrobacterium tumefaciens.*

Biolistics involves bombarding plant cells with tiny (4-μm) microprojectiles made of gold or tungsten. These microprojectiles are coated with DNA and are propelled at high velocity from a particle gun or 'gene gun' into plant tissue or cells. In this method, the projectile penetrates the cell wall and carries the transgene into the cell nucleus.

A. tumefaciens is naturally able to transform a wide variety of plant species. Mature differentiated plant tissue (an explant) is exposed to *A. tumefaciens* bacteria harboring a 'foreign' gene. The bacterial infection results in foreign DNA from the bacterium being transferred into the genome of the host plant, and results in a crown gall tumor. This naturally occurring process can easily be exploited to produce a transgenic plant.

Plasmids are often used as vectors to transfer DNA into plant cells. In particular, the tumor-inducing (Ti) plasmid of *A. tumefaciens* is a common vector. Plasmids are extrachromosomal, autonomously replicating, circular double strands of DNA that can occur in high copy number in a bacterial cell. It is possible to construct a recombinant Ti plasmid by inserting an effect gene, regulatory sequences (such as transcriptional promoters and terminators), along with a selectable marker gene (such as antibiotic or herbicide resistance) into the circular plasmid.

After the recombinant plasmid has been constructed using *in vitro* methods, leaf disks or protoplasts are infected with recombinant *A. tumefaciens* cells. The infection process incorporates the foreign gene and other genetic elements into the host-plant genome. The host cells are then regenerated from undifferentiated callus tissue into a transgenic plant in tissue culture. Only some of the cells receive the gene of interest, so it is necessary for explants to be grown up in a selective medium.[118]

In order for any gene to synthesize a protein, it must contain certain genetic elements such as promoter and terminator sequences. These regulatory regions signal where the DNA sequence that encodes a product (i.e., a gene) begins and ends. The recombinant

DNA construct will often contain an effect gene and a selectable marker gene (such as antibiotic or herbicide resistance), both of which are bracketed by promoter and terminator sequences. A plasmid vector carries this cassette of genetic information into the plant genome by one of the above methods.

Multiple or 'stacked' traits are sometimes introduced into a single plant. These could include resistance to multiple viruses, fungal resistance, etc. Each of these stacked-trait genes usually has an associated promoter and terminator sequence. Obtaining information about particular gene constructs, including marker and regulatory sequences, is vital for PCR testing to detect GMOs in a crop or food sample. The required sequence information can be inferred by restriction mapping of the recombinant plasmid or, more commonly, by DNA sequencing.

GMO screening often relies on the common genetic elements that are present in many commercial GMOs. Many genetically modified plants use common regulatory sequences and/or marker genes, which makes it possible to simultaneously screen for many GMOs by detecting these sequences. The cauliflower mosaic virus (CaMV) 35S-promoter and the *A. tumefaciens nos*-terminator are examples of two DNA sequences that are present in many commercial GMOs.

A positive result for one of these sequences does not necessarily indicate that the test sample contains GM material. Since the 35S-promoter comes from a virus that infects cauliflower, positive results from plants that belong to the genus *Brassica* would need to be carefully evaluated. Likewise, the *nos*-terminator originated in *A. tumefaciens* and this soil bacterium has a broad spectrum of potential hosts. *Nos*-positive results must be confirmed to rule out bacterial contamination. Testing for these common genetic elements only serves as a GMO screening; it is necessary to apply a specific test to determine which GMO is present in the sample. The following list gives some genetic elements that are commonly detected in GMO screening tests:

- *CaMV 35S* promoter: a promoter sequence from the CaMV
- *nos* terminator: nopaline synthase, a terminator sequence from *A. tumefaciens*
- *bar* gene: a herbicide resistance selectable marker from *Streptomyces hygroscopicus* that encodes phosphinothricin acetyltransferase
- *pat* gene: phosphinothricin acetyltransferase, a herbicide resistance selectable marker
- *npt II*: neomycin phosphotransferase, an antibiotic resistance selectable marker.[119]

For PCR analysis of a specific GMO, it is necessary to have sequence information about the gene construct, so primers can be designed to be specific to a gene or to a sequence that bridges genetic elements of the specific construct. An example is the specific test for the genetic modification in Roundup Ready soybeans. The target sequence is the transition that links the transit peptide gene from petunia to the 35S promoter region. This transition DNA sequence is specific to Roundup Ready soybeans.

Table 10 lists United States Food and Drug Administration (FDA) submissions in 2000 for commercial GMOs, including the food, gene, source and intended effect.[120]

Table 10 Commercial GMOs

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Corn* DowAgro/2000	Cry1F protein, phosphinothricin acetyltransferase (PAT)	*Bacillus thuringiensis*, *Streptomyces viridochromogenes*	Resistance to certain lepidopteran insects; tolerance to the herbicide glufosinate
Corn Monsanto/2000	5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS)	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Corn Aventis/1999	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Rice Aventis/1999	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Canola Rhone-Poulenc/1999	Nitrilase	*Klebsiella ozaenae* subsp. *ozaenae*	Tolerance to the herbicide bromoxynil
Cantaloupe Agritope/1999	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Canola BASF/1997	Phytase	*Aspergillus niger* van Tieghem	Degradation of phytate in animal feed
Canola AgrEvo/1998	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1998	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Sugar beet Monsanto and Novartis/1998	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Soybean AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Tomato* Calgene/1997	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to certain lepidopteran insects
Corn Monsanto/1997	Modified EPSPS	Corn	Tolerance to the herbicide glyphosate
Flax University of Saskatchewan/1997	Acetolactate synthase (csr-1)	*Arabidopsis*	Tolerance to the herbicide sulfonylurea
Potato* Monsanto/1997	CryIIIA, PVY coat protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato virus Y (PVY)	Resistance to Colorado potato beetle and PVY
Potato* Monsanto/1997	CryIIIA, PLRV replicase	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt), potato leafroll virus (PLRV)	Resistance to Colorado potato beetle and PLRV
Cotton* Calgene/1997	Nitrilase, Cry1Ac protein	*Klebsiella pneumoniae* subsp. ozaene, *Bacillus thuringiensis* var. *kurstaki* (Btk)	Tolerance to the herbicide bromoxynil, resistance to certain lepidopteran insects
Corn* AgrEvo/1998	Cry9C protein, PAT	*Bacillus thuringiensis* subsp. *tolworthi* (Bt), *Streptomyces hygroscopicus*	Resistance to several lepidopteran insects, tolerance to the herbicide glufosinate

Sugar beet AgrEvo/1998	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Corn Pioneer Hi-Bred/1998	DNA adenine methylase (DAM), PAT	*Escherichia coli*, *Streptomyces viridochromogenes*	Male sterility, tolerance to glufosinate
Canola AgrEvo/1997	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Radicchio Bejo Zaden/1997	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Squash* Seminis Vegetable Seeds/1997	Coat proteins from CMV, ZYMV and WMV2	Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV2)	Resistance to the viruses CMV, ZYMV and WMV2
Papaya* University of Hawaii/1997	PRV coat protein	Papaya ringspot virus (PRSV)	Resistance to PRSV
Corn* Dekalb Genetics/1996	CryIAc	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Soybean DuPont/1996	GmFad2-1 gene to suppress endogenous GmFad2-1 gene, which encodes delta-12 desaturase	Soybean	High oleic acid soybean oil
Corn* Monsanto/1996	CryIAb protein, EPSPS, glyphosate oxidoreductase	*Bacillus thuringiensis* subsp. *kurstaki* (Btk), *Agrobacterium* sp. strain CP4, *Ochrobactrum anthropi*	Resistance to European corn borer, tolerance to the herbicide glyphosate
Corn Monsanto/1996	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Potato* Monsanto/1996	CryIIIA protein	*Bacillus thuringiensis* var. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Oilseed rape Plant Genetic Systems/1995	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Oilseed rape (Canola) Plant Genetic Systems/1995	Barstar, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Fertility restorer, tolerance to glufosinate
Oilseed rape Plant Genetic Systems, America/1996	Barnase, PAT	*Bacillus amyloliquefaciens*, *Streptomyces hygroscopicus*	Male sterility, tolerance to glufosinate
Cotton Dupont/1996	Acetolactate synthase (ALS)	*Nicotiana tabacum* cv. *Xanthi* (tobacco)	Tolerance to the herbicide sulfonylurea
Corn Dekalb Genetics/1995	PAT	*Streptomyces hygroscopicus*	Tolerance to the herbicide glufosinate
Corn* Monsanto/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer
Corn* Northrup King/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to European corn borer

Table 10—*Continued*

Food[a] Company/year	Gene, gene product, or gene fragment	Source	Intended effect
Tomato Agritrope/1996	*S*-Adenosylmethionine hydrolase	*Escherichia coli* bacteriophage T3	Delayed fruit ripening due to reduced ethylene synthesis
Corn AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Cotton Monsanto/1995	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Oilseed rape (Canola) Calgene/1992	12:0 Acyl carrier protein thioesterase	*Umbellularia californica* (California Bay)	High-laurate canola oil
Corn* Ciba-Geigy/1995	CryIAb protein	*Bacillus thuringiensis* subsp. *kurstak*i (Btk)	Resistance to European corn borer
Oilseed rape (Canola) AgrEvo/1995	PAT	*Streptomyces viridochromogenes*	Tolerance to the herbicide glufosinate
Oilseed rape (Canola) Monsanto/1995	EPSPS, glyphosate oxidoreductase (GOX)	*Agrobacterium* sp. strain CP4, *Achromobacte*r sp. strain LBAA	Tolerance to the herbicide glyphosate
Cotton* Monsanto/1994	CryIAc protein	*Bacillus thuringiensis* subsp. *kurstaki* (Btk)	Resistance to cotton bollworm, pink bollworm and tobacco budworm
Tomato DNA Plant Technology/1994	A fragment of the gene encoding aminocyclopropanecarboxylic acid synthase (ACCS) to suppress the endogenous ACCS enzyme	Tomato	Delayed ripening due to reduced ethylene synthesis
Squash* Asgrow/1994	ZYMV and WMV2 coat proteins	ZYMV and WMV2	Resistance to ZYMV and WMV2
Potato* Monsanto/1994	CryIIIA protein	*Bacillus thuringiensis* subsp. *tenebrionis* (Btt)	Resistance to Colorado potato beetle
Cotton Calgene/1994	Nitrilase	*Klebsiella ozaenae*	Tolerance to the herbicide bromoxynil
Tomato Zeneca/1994	A fragment of the polygalacturonase (PG) gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation
Tomato Monsanto/1994	1-Aminocyclopropane-1-carboxylic acid deaminase (ACCD)	*Pseudomonas chloraphis*	Delayed softening due to reduced ethylene synthesis
Soybean Monsanto/1994	EPSPS	*Agrobacterium* sp. strain CP4	Tolerance to the herbicide glyphosate
Tomato Calgene/1991	Antisense PG gene to suppress the endogenous PG enzyme	Tomato	Delayed softening due to reduced pectin degradation

[a] An asterisk indicates that the modified plant produces a pesticidal substance that is regulated by the United States Environmental Protection Agency (USEPA).

3.2 Basic principles of the PCR

DNA is the molecule that encodes genetic information. DNA is a double-stranded molecule with two sugar–phosphate backbones held together in the shape of a double helix by weak hydrogen bonds between pairs of complementary nitrogenous bases. The four nucleotides found in DNA contain the nitrogenous bases adenine (A), guanine (G), cytosine (C) and thymine (T). A base sequence is the order of nucleotide bases in a DNA molecule. In nature, base pairs (bp) form only between A and T and between G and C; hence the base sequence of each single strand can be deduced from that of its complementary sequence.

The PCR is a method for amplifying a DNA base sequence in vitro using a heat-stable DNA polymerase and two primers, complementary to short sequences flanking the target sequence to be amplified. A primer is a short nucleotide chain, about 20 bp in length, which anneals to its complementary sequence in single-stranded DNA. DNA polymerase, an enzyme that aids in DNA replication, adds new deoxyribonucleotides to the extensible (3′) end of the primer, thereby producing a copy of the original target sequence. Taq polymerase (isolated from a thermophilic bacterium called *Thermus aquaticus*) is the most common heat-stable DNA polymerase used in the PCR.

A PCR cycle involves DNA denaturation, primer annealing and strand elongation. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, the PCR produces rapid and highly specific amplification of the target sequence. Repeated rounds of thermal-cycling result in exponential amplification of the target sequence. Theoretically, 2^n copies of the target can be generated from a single copy in n cycles. There is therefore a theoretical quantitative relationship between number of cycles and starting copy number. This will be covered in more detail in the discussion of real-time PCR.

3.2.1 Isolation and purification of the template DNA

The quantity, quality and purity of the template DNA are important factors in successful PCR amplification. The PCR is an extremely sensitive method capable of detecting trace amounts of DNA in a crop or food sample, so PCR amplification is possible even if a very small quantity of DNA is isolated from the sample. DNA quality can be compromised in highly processed foods such as pastries, breakfast cereals, ready-to-eat meals or food additives owing to the DNA-degrading action of some manufacturing processes. DNA purity is a concern when substances that inhibit the PCR are present in the sample. For example, cocoa-containing foodstuffs contain high levels of plant secondary metabolites, which can lead to irreversible inhibition of the PCR. It is important that these substances are removed prior to PCR amplification. Extraction and purification protocols must be optimized for each type of sample.

Several standard DNA isolation kits are commercially available, including the QIAamp DNA Stool Mini Kit and the DNeasy Plant Mini Kit made by Qiagen. Both of these products are based on silica gel membrane technology and allow for the extraction of total DNA from processed foods and raw foodstuffs, respectively. In

both methods, the cellular components of the samples are first lysed; next the isolated DNA is bound to a membrane gel matrix and washed thoroughly. DNA is then eluted. The DNA Stool Mini Kit includes an extra pre-purification step to remove PCR inhibitors.[121]

Classical approaches to plant DNA isolation aim to produce large quantities of highly purified DNA. However, smaller quantities of crudely extracted plant DNA are often acceptable for PCR analysis. Another efficient method for preparation of plant DNA for PCR is a single-step protocol that involves heating a small amount of plant tissue in a simple solution. Several factors influence nucleic acid release from tissue: salt, EDTA, pH, incubation time and temperature. These factors must be optimized for different sample substrates. EDTA in the sample solution binds the Mg^{2+} cofactor required by the Taq polymerase in the PCR, so the EDTA concentration in the solution, or the Mg^{2+} concentration in the PCR, must be carefully optimized.

An optimized single-step protocol for the extraction of leaf tissue or seed embryos is given here. The template preparation solution (TPS) contains:

100 mM Tris–HCl, pH 9.5
1 M KCl
10 mM EDTA

1. To a sterile 1.7-mL microcentrifuge tube containing 20 μL of TPS, add a maximum of a 2-mm^2 piece of leaf or 0.5-mg piece of embryo and incubate at 95 °C for 10 min.
2. Add a 1-μL portion of the supernatant (or dilution thereof, if inhibitors are present) to the 50-μL PCR reaction.

Making sure that the sample size does not exceed the maximum area or weight is important to minimize the amounts of interfering substances that are coextracted. If the leaf sample is larger than 2 mm^2, coextractive substances can inhibit the PCR assay. Regardless of which extraction method is used, it is important that the PCR assay is evaluated for coextractive interferences or inhibitors.[122]

3.2.2 Components of a PCR

The components necessary for a PCR are assembled in what is known as a mastermix. A PCR mastermix contains water, buffer, $MgCl_2$, dNTPs, forward and reverse primers and DNA polymerase (enzyme). After the mastermix has been assembled, template DNA is added.

1. *Water*: The water used in the assay should be deionized, ultrafiltered and sterile.
2. *Buffer*: The PCR buffer is usually provided as a 10-fold solution and is designed to be compatible with the enzyme. Common buffer components are: 500 mM KCl; 100 mM Tris–HCl, pH 9.3; 1–2% Triton X-100; 0.1% Tween.
3. *$MgCl_2$*: 0.5–3.5 mM $MgCl_2$ salt must be added to the assay, as Mg^{2+} is required as a cofactor for the DNA polymerase.
4. *dNTPs*: Deoxynucleoside triphosphates (dATP, dTTP, dCTP, dGTP) are the nucleotide building blocks for the synthesis of new DNA. The dNTPs are sensitive to repeated freeze–thaw cycles and are usually stored in small aliquots (10 mM pH 7.0); concentrations of 20–200 mM are needed in the assay; too high a

concentration can lead to mispriming and misincorporation of nucleotides. All four nucleotides must have the same concentration in the assay.

5. *Primers*: The primers are short (15–30) oligonucleotide sequences designed to base pair or anneal to complementary sequences that flank the DNA target sequence to be amplified. The primers are added at 0.1–1 μM in the assay.
6. *Enzyme*: Taq polymerase (or some other enzyme) adds new deoxyribonucleotides during strand elongation. Taq is added to the assay at 1 unit per 50 μL of reaction mixture.
7. *DNA*: The template DNA is isolated from cells by some sort of extraction procedure. This is usually the last thing added to the reaction before the tube is placed in the thermal cycler.[123]

3.2.3 Contamination control

Because the PCR exponentially copies the target molecule or molecules, amplicon contamination in the laboratory is a serious concern. It is recommended that the mastermix is prepared in an isolated area, such as a PCR station equipped with a UV light. This work area should be exposed to UV radiation after use to destroy any DNA contaminants. The use of dedicated pipets and filtered pipet tips is also recommended. The template DNA should be prepared and added to the reaction in an area that is isolated from the mastermix preparation hood. The thermal cycling and gel electrophoresis should be conducted in a third work area and care should be taken not to introduce amplified PCR products into the mastermix or template preparation work areas.

3.2.4 Thermal cycling

Once the reaction tube has been placed in the thermal cycler, there are normally three steps in a PCR cycle:

1. *Denaturation step*. This step separates the double-stranded DNA into complementary single strands. Also called melting, this usually occurs at a temperature of about 95 °C for 30 s or 97 °C for 15 s.
2. *Annealing step*. The second step is primer annealing, where the forward and reverse primers find their complementary sequences and bind, forming short double-stranded segments. The annealing temperature (T_a) can be estimated from the melting temperature (T_m) by the following equations:

$$T_a = T_m - 5\,^{\circ}\mathrm{C} \tag{1}$$

$$T_m = (A + T) \times 2 + (\mathrm{C} + \mathrm{G}) \times 4 \tag{2}$$

3. *Elongation step.* The third step is strand elongation, where the DNA polymerase synthesizes new DNA strands starting at the primer sequences. Under optimum conditions, approximately 60 bp are synthesized per second. Typically, elongation takes place at about 72 °C.

The number of PCR cycles depends on the number of source molecules. For 10^5 source molecules, 25–30 cycles are required; for 10^4 source molecules, 30–35 cycles; and for 10^3 source molecules, 35–40 cycles. Running more than 40 cycles can cause the formation of unspecific fragments and does not normally yield any more of the target sequence.[123]

3.2.5 Gel electrophoresis

After amplification, it is necessary to visualize the PCR products. Agarose gel electrophoresis is a technique for separating DNA fragments by size. Purified agar (isolated from seaweed) is cast in a horizontal slab. The agarose slab is submerged in a buffer solution and samples are loaded into wells in the gel. An electric current is applied to electrodes at opposite ends of the gel to establish an electrical field in the gel and the buffer. Because the sugar–phosphate DNA backbone is negatively charged, the fragments migrate by size through the pores in the agarose toward the positive electrode. The addition of an intercalating dye such as ethidium bromide causes bands on the gel to fluoresce under UV radiation.

3.2.6 Multiplex PCR

It is possible to amplify and detect multiple DNA sequences in a single reaction tube by using multiple primer pairs, which recognize and bind to the flanking regions of different specific target sequences. Since the PCR products (amplicons) are separated and visualized according to fragment size, it is important to be sure that the fragments produce bands that can be resolved on a gel during electrophoresis. It is also important to design primers that are not likely to compete or bind to each other to form primer dimers.

3.2.7 Results and data interpretation

Smaller nucleic acid fragments migrate more rapidly than larger ones, hence migration distance can be related to fragment size by comparing bands in sample lanes with a molecular marker containing reference DNAs of known lengths run on the same gel. Solutions are loaded into wells at the top of the gel and the migration distance from the well to the band front is related to the size of the DNA fragment.

The gel photograph in Figure 19 shows seven lanes of data. The 100-bp molecular marker was loaded into lane 1. Sample solutions after PCR were loaded into lanes 2–6. These plant samples were assayed to determine transgenic status. In this multiplex PCR assay, three primer sets were used to amplify three target DNA sequences: top band – species-specific endogenous gene; middle band – introduced effect gene (transgene); bottom band – selectable marker gene (transgene).

The presence of the band for the species-specific endogenous gene in all sample lanes demonstrates that the PCR amplification was successful. It is clear that the plant sample in lane 3 is negative for the transgene of interest, because the only band present is the endogenous species-specific gene. It is clear that the plant samples in lanes 2, 4, 5 and 7 are all positive for the transgene of interest because all three of the target sequences are visible on the gel.

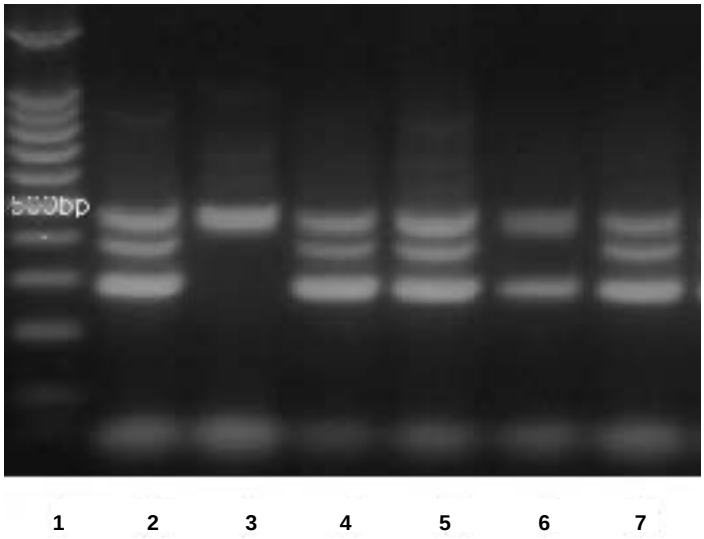


Figure 19 Sample gel of the results of a PCR. Lane 1 is a 100-bp molecular marker; lanes 2–6 are samples. The presence of the top bands (the species-specific endogenous gene) demonstrates that the PCR amplification was successful. Lack of the middle band (the introduced effect gene) and the bottom band (the selectable marker gene) in lane 3 indicates that sample is negative for the effect gene. Presence of all three bands in the remaining lanes indicates the samples are positive for the effect gene

The plant sample in lane 6 is also positive for the transgene of interest. Because the band for the effect gene (middle band) is typically fainter than the band for the selectable marker gene (bottom band), it appears that for lane 6, the PCR product amplification for the effect gene is below the assay detection threshold. Because the selectable marker is clearly present and the PCR amplification worked, lane 6 can be interpreted as a positive result for the transgene of interest.

3.2.8 PCR controls

There are three types of PCR controls, endogeneous reference genes and negative and positive controls. Primers that amplify a species-specific endogenous reference gene are used as internal controls in the PCR. For example, in a soybean assay, the soy lectin gene may be used as the species-specific reference gene (Table 11).[121] Maize invertase can be used as the endogenous reference gene in corn (Table 12).[121]

Table 11 Primer sequences for PCR analysis of Roundup Ready (RR) Soy

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Lectin	GACGCTATTGTGACCTCCTC	
Lectin	GAAAGTGTCAAGCTTAACAGCCGACG	318
EPSPS RR Soy-specific	TGGCGCCCAAAGCTTGCATGGC	356
EPSPS RR Soy-specific	CCCCAAGTTCCTAAATCTTCAAGT	

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

Table 12 Primer sequences for PCR analysis of Bt corn[a]

Primer	Sequence (5′–3′)[a]	Length of amplicon (bp)
Invertase	CCGCTGTATCACAAGGGCTGGTACC	
Invertase	GGAGCCCGTGTAGAGCATGACGATC	226
Cry1A(b)	ACCATCAACAGCCGCTACAACGACC	
Cry1A(b)	TGGGGAACAGGCTCACGATGTCCAG	184

[a] Standard one-letter amino acid abbreviation (see list of Abbreviations and Acronyms).

These reference genes demonstrate that the DNA isolated was of sufficient quality and quantity for PCR amplification. It is assumed that in the course of food processing, the species-specific reference gene and the transgene are degraded in a similar manner. It is also assumed that effects of the matrix on PCR amplification will be similar. The reduced amplification efficiency of both genes presumably has no effect on the ratio of their amounts, which reflects the ratio of modified and unmodified DNA.

Negative controls demonstrate the absence of laboratory contamination or sample cross-contamination. DNA extracts from nontransgenic plants, clean buffer and mastermix with no template DNA added are common negative controls that are run concurrently with the test samples in the PCR.

Positive controls demonstrate adequate amplification and may be used to quantify the sensitivity of the reaction. One approach is to add known amounts of reference material [e.g., soybean and corn powder containing 0.1% (w/w) genetically altered material] to the standard PCR and to run these concurrently with the test samples. Plant genomic DNA and GMO genomic DNA may also be used as positive controls in the PCR.

3.2.9 Primer design

Primer design is one of the most important aspects of a robust PCR assay. In general, primers should be designed such that they are not able to form secondary structures such as stemloop or hairpin configurations. A primer must not be complementary at the 3′ end, as this will cause primer dimers to form. All primers should have similar melting temperatures and should not contain stretches of individual nucleotides. There are software programs available to assist in primer design, but it is crucial that primers are tested in the assay, especially in a multiplex system.

3.2.10 PCR confirmatory techniques

Presented below are four increasingly stringent confirmatory techniques for PCR and a brief discussion of considerations, limitations and advantages of each. These four techniques are agarose gel electrophoresis, restriction analysis, Southern blotting and sequencing.

Agarose gel electrophoresis can be used to determine whether the PCR amplicon is the expected size. The density of the gel should be chosen to ensure resolution of

the amplicon, and the molecular weight marker should be chosen to encompass the expected size range of the amplicon. A limitation to this approach is that it gives an indication only of the size of the amplification product, not its identity. An advantage is that the technique is quick and easy, allowing for screening of many samples within a short period of time.

Restriction analysis utilizes known restriction enzyme cleavage sites within the DNA sequence of interest. Knowing the sequence of the target PCR product, one can cleave the DNA with appropriate restriction enzymes and separate those fragments by agarose gel electrophoresis. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen to appropriately resolve and identify the size of the resultant DNA fragments. This type of analysis will give an indirect indication of the identity of the amplicon based solely on common restriction sites and size. Using the known restriction enzyme cleavage sites gives more conclusive data than simple gel electrophoresis, because the recognition site must be present to produce a DNA fragment of the predicted size. Restriction analysis is easily performed on a large number of samples in a short period of time.

Southern blotting consists of agarose gel electrophoresis of the PCR product followed by transfer of the DNA to a solid support matrix, and hybridization with a labeled DNA probe. This technique allows for the determination of the amplicon size and infers specificity related to the DNA probe. As with agarose gel electrophoresis, the density of the gel and molecular weight markers must be chosen appropriately for the size of amplicon being analyzed. It is important that the DNA probe be adequately characterized to ensure its specificity to the targeted DNA sequence. The Southern blotting technique is a lengthy process, but this technique allows for the confirmation of reactivity to a specific DNA probe, giving more confidence about the identity of the PCR product.

Sequencing the amplicon is the most conclusive confirmatory technique. The main consideration is that the DNA must be appropriately purified to achieve unambiguous sequencing data. However, sequencing requires expensive laboratory equipment that may not be available in all labs. Sequencing does not depend upon the specificity of a probe, or restriction enzyme, but gives a direct identification of the amplicon of interest.

3.3 Basic principles of real-time PCR

Real-time quantitative PCR offers an approach to DNA detection by monitoring the accumulation of PCR products as they are generated. A single copy of a target DNA sequence can yield 2^n copies after n cycles. Hence, theoretically, there is a relationship between starting copy number and amount of PCR product at any given cycle (Figure 20, line A). In reality, replicate reactions often yield widely different amounts of PCR product (Figure 20, line B). This is due to reagents and enzyme activity limiting the reaction. It is difficult to quantify the starting amount of target DNA based on the endpoint. Real-time PCR has the potential to decrease the variability of the measurement by using kinetic rather than endpoint analysis of the PCR process.

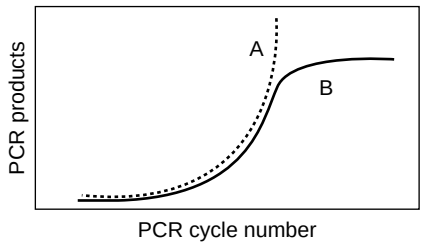


Figure 20 Plot of PCR products produced against the number of amplification cycles. (A) Theoretical PCR product amplified and (B) actual PCR product amplified

3.3.1 Intercalating dyes

The first real-time systems detected PCR products as they were accumulating using DNA binding dyes, such as ethidium bromide.[124,125] UV radiation was applied during thermal cycling, resulting in increasing amounts of fluorescence, which was captured with a charge-coupled device (CCD) camera. The increase in fluorescence ($\Delta_n R$) was plotted against cycle number to give a picture of the kinetics of the PCR process rather than merely assaying the amount of PCR product that had accumulated at a fixed endpoint. These binding dyes are nonspecific, because a fluorescent signal is generated for any double-stranded DNA present. The presence of double-stranded DNA could be due to mispriming or the formation of primer dimer artifacts rather than specific amplification of the target sequence. Nonetheless, DNA binding dyes are very useful in real-time PCR when specificity is not a concern. Examples of commonly used intercalators are ethidium bromide and SYBR Green.[126]

3.3.2 Fluorogenic probes

With fluorogenic probes, it is possible to detect specifically the target sequence in real-time PCR because specific hybridization is required to generate fluorescence. A typical fluorogenic probe is an oligonucleotide with both a reporter and a quencher dye attached. The probe typically binds to the target sequence between the two primers. The proximity of the quencher in relation to the reporter molecule reduces the Forster resonance energy transfer (FRET) of the fluorescent signal emitted from the reporter. There are also a wide range of fluorophores/quenchers and several different hybridization probe strategies available (Table 13).

The three main categories of hybridization probes for real-time PCR are (1) cleavage based assays such as TaqMan, (2) displaceable probe assays such as Molecular Beacons and (3) probes which are incorporated directly into primers such as Scorpions.

Table 13 Common fluorophores/quenchers

DABCYL	4-(4-Dimethylaminophenylazo)benzoic acid
FAM	Fluorescein
TET	Tetrachloro-6-carboxyfluorescein
HEX	Hexachloro-6-carboxyfluorescein
TAMRA	Tetramethylrhodamine
ROX	Rhodamine-X

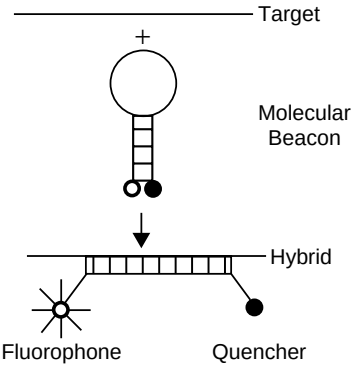


Figure 21 Schematic of the Molecular Beacon

3.3.3 Examples of fluorescent PCR systems

The TaqMan system is also called the fluorogenic 5′ nuclease assay. This technique uses the 5′ nuclease activity of Taq polymerase to cleave an internal oligonucleotide probe. The probe is labeled with both a fluorescent reporter dye and a quencher. The assay results are detected by measuring changes in fluorescence that occur during the amplification cycle as the fluorescent probe is cleaved, uncoupling the dye and quencher labels. The increase in the fluorescent signal is proportional to the amplification of target DNA.

The Molecular Beacons system uses probes that are configured in the shape of a stem and loop. In this conformation, the probe is 'dark' (background level fluorescence) because the stem hybrid keeps the fluorophore in close proximity to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, resulting in increased fluorescence proportional to the amplification of target DNA (Figure 21).

The Scorpions system combines a primer, a specific hybridization probe, fluorophore and quencher in a single molecule. When the Scorpions primer is in a stem and loop conformation, the fluorophore and quencher are in close proximity. The initial heating step denatures the template and also the stem of the Scorpions primer. The primer anneals to the template and strand elongation occurs, producing a PCR amplicon. This double-stranded DNA is denatured and the specific hybridization probe (sequence originally within the loop of the stem/loop) reaches back and hybridizes to the PCR product, binding to the target in an intramolecular manner. The new conformation separates the fluorophore and quencher, resulting in an increase in the fluorescent signal that is proportional to the amplification of target DNA.[127]

3.3.4 Quantitative results/data interpretation

A method for quantitation of the amount of target involves measuring threshold cycle (C_T) and use of a calibration curve to determine starting copy number. The parameter C_T is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. A plot of the log of initial target copy number for a set of standards versus C_T is a straight line (Figure 22).[125] Thus, when the percentage of GMOs in the sample

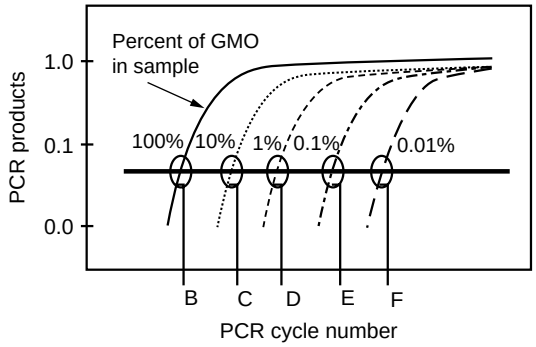


Figure 22 Real-time quantitation of PCR products. The straight line represents the threshold fluorescence value. Each curved line is a plot of the PCR products formed against the number of cycles for different samples. For samples containing 100% GMO, only *B* cycles are required to reach the threshold fluorescence. Samples containing 0.01% GMO will require *F* cycles before the threshold is attained

is 100%, the threshold fluorescence will be reached after only *B* cycles, whereas the sample containing 0.01% of GMO will reach the threshold after *F* cycles.

The use of C_T values also expands the dynamic range of quantitation because data are collected for every PCR cycle. A linear relationship between C_T and initial DNA amount has been demonstrated over five orders of magnitude, compared with the one or two orders of magnitude typically observed with an endpoint assay.[126]

3.4 Applications of PCR to agricultural biotechnology

3.4.1 Research and development

The PCR technique is very useful during all stages of the research and development of biotech crops. PCR analysis is used for gene discovery, event selection, screening, transformant identification, line selection and plant breeding. Quantitative real-time PCR is used to determine the number of transgene copies inserted in experimental plants.

3.4.2 Regulatory submissions

PCR is used to support regulatory submissions. For example, a petition for nonregulated status for a biotech crop must contain the following information:

- rationale for development of product
- description of crop
- description of transformation system
- the donor genes and regulatory sequences
- genetic analysis and agronomic performance
- environmental consequences of introduction
- adverse consequences of introduction
- references.

PCR analysis is one of the techniques used to generate data for the genetic analysis requirement.

3.4.3 Food and commodity testing

There are commercial testing laboratories that offer PCR testing of commodities and food for GMO content. Testing of bulk commodities such as corn grain requires a large sample size. A 2500-g sample is required to have a 99.9% probability of detecting 0.1% GMO content in a sample. The sampling strategy must produce a statistically valid sample for the test results to be meaningful. The entire 2500-g sample would typically be ground, and duplicate 10-g subsamples of raw corn or soy would be extracted. For processed or mixed foods, duplicate 2-g subsamples would typically be extracted.

These PCR laboratories often offer GMO screening, specific tests for certain commercial GMOs and real-time quantitative testing. The different approaches vary widely in cost and the choice would depend on the testing objective.

3.5 Recent advances in nucleic acid amplification and detection

Many nucleic acid detection strategies use target amplification, signal amplification or both. Invader, branched DNA (bDNA) and rolling circle amplification (RCA) are three approaches.

Invader is a signal amplification approach. This cleavage-based assay uses two partially overlapping probes that are cleaved by an endonuclease upon binding of the target DNA. The Invader system uses a thermostable endonuclease and elevated temperature to evoke about 3000 cleavage events per target molecule. A more sensitive homogeneous Invader assay exists in which the cleaved product binds to a second probe containing a fluorophore and quencher. The second probe is also cleaved by endonuclease, generating 10^7 fluorescence events for each target molecule, which is sensitive enough to detect less than 1000 targets.[128]

bDNA achieves signal amplification by attaching many signal molecules (such as alkaline phosphatase) to a DNA dendrimer. Several tree-like structures are built in each molecular recognition event. The Quantiplex bDNA assay (Chiron) uses a dioxetane substrate for alkaline phosphatase to produce chemiluminescence.[127]

The linear RCA method can use both target and signal amplification. A DNA circle (such as a plasmid, circular virus or circular chromosome) is amplified by polymerase extension of a complementary primer. Up to 10^5 tandemly repeated, concantemerized copies of the DNA circle are generated by each primer, resulting in one single-stranded, concantemerized product.[129]

4 Biosensors: immunosensors

The development of immunosensors is one of the most active research areas in immunodiagnostics. A large number of immunosensors, which combine the sensitivity and specificity of immunoassays with physical signal transduction, have been developed

in recent years for pesticide analysis. A classical biosensor consists of three components, including a receptor (an antibody or binding protein), a transducer (e.g., an optical fiber or electrode) and signal processing electronics. The receptor is usually immobilized to the transducer surface, which enables it to detect interaction with analyte molecules. In contrast to immunoassays, immunosensors commonly rely on the reuse of the same receptor surface for many measurements. Direct signal generation potentially enables real-time monitoring of analytes, thus making immunosensors suitable tools for continuous environmental monitoring.

There are several classes and subclasses of immunosensors, each with advantages for environmental analysis. Piezoelectric sensors (including bulk acoustic and surface acoustic wave) use an external alternating electric field to directly measure the antibody–antigen interaction. Electrochemical sensors (including potentiometric, amperometric, capacitative and conductimetric) may offer inexpensive analytical alternatives for effluent monitoring.[130,131] Optical sensors (including fiber-optic, evanescent wave biosensors and Mach–Zehnder interferometer sensors) measure the absorption or emission of a wavelength of light and base detection on fluorescence, absorbance, luminescence or total internal reflectance fluorescence.[132,133]

Surface plasmon resonance (SPR) is an optical electronic technique in which an evanescent electromagnetic field generated at the surface of a metal conductor is excited by light of a certain wavelength at a certain angle. An immunosensor has been developed for the detection of atrazine using SPR.[134] Moreover, a grating coupler immunosensor was evaluated for the measurement of four *s*-triazine herbicides.[135] One could detect terbutryn in the range 15–60 nM using this biosensor. Because antibody-based biosensors have no associated catalytic event to aid in transduction, they are far more complex than enzyme-based biosensors. In addition, they do not release their ligand quickly, leading to a slow response. Theoretically, biosensors are capable of continuous and reversible detection, but reversibility is difficult to achieve in practice because sensitive antibody–antigen interactions have high affinity constants. Because cost and time are critical factors in environmental monitoring, it is likely that the development of small-probe antibody-based biosensors yielding continuous readouts of an analyte at low concentration will not be rapid. However, research in the sensor field is certain to give improvements in many aspects of immunoassay technology, and antibody–hapten and receptor–ligand binding assays are being coupled to biological and physical transducers in many ingenious ways.

4.1 Biological transducers

With enzymes, binding proteins or receptors, it is attractive to use biological transduction. A simple example is acetylcholinesterase for the detection of organophosphate and carbamate insecticides. Binding of these materials to the enzyme inhibits it, thus blocking substrate turnover. Similar approaches can be used for herbicide detection. Coupling a receptor to its natural responsive element also can provide a valuable biosensor. This could be induction of natural proteins such as vitellogenen by estradiol or the responsive element could be moved upstream of luciferase, a fluorescent protein or other easily detected biological molecules.[136]

5 Conclusion

As described by Hammock and Mumma,[8] there are many unique applications for immunodiagnostics in pesticide chemistry. Such uses include human monitoring, field monitoring, analysis of chirality, analysis of complex molecules and analytical problems where large numbers of samples must be processed quickly. Such applications are expanding as we see the development of more complex and nonvolatile pesticide chemicals and the need to monitor polar metabolites, environmental degradation products and GMOs. However, other analytical technologies are improving. For example, liquid chromatography/mass spectrometry (LC/MS) technologies increasingly can handle complex molecules and, like immunoassay, tandem mass spectrometry (MS/MS) technologies avoid the need for many cleanup steps. Hence, many of the traditional applications of immunoassay will be replaced by other technologies if immunochemistry remains static. Active research on new formats and new applications of immunoassays argues for a continued place for the technology in the repertoire of environmental chemists. Coupled immunochemical techniques are promising where, for example, antibodies are used as sensitive, selective detection systems for HPLC[137] or for immunoaffinity procedures preceding MS[138] or other analyses.

Although immunoassays can compete effectively with other technologies in the analysis of small molecules, a major strength of the technology is in the analysis of peptides and proteins. With the expanded use of GMOs in agriculture, all of which to date are expressing novel proteins, there is a new and important application for immunoassay. The technology will be important for GMO development, product stewardship and quality control. With some public concern over the safety of GMOs, there is a commercial need for high-throughput and for field analysis of food products for GMO content. High throughput and field analysis are two major strengths of immunoassay technology, making it an ideal technology for monitoring indicators of food quality. Food quality monitoring, then, represents a major market for this technology.

6 Abbreviations

A	adenine
Ab	antibody
ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
ACCS	aminocyclopropane carboxylic acid synthase
Ag	antigen
ALS	acetolactate synthase
bDNA	branched DNA
bp	base pairs
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
C	cytosine
CaMV	cauliflower mosaic virus
CCD	charge-coupled device
CD	compact disk

CMC	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as Morpho CDI)
CMV	cucumber mosaic virus
C_T	threshold cycle
DAM	DNA adenine methylase
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
FIIA	flow injection immunoassay
FRET	Forster resonance energy transfer
G	guanine
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GE	genetically engineered
GLC	gas–liquid chromatography
GM	genetically modified
GMO	genetically modified organism
GOX	glyphosate oxidoreductase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
I_{50}	the concentration of analyte that inhibits the immunoassay by 50%
IgG	immunoglobulin G
K_A	equilibrium binding constant for the binding of analyte to antibody
K_H	equilibrium binding constant for the binding of hapten to antibody
KLH	keyhole limpet hemocyanin
LC/MS	liquid chromatography/mass spectrometry
LLD	lower limit of detection
LOQ	limit of quantitation
LPH	horseshoe crab hemocyanin
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MBS	*m*-maleimidobenzoyl-*N*-hydroxysuccinimide
Morpho CDI	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (same as CDI)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NHS	*N*-hydroxysuccinimide
NPTII	neomycin phosphotransferase II

OD	optical density
PAT	phosphinothricin acetyltransferase
PBA	phenoxybenzoic acid
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PG	polygalacturonase
PRSV	papaya ringspot virus
QC	quality control
RCA	rolling circle amplification
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
T	thymine
T_a	annealing temperature
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T_m	melting temperature
Ti	tumor-inducing
TOF	time-of-flight
TPS	template preparation solution
USDA	United States Department of Agriculture
USDA GIPSA	United States Department of Agriculture Grain Inspection Protection Service
USEPA	United States Environmental Protection Agency
UV	ultraviolet
UV/VIS	ultraviolet/visible
WMV2	watermelon mosaic virus2
ZYMV	zucchini yellow mosaic virus
λ_{max}	wavelength of maximum absorption

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A Sensitive Class Specific Immunoassay for the Detection of Pyrethroid Metabolites in Human Urine

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The pyrethroids are one of the most heavily used insecticide classes in the world. It is important to develop sensitive and rapid analytical techniques for environmental monitoring and assessment of human exposure to these compounds. Because major pyrethroids contain a phenoxybenzyl group and phenoxybenzoic acid (PBA) is a common metabolite form or intermediate, PBA might be used as a biomarker of human exposure to pyrethroids. A sensitive and selective immunoassay for the common pyrethroid metabolite PBA was developed. Rabbits were immunized with 3-[4-(3-carboxyphenoxy)phenoxy] N-thyroglobulin ethylamine. All sera were screened against numerous coating antigens. The assay with the least interference and the best sensitivity was optimized and characterized. The average IC_{50} for free PBA was 1.65 ng/mL. No cross-reactivity was measured to parent pyrethroids and other metabolites. Urine matrix effects can be eliminated by simple dilution. Results from urine samples from exposed workers suggest that this PBA immunoassay might be suitable as a monitoring tool for human exposure to pyrethroids.

Introduction

Pyrethroids have been widely used in agriculture, forestry, horticulture, animal and public health, and homes all around the world (*1*). They are likely to become more widely used as organophosphate insecticides are phased out due to the concerns regarding their safety. A variety of personnel are exposed to pyrethroids during manufacture and application, diet, and drinking water. Although these compounds are widely considered safe for humans (*2*), numerous studies have shown that very high exposure to pyrethroids might cause potential problems to man. Such effects include suppressive effects on the immune system (*3*, *4*), endocrine disruption (*5*), lymph node and splenic damage, and carcinogenesis (*6*). Therefore, it is important to develop a rapid, sensitive, and efficient analytical method for both toxicological and epidemiological monitoring.

A generalized pathway of pyrethroid metabolism in mammals is shown in Figure 1. Pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage, followed by various species-dependent conjugations such as to glucuronide, glycine, taurine, and sulfate (*7*−*10*). Current analytical methods for pyrethroid metabolites in urine rely upon multistep sample cleanup procedures including hydrolysis, extraction, and derivatization. Samples are then analyzed using either HPLC (*11*) or GC-MS with a detection limit of 0.5 μg/L urine (*12*, *13*). These methods are expensive and relatively time-consuming. Immunoassay is a leading method for rapid assessment of exposure to agrochemicals by detecting key urinary biomarkers of exposure.

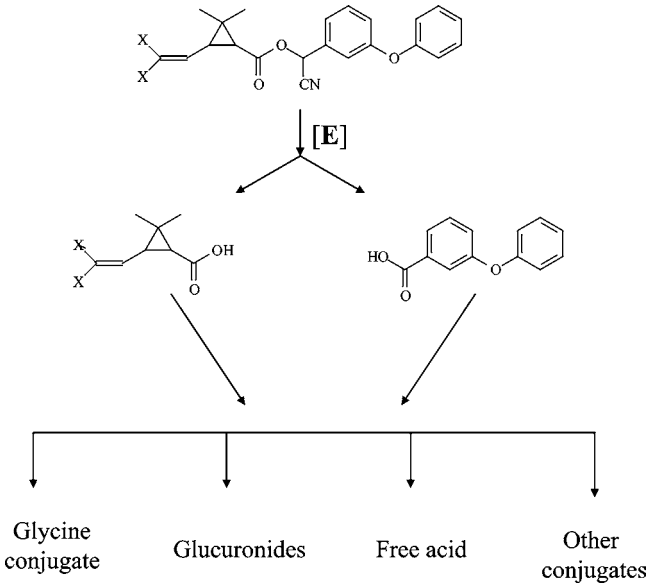

Figure 1. Pyrethroid metabolism pathway in mammals.

Because major pyrethroids such as permethrin, cypermethrin, and cyfluthrin contain the PB[1] group and PBA is a common metabolite form or intermediate, PBA conjugates might be suitable biomarkers of human

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[1] Abbreviations: Ab, antibody; BSA, bovine serum albumin; cAg, coating antigen; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; FAB-HRMS, fast atom bombardment high-resolution mass spectrum; FPBA, 4-fluoro-3-phenoxybenzoic acid; FPBAG, *N*-(4-fluoro-3-phenoxybenzoyl)glycine; GAR-HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; IC_{50}, the concentration of analyte giving 50% inhibition; LOD, limit of detection; LOQ, limit of quantitation; NHS, *N*-hydroxysuccinimide; PB, phenoxybenzyl; PBA, phenoxybenzoic acid; PBAG, *N*-(3-phenoxybenzoyl)glycine; PBST, phosphate-buffered saline with 0.05% of Tween 20; RT, room temperature; TMB, tetramethylbenzidine.

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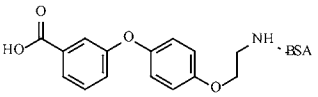
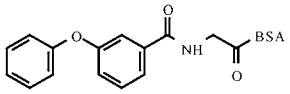
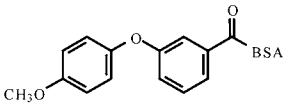
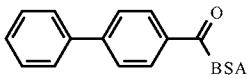
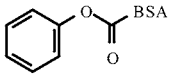
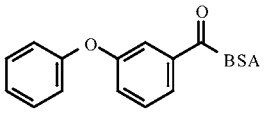
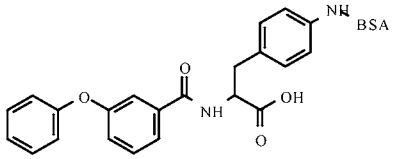
Table 1. Titration Summary for anti-PBA Antisera[a]

Coating Antigens	Structures	Ab 294	Ab 295
cAg **01** Compound **6**-BSA		++++	+++
cAg **02** PBA-glycine-BSA		+++	+
cAg **03** 4-methoxy-PBA-BSA		+	-
cAg **04** 4-biphenyl benzoic acid-BSA		-	-
cAg **05** phenoxyacetic acid-BSA		-	-
cAg **06** 3-PBA-BSA		+++	+
cAg **07** *N*-(3-Phenoxybenzoyl)-4-amino-*L*-phenylalanine-BSA		+++	+

[a] The data shown are at a cAg concentration of 0.1 μg/mL and an Ab dilution of 1:10 000; −, absorbance < 0.25; +, absorbance 0.25−0.50; ++, absorbance 0.50−0.75; +++, absorbance 0.75−1.00; and ++++, absorbance > 1.00.

exposure. In the previous study, we developed a sensitive and selective immunoassay for detecting one of the possible pyrethroid metabolites, a PBA−glycine conjugate (*14*). However, no study has specifically determined the nature of the conjugates of pyrethroid metabolites in humans. More likely, the pyrethroid metabolite profile will vary from individual to individual. An assay detecting a single conjugate such as PBA−glycine may not be sufficient for exposure monitoring. A sensitive and specific PBA assay will be more significant since it allows us to detect all conjugation forms after a hydrolysis treatment, and it will complement conjugate specific assays. A PBA assay could be used as a generic biomarker of human exposure to pyrethroids. In this study, an immunoassay to PBA was developed and characterized. Urine matrix was evaluated, and the resulting assay was applied to urine samples from exposed workers.

Experimental Procedures

Chemicals. The standards cypermethrin, fluvalinate, fenvalerate, and permethrin were obtained from Riedel de Haen (Seelze, Germany). Esfenvalerate was synthesized as described by Shan et al. (*15*) with a purity of >99% based on analytical data. BSA, thyroglobulin (Thyr), Tween-20, 3,3′,5,5′-TMB, and GAR-HRP were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), and Lancaster Synthesis, Inc. (Windham, NH). Preparation of cAgs cAg **02**, cAg **06**, and cAg **07** was reported earlier (*14*) (Table 1). FPBA was prepared in this laboratory with a purity of 96.4%. Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel, packed in glass columns of such diameter to give a column height/diameter ratio of ∼7. The → notation denotes a stepwise solvent gradient.

Instruments. Proton NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from the internal standard, tetramethylsilane. TLC utilized 0.2 mm precoated silica gel 60 F254 on glass (E. Merck, Darmstadt, Germany), and detection was made by UV light or iodine vapor stain. Fast atom bombardment high-resolution mass spectra (FAB-MS) were obtained on a ZAB-2SE mass spectrometer (VG Analytical, Wythenshawe, U.K.), using high energy cesium ions at a density flux of 1−2 mA and 35−38 kV to generate secondary $[MH]^+$ ions. The liquid matrix was glycerol or 3-nitrobenzyl alcohol, and cesium iodide was used for mass calibration at a dynamic resolution of 5000:1. ELISAs were carried out with 96 well microtiter plates and read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Synthesis and Verification. Syntheses of hapten **6** and FPBA−glycine were carried out as outlined in Figures 2 and 3. All reactions were straightforward using well-known procedures, and yields were good. NMR spectral data supported all structures, and mass spectra further supported the structure of **6**.

1. Synthesis of Hapten 6. 1.1. 3-(4-Methoxyphenoxy)-benzoic Acid (1). 3-(4-Methoxyphenoxy)benzaldehyde (5.0 g) was mixed with 50 mL of distilled water containing 1.0 g of K_2CO_3. At RT, a catalytic amount of tetrabutylammonium chloride was added, and then, 1.0 g of $KMnO_4$ was added in portions with vigorous stirring. The reaction mixture turned to a purple color and was stirred for another 60 min. A small amount of solid $NaHSO_3$ was added and stirred for 15 min to remove unreacted $KMnO_4$. The mixture was filtered through Celite, and the filtrate was acidified with 1 N HCl to precipitate the acid. Then, the precipitate was filtered and dried to give 4.02 g of

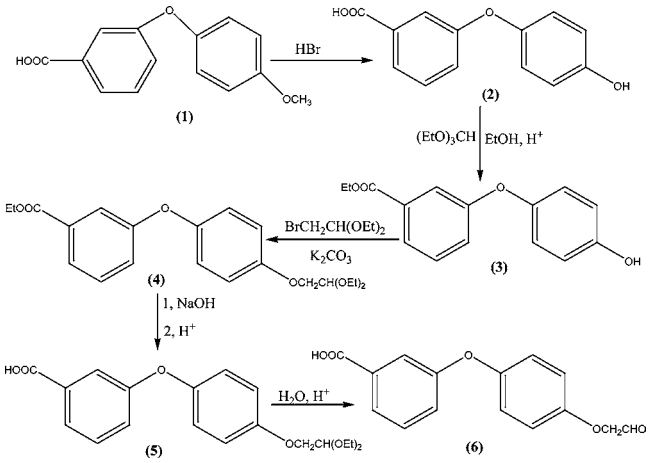


Figure 2. Scheme for synthesis of hapten 6.

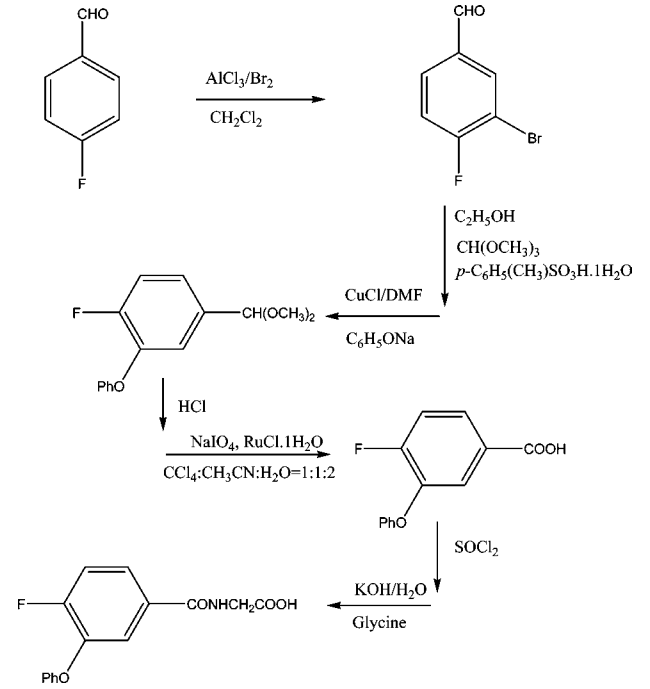


Figure 3. Scheme for synthesis of FPBA-glycine.

compound **1**. TLC R_f 0.49 (ethyl acetate:hexane = 1:1, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 3.77 (S, 3H, CH_3O), 6.98−7.08 (m, 4 H, Ar), 7.21−7.65 (m, 4 H, Ar), 13.1 (bs, 1 H, COOH).

1.2. 3-(4-Hydroxyphenoxy)benzoic Acid (2). A mixture of **1** (3.04 g, 12.4 mmol), concentrated hydrobromic acid (30 mL), and xylenes (1.5 mL) was refluxed for 12 h. The mixture was cooled, diluted with water, and extracted with CH_2Cl_2 containing a small amount of ethyl acetate to effect solution of all solids. The organic phase was washed twice with water, dried (Na_2SO_4), and stripped to a white solid. This was flash chromatographed on silica gel (75 g) (25 → 100% EtOAc in hexane + 1.5% acetic acid) to recover 7% of the starting material and 2.18 g (76%) of **2** as a white solid; mp 156−157.5 °C. TLC R_f 0.4 (EtOAc:hexane = 1:1, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 6.80−6.97 (m, 4 H, Ar), 7.18−7.63 (m, 4 H, Ar), 9.5 (bs, 1 H, OH), 13.1 (bs, 1 H, COOH).

1.3. Ethyl 3-(4-Hydroxyphenoxy)benzoate (3). A mixture of **2** (2.18 g, 9.47 mmol), anhydrous ethanol (35 mL), triethyl orthoformate (1.96 g, 13.2 mmol), and concentrated H_2SO_4 (0.5 mL) was heated under N_2 at 60−65 °C for 20 h and then poured into water and extracted with EtOAc/hexane. The organic phase was washed with $NaHCO_3$ solution and water, dried (Na_2SO_4), and stripped to an oil. This was flash chromatographed on silica gel (35 g) (20 → 30% EtOAc in hexane + 1.5% acetic acid). Fractions containing product were combined, washed with $NaHCO_3$ solution, and stripped to recover 2.43 g (98%) of **3** as a colorless oil. TLC R_f 0.33 (EtOAc:hexane = 1:3, 1.5% acetic acid). ^{1}H NMR (DMSO-d_6): δ 1.37 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 4.36 (q, J = 7.1 Hz, 2 H, OCH_2CH_3), 5.25 (s, 1 H, OH), 6.82−6.95 (m, 4 H, Ar), 7.12−7.73 (m, 4 H, Ar).

1.4. Ethyl 3-(4-(2,2-Diethoxyethoxy)phenoxy)benzoate (4). A mixture of **3** (800 mg, 3.10 mmol), bromoacetaldehyde diethyl acetal (656 μL, ∼1.4 X), anhydrous K_2CO_3 (0.96 g, 1.5 X), dimethyl formamide (6.4 mL), and KI (1−2 mg) was magnetically stirred under N_2 at 125 °C for 4.5 h. The cooled mixture was diluted with EtOAc (4 mL) and water (10 mL), and the aqueous phase was extracted with additional solvent. The combined organic extract was washed with water, dried (Na_2SO_4), and stripped to a tan oil. This was flash chromatographed on silica gel (30 g), (5 → 30% EtOAc in hexane) to recover 268 mg (34%) of **3** and 618 mg (80% based on recovered **3**) of **4**. TLC R_f 0.37 (EtOAc:hexane = 1:4). ^{1}H NMR ($CDCl_3$): δ 1.26 (t, J = 7.0 Hz, 6 H, CH_2CH_3 acetal), 1.37 (t, J = 7.1 Hz, 3 H, CH_2CH_3 ester), 3.65 (dq, J = 7.0, 9.4 Hz, 2 H, $HCHCH_3$ acetal) 3.78 (dq, J = 7.1, 9.4 Hz, 2 H, $HCHCH_3$ acetal), 4.00 (d, J = 5.2 Hz, 2 H, CH_2OAr), 4.35 (q, J = 7.1 Hz, 2 H, CH_2CH_3 ester), 4.85 (t, J = 5.2 Hz, 1H, CH), 6.90−6.98 (m, 4 H, Ar), 7.10−7.74 (m, 4 H, Ar).

1.5. 3-(4-(2,2-Diethoxyethoxy)phenoxy)benzoic Acid (5). A mixture of **4** (400 mg, 1.15 mmol), 50% NaOH solution (940 mg, 4X), water (0.4 mL), 1,2-dimethoxyethane (glyme) (3.6 mL), and 40% Triton B in methanol (1 mL) was stirred under N_2 and heated at 75−80 °C for 10 h. The cooled solution was partially stripped of solvent, diluted with water (homogeneous), acidified to pH 3, and immediately extracted with EtOAc and water, washed, dried, and stripped to give 368 mg of white solid. This was recrystallized from hot butyl chloride with dilution with hexane to return 341 mg (92%) of **5**; mp 101.5−102.5 °C. TLC R_f 0.17 (EtOAc: hexane = 1:4, 1.5% acetic acid). ^{1}H NMR ($CDCl_3$): δ 1.26 (t, J = 7.0 Hz, 6 H, CH_2CH_3), 3.66 (dq, J = 7.1, 9.3 Hz, 2 H, $HCHCH_3$), 3.79 (dq, J = 9.3 Hz, 2 H, $HCHCH_3$), 4.01 (d, J − 5.2 Hz, 2 H, CH_2O), 4.85 (t, J = 5.2 Hz, 1 H, CH), 6.91−7.00 (m, 4 H, Ar), 7.17−7.79 (m, 4 H, Ar).

1.6. 3-((2-Oxoethoxy)ethoxy)phenoxybenzoic Acid (6). A solution of **5** (400 mg, 1.15 mmol) in glyme (4 mL) was treated with 3 N HCl (2 mL) and stirred under N_2 and heated at 75−80 °C for 30 min. The initial two phase reaction mixture became homogeneous after 5 min. Most of the solvents were stripped off under reduced pressure, and the residue was diluted with water and extracted with EtOAc. The extract was washed, dried (Na_2SO_4), and stripped. The gummy residue was flash chromatographed on 13 g of silica gel (10 → 70% EtOAc in hexane) to return 287 mg (90%) of the aldehyde (**6**) as a white solid, mp 143−147 °C, with a sintering from 139 °C. TLC R_f 0.56 (1.5% acetic acid in EtOAc). ^{1}H NMR (DMSO-d_6): δ 4.88 (s, 2 H, CH_2), 6.97−7.67 (m, 8 H, Ar), 9.7 (s, 1 H, CHO), 11.1 (bs, 1 H, COOH). FAB-MS m/z calculated for $\{M + H\}^+ = C_{15}H_{13}O_5$ 273, observed 273.

2. Synthesis of FPBA−Glycine. 2.1. Preparation of 3-Bromo-4-fluorobenzaldehyde (*16*). A solution of 4-fluorobenzaldehyde (2.48 g, 20 mmol) in dry methylene chloride (5 mL) was added to an ice-cooled suspension of powdered aluminum trichloride (2.66 g, 20 mmol) in dry CH_2Cl_2 (15 mL), in which bromine (3.4 g, 1.1 mL, 21.25 mmol) was added dropwise. The mixture was refluxed for 16 h at 45 °C, cooled, and carefully poured into ice H_2O and then extracted with CH_2Cl_2 (3 × 10 mL). The combined organic phase was washed sequentially with saturated sodium metabisulfite (20 mL) and H_2O (20 mL) and saturated with NaCl (20 mL) and then dried with magnesium sulfate. The resultant dark red oil, after removal of solvent under reduced pressure, was run on flash chromatography (hexane:EtOAc, 6:1) to give a sticky oil (1.60 g). Crystallization in the mixture (hexane:EtOAc, 20:1) gave the title compound (1.3 g) as a white solid. Yield: 32% (95% purity). R_f = 0.52 (hexane:EtOAc, 10:1). mp 30−32 °C. ^{1}H NMR ($CDCl_3$): δ 9.95 (s, 1H, C*H*O), 7.30−8.12 (m, 3H, Ar). m/z: 203 (M^+, 100).

2.2. Preparation of 3-Phenoxy-4-fluorobenzaldehyde. The title compound was prepared according to the procedure of Maurer (*17*), which included two steps: protection of the aldehyde and Williamson condensation. A mixture of 3-bromo-4-fluorobenzaldehyde (0.74 g, 5 mmol), triethyl orthoformate (0.83 mL, 5 mmol) and *p*-toluenesulfonic acid monohydrate (0.1 g), and absolute ethanol (20 mL) was refluxed overnight. A few pellets of sodium hydroxide were added, and then, the mixture was refluxed for 20 more minutes. After it was cooled, the reaction mixture was filtered and evaporated under reduced pressure to give a sticky cream. The cream was dissolved in 20 mL of ethyl ether and filtered through a pad of sodium sulfate. The ether was removed under reduced pressure. The resultant oil was dried overnight under vaccum and used in the next step.

Dimethylformate (10 mL) was distilled at 160 °C from a mixture of dimethylformate (60 mL) and phenol (0.7 g, 7.5 mmol). After the mixture was cooled, sodium hydride (0.32 g, 8 mmol) was added by portions and the reaction continued for 30 min at RT, and then, copper(I) chloride (0.2 g) was added. The reaction mixture was heated to 155 °C, and the protected aldehyde (5 mmol) was added over 10 min, and then, it was refluxed for 17 h at 155−165 °C. Subsequently, the reaction mixture was filtered, washed with 5% potassium carbonate, and extracted with petroleum ether (3 × 20 mL). Then, the ether layer was dried overnight with magnesium sulfate. After removal of solvent, the oil was deprotected in 10 mL of acetone containing hydrochloric acid (1.7 mL, 5.1 mmol). After removal of acetone, the residue was washed with 5% potassium carbonate and extracted with petroleum ether (3 × 20 mL), which was then dried with magnesium sulfate. Finally, after removal of the ether, the resultant oil was run on chromatography with a mobile phase (hexane:EtOAc, 20:1) to give the title compound (0.75 g). Yield: 69.4%, purity 92.3%. ^{1}H NMR: ($CDCl_3$): δ 9.92 (s, 1H, C*H*O), 6.89−7.53 (m, 8H, Ar). MS: *m*/*z* 216 (M^+, 100).

2.3. Preparation of FPBA (*18*). Sodium periodate (0.35 g, 1.6 mmol) was added to a mixture containing carbon tetrachloride (2 mL), acetonitrile (2 mL), water (4 mL), and 4-fluoro-3-phenoxy benzaldehyde (0.178 g, 0.8 mmol). The mixture was vigorously stirred while ruthenium chloride hydrate (0.15 g, 0.7 mmol) was added. The reaction was continued at RT until the starting material disappeared by TLC analysis. The reaction mixture was extracted with methylene chloride (3 × 10 mL). The combined organic phase was dried with magnesium sulfate, filtered, and evaporated under reduced pressure to give a brown oil. The oil was dissolved in diethyl ether and washed with 10% sodium hydroxide solution (2 × 10 mL). Acidification of the aqueous phase gave a white solid, which was reextracted with diethyl ether. The ether layer was washed with water and dried over magnesium sulfate, and the solvent was evaporated under reduced pressure to give the title compound (0.136 g) as a white solid. Yield: 71.2%. Purity: 96.4%. mp 99−100 °C. R_f = 0.39 ($CHCl_3$:CH_3OH:acetic acid, 20:1:0.1). ^{1}H NMR (DMSO-d_6): δ 7.05−7.77 (m, 8H, Ar), 12.30 (S, 1H, COO*H*). *m*/*z* ($[M + 1]^+$) = 232.04.

2.4. FPBAG. Thionyl chloride (0.41 g, 3.45 mmol) under N_2 protection was added to a mixture of FPBA (0.4 g, 1.72 mmol), chloroform (3 mL), and dimethylformate (0.5 mL). The mixture was heated to 60−65 °C for 1 h, and the solvent was stripped under reduced pressure. A few drops of toluene were added and then restripped under reduced pressure to give crude 4-fluoro-3-phenoxybenzoyl chloride. The crude product was added in portions to a vigorously stirred ice-cooled solution containing glycine (0.143 g, 1.9 mmol) and potassium hydroxide (3 mL, 2 N). Meanwhile, a potassium hydroxide solution (3 mL, 2 N) was added dropwise over 10 min to keep the aqueous phase slightly basic. The reaction mixture was acidified (3 N HCl) and extracted with diethyl ether. The combined ether was washed with water and evaporated to give a sticky oil. Chromatography (hexane:EtOAc, 1:1 → 0:1) of the oil gave the title compound (FPBAG) as a sticky oil (0.212 g). Purity: 90.1%. ^{1}H NMR (DMSO-d_6): δ 7.01−7.67 (m, 8 H, Ar), 8.01 (t, 1H, N*H*), 3.97 (d, 2H, CH_2), 12.10 (s, 1H, COO*H*). MS ($[M + 1]^+$) = 289.17.

Conjugation of Hapten 6-Thyroglobulin/BSA. The conjugate was synthesized using Schiff base formation and the reductive amination method (*19*). Fifty milligrams of thyroglobulin or BSA was dissolved in 10 mL of carbonate buffer (pH 9). Then, compound **6** (0.05 mmol) was added with gentle stirring followed by addition of 100 μL of 5 M cyanoborohydride in 1 N NaOH. The reaction mixture was stirred for 3.5 h at RT. The unreacted aldehyde sites were blocked by adding 200 μL of 3 M ethanolamine solution (pH adjusted to approximately 9 with 2 N HCl) and reacted for 15 min at RT. The solution was then dialyzed against PBS over 72 h at 4 °C and stored at −20 °C. The **6**−Thyr conjugate was used for immunization, and the **6**−BSA (cAg**01**) was used as a cAg.

Preparation of cAgs cAg 03, cAg 04, and cAg 05. The conjugate was synthesized using an activated ester method (*20*). Compounds 3-(4′-methoxyphenoxy)benzoic acid, 4-phenylbenzoic acid, or phenoxyacetic acid (0.025 mmol) were dissolved in 1.6 mL of dry DMF, and then, 6 mg (0.05 mmol) of NHS and 5.8 mg (0.03 mmol) of EDC were added. The reaction mixture was stirred overnight at RT. Forty milligrams of BSA was dissolved in 12 mL of PBS and 4 mL of carbonate buffer (pH 9). The activated hapten was added dropwise to the protein solution. The mixture was stirred for 30 min at RT and 7 h at 4 °C. The solution was then dialyzed against PBS over 72 h at 4 °C and stored at −20 °C.

Immunization. The production of Ab was made following the protocol reported earlier (*14*). Briefly, two New Zealand white rabbits were immunized (rabbit nos. 294 and 295) with **6**−Thyr. The antigen solutions (100 μg in PBS) were emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. After 1 month, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1 v/v). Booster injections were given at 4 week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C. The results of Ab characterization were obtained from sera of terminal bleeds after four boosters. These terminal bleeds were used for ELISA development.

ELISA. The competitive inhibition ELISA format in this study was based on methods described by Voller et al. (*21*). Microplates were coated overnight at 4 °C with 100 μL/well of the appropriate cAg concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After the plates were washed with PBST (PBST: 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KCl, and 0.05% Tween-20), the plate was incubated with 200 μL per well of a 0.5% BSA solution in PBS for 30 min at RT. After another washing step, 100 μL per well of antiserum diluted in PBS per well (for titration experiment) or 50 μL/well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 μL/well of standard analyte or sample solution were added and incubated for 1 h. The standard analyte concentrations ranged from 0.05 to 5 μg/L. Following a washing step, the secondary Ab GAR-HRP (diluted 1:3000 in PBS with 0.05% Tween 20, 100 μL/well) was added and incubated for 1 h at RT. The plates were washed again, and 100 μL/well of substrate solution (3.3 μL of 30% H_2O_2, 400 μL of 0.6% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) was added. The color development was stopped after 15 min with 50 μL/well of 2 M H_2SO_4. The absorbance was measured using the dual wavelength mode at 450 nm minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$ where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is IC_{50}, and D is the minimum absorbance at infinite concentration.

Assay Optimization. The assay conditions were optimized in such a way that the IC_{50} values were minimized. This goal was achieved by screening antibodies and antigens in a two-dimensional titration for best dilution of cAg and antiserum. Then, competitive inhibition curves were measured for different Ab and antigen combinations, and the one with the lowest IC_{50} was selected for further assay development.

Cross-Reactivity (CR). The optimized assay was submitted to cross-reactivity studies by using the standard solution of the analytes and other structurally related compounds (listed in Table 3). The CR was obtained from the IC_{50} values of metabolite standard and the related compounds from the same plate where

$$\%CR = (IC_{50} \text{ of metabolite}/IC_{50} \text{ of tested compound}) \times 100$$

Matrix Effects. 1. pH. The pH effect was tested by preparing analytes in phosphate buffers at pH 4, 6, and 8.

2. Ionic Strength. The effect of ionic strength on the quantitation of PBA was studied by preparing analyte standard solutions in 0.1, 0.3, 0.5, and 0.7 M PBS all at pH 7.5.

3. Urine. The effects of urine matrix on the quantitation of PBA were evaluated by preparing analyte standard solutions in a buffer with different concentrations of urine (0, 1, 2, 4, or 10% of urine). Urine samples tested in this study were from healthy individuals without known exposure to pyrethroids.

Urine Sample Analysis. Urine samples collected from persons exposed to cyfluthrin were used in this study (provided by Dr. Gabriele Leng). In Dr. Leng's lab, these urine samples have been analyzed for the total FPBA after hydrolysis via GC-MS. To test which form of conjugate is predominant in the urine, these samples were tested by different immunoassays including the PBA−glycine ELISA (*14*). Each urine sample was split into three aliquots. One aliquot was analyzed directly by PBA and PBA−glycine ELISAs to determine the free FPBA and FPBA−glycine conjugate in urine. The second aliquot was incubated with glucuronidase and then measured with the PBA ELISA to determine the FPBA−glucuronide conjugate in urine (the difference in FPBA concentration measured before and after enzyme treatment). The third aliquot was hydrolyzed as described by Leng et al. (*22*) followed by the PBA ELISA to measure total free FPBA and FPBA conjugates in urine.

Results and Discussion

Hapten Design and Synthesis. No study has reported which conjugate is predominant for pyrethroid metabolites in human urine. The predominate conjugate may vary with an individual, exposure level, and other factors. In mammals, the major "detoxification" reaction for carboxylic acid-containing xenobiotics is conjugation, either with an amino acid to form a peptide or with glucuronic acid to form a glycoside (*23*, *24*). Therefore, PBA, a main breakdown metabolite of pyrethroids, may undergo either amino acid conjugation or glucuronidation in humans. A specific assay for each possible conjugate may be ideal but not economically feasible for exposure monitoring. One alternative way is to hydrolyze all possible conjugates to free PBA and measure the total PBA in urine. This would allow monitoring of human exposure to total pyrethroids.

To generate a specific Ab for an analyte, it is important to maintain the structure of target compound with as little change as possible when it is modified and coupled to a carrier protein. In this respect, it is prudent to attach the handle as distal as possible from the structure that defines the target (including carboxylic acid group). The PBA molecule contains a carboxylic acid group, which can be directly conjugated to the carrier protein. However, such a strategy may result in antibodies with low sensitivity and high CR to parent pyrethroid molecules. A monoclonal Ab (from Shell Chemical Co.) generated using such a strategy was tested and its IC_{50} for PBA was about 3.0 ppm. This suggests that the carboxylic acid group might be important to develop a more sensitive and selective PBA assay. In the previous study for a PBA−glycine conjugate immunoassay, we successfully developed a highly sensitive ELISA by designing a hapten with an attachment of a handle at the α-position of the glycine and leaving the carboxylic acid group unchanged. Similarly, in this study, haptens for PBA had the handle attached to the 4-position of distal phenyl group (Figure 2).

Table 2. Selected Competitive ELISA Results for Screening from a Single Data Set

Ab/antigen	OD_{max} (A)	slope (B)	IC_{50} (C) (ng/mL)	OD_{min} (D)
Ab294/cAg**06**	0.89	1.16	1.28	0.06
Ab294/cAg**02**	0.95	1.33	2.83	0.06
Ab294/cAg**07**	0.85	0.96	5.78	0.06
Ab294/cAg**01**	0.95	1.10	121	0.06
Ab295/cAg**01**	0.65	1.00	145	0.07

Screening of the Sera and Assay Optimization. A checkerboard titration system was used for screening of Ab and antigen combinations (*25*). The antisera of two rabbits were tested against seven cAgs. Both antisera showed higher titer in the homologous system than in heterologous systems. The main reason the titer is higher in the homologous system is that the identity of the hapten in the cAg and immunogen is identical. Recognition by both antibodies for the related biphenyl cAg or the partial molecule, phenoxyacetic acid, was poor suggesting that the PBA structure was required for recognition (Table 1). Ab 294 showed higher titer on all cAgs tested than Ab 295; thus, only Ab 294 was used for further screening.

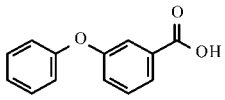
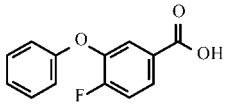
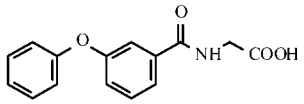
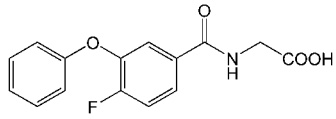
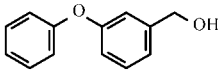
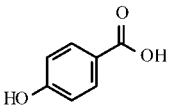
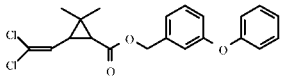
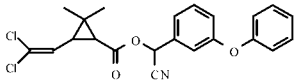
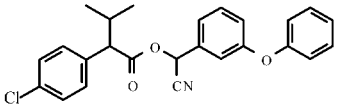
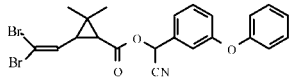
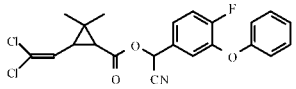
All heterologous combinations tested showed a very low IC_{50} (<6.0 μg/L) against the target analyte, which ranged from 6.0 to 1.28 μg/L (Table 2). The homologous systems had higher IC_{50} values and were less desirable than the heterologous systems. For example, with Ab 294, the IC_{50} for PBA in a homologous system was about 50 times higher than that in the heterologous systems (Table 2). Of the cAgs screened, the BSA conjugate of PBA (cAg**06**) yielded the most sensitive assay. In this study, only Ab 294/cAg**06** was used for further assay development.

CR. Esfenvalerate, cypermethrin, permethrin, deltamethrin, cyfluthrin, their metabolites, and other structurally related compounds were tested for CRs (Table 3). Ab 294 is highly selective for the target analyte PBA and the related cyfluthrin metabolite FPBA (72%). In all cases, the CR of the parent compound, metabolites, and other tested compounds was negligible. Although PBA−glycine, PB alcohol, and other tested pyrethroids contain the PB group, which is present in the immunogen hapten, they did not interfere in the assay.

Matrix Effects. Because this assay was intended to analyze urine samples, it was necessary to study the influence of pH and ionic strength in urine samples. This assay showed good tolerance to the pH of the solution (Figure 4). As compared with PBS buffer (0.15 M, pH 7.5), no significant influences were observed at pH 5−8 indicating that a slight difference in the pH of sample or buffer (in this range) would not affect the accuracy of PBA quantitation.

The assay is very sensitive to higher concentrations of salt in solution (Figure 5). At an ionic strength of 0.3 M PBS, the binding between Ab and antigen was suppressed about 30%. These results are consistent with the previous report about atrazine mercapturic acid and assumed that there was a disruption of Ab−antigen interaction (*26*).

Table 3. Summary of Cross-Reactivities of PBA Assay

Chemicals	Structures	IC_{50} (ng/mL)	CR (%)
3-Phenoxybenzoic acid (PBA)		1.65	100
4-Fluoro 3-phenoxybenzoic acid (FPBA)		2.28	72
PBA-glycine conjugate (PBAG)		>10000	0
FPBA-glycine conjugate (FPBAG)		>10000	0
3-Phenoxybenzyl alcohol		>10000	0
4-Hydroxybenzoic acid		>10000	0
Permethrin		>10000	0
Cypermethrin		>10000	0
Esfenvalerate		>10000	0
Deltamethrin		>10000	0
Cyfluthrin		>10000	0

The effect of a urine matrix was evaluated as well. In tests with four different urine samples, this assay could tolerate 2% of urine with little alteration in the standard curve (Figure 6). Therefore, dilution prior to ELISA would be necessary for the assay. Considering variation among different urine samples, a 100× dilution of the urine before the assay was chosen. Alternatively, differential partitioning or solid phase extraction could be used to clean up the sample. In recovery experiments for the direct dilution of urine samples, urine matrix had a little effect on accurate quantitation for urines spiked with PBA ≥ 5.0 μg/L. The recovery rate for PBA at 5.0 μg/L is 110.0 ± 10.5%. According to the recommended LOQ determination guideline (*27*), the approximate LOQ was 5.0 μg/L for the assay with no clean up. This is consistent with the estimated concentration that corresponded to the absorbance of the control (zero analyte) minus three times the standard deviation of control, when standard analytes were prepared in a buffer system with 2% blank urine.

Assay Validation and Application. The assay validation was performed in a blind fashion by direct dilution

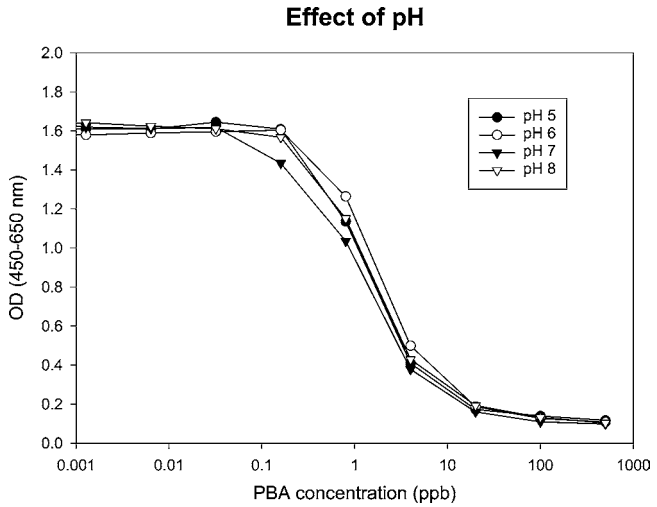


Figure 4. ELISA competition curves of PBA prepared at various pH values. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).

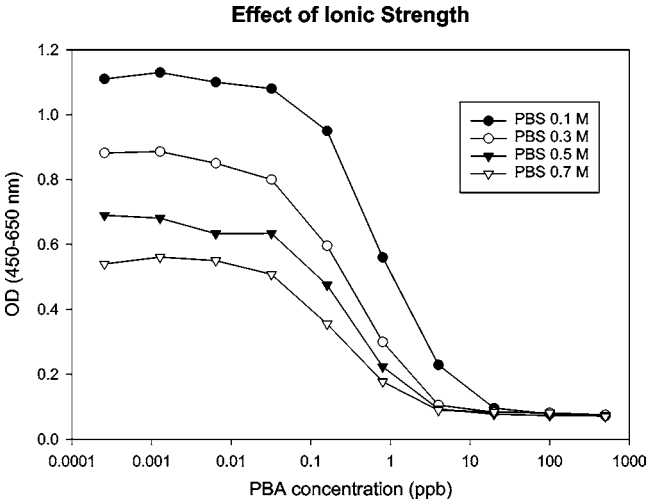


Figure 5. ELISA competition curves of PBA prepared at various ionic strengths. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).

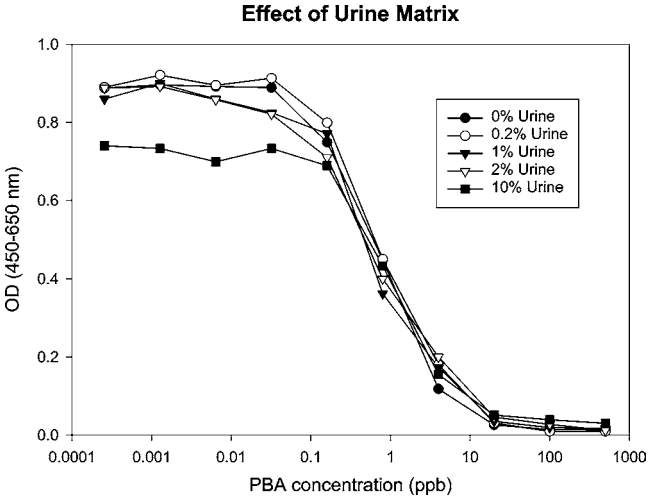


Figure 6. ELISA competition curves of PBA prepared at various urine concentrations. Reagent concentrations: cAg (cAg**06**, PBA-BSA) (0.5 μg/mL); antiserum (Ab294, 1/10 000, final concentration in wells); and GAR-HRP (1/3000).

of the urine samples, which were spiked with PBA concentrations ranging from 0 to 160 μg/L (Figure 7). The linear regression analysis of ELISA results showed a good correlation ($R^2 = 0.900$). All recoveries were over 86% of the spiked values. These results demonstrated that these assays are suitable for the quantitative detection of pyrethroid metabolite (as total PBA) at trace levels in urine samples.

Two positive human urine samples (I and II) from the laboratory of Dr. Gabriele Leng were tested using the PBA ELISA and the PBA−glycine ELISA (*14*). In the PBA−glycine assay, PBA−glycine was used as reference standard. On the basis of the structural similarity between PBA and FPBA (Table 4) and high CR of FPBA in the PBA assay (72%), the FPBA−glycine should be highly cross-reactive in the PBA−glycine assay. No FPBA−glycine was detected (<2.0 ng/mL as PBA−glycine) in either urine sample. A moderate amount of FPBA−glucuronide was detected in these urine samples. This conjugate is about 7.2 (8.4 ng/mL) and 6.2% (2.4 ng/mL) of the total FPBA in urine samples I and II, respectively. Interestingly, the major FPBA-containing

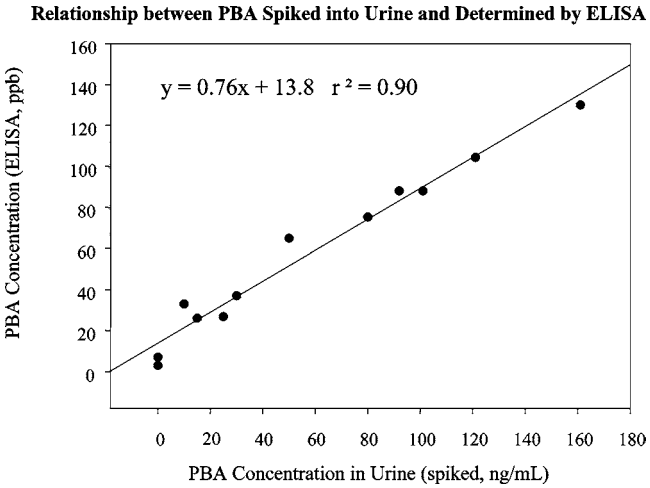


Figure 7. Relationship between spiked PBA in urine and measured by ELISA. Blank urine samples were provided by lab personnel without known exposure to pyrethroids.

Table 4. Summary of Cyfluthrin Metabolites in Human Urine

	prehydrolysis				posthydrolysis	
	FPBAG (ng/mL)	glucuronide (ng/mL)	FPBA (ng/mL)	others (ng/mL)	FPBA (ng/mL) ELISA	FPBA (ng/mL) GC-MS
urine I	ND[a]	8.4	68.7	20.5	117	97.6
urine II	ND	2.4	28.2	4.0	39	34.6

[a] ND: not detectable at the detection limit of PBA-glycine ELISA (5 ng/mL).

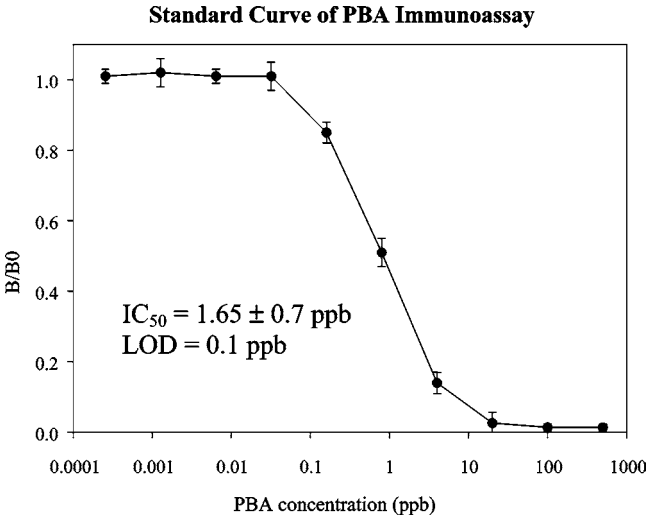


Figure 8. ELISA inhibition curve for PBA using antiserum 294 (diluted 1:10 000, final dilution in well), cAg PBA-BSA (cAg**06**) (0.5 μg/mL), and GAR-HRP (1:3000). This standard curve represents the average of 23 curves.

metabolite in these urine samples is free FPBA, which was 59 (68.7 ng/mL) and 72% (28.2 ng/mL) of the total FPBA metabolites in urine samples I and II, respectively. This may be due to the degradation of FPBA conjugates during storage because the urine samples were stored at 0−4 °C for 3−4 months prior to this study. A further study using freshly collected urine samples is needed to determine whether free FPBA or PBA is the predominant form of pyrethroid metabolite in urine. If confirmed, PBA or FPBA can be used directly as a biomarker of human exposure to pyrethroids. A good agreement between ELISA and GC-MS results was observed (Table 4)

suggesting that this assay is suitable for human urine monitoring.

Conclusions. A sensitive and selective immunoassay for PBA has been developed by using **6** as the immunizing hapten and PBA as the cAg hapten. ELISA cAg**06**/294 has a very low IC_{50} value and also exhibits good performance characteristics at various pH values. The assay is sensitive to urine matrix and a 50× dilution is needed before analysis. The optimized assay using antiserum 294 (diluted 1:10 000, final dilution in well) and cAg**06** (0.5 μg/L) gave an IC_{50} value of 1.65 ± 0.7 ng/mL with a lower detection limit of 0.1 ng/mL (Figure 8). This ELISA was successfully applied to quantitate low parts per billion (ppb or ng/mL) of PBA in urine. A good correlation between ELISA and GC-MS results was achieved in the samples from exposed workers suggesting that this PBA immunoassay is useful for human exposure monitoring and toxicological studies.

Acknowledgment. We thank Axel Ehmann (Mass Search, Inc., Modesto, CA) and A. Daniel Jones (Pennsylvania State University) for mass spectral determination and the Department of Chemistry (UC Davis) for the use of their NMR instrument. Dr. Gabriele Leng of the Medical Department, Bayer AG, Leverkusen, Germany, is thanked for providing positive human urine samples and GC-MS results. This project was funded by the National Institute of Environmental Health Sciences Superfund Basic Research Program, P42 ES04699; the National Institute of Environmental Health Sciences Center, P30 ESO5707; U.S. Army Medical Research and Materiel Command DAMD17-01-1-0769; and NIOSH/Western Center for Agricultural Health and Safety, University of California, Davis.

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Hapten and Antibody Production for a Sensitive Immunoassay Determining a Human Urinary Metabolite of the Pyrethroid Insecticide Permethrin

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Permethrin is the most popular synthetic pyrethroid insecticide in agriculture and public health. For the development of the enzyme-linked immunosorbent assay (ELISA) to evaluate human exposure to permethrin, the glycine conjugate (DCCA-glycine) of a major metabolite, *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), of permethrin was established as the target analyte. Four different types of the *cis*- and *trans*-isomers of immunizing haptens were synthesized as follows: *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine (hapten **3**), *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (hapten **5**), *N*-(*N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)-amino-6-(2,4-dinitrophenyl)aminohexanoic acid (hapten **9**), and *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic acid (hapten **24**). Sixteen polyclonal antibodies produced against each *cis*- or *trans*-hapten−thyroglobulin conjugate as immunogens were screened against numerous hapten−bovine serum albumin conjugates as coating antigens. Six ELISAs with both a heterologous hapten structure and a heterologous hapten configuration (*cis*/*trans* or *trans*/*cis)* between antibody and coating antigen showed a high sensitivity for the target analyte. The IC_{50} was 1.3, 2.1, and 2.2 μg/L for the *trans*-target analyte and 0.4, 2.3, and 2.8 μg/L for the *cis*-target analyte. The immunizing haptens, except for hapten **5**, provided the target specific antibodies. Molecular modeling of the haptens supported the selection of reasonable immunizing haptens that best mimicked the target analyte. Hapten **5** was suitable as a coating antigen rather than as an immunogen since it had a different geometry. Very low cross-reactivities were measured to permethrin, its free metabolite (DCCA), PBA-glycine conjugate, and glycine. The ELISA will be optimized for the detection of total *cis*/*trans*-DCCA-glycine in human urine samples.

KEYWORDS: ELISA; permethrin; glycine conjugate; human exposure; hapten

INTRODUCTION

The pyrethroid insecticide permethrin acts as a neurotoxin to control a wide range of insects in agriculture, forestry, homes, horticulture, and public health around the world (*1*−*5*). Permethrin is very nontoxic to mammals, whereas it is highly toxic to some nontarget insects such as honeybees and other beneficial insects (*6*). This compound has apparent toxic effects to some aquatic species such as fish, aquatic insects, crayfish, and shrimp at parts per billion levels (*7*−*9*). Its high octanol−water partition coefficient suggests that it may have a tendency to bioaccumulate in living organisms (*10*). In the study on the neonatal effect of the exposure, Cantalamessa (*11*) suggested that permethrin is more acutely toxic to children than to adults. Because permethrin causes lung and liver tumors in mice, the U.S. Environmental Protection Agency has classified permethrin as a potential carcinogen (*12*). It also belongs to categories of endocrine disrupting compounds (*13*), priority pollutants (*14*, *15*), and environmental contaminants.

Permethrin is the most popular insecticide among the pyrethroid insecticides in the United States as the active ingredient in personal care products, such as shampoos and lotions for lice. Permethrin residues have been found in ground and surface waters throughout the United States. It is also detected in agricultural products, particularly spinach, tomatoes, celery, lettuce, and peaches, and peach and plum baby food (*16*). The most common route of exposure to pyrethroid insecticides for the general population is through drinking water and the ingestion of foods such as vegetables and fruits onto which the insecticide has been applied. Farmers, pesticide applicators, manufacturers, and military service personnel may also receive additional occupational exposure through dermal contact and

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inhalation. For humans, pyrethroid insecticides are much less toxic than many other insecticides. The ADI values (acceptable daily intake for man) range from 0.01 (deltamethrin) to 0.05 (permethrin and cypermethrin) mg/kg body weight per day (*17*, *18*). However, overexposure causes reversible symptoms such as headache, dizziness, nausea, irritation of the skin and nose, and paraesthesia (*19*, *20*).

In mammals, permethrin, like other pyrethroid insecticides, is rapidly metabolized by ester cleavage to its constituent acids (*cis*- and *trans*-DCCA) and alcohol (PBOH) and subsequent oxidation of the alcohol to PBA. There is little tendency for it to accumulate in tissues. These metabolites might be partially conjugated to glycine and/or glucuronic acid and then finally excreted in the urine (*21*−*23*), which is a common pathway for xenobiotics.

In humans, the major metabolic pathway is ester hydrolysis followed by conjugation. Ring hydroxylation, oxidation at the *gem*-dimethyl group, and other oxidation pathways result in minor metabolites (*24*). The major permethrin metabolites, *cis*- and *trans*-DCCA, and PBA were determined to serve as biomarkers in the urine of exposed people (*25*). While little is known about the conjugates of pyrethroid metabolites in humans, it has been well-established that glycine is the most common amino acid used in conjugation reactions with free carboxylic acids of xenobiotics (*26*). The glucuronide and glycine conjugates of DCCA in rat and mouse and the glucuronide, glycine, serine, glutamine, and glutamic acid conjugates of DCCA in cockroach, housefly, and cabbage looper have been found (*27*).

Analytical methods for detection of pyrethroid metabolites in biological samples such as urine and blood include some sample preparation steps such as acid hydrolysis, liquid−liquid or solid phase extraction, and derivatization with high-performance liquid chromatography or gas chromatography with mass spectrometry (*25*, *28*−*30*).

Although the instrumental methods are very sensitive for these metabolites, they are time-consuming and expensive and not suitable for a routine and rapid analysis. Immunoassay techniques are widely used in clinical diagnostics, environmental monitoring, food quality, agriculture, and field or on-site test of personnel. An example of the latter is deployed soldiers exposed to toxic chemicals. The assays are rapid, sensitive, and selective analytical tools to determine trace chemicals such as agrochemicals and their metabolites as key urinary biomarkers of exposure (*27*).

A sensitive ELISA with an IC_{50} value of 0.4 μg/L and a detection range of 0.04−10 μg/L in buffer was developed for the glycine conjugate of the pyrethroid metabolite PBA (PBA-glycine) (*32*). The limit of quantitation was 1 μg/L in real human urine.

It is likely that the relative amount of DCCA-glycine conjugate is higher than that of the PBA-glycine, because the PBOH that is directly hydrolyzed from the parent can enter several metabolic pathways including direct conversion to the glucuronide conjugate prior to further oxidation to PBA. Therefore, DCCA-glycine can exist in high abundance and then be a biomarker as an indicator of exposure to this insecticide and a good target analyte for biomonitoring in human urine.

The preparation of haptens as immunogens and coating antigens is one of the major stages in the development of immunoassays for small molecules (*31*, *33*). The synthesis of haptens can sometimes require extensive investment of time even before Ab production. Therefore, the hapten chemistry should be thoroughly considered in order to develop sensitive and selective immunoassays. In this study, the authors report the syntheses of *cis*/*trans*-haptens and their use for Ab production and ELISA development. Because the target analyte possesses separate *cis*- and *trans*-isomers of DCCA-glycine, optimal haptens would produce antibodies specific to each isomer of the target analyte as well as for use as coating antigens. Various haptens with different *cis*- or *trans*-configurations were synthesized and used to produce polyclonal antibodies in rabbits. The screening of the antibodies against the various coating antigens and characterization of the antibodies are reported.

MATERIALS AND METHODS

Chemicals. The *cis*- and *trans*-DCCA, as the starting materials for hapten syntheses, were prepared, according to published synthetic procedures (*34*−*36*). Organic materials for the synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was by ultraviolet light or iodine vapor stain. Column chromatographic separations were carried out using Baker silica gel (40 μm average particle size) using the indicated solvents where the → notation denotes a stepwise concentration gradient. The coupling reagents were purchased from Aldrich. BSA, Thyr, GAR-HRP as the second Ab, Tween 20, and 3,3′,5,5′-TMB were purchased from Sigma Chemical Co. (St. Louis, MO).

Instruments. ^{1}H nuclear magnetic resonance (NMR) spectra of compounds synthesized were obtained on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, ME) using tetramethylsilane as an internal standard. Electrospray mass spectra in the positive (MS-ESI$^+$) or negative (MS-ESI$^-$) mode were recorded by a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, United Kingdom). Melting points were determined on a Thomas-Hoover Uni-Melt apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. R_f values refer to TLC on silica gel 60 F254, precoated plates (Merck) with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor. ELISA was performed on 96 well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Menlo Park, CA) in the dual wavelength mode (450−650 nm).

Hapten Synthesis and Verification. The proposed target haptens are shown in **Figure 1**. Because the target DCCA-glycine is of a small molecular weight (MW 265) and requires conjugation to carrier proteins in order to be immunogenic, various haptens with the carboxylic group or amine group were synthesized. The reactions followed the procedures used in previous publications (*32*, *37*).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine (*cis*/*trans*-DCCA-glycine, *cis*/*trans*-Hapten 3; Figure 2).** Thionyl chloride ($SOCl_2$, 3 mL) was added to *cis*-DCCA (1500 mg, 7.17 mmol) in a 10 mL round bottomed flask. The mixture was stirred under N_2 at 65 °C for 1.5 h. The solution was concentrated under reduced pressure to remove excess $SOCl_2$. Hexane was added, and then the solution was concentrated again. The crude acid chloride, *cis*-**2**, was obtained as a pale yellow liquid. It was added dropwise to a vigorously stirred ice-cooled solution of glycine in 10 mL of 2 N KOH. A 1 N solution of KOH was added to keep the mixture slightly basic. After 3 h, the odor of the acid chloride was no longer detectable. The mixture was acidified with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was recrystallized from the mixture of ethyl acetate and *tert*-butyl chloride to give 1084 mg (56%) of *cis*-DCCA-glycine, *cis*-**3**, as a white solid; mp 141−143 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.85. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 1H, CH_3), 1.32 (s, 3H, CH_3), 1.85 (d, J = 8.5 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, C═C−CH), 3.78 (t, J = 5.8 Hz, 2H, NCH_2), 6.06 (d, J = 8.9 Hz, 1H, C═CH), 8.40 (t, J = 5.8 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 264.09.

The *trans*-isomer of compound **3** (1023 mg, 53%) was prepared as a white solid from the *trans*-DCCA by the same method as described

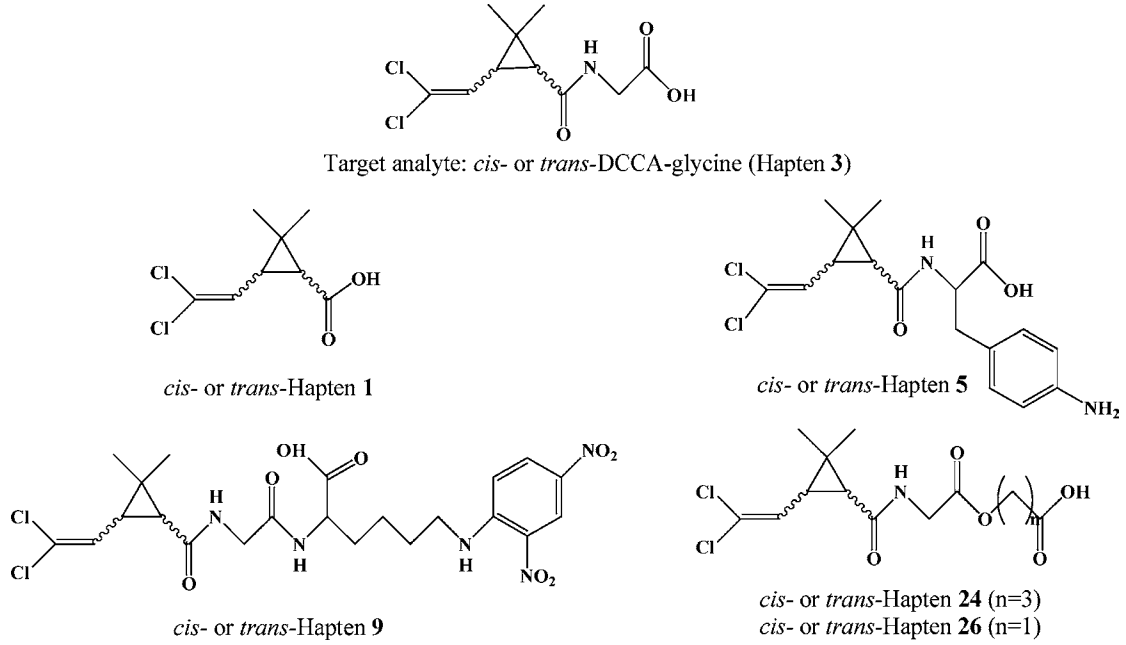

Figure 1. Target analyte and haptens designed.

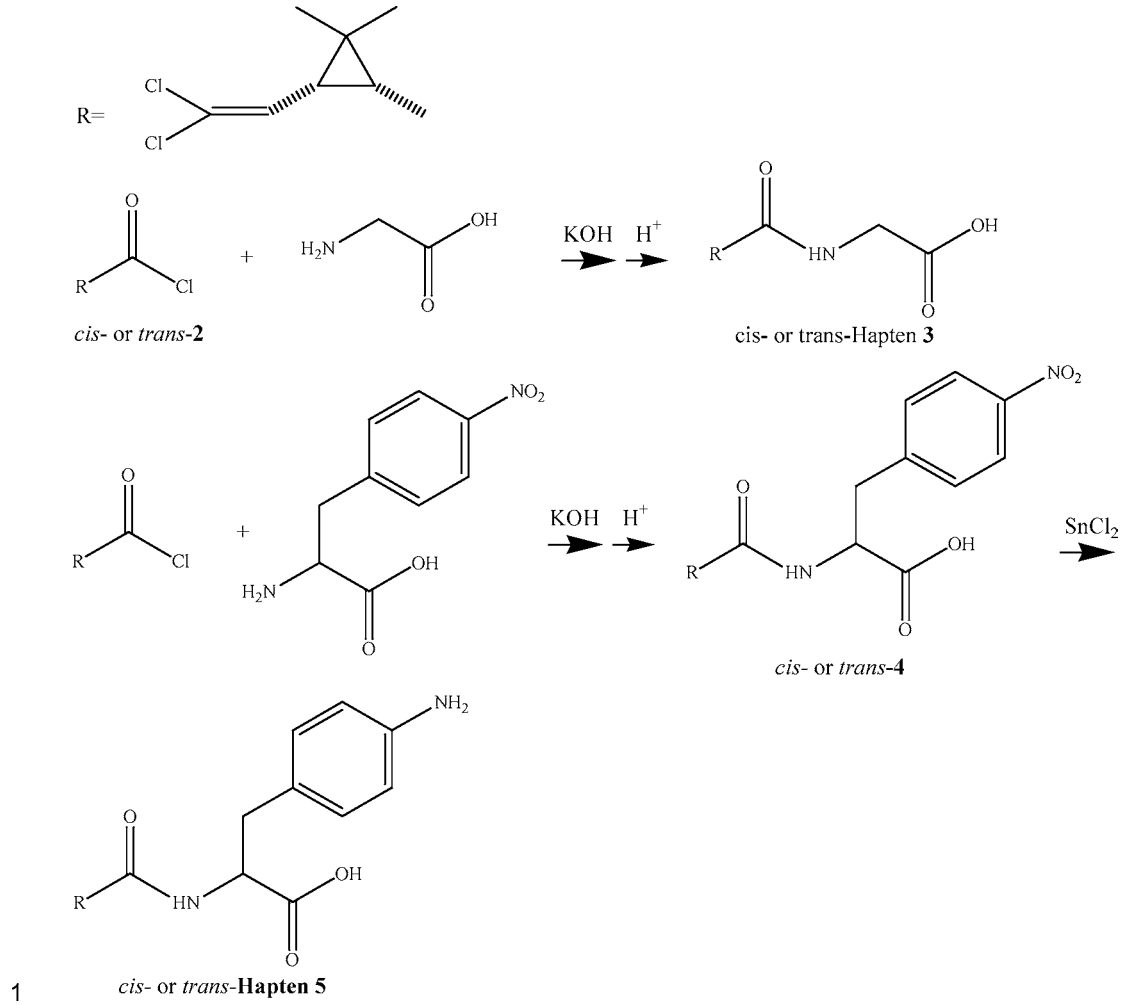

Figure 2. Scheme for the synthesis of the target analyte (*cis*/*trans*-DCCA-glycine, hapten **3**) and *cis*/*trans*-hapten **5**.

above for the *cis*-isomer of compound **3**; mp 178−179 °C. TLC (ethyl acetate/hexane/acetic acid [1:1:0.1, v/v/v)] R_f, 0.75. ^{1}H NMR (DMSO-d_6): δ 1.14 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.83 (d, J = 5.3 Hz, 1H, CHCO), 2.04 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C=C−CH), 3.78 (t, 5.6 Hz, 2H, NCH_2), 6.04 (d, J = 8.3 Hz, 1H, C=CH), 8.36 (t, J = 5.8 Hz, 1H, NH). MS-ESI$^-$ *m*/*z* calcd for $[M - H]^- = C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 263.95.

***cis*/*trans*-Compound 4 (Figure 2).** Crude acid chloride, *cis*-**2** (0.06 mmol), was added dropwise to a vigorously stirred ice-cooled solution of 4-nitro-L-phenylalanine (139 mg, 0.66 mmol) in 10 mL of 2 N KOH.

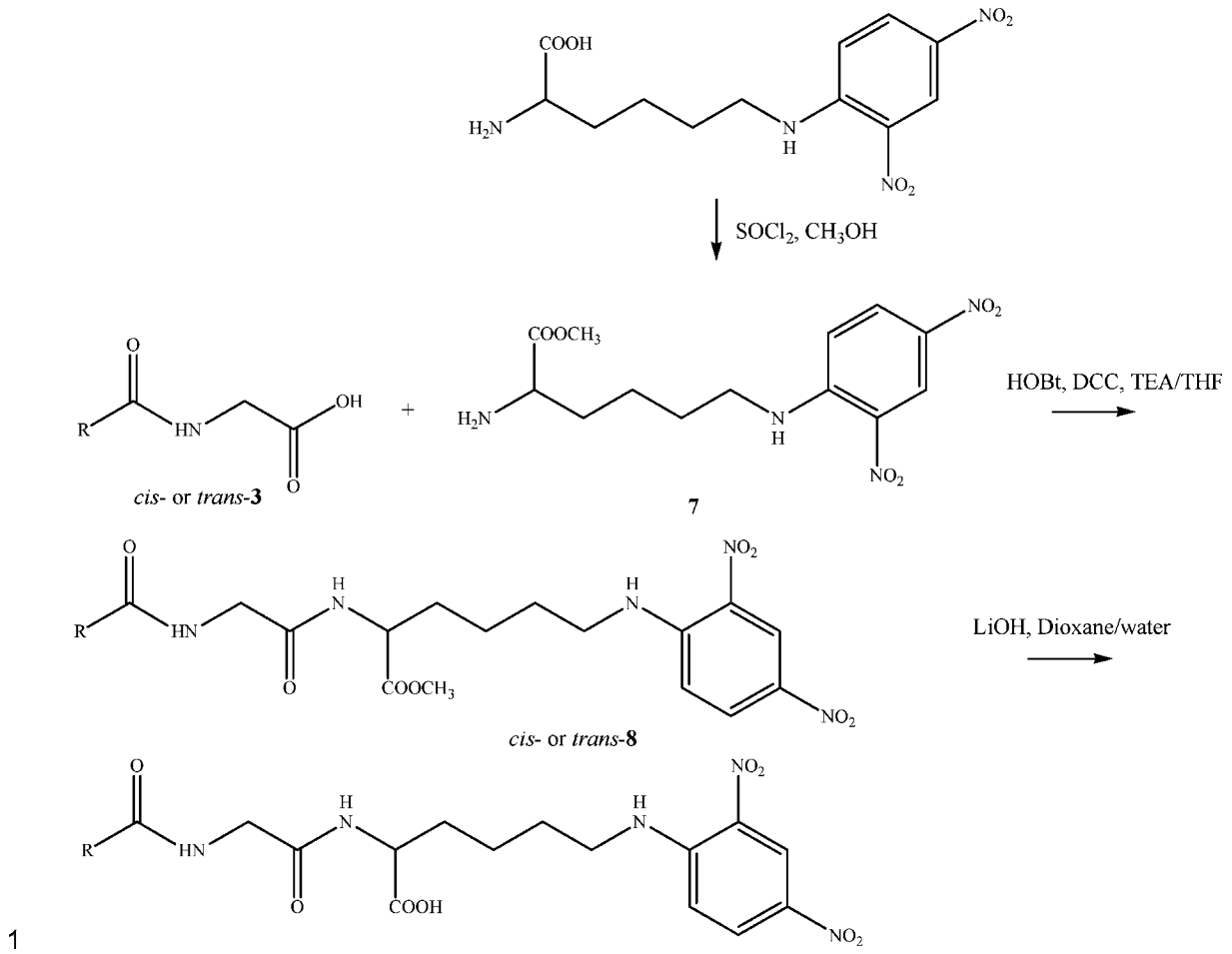

Figure 3. Scheme for the synthesis of *cis*/*trans*-hapten **9**.

Over the course of 30 min, 1 N KOH was added to keep the mixture slightly basic. After 3 h, the odor of the acid chloride was no longer detectable. The mixture was acidified with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by using preparative TLC on silica gel with the mixture of ethyl acetate/hexane/acetic acid (1:1:0.01, v/v/v) as an eluent to give 180 mg of *cis*-compound **4** as a yellow oil. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.19. ^{1}H NMR ($CDCl_3$): δ 1.22 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.63 (d, J = 8.5 Hz, 1H, CHCO), 2.27 (dd, J = 8.7, 8.8 Hz, 1H, C═C−CH), 3.22−3.26 (m, 2H, NCHCH_2Ar), 4.95 (dt, J = 7.1, 5.8 Hz, 1H, NCHCO), 5.63 (d, J = 8.9 Hz, 1H, C═CH), 7.26−7.40 (m, 4H, Ar), 8.20 (d, J = 7.1 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{17}H_{18}Cl_2N_2O_5$, 399.06; observed, 399.24.

The *trans*-isomer of compound **4** (150 mg) was prepared as a yellow oil from the *trans*-acid chloride by the same method as described above for the *cis*-isomer of compound **4**. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.20. ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.63 (d, J = 5.4 Hz, 1H, CHCO), 2.26 (dd, J = 5.4, 8.4 Hz, 1H, C═C−CH), 3.21−3.25 (m, 2H, NCHCH_2Ar), 4.91 (dt, J = 7.3, 5.5 Hz, 1H, NCHCO), 5.63 (d, J = 8.4 Hz, 1H, C═CH), 7.28−7.42 (m, 4H, Ar), 8.23 (d, J = 7.2 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{17}H_{18}Cl_2N_2O_5$, 399.06; observed, 399.24.

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (*cis*/*trans*-Hapten 5; Figure 2).** The mixture of *cis*-compound **4** (261 mg, 0.65 mmol) and stannous chloride dihydrate (733 mg, 3.25 mmol) in 2 mL of ethanol was stirred at 70 °C in an oil bath for 30 min. The mixture was cooled and poured into the slurry of water, ethyl acetate, and Celite. $NaHCO_3$ was added in portions. The neutral mixture was filtered, and the solids on the filter and in the flask were washed with water and ethyl acetate. The ethyl acetate phase was separated and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The oil residue was purified by using preparative TLC on silica gel with the mixture of methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v) as an eluent to give 112 mg (47%) of *cis*-hapten **5** as a yellow solid; mp 93−97 °C. TLC [methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v)] R_f, 0.59. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.86 (d, J = 8.6 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 3.32−3.48 (m, 2H, NCHCH_2Ar), 4.28 (dt, J = 7.0, 5.6 Hz, 1H, NCHCO), 6.05 (d, J = 8.8 Hz, 1H, C═CH), 6.99−8.13 (m, 4H, Ar), 8.40 (d, J = 7.1 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{17}H_{20}Cl_2N_2O_3$, 369.09; observed, 369.10.

By the same method as described above for the *cis*-isomer of hapten **5**, 98 mg (41%) of *trans*-hapten **5** was prepared as a yellow solid from the *trans*-compound **4**; mp 102−105 °C. TLC [methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v)] R_f, 0.59. ^{1}H NMR (DMSO-d_6): δ 1.20 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.75 (d, J = 5.0 Hz, 1H, CHCO), 2.11 (dd, J = 5.5, 8.4 Hz, 1H, C═C−CH), 3.34−3.50 (m, 2H, NCHCH_2Ar), 4.52 (dt, J = 7.3, 5.5 Hz, 1H, NCHCO), 6.07 (d, J = 8.5 Hz, C═CH), 6.99−8.13 (m, 4H, Ar), 8.23 (d, J = 7.2 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{17}H_{20}Cl_2N_2O_3$, 369.09; observed, 369.04.

***cis*/*trans*-Compound 8 (Figure 3).** Compound **7** as a yellow solid was synthesized according to Watanabe et al. (*37*). TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.63.

In a 25 mL flask, *cis*-DCCA-glycine (79.84 mg, 0.3 mmol), 1-hydroxy-1H-benzotriazole monohydrate (81.08 mg, 0.2 mmol), and compound **7** (97.89 mg, 0.3 mmol) were dissolved in 2 mL of dry tetrahydrofuran (THF), and then, 41.8 μL of triethylamine (0.3 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (68.09 mg, 0.33 mmol) dissolved in 500 μL of dry 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 and then was filtered to remove the dicyclohexylurea. Ethyl acetate (50 mL) was added and was washed with saturated sodium bicarbonate

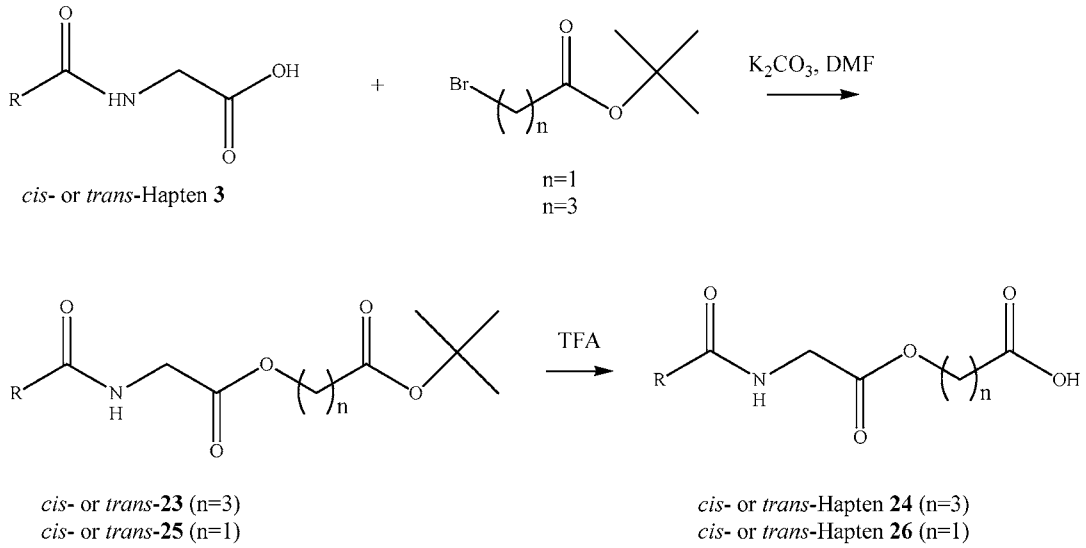

Figure 4. Scheme for the synthesis of *cis*/*trans*-haptens **24** and **26**.

solution, saturated ammonium chloride solution, saturated sodium bicarbonate solution, and water (50 mL of each). After the organic phase was dried over anhydrous sodium sulfate, ethyl acetate was removed under reduced pressure. The resultant residue was recrystallized from ether to give 140 mg of *cis*-compound **8** as a yellow solid. TLC [methanol/methylene chloride (0.5:9.5, v/v)] R_f, 0.60. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.51−1.96 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.27 (dd, J = 8.6 Hz, 8.7 Hz, 1H, C═C−CH), 3.80 (s, 3H, OCH_3), 4.01 (t, 5.8 Hz, 2H, NCH_2CO), 4.08−4.18 (m, 1H, CHCO), 4.65 (q, J = 14.7 Hz, 2H, CH_2CH), 5.64 (d, J = 8.9 Hz, 1H, C═CH), 6.82 (t, J = 5.8 Hz, 1H, CONHCH$_2$), 6.92−8.58 (m, 3H, Ar). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{23}H_{29}Cl_2N_5O_8$, 574.14; observed, 574.12.

By the same method as described above for the *cis*-isomer of compound **8**, 158 mg of the *trans*-isomer of compound **8** was prepared as a yellow solid from the *trans*-DCCA-glycine, *trans*-**3**. TLC [methanol/methylene chloride (0.5:9.5, v/v)] R_f, 0.60. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.51−1.94 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.25 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C═C−CH), 3.78 (s, 3H, OCH_3), 4.05 (t, 5.7 Hz, 2H, NCH_2CO), 4.08−4.18 (m, 1H, CHCO), 4.64 (q, J = 14.5 Hz, 2H, CH_2CH), 5.63 (d, J = 8.3 Hz, 1H, C═CH), 6.80 (t, J = 5.7 Hz, 1H, CONHCH$_2$), 6.91−8.57 (m, 3H, Ar). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{23}H_{29}Cl_2N_5O_8$, 574.14; observed, 574.12.

***N*-(*N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)amino-6-(2,4-dinitrophenyl)aminohexanoic Acid (*cis*/*trans*-Hapten 9; Figure 3).** The hydrolysis reaction of *cis*-compound **8** (100 mg, 0.17 mmol) was conducted in 10 mL of 1,4-dioxane/water (1:1, v/v) with 10 equiv of lithium hydroxide monohydrate. After 4.5 h, the resulting mixture was acidified to pH 4 with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was washed with distilled water (30 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was purified by using preparative TLC on silica gel with the mixture of methanol/methylene chloride (1:9, v/v) as an eluent to give 80 mg (84%) of *cis*-hapten-**9** as a yellow solid; mp 50−55 °C. TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.59. ^{1}H NMR ($CDCl_3$): δ 1.18 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.53−1.95 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.25 (dd, J = 8.6 Hz, 8.7 Hz, 1H, C═C−CH), 3.97 (t, 5.8 Hz, 2H, NCH_2CO), 4.08−4.15 (m, 1H, CHCO), 4.63 (q, J = 14.7 Hz, 2H, CH_2CH), 5.63 (d, J = 8.9 Hz, 1H, C═CH), 6.78 (t, J = 5.8 Hz, 1H, CONHCH$_2$), 6.90−8.55 (m, 3H, Ar). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{22}H_{27}Cl_2N_5O_8$, 558.12; observed, 558.29.

By the same method as described above for the *cis*-isomer of hapten **9**, 75 mg (79%) of *trans*-hapten **9** was prepared as a yellow solid from the *trans*-isomer of compound **8**; mp 65−70 °C. TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.59. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.50−1.91 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.23 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C═C−CH), 4.01 (t, 5.7 Hz, 2H, NCH_2CO), 4.03−4.16 (m, 1H, CHCO), 4.61 (q, J = 14.5 Hz, 2H, CH_2CH), 5.61 (d, J = 8.3 Hz, 1H, C═CH), 6.83 (t, J = 5.7 Hz, 1H, CONHCH$_2$), 6.93−8.55 (m, 3H, Ar). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{22}H_{27}Cl_2N_5O_8$, 558.12; observed, 558.35.

***cis*/*trans*-Compound 23 (Figure 4).** The mixture of the *cis*-DCCA-glycine, *cis*-**3** (150 mg, 0.56 mmol), 4-bromo-butyric acid *tert*-butyl ester (249.88 mg, 1.12 mmol), and potassium carbonate (111.63 mg, 0.616 mmol) in 1 mL of anhydrous DMF was reacted at 100 °C for 3 h. The resulting mixture was filtered to remove excess K_2CO_3 and HBr produced in the reaction. The filtrate diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to obtain 179 mg of *cis*-compound **23** as a transparent oil. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.67. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.45 (s, 9H, $3CH_3$), 1.50 (d, J = 8.5 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 1.90−2.05 (m, 2H, $CH_2CH_2CH_2$), 2.31 (t, 2H, $CH_2CH_2CH_2$), 4.03 (t, 2H, C$H_2CH_2CH_2$), 4.20 (d, J = 5.7 Hz, 2H, NCH_2COO), 6.01 (bs, 1H, NH), 6.41 (d, J = 8.8 Hz, 1H, C═CH).

By the same method as described above for the *cis*-isomer of compound **23**, the *trans*-isomer of compound **23** (179 mg) was prepared as a transparent oil from the *trans*-isomer of DCCA-glycine, *trans*-**3**. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.64. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.44 (s, 9H, $3CH_3$), 1.61 (d, J = 5.4 Hz, 1H, CHCO), 2.05 (dd, J = 5.4, 8.7 Hz, 1H, C═C−CH), 1.92−2.07 (m, 2H, $CH_2CH_2CH_2$), 2.35 (t, 2H, $CH_2CH_2CH_2$), 4.10 (t, 2H, C$H_2CH_2CH_2$), 4.18 (d, J = 5.7 Hz, 2H, NCH_2COO), 6.10 (bs, 1H, NH), 6.42 (d, J = 8.3 Hz, 1H, C═CH).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic Acid (*cis*/*trans*-Hapten 24; Figure 4).** Trifluoroacetic acid (TFA) (0.5 mL) was added to the *cis*-ester **23**, and the mixture was allowed to stand at ambient temperature for 15 min. The TFA was stripped off under vacuum, and ethyl acetate was added twice and stripped to remove residual TFA. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:1, v/v). Fractions containing pure product by TLC were stripped under high vacuum, and the residue was recrystallized from ethyl acetate and *tert*-butyl chloride to give 37 mg (19%) of *cis*-hapten-**24** as a white solid; mp 85−88 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.55. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3),

1.27 (s, 3H, CH_3), 1.71 (d, J = 8.5 Hz, 1H, CHCO), 1.96−2.02 (m, 3H, C═C−CH and $CH_2CH_2CH_2$), 2.40 (t, 2H, $CH_2CH_2CH_2$), 3.99 (t, 2H, $CH_2CH_2CH_2$), 4.23 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.72 (bs, 1H, NH), 6.38 (d, J = 8.8 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{14}H_{18}Cl_2NO_5$, 352.06; observed, 352.09.

By the same method as described above for the *cis*-isomer of hapten **24**, 150 mg (76%) of *trans*-hapten **24** from the *trans*-isomer of ester **23** was prepared as a white solid; mp 103−104 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.55. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.52 (d, J = 5.4 Hz, 1H, CHCO), 2.09−2.17 (m, 3H, C═C−CH and $CH_2CH_2CH_2$), 2.57 (t, 2H, $CH_2CH_2CH_2$), 4.12 (t, 2H, $CH_2CH_2CH_2$), 4.30 (d, J = 5.7 Hz, 2H, NCH_2COO), 5.70 (d, J = 8.8 Hz, 1H, C═CH), 6.21 (bs, 1H, NH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{14}H_{18}Cl_2NO_5$, 352.06; observed, 352.19.

***cis*/*trans*-Compound 25 (Figure 4).** The mixture of *cis*-DCCA-glycine, *cis*-**3** (100 mg, 0.38 mmol), *tert*-butyl bromoacetate (148.25 mg, 0.76 mmol), and potassium carbonate (76.11 mg, 0.418 mmol) in 1 mL of anhydrous DMF was reacted at 100 °C for 3 h. The resulting mixture was further prepared by the same method as described above for hapten **23**. The residue was chromatographed on silica gel eluting with the ethyl acetate/hexane (1:3, v/v) mixture. The fractions containing pure product identified by TLC were stripped under high vacuum. After toluene was added, the solvent was removed under reduced pressure to give 126 mg of *cis*-compound **25** as a white solid. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.64. ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.48 (s, 9H, $3CH_3$), 1.61 (d, J = 8.5 Hz, 1H, CHCO), 2.02 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 4.16 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.58 (s, 2H, $COOCH_2COO$), 6.07 (bs, 1H, NH), 6.40 (d, J = 8.7 Hz, 1H, C═CH).

By the same method as described above for the *cis*-isomer of compound **25**, 138 mg of the *trans*-isomer of compound **25** was prepared as a white solid from the *trans*-DCCA-glycine. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.53. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.50 (s, 9H, $3CH_3$), 1.53 (d, J = 5.4 Hz, 1H, CHCO), 2.03 (dd, J = 5.3, 8.3 Hz, 1H, C═C−CH), 4.12 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.70 (s, 2H, $COOCH_2COO$), 6.23 (bs, 1H, NH), 6.41 (d, J = 8.7 Hz, 1H, C═CH).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-oxoacetic Acid (*cis*/*trans*-Hapten 26; Figure 4).** TFA (0.5 mL) was added to the *cis*-ester **25**, and the mixture was allowed to stand at ambient temperature for 15 min. By the same method as described above for hapten **24**, the mixture was further prepared, chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:3 → 1:1, v/v), and recrystallized to give 65 mg (53%) of *cis*-hapten **26** as a white solid; mp 120−123 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.38. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.61 (d, J = 8.5 Hz, 1H, CHCO), 2.04 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 4.18 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.58 (s, 2H, $COOCH_2COO$), 6.17 (bs, 1H, NH), 6.42 (d, J = 8.7 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{12}H_{15}Cl_2NO_5$, 324.03; observed, 324.03.

By the same method as described above for *cis*-hapten **26**, 75 mg (61%) of the *trans*-isomer of hapten **26** was prepared as a white solid from the *trans*-compound **25**; mp 123−125 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.15. ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.43 (d, J = 5.4 Hz, 1H, CHCO), 2.13 (dd, J = 5.3, 8.3 Hz, 1H, C═C−CH), 4.13 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.77 (s, 2H, $COOCH_2COO$), 6.25 (bs, 1H, NH), 6.38 (d, J = 8.7 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{12}H_{15}Cl_2NO_5$, 324.03; observed, 324.03.

Hapten Conjugation. Hapten−protein conjugates were synthesized using the water soluble carbodiimide method (*38*) for haptens with a carboxylic acid and the diazotization method (*39*) for haptens with an amine group. For immunogens, *cis/trans*-DCCA-glycine (*cis/trans*-hapten **3**), *cis/trans*-hapten **5**, *cis/trans*-hapten **9**, and *cis/trans*-hapten **24** were conjugated to Thyr. For coating antigens, *cis/trans*-DCCA and *cis/trans*-hapten **26** as well as the above haptens were conjugated to BSA.

*Diazotization Method for Conjugation of cis/trans-Hapten **5** with Amine Group to Proteins. cis/trans*-Hapten **5** (0.10 mmol) was dissolved in 4 drops of ethanol and treated with 1 mL of 1 N HCl. The resulting solution was stirred in an ice bath as 0.5 mL of 0.20 M sodium nitrite was added. DMF (0.4 mL) was then added dropwise to give a homogeneous solution, which was divided into two equal aliquots. Twenty-five milligrams of Thyr or BSA was dissolved in 5.5 mL of 0.05 M borate buffer (pH 9.6) and 1.0 mL of DMF. Aliquots of the activated hapten solution were added dropwise to the two stirred protein solutions. The reaction mixture was stirred at 4 °C overnight, and the resulting yellow conjugates were purified by exhaustive dialysis in normal strength phosphate-buffered saline (1 × PBS), which was changed for a new buffer twice a day for 4 days. Finally, the conjugates were dispensed into the 2 mL cryogenic vials and stored at −80 °C.

NHS Method for Conjugation of Haptens with Carboxylic Acid to Proteins. cis/trans-DCCA-glycine and *cis/trans*-haptens **9**, **24**, and **26** (0.04 mmol) were dissolved in 1 mL of dry DMF with sulfo-NHS (0.06 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.05 mmol). After the mixture was stirred at room temperature overnight, the activated hapten was added slowly to the protein solution (25 mg of protein in 6 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was purified as described above.

Immunization and Antiserum Preparation. The immunization was made by following the protocol reported previously (*40*, *41*). Two female New Zealand white rabbits were immunized for each isomer of immunogen (rabbits 3700 and 3701 for *cis*-hapten **3**−Thyr, rabbits 3702 and 3703 for *trans*-hapten **3**−Thyr, rabbits 3704 and 3705 for *cis*-hapten **5**−Thyr, rabbits 3706 and 3707 for *trans*-hapten **5**−Thyr, rabbits 3708 and 3709 for *cis*-hapten **9**−Thyr, rabbits 3710 and 3711 for *trans*-hapten **9**−Thyr, rabbits 369 and 3696 for *cis*-hapten **24**−Thyr, and rabbits 3697 and 3698 for *trans*-hapten **24**−Thyr). Each immunogen (100 μg) in 0.5 mL of 0.85% saline was emulsified with an equal volume of Freund's complete adjuvant, and then, the emulsion was injected subcutaneously. After 3 weeks, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1, v/v). The boosts were given every 3 weeks, and blood samples were drawn 7 days after each boost to check the titer of antibodies. The final serum was collected 5 months following the first immunization. The blood was collected into the Vacutainer tube with a serum separation gel. The antiserum was obtained by centrifugation and stored at −80 °C. The antiserum was used without any purification.

ELISA. *Buffer Solutions.* Normal strength PBS [1 × PBS; 8 g/L of sodium chloride (NaCl), 0.2 g/L of potassium phosphate, monobasic (KH_2PO_4), 1.2 g/L of sodium phosphate dibasic anhydrous (Na_2HPO_4), and 0.2 g/L of potassium chloride (KCl), pH 7.5], PBST [1 × PBS containing 0.05% (v/v) Tween 20, pH 7.5], 0.1 × PBST (PBS diluted 1:10 with distilled water and containing 0.05% Tween 20), carbonate buffer [1.59 g/L sodium carbonate (Na_2CO_3), 2.93 g/L sodium hydrogen carbonate ($NaHCO_3$), pH 9.6], 0.05 M borate buffer (19.1 g/L $Na_2B_4O_7 \cdot 10H_2O$, pH 8), and 0.05 M acetate buffer (14.71 g/L $Na_3C_6H_5O_7 \cdot 2H_2O$) were used for immunoassay.

ELISA. Indirect ELISA and competitive indirect ELISA were performed according to the method of Voller et al. (*42*). The 96 well microtiter plates were coated overnight at 4 °C with 100 μL/well of the appropriate coating antigen concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After it was washed five times with PBST, the plate was incubated with 200 μL/well of a 0.5% BSA solution in PBS for 1 h at room temperature. After another washing step, 100 μL/well of antiserum diluted in PBST per well (for titration experiment) or 50 μL/well of antiserum diluted in PBST and 50 μL/well of standard analyte and sample solution were added, mixed for 30 s in the reader, and incubated for 1 h at room temperature. The standard analyte concentrations ranged from 0.003 μg/L to 5 mg/L. After the plate was washed, 100 μL/well of the secondary Ab GAR-HRP (1:5000 in PBST) was added and incubated for 1 h at room temperature. The plate was washed, and 100 μL/well of a substrate solution (0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% 3,3′,5,5′-TMB in DMSO added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL/well of 2 N sulfuric acid. The absorbance was measured using a dual wavelength mode at 450 minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte

concentration, which were fitted to a four parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$ where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is the IC_{50}, and D is the minimum absorbance at infinite concentration. The IC_{50} value was expressed as the sensitivity of the immunoassay.

Molecular Modeling. Molecular modeling was done using the CS Chemoffice 6.0 software package (CambridgeSoft Corporation, Cambridge, MA). The geometries optimized of the *cis*-target analyte and *cis*-haptens at their minimum energy levels with the minimum RMS gradient of 0.1 were calculated by semiempirical quantum mechanics MNDO model (*43*) with a wave function of close shell (restricted). The best immunizing and coating haptens on the target analyte are evaluated with both real data (**Table 2**) and modeling.

Cross-Reactivity (CR). The CR studies were evaluated by using the standard solution of the permethrin metabolites and other structurally related compounds. The test compounds are listed in **Table 3**. The CR was calculated as (IC_{50} of the target analyte/IC_{50} of the tested compound) $\times$ 100.

RESULTS AND DISCUSSION

Hapten Design and Synthesis. Possessing two chiral centers in the cyclopropane ring of the permethrin molecule, permethrin has a mixture of four different optical and geometrical isomers. It is mainly classified into two pairs of isomers with different geometries, referred to as the *cis*- and the *trans*-isomers. The target analyte, *cis*/*trans*-DCCA-glycine, in itself cannot be used as an immunogen because of its low molecular weight (MW 265.1). Therefore, haptens mimicking the target analyte and containing reactive groups such as a −COOH or $-NH_2$ group for conjugation to carrier proteins were synthesized to develop an immunoassay. One of the strategies for designing the immunizing hapten was to link the spacer through the −COOH group of the target molecule and leave the two Cl atoms and the amide bond (−RCONH−) and carbonyl group (−RCO−) in the molecule to improve Ab specificity. The target analyte (DCCA-glycine) molecule also possesses a cyclopropane ring that is also divided into the *cis*- and *trans*-isomers such as the parent permethrin. As another strategy, the haptens were designed so that each individual *cis*- and *trans*-isomer was obtained. This is both for the production of Ab specific to each isomer of the target and for the enhancement of sensitivity with heterologous *cis*/*trans* or *trans*/*cis* configurational immunoassay format that for example uses the *cis*-isomer for coating antigen with Ab raised against the *trans*-isomer of the immunogen.

For the production of a specific Ab capable of recognizing *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine, four different types of haptens as immunizing antigens were prepared. The target analyte (*cis*/*trans*-DCCA-glycine) with the carboxylic acid also served as *cis*/*trans*-hapten **3** with no spacer. It was synthesized via the Schotten−Boumann reaction with a synthetic procedure similar to that used for the PBA-glycine conjugate (*32*) (**Figure 2**). That is, the acid chloride of DCCA was reacted with glycine in alkaline solution and then acidified to obtain *cis*/*trans*-DCCA-glycine. *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (hapten **5**) was synthesized to preserve the carboxylic acid group of the target acid. Briefly, the acid chloride of *cis*/*trans*-DCCA was also reacted via the Schotten−Boumann reaction with 4-nitro-L-phenylalanine to give the *N*-acylated nitro intermediate **4**. Subsequent reduction of the nitro group with stannous chloride gave hapten **5**. To get high titer of Ab more rapidly (*37*), *N*-(*N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)amino-6-(2,4-dinitrophenyl)-aminohexanoic acid (hapten **9**) was synthesized by coupling *N*ϵ-2,4-dinitrophenyl-L-lysine to the target analyte according to the synthetic method by Watanabe et al. (*37*) (**Figure 3**). Finally, as a common modification of the acid moiety on the target, *cis*/*trans*-DCCA-glycine was reacted with bromo-butyric acid *tert*-butyl ester to give ester intermediate **23**. Subsequent alkaline hydrolysis with lithium hydroxide and acidification gave the four carbon spacer linker of *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic acid (hapten **24**) (**Figure 4**).

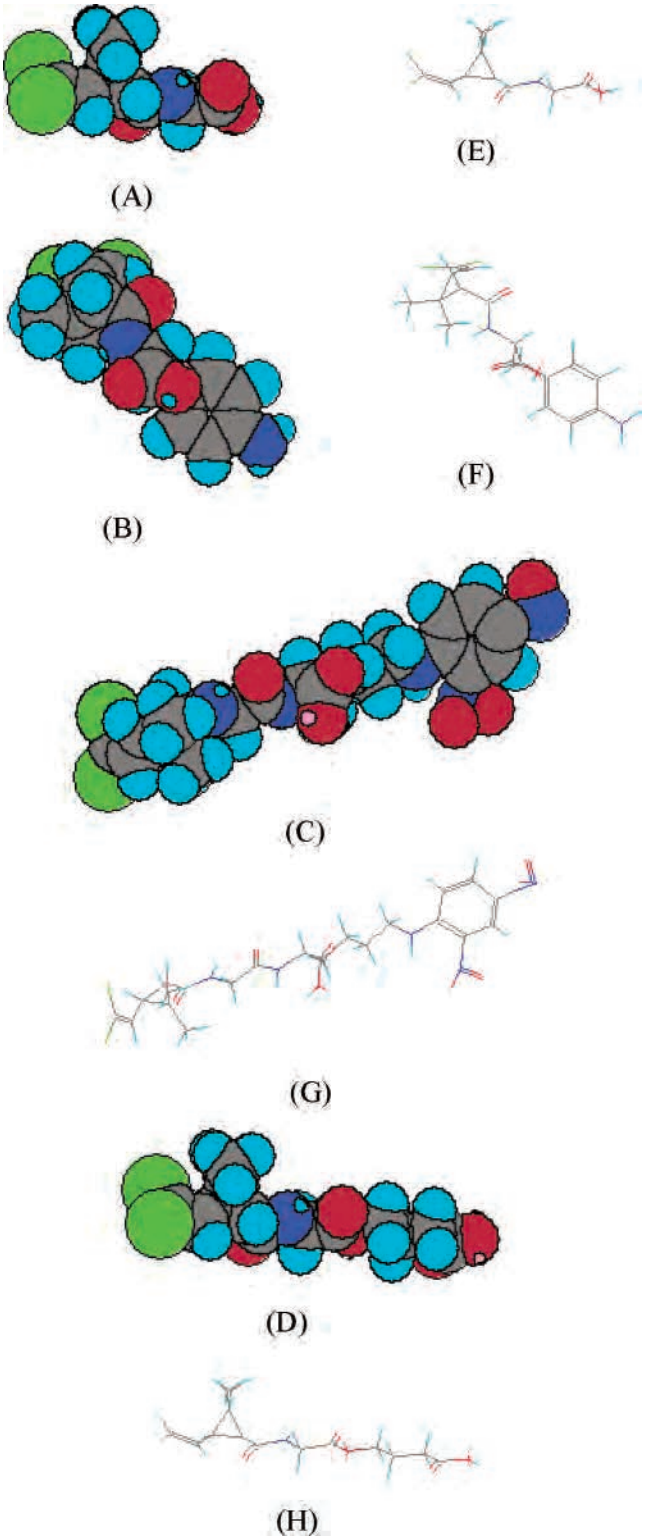

Figure 5. Optimized molecular model of (**A**,**E**) DCCA-glycine, (**B**,**F**) hapten **5**, (**C**,**G**) hapten **9**, and (**D**,**H**) hapten **24**; **A**–**D**, space filling models, and **E**–**H**, stick models.

Table 1. Antiserum Titer Response of Rabbits against Various Coating Antigens[a]

		coating antigen											
immunogen	antiserum	*cis*-**1**–BSA	*trans*-**1**–BSA	*cis*-**3**–BSA	*trans*-**3**–BSA	*cis*-**5**–BSA	*trans*-**5**–BSA	*cis*-**9**–BSA	*trans*-**9**–BSA	*cis*-**24**–BSA	*trans*-**24**–BSA	*cis*-**26**–BSA	*trans*-**26**–BSA
cis-hapten **3**–Thyr	3700	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3701	+++	+++	+++[b]	+++	+++	++	+++	+++	+++	+++	+++	+++
trans-hapten **3**–Thyr	3702	+++	+++	+++	+++[b]	++	+++	+++	+++	+++	+++	+++	+++
	3703	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++
cis-hapten **5**–Thyr	3704	++	++	++	++	+++[b]	+++	++	++	+++	+++	+++	+++
	3705	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++
trans-hapten **5**–Thyr	3706	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++
	3707	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++
cis-hapten **9**–Thyr	3708	+	++	+++	+++	–	–	+++[b]	+++	+++	+	+	+++
	3709	++	+++	+++	+++	+	–	+++[b]	+++	+++	++	+++	+++
trans-hapten **9**–Thyr	3710	–	++	+	+	–	–	+++	+++[b]	++	+++	+	+++
	3711	+	++	++	+++	–	+	+++	+++[b]	+	++	+	+++
cis-hapten **24**–Thyr	3696	+++	+++	+++	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++
	369	+++	+++	+++	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++
trans-hapten **24**–Thyr	3698	++	+++	+++	+++	–	+	++	+++	+++	+++[b]	+++	+++
	3699	+++	+++	+++	+++	–	++	+++	+++	+++	+++[b]	+++	+++

[a] The data shown are at a coating antigen concentration of 1 μg/mL and an Ab dilution of 1:32 000; –, absorbance < 0.3; +, absorbance 0.3–0.6; ++, absorbance 0.6–0.9; and +++, absorbance > 0.9. [b] Homologous systems.

Additionally *cis*/*trans*-hapten **26** with a carboxylic acid was synthesized according to the same method as hapten **24**. *cis*/*trans*-DCCA-glycine was reacted with bromo-acetic acid *tert*-butyl ester to give ester intermediate **25**. The alkaline hydrolysis and acidification gave *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-2-oxoacetic acid (hapten **26**) with a shorter two carbon space linker to serve as a coating antigen.

Molecular Modeling of Haptens. Although the planar chemical structures of haptens are shown to completely mimic the target analyte, their geometries can be different. The geometry study of the molecules is useful to select an immunizing hapten to produce Ab against the target analyte (*44*−*46*). The immunizing haptens synthesized were characterized by computing the molecules at their minimum energy levels. Observing the molecular models (**Figure 5**), the geometries of hapten **24** and the target analyte were completely matched, and the geometry of hapten **9** showed a small difference in the cyclopropane ring moiety of the molecules of the target and hapten **9**. On the contrary, that of hapten **5** did not exhibit any similar geometry to the model of the target analyte.

Hapten Conjugation. For immunogens, haptens with a carboxylic acid group were conjugated to Thyr by the sulfo-NHS ester method and with an amino group by the diazotization method. The sulfo-NHS conjugation method was used to obtain the more stable sulfo-NHS ester intermediate as compared to an NHS ester and thus more increased coupling yield (*47*). In this study, the carrier protein Thyr was chosen as an immunizing protein (*48*). For coating antigens, haptens were conjugated to BSA by the NHS ester and diazotization methods. This avoids recognition of protein−urea adducts that form with the use of water soluble diimides. *trans*-Hapten **9**−protein conjugates were confirmed by the UV/vis spectrophotometry. *trans*-Hapten **9** showed a characteristic peak at 360 nm distinguishing it from that of each protein. The hapten−protein conjugates also showed a peak at 360 nm. These results indicate that hapten was successfully conjugated to the protein by the sulfo-NHS method. In addition, the yellow color of the hapten **5**− and **9**−protein conjugates was an indicator of a successful coupling reaction, whereas haptens **1**, **3**, and **24** as white solids did not show any characteristic peak distinguished from that of protein. From the available amino groups according to the TNBS method of Habeeb (*49*), 20−61% of the *cis*/*trans*-haptens **1**, **3**, and **24** were conjugated in the hapten−Thyr or −BSA conjugates, suggesting successful hapten loading on the proteins.

Titers and Screening of the Antisera. For the production of Ab specific to the *cis*/*trans*-target analyte, the *cis*/*trans*-hapten−Thyr conjugates were injected a total of seven times into each rabbit. The antisera collected after each boost were subjected to titration by the homologous indirect ELISA. All of the antisera showed relatively constant high titers after the fifth immunization (data not shown). These results indicate that specific antibodies in the rabbit antiserum were produced against each immunogen. The final serum was collected 5 months following the first immunization and was used for the subsequent screening in search of antibodies specific to the target compound. Despite showing high titers of antisera after the fifth immunization, rabbits were boosted twice more to raise more sensitive and specific antibodies. None of the antisera demonstrated any significant affinity for BSA itself as a coating antigen.

The checkerboard titration was used for the screening of Ab and antigen combinations. All antisera had high titers in homologous systems, but in the heterologous systems, antisera produced against hapten **9**−Thyr and *trans*-hapten **24**−Thyr conjugates showed especially low titers (absorbance < 0.3) against the coating antigens, hapten **5**−BSA and *cis*-hapten **5**−BSA, respectively (**Table 1**).

All of the 16 antisera were screened against 12 coating antigens via the simple inhibition test at the two concentrations (10 and 1000 μg/L) of each individual *cis*- or *trans*-target analyte. There was no or low inhibition in homologous systems, whereas there were much higher inhibitions in heterologous systems (data not shown). The combinations of Ab and coating antigen with over 80% inhibition at the concentration of 1000 μg/L of the target analyte and with over 20% inhibition at the concentration of 10 μg/L were again screened at the 10 different concentrations of each target analyte.

As shown in **Table 2**, none of the antibodies generated against hapten **5**−Thyr had IC_{50} values below 3 μg/L for either the *trans*- or the *cis*-target analyte. The antisera specific to the target *trans*-DCCA-glycine were mainly raised against *trans*-haptens **3**−, **9**−, and **24**−Thyr except for *trans*-hapten **5**−Thyr. This result suggests that because the spacer arm of hapten **5** possessed a bulky aminophenyl functional group that may serve as an antigenic determinant, the hapten **5**−Thyr conjugate appeared

Table 2. Combination Data for Screening with Competitive Indirect ELISA against *cis*- or *trans*-DCCA-glycine[a]

analyte	immunogen	combination: antiserum/coating antigen	IC_{50} (μg/L)
trans-DCCA-glycine	*trans*-hapten **3**–Thyr	Ab 3702/*cis*-hapten **5**–BSA	26
		Ab 3703/*cis*-hapten **5**–BSA	1.2
	trans-hapten **5**–Thyr	Ab 3706/*cis*-hapten **9**–BSA	42
		Ab 3706/*cis*-hapten **26**–BSA	50
		Ab 3707/*cis*-hapten **1**–BSA	52
		Ab 3707/*cis*-hapten **9**–BSA	7.6
		Ab 3707/*cis*-hapten **26**–BSA	16
	trans-hapten **9**–Thyr	Ab 3710/*cis*-hapten **5**–BSA	1.2
		Ab 3710/*trans*-hapten **5**–BSA	5.8
		Ab 3711/*cis*-hapten **1**–BSA	81
		Ab 3711/*trans*-hapten **5**–BSA	25
	trans-hapten **24**–Thyr	Ab 3698/*cis*-hapten **3**–BSA	93
		Ab 3698/*cis*-hapten **5**–BSA	2.0
		Ab 3698/*trans*-hapten **5**–BSA	14
		Ab 3699/*cis*-hapten **1**–BSA	15
		Ab 3699/*cis*-hapten **5**–BSA	2.0
		Ab 3699/*trans*-hapten **5**–BSA	4.1
		Ab 3699/*cis*-hapten **9**–BSA	8.7
cis-DCCA-glycine	*cis*-hapten **3**–Thyr	Ab 3700/*cis*-hapten **5**–BSA	18
		Ab 3700/*trans*-hapten **5**–BSA	5.8
		Ab 3701/*cis*-hapten **5**–BSA	39
		Ab 3701/*trans*-hapten **5**–BSA	3.5
	cis-hapten **5**–Thyr	Ab 3704/*cis*-hapten **1**–BSA	84
	cis-hapten **9**–Thyr	Ab 3709/*cis*-haptne **1**–BSA	10
		Ab 3709/*trans*-hapten **1**–BSA	3.5
		Ab 3709/*trans*-hapten **3**–BSA	92
		Ab 3709/*cis*-hapten **5**–BSA	2.9
		Ab 3709/*trans*-hapten **5**–BSA	0.7
		Ab 3709/*trans*-hapten **26**–BSA	23
	cis-hapten **24**–Thyr	Ab 369/*cis*-hapten **5**–BSA	35
		Ab 369/*trans*-hapten **5**–BSA	3.7

[a] The analytes were prepared in the PBS solution.

to produce spacer specific antibodies, which resulted in lower specificity to the target analyte as compared to other Ab groups. Showing low IC_{50} values (<2 μg/L) for the *trans*-target analyte, the Ab coating antigen combinations of Ab 3703/*cis*-hapten **5**−BSA, Ab 3710/*cis*-hapten **5**−BSA, and Ab 3698/*cis*-hapten **5**−BSA were chosen for more study. The antisera specific to *cis*-DCCA-glycine were also generated against the *cis*-haptens **3**−, **9**−, and **24**−Thyr. Showing low IC_{50} values (<3 μg/L) for the *cis*-target analyte, the combinations of Ab 3701/*trans*-hapten **5**−BSA, Ab 3709/*trans*-hapten **5**−BSA, and Ab 369/*trans*-hapten **5**−BSA were also chosen. These results suggest that the structure of hapten **5** was not suitable to produce the Ab specific to the target but was suitable as a coating antigen in a heterologous system. With respect to the isomeric configuration of the molecule, the heterologous *cis*/*trans* or *trans*/*cis* configuration systems between Ab and coating antigen were much more sensitive than homologous configuration systems. This result was consistent with studies of immunoassays for the pyrethroids permethrin and cypermethrin (*36*, *50*). These assays also used the heterologous configuration. For an example, the combination of the Ab raised against the *trans*-permethrin hapten was used with a *cis*-coating antigen for a sensitive immunoassay.

Inhibition curves of the target analyte with six combinations of the antiserum and the coating antigen (**Figure 6**) resulted in IC_{50} values of 1.3, 2.1, and 2.2 μg/L for *trans*-DCCA-glycine and 0.4, 2.3, and 2.8 μg/L for *cis*-DCCA-glycine under unoptimized conditions, indicating that the sensitive immunoassay for the analysis of the target analyte can be developed.

CR. As seen in **Table 3**, the antibodies generated against the immunizing haptens of the *trans*-configuration had a higher

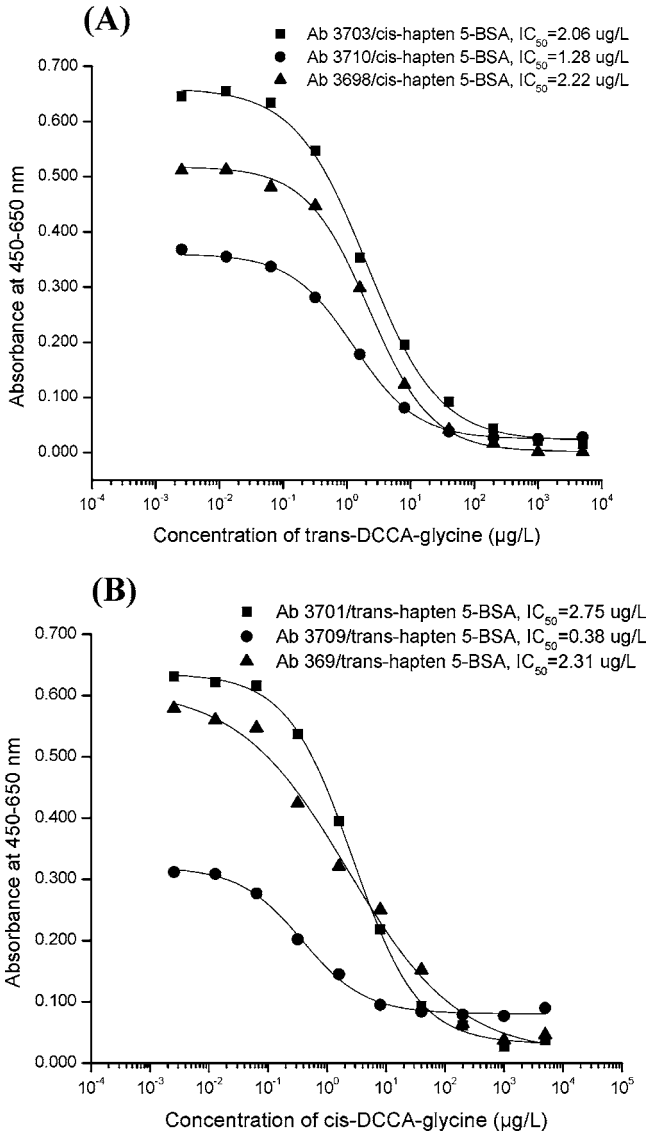

Figure 6. Six ELISA inhibition curves for *trans*-DCCA-glycine (**A**) and *cis*-DCCA-glycine (**B**) under unoptimized conditions. The reagent concentrations were as follows: the assay for *trans*-DCCA-glycine using Ab 3703, 1:32 000; Ab 3710, 1:8000; and Ab 3698, 1:16 000; and coating antigen *cis*-hapten **5**–BSA, 1, 5, and 2 μg/mL, respectively. The assay for *cis*-DCCA-glycine using Ab 3701, 1:16 000; Ab 3709, 1:8000; and Ab 369, 1:16 000; and coating antigen *trans*-hapten **5**–BSA, 1 μg/mL. The dilution ratios of the Ab are the final concentrations in the wells.

affinity for the *trans*-configuration of target analyte than the *cis*-configuration, whereas the antibodies generated against the *cis*-configuration had a higher affinity for the *cis*-target analyte. The CRs of the tested *trans*-antibodies to *cis*-DCCA-glycine were below 28%. The Ab 3703 showed relatively higher CR to *cis*-DCCA-glycine than that of other testes antibodies, Ab 3710 and Ab 3698, indicating the mixture of *cis*/*trans*-DCCA-glycine can be analyzed using this Ab. Additionally, the shorter linker of hapten presents the hapten relatively close to the protein carrier surface; thus, the Ab produced against the hapten can provide broader selectivity to the structurally related compounds (*47*). This theory was applicable to Ab 3703. Antibodies produced against the *trans*-isomer of haptens **9** and **24** with longer linkers showed a lower CR to the *cis*-target analyte. The Ab (Ab 3703) raised against *trans*-hapten **3** (*trans*-DCCA-glycine) with no spacer displayed a better recognition to the *cis*-isomer of the target analyte than that of other antibodies

Table 3. CR (%) of the Selected Antibodies to Permethrin Metabolites and Other Structurally Related Compounds[a]

	IC_{50} (CR [%])		
compound	Ab 3703/*cis*-**5**–BSA	Ab 3710/*cis*-**5**–BSA	Ab 3698/*cis*-**5**–BSA
trans-DCCA-glycine	1.9 (100)	2.6 (100)	0.8 (100)
cis-DCCA-glycine	6.7 (28)	18 (15)	37 (2)
trans-DCCA	430 (0.40)	808 (0.30)	>8200 (<0.01)
permethrin	4475 (0.04)	2357 (0.11)	2357 (0.04)
glycine	>19 000 (<0.01)	>26 000 (<0.01)	>8200 (<0.01)
PBA-glycine	>19 000 (<0.01)	>26 000 (<0.01)	>8200 (<0.01)

	IC_{50} (CR [%])		
	Ab 3701/*trans*-**5**–BSA	Ab 3709/*trans*-**5**–BSA	Ab 369/*trans*-**5**–BSA
cis-DCCA-glycine	3.1 (100)	0.7 (100)	6.2 (100)
trans-DCCA-glycine	194 (1.61)	179 (0.38)	322 (1.93)
cis-DCCA	590 (0.53)	22 (3.00)	2947 (0.21)
permethrin	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)
glycine	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)
PBA-glycine	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)

[a] The antibodies, Ab 3703, Ab 3710, and Ab 3698, were produced against the *trans* configuration of immunogens, and the antibodies, Ab 3701, Ab 3709, and Ab 369, were produced against the *cis* configuration of immunogens. The compounds were prepared in the PBS solution.

(Ab 3710 and 3698). The ELISA combined with Ab 3703 and coating antigen *cis*-hapten **5**−BSA can be good for the detection of total *cis*/*trans*-DCCA-glycine. All of the antibodies showed no or very little CRs to free permethrin metabolite (DCCA), permethrin, PBA-glycine conjugate, and glycine, suggesting that the antibodies are very selective for the target structure.

Unlike the above antibodies produced against the *trans*-immunogens, the antibodies, Ab 3701, Ab 3709, and Ab 369, generated against the *cis*-configuration, had a low CR to the *trans*-configuration. The antibodies showed a high selectivity for the *cis*-target analyte. These antibodies also showed very low CRs to other structurally related compounds. On the basis of these results, it is thought that the most antigenic determinants of the target analyte for the Ab production are the glycine moiety (−$NHCH_2COO$−), and the *cis*- or *trans*-isomeric configuration of the structure.

Discussion. The toxicity of permethrin is dependent on its three-dimensional configuration. The *cis*-isomer is more toxic than the *trans*-isomer. However, *trans*-permethrin is metabolized more rapidly than *cis*-permethrin in mammals (*2*, *51*). The *trans*-permethrin predominates (60−75%) in the commercial product. The amount of *trans*-DCCA metabolite not conjugated and/or after acid hydrolysis of conjugates of permethrin in human urine ranged from 65 to 87% (*52*, *53*). The ratios of conjugates of *cis*- and *trans*-DCCA might be similar to that of free *trans*-DCCA. No information is reported about the ratios of the free conjugates of the metabolites. The immunoassay with *cis* or *trans* specific Ab will be developed for separate analysis of each *cis*- and *trans*-isomer as well as the analysis of total isomers, depending upon CR studies of different ratios of the *cis*- and *trans*-mixtures.

On the basis of the optimized model of the hapten molecules, all of the immunizing haptens except for hapten **5** were fairly good matches to the geometry of the target analyte. As a result, they generated specific antibodies to the target, which resulted in a sensitive and selective immunoassay. Therefore, using a molecular modeling technique can help with reasonable hapten designs to develop a sensitive immunoassay. Finally, a very sensitive ELISA has been obtained by using both heterologous hapten structure and configuration systems between antibodies and coating antigen. The IC_{50} values are as low as 1.3−2.2 μg/L for *trans*-DCCA-glycine and 0.4−2.8 μg/L for *cis*-DCCA-glycine. Depending in part on the application, the optimum Ab for the assay will be selected. The Ab 3703 generated against the *trans*-immunogen provided broad selectivity for the *cis*-target analyte. The ELISA with the combination of Ab 3703 and coating antigen, *cis*-hapten **5**−BSA, can be good for detection of the *cis*- and *trans*-isomers of DCCA-glycine in the sample. The immunoassay will be optimized with the parameters such as detergent, solvent content, pH, and ionic strength in the assay buffer for the best sensitivity and reproducibility, providing a powerful tool for human monitoring for pyrethroid exposure.

ABBREVIATIONS USED

Ab, antibody; Thyr, thyroglobulin; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment mass spectrum; GAR-HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition; sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt; OD, optical density; PBST, phosphate-buffered saline with 0.05% of Tween 20; TMB, tetramethylbenzidine; DCCA, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; DCCA-glycine, *N*-(3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-glycine; PBOH, 3-phenoxybenzyl alcohol; PBA, 3-phenoxybenzoic acid.

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Development of a class selective immunoassay for the type II pyrethroid insecticides

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Abstract

A general and broad class selective enzyme-linked immunosorbent assay was developed for the type II pyrethroid insecticides, such as cypermethrin, deltamethrin, cyhalothrin, cyfluthrin, fenvalerate, esfenvalerate and fluvalinate. Polyclonal antibodies were generated by immunizing with a type II pyrethroid immunogen ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis*,*trans*-2,2-dimethyl-3-carboxyl-cyclopropanecarboxylate) conjugated with thyroglobulin. Antisera were screened against nine different coating antigens. The antibody–antigen combination with the most selectivity for type II pyrethroids such as cypermethrin was further optimized and tested for tolerance to co-solvent, pH and ionic strength changes. The IC_{50}s of the optimized immunoassay were 78 $\mu g\,l^{-1}$ for cypermethrin, 205 $\mu g\,l^{-1}$ for cyfluthrin, 120 $\mu g\,l^{-1}$ for cyhalothrin, 13 $\mu g\,l^{-1}$ for deltamethrin, 6 $\mu g\,l^{-1}$ for esfenvalerate, 8 $\mu g\,l^{-1}$ for fenvalerate and 123 $\mu g\,l^{-1}$ for fluvalinate. No cross-reactivity was measured for the type I pyrethroids such as permethrin, bifenthrin, phenothrin, resmethrin and bioresmethrin. This assay can be used in monitoring studies to distinguish between type I and II pyrethroids.

Keywords Pyrethroid; Insecticide; Pesticide class; Type II; Immunoassay; ELISA

1. Introduction

Synthetic pyrethroids are used widely in domestic, public health, agriculture, forestry, and veterinary applications [1]. The increasing use of synthetic pyrethroids, compared with that of other classes of insecticides, is attributed to their remarkably high insecticidal activities and low toxicity to mammals [2,3]. Although pyrethroids are thought to be safe for humans, reversible symptoms of poisoning and suppressive effects on the immune system have been reported after exposure [4–6]. Some pyrethroids may cause lymph node and splenic damage as well as carcinogenesis [7]. Because pyrethroids offer significant advantages to the agricultural ecosystem if used carefully, but have a potential for environmental damage, a sensitive, selective, and rapid method for monitoring residue levels of pyrethroids in aquatic ecosystems is desirable.

The synthetic pyrethroids and natural pyrethrins can be divided into two groups of compounds on the basis of chemical structure and mechanism of action at insect target sites. The type I compounds are simple cyclic alcohol esters of 2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylic acid. The type II compounds are esters of an arylcyanohydrin. The type I and II pyrethroids have different toxicological effects, and may have slightly different mechanisms of action at the insect neuron [1], thus the ability to monitor these two compound groups selectively would be an advantage.

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There are many methods for the detection of pyrethroids, such as high-performance liquid chromatography (HPLC) and gas chromatography with electron capture detector (GC-ECD) [8–10]. In spite of good sensitivity, the procedure for sample preparation in such methods is complicated, relatively time-consuming and expensive. In addition, these methods are not suitable for high throughput screening. Therefore, another effective and rapid method for analysis of several pyrethroids at residue levels is desirable. Hammock and Mumma [11] reported that immunoassay could be used for pesticide residue analysis and screening. Owing to the lower cost and higher sample throughput compared to instrumental analyses, enzyme linked immunosorbent assay (ELISA) is an alternative quantitative method. It also has the potential for use as a preliminary screen to reduce the number of samples that undergo subsequent instrumental analyses. ELISA can simplify the quantification of pesticide residues in complex matrices, such as sediments and soils or animal or plant tissues, and it may be possible to reduce the harmful organic solvents used for extraction [12].

Several immunoassays for pyrethroids have been reported. Stanker et al. [13] produced monoclonal antibodies against an immunogen containing the phenoxybenzyl moiety and a cyclopropane ring, and applied this method to detect permethrin in meat extracts. Skerritt et al. [14] described an ELISA format using the same antibodies to detect permethrin in grain and flour extracts. Class selective assays have been developed for those pyrethroids containing the chrysanthemic acid moiety [15]. Pullen and Hock [16] used the permethric acid moiety as a hapten to detect both permethrin (type I) and cypermethrin (type II). An assay that was highly selective for type I over type II pyrethroids has also been developed [17]. For this assay a permethrin-like hapten with a rigid double bond in a long linker attached to the permethric acid portion of the molecule was used. Class selective and compound selective immunoassays for the type II pyrethroid insecticides have also been developed [18,19]. For these assays the sensitivity is in the hundreds of ng ml^{-1}. However, if the analyte is isomerized the sensitivity increases to the tens of ng ml^{-1}. Selectivity was also greater with the isomerized analyte. The assay reported here has similar sensitivity to the isomerized assays, but does not require the isomerization step, and is more selective for type II pyrethroids than the previously reported assay.

In some situations, such as the analysis of storm water runoff, the ability to determine which pyrethroid is present is important. Thus, having a series of assays ranging in selectivity from broad to specific is useful. A class-selective immunoassay that can distinguish between types I and II pyrethroids is desirable. A class selective immunoassay for the type I pyrethroid insecticides has been already developed in this laboratory [17]. Using a similar hapten design strategy, a general and broad selective immunoassay for the type II pyrethroids is described here.

2. Experimental

2.1. Materials

2.1.1. Reagents

Permethrin (3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) (*cis:trans* = 25:75), esfenvalerate ((*S*)-α-cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate), fenvalerate ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate), cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), cyfluthrin ((*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl (*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate), fluvalinate ((*RS*)-α-cyano-3-phenoxybenzyl *N*-(2-chloro-α,α,α-trifluoro-*p*-tolyl)valinate), phenothrin (3-phenoxybenzyl (*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate), resmethrin (5-benzyl-3-furylmethyl (*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate) and bioresmethrin ((5-benzyl-3-furyl)methyl (*R*)-*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate) were purchased from Reidel de Haen (Seelze, Germany) (Fig. 1). Bifenthrin (2-methylbiphenyl-3-ylmethyl 3-(2-chloro-3,3,3, trifluoro-propenyl)-2,2-dimethyl-cyclopropanecarboxylate and cyhalothrin (cyano-(3-phenoxy-phenyl)-methyl 3-(2-chloro-3,3,3-trifluoro-propenyl)-2,2-dimethylcyclopropanecarboxylate were purchased from Chem Service Inc. (West Chester, PA) (Fig. 1). Hapten **V** (3-phenoxybenzoic acid; Table 1), and 3-phenoxybenzyl alcohol were purchased from Aldrich (St. Louis, MO) (Fig. 1). Coating antigen haptens **IV**, **VI**, **VII**, and **VIII** (Table 1) were prepared in the laboratory [20]. Methanol (GC Resolve grade) was obtained from Fisher Scientific (Pittsburgh, PA), and dimethyl sulfoxide (DMSO; 99.8%) was purchased from Aldrich (St. Louis, MO). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich), *N,N*-dimethylformamide (DMF; Aldrich) and *N*-hydroxysulfosuccinimide sodium salt (Fluka, Buchs, Switzerland) were used for the production of conjugate with protein. Thyroglobulin (THY), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma (St. Louis, MO) as carrier proteins, and goat anti-rabbit immunoglobulin conjugated to horseradish-peroxidase (HRP), Tween 20 and 3,3′,5,5′-tetramethylbenzidine (TMB) were also purchased from Sigma (St. Louis, MO). Water used was purified by a NANOpure II system (Barnstead, Newton, MA). Thin layer chromatography (TLC) was performed on 0.2 μm silica gel 60-F254 glass plates from E. Merck (Darmstadt, Germany). Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel, packed in glass columns of such diameter to give a column height:diameter ra-

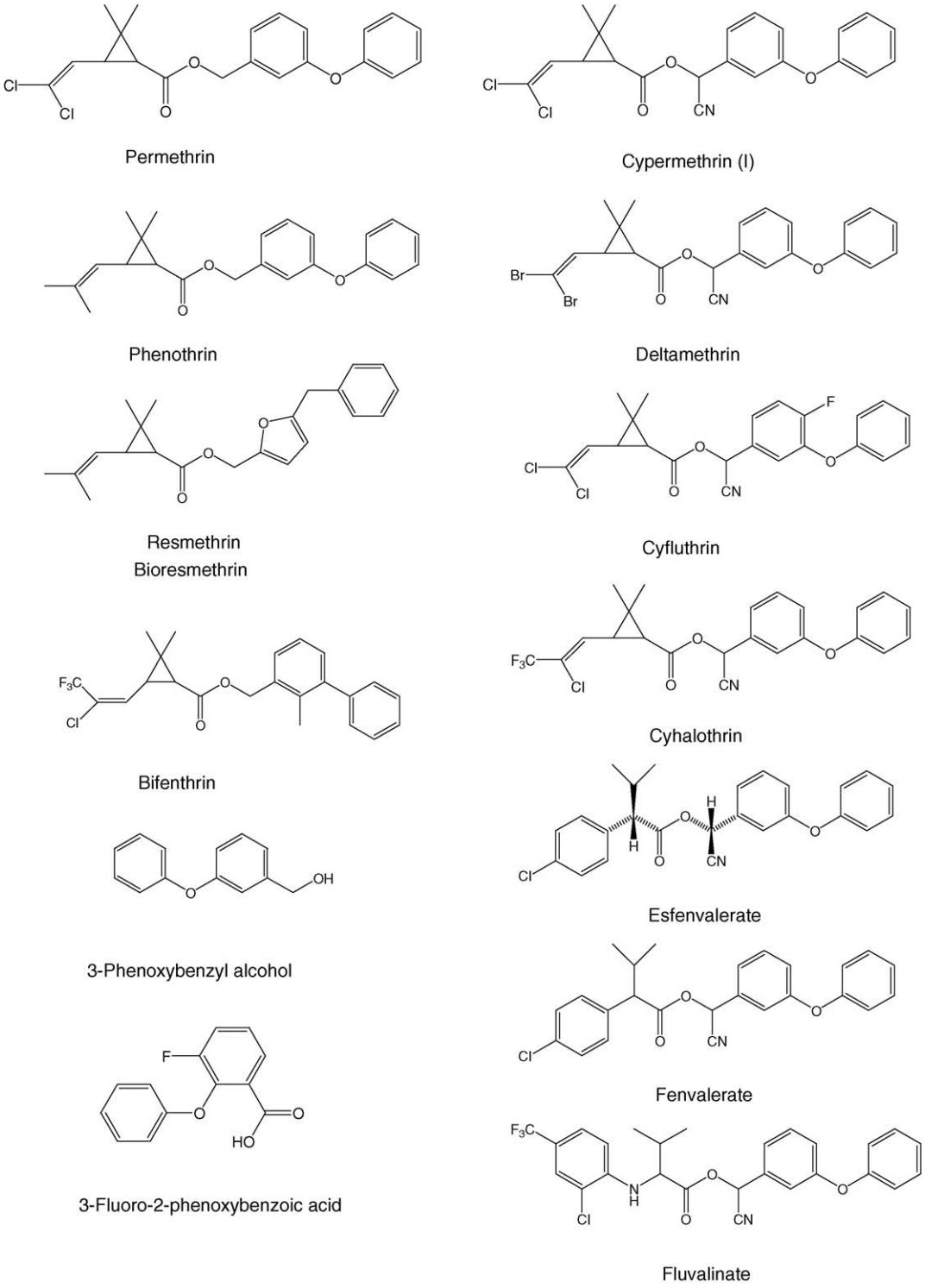


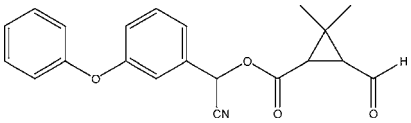
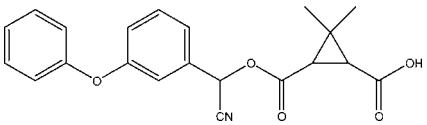
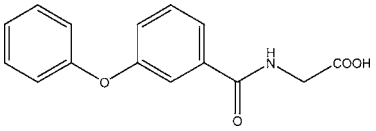
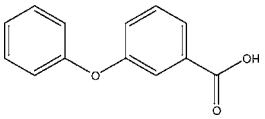
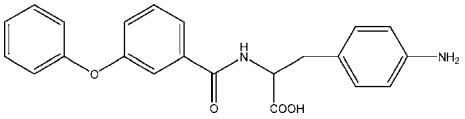
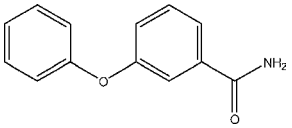
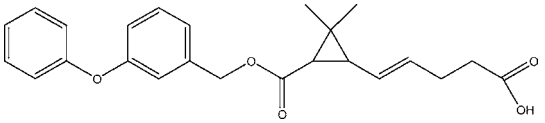
Fig. 1. Structures of the pyrethroids and pyrethroid metabolites.

tio of ∼7. The → notation denotes a stepwise solvent gradient.

2.1.2. Instruments

Proton NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from internal standard tetramethylsilane. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark) and the absorbances were read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450–650 nm). The charac-

Table 1
Structure of coating antigen haptens[a]

Haptens	Structure
II	
III	
IV	
V	
VI	
VII	
VIII	

[a] Haptens were conjugated with BSA or OVA as described in Section 2.

terization of hapten with protein conjugate was done with a Shimadzu UV-2101PC UV–vis scanning spectrophotometer (Shimadzu, Kyoto, Japan).

2.2. Methods

2.2.1. Hapten synthesis and verification

Syntheses of the haptens **II** and **III** were carried out as outlined in Fig. 2. All reactions were straightforward using well-known procedures, and yields were good. NMR spectral data supported all structures, and mass spectra further supported the structure of target molecules.

*2.2.1.1. (RS)-α-Cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate (**I**) [21].* (Note: the acid used in this preparation was a sample labeled as ±-*cis*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid; however, NMR examination of the product, as noted below, indicated it to be about 13% of the *trans* isomer.)

A sample of ZnI_2 (0.5 mg) was added under N_2 with stirring and ice cooling to a solution of 3-phenoxybenzaldehyde (1.98 g, 0.01 M) in chloroform, and about one-half of a sample of cyanotrimethylsilane (1.47 ml, 0.011 M). The temperature rose slowly to ∼30 °C. The mixture was cooled to ∼10 °C and the remainder of the cyanotrimethylsilane was injected giving a second mild exotherm. After warming the mixture briefly to 40 °C, and allowing to stand at ambient temperature for 45 min, the mixture was poured into a mixture of glyme (15 ml) and 3N HCl (3.5 ml). There was a mild immediate exotherm in the two phase mixture which was then stirred for 30 min, diluted with water, phase separated, and the aqueous phase was extracted twice with chloroform. The combined organic phase was washed twice with water, dried briefly over magnesium sulfate and stripped to a tan oil.

Meanwhile, a sample of the acid (1.68 g, 0.01 M) in chloroform (4 ml) plus dimethylformamide (0.5 ml) was treated with thionyl chloride (1.46 ml, 0.02 M) and stirred and heated in an oil bath at 60–65 °C under a short spiral bantam ware distillation column. Gas evolution stopped after ∼15 min. After 45 min, the oil bath temperature was increased to ∼110 °C to distill out most of the chloroform. Cyclohexane (5 ml) was added and distilled out to a pot temperature of 135 °C. A second 5 ml sample of cyclohexane was added and distilled out in the same way. The residual acid chloride, as a colorless oil, was dissolved in chloroform (4 ml), then added to a stirred and ice cooled solution of the above cyanohydrin in chloroform (2.5 ml). After 1 min, pyridine (1.01 ml, 0.0125 M) was injected over ∼2 min. The mixture was stirred for 30 min, then washed with dilute HCl solution (acidic wash), twice with water, dried ($MgSO_4$) and stripped. Flash chromatography on silica gel (25 g) eluting with 50 ml each of 25, 50, and 75% methylene chloride in hexane cleanly separated a small amount of unreacted cyanohydrin from product. Stripping product fractions gave 2.54 g of (**I**) as a mixture of isomers.

A 200 mg sample of **I** was separated by radial chromatography to give 161 mg of the higher R_f isomer mixture and 23 mg of the lower R_f isomer. The NMR spectra of these two were both consistent with the desired structure.

*2.2.1.2. (RS)-α-Cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-formyl cyclopropanecarboxylate (**II**) and (RS)-α-cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-carboxylcyclopropanecarboxylate (**III**).* The target compounds were prepared using ozonolysis of compound **I**. Compound **I** (0.305 g) was dissolved in 25 ml anhydrous methanol in a tubular reactor equipped with a magnetic stirrer, the reactor flask was placed in a liquid nitrogen bath. A stream of ozone-rich oxygen (from an ozone generator) was passed into the stirred, cold solution with a flow rate of 0.0248 l min^{-1}. The reaction solution was monitored by TLC (hexane:ethyl acetate = 4:1). After 60 min, the reactant was nearly gone and the ozonation was stopped. Acetic acid (1.2 ml) was added as the temperature was warmed to 30 °C by water bath. Then zinc powder (2.0 g) was added a small portion at a time, and the mixture was stirred overnight

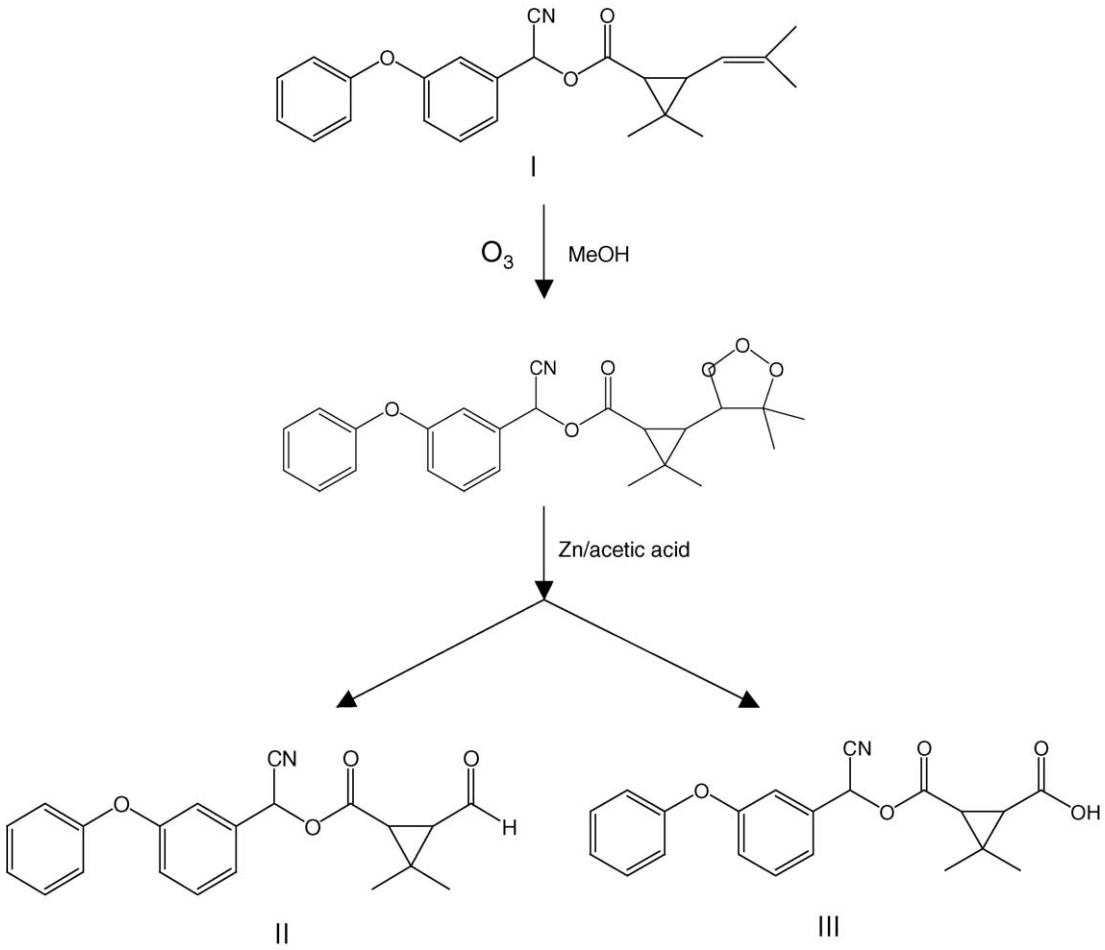


Fig. 2. Synthetic scheme for the preparation of haptens **II** and **III**.

at room temperature. The mixture was filtered to remove the precipitate. The solution was condensed to about 5 ml under vacuum. Flash chromatography on 40 g of silica gel (hexane → CH_2Cl_2) and high vacuum stripping gave the pure gummy aldehyde (**II**) 97 mg (35%) TLC $R_f = 0.65$, $M^+ = 349$. ^{1}H NMR ($CDCl_3$): δ 1.15 (d, 3H, CH_3), 1.25 (d, $J = 6.5$ Hz, 3H, CH_3), 1.64 (m, 1H, CH), 2.10 (d, 1H, CHC(O)O), 6.40 (s, 1H, CHCN), 7.0–7.8 (m, 10H, Ar), 9.92 (s, CHO), and further oxidized acid product (**III**), 40 mg (14%) TLC $R_f = 0.45$ CH_2Cl_2, $M^+ = 366$. ^{1}H NMR ($CDCl_3$): δ 1.10 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.62 (m, 1H, CH), 2.08 (d, 1H, CH), 6.35 (s, 1H, CHCN), 6.9–7.5 (m, 10H, Ar).

2.2.2. Production of hapten–protein conjugates

Hapten **II**-THY/BSA/KLH was synthesized using the Schiff base formation and reductive amination method [22]. Sixty milligrams of BSA (or 50 mg THY or 24 mg KLH) was dissolved in 5 ml of PBS (pH 7.4). Then compound **II** (0.02 mmol) in 200 μl DMSO was added with gentle stirring and followed by addition of 50 μL of 5 M sodium cyanoborohydride in 1N NaOH. The reaction mixture was stirred for 3 h at room temperature. The unreacted aldehyde sites were blocked by adding 100 μl of 3 M ethanolamine solution and reacted for 15 min at room temperature. The solution was then dialyzed against PBS over 48 h at 4 °C and stored at −20 °C. The **II**-THY and **II**-KLH conjugates were used for immunization and the **II**-BSA was used as a coating antigen.

Hapten **III**-THY/BSA was synthesized by conjugating hapten **III** with carrier proteins using an activated ester method [23]. Hapten **III** (45 μM) was dissolved in 1.0 ml of anhydrous DMF with equimolar (0.04 mmol) concentration of *N*-hydroxysuccinimide and a 10% molar excess of dicyclohexylcarbodiimide. After 3 h of stirring at room temperature, the precipitated dicyclohexylurea was removed by centrifugation, and the DMF supernatant was divided into two aliquots and added to protein solutions as below.

Fifty milligrams of BSA or THY was dissolved in 5 ml of borate buffer (pH 8.7) in a 10 ml glass vial with a Teflon stir bar. This solution was allowed to stir vigorously, and 1.05 ml of DMF was added very slowly to bring the DMF to 20%. Activated hapten then was added to the protein solution a few microliters each time at room temperature over about 20 min to finish this step. Then the mixture was stirred at 4 °C for 6 h. The solution was dialyzed against PBS for 72 h at 4 °C and stored at −20 °C. Hapten **III**-THY was used as

an immunogen and hapten **III**-BSA was used as a coating antigen.

2.2.3. Immunization and antiserum preparation

Cypermethrin antisera was obtained following the protocol described previously [24]. New Zealand white rabbits were immunized intradermally with each immunogen hapten **II**-KLH (rabbits #3540, and #3541), **II**-THY (rabbits #3543 and #3545) and **III**-THY (rabbits #3546 and #3547). One month after an initial immunization with 100 μg of the immunogen protein emulsified with Freund's complete adjuvant (1:1, v:v), further injections of 100 μg of the immunogen emulsified with Freund's incomplete adjuvant were given (1:1, v:v). Booster injections were given at 4-week intervals. The rabbits were bled 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C and stored at −20 °C. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters.

2.2.4. Enzyme-linked immunosorbent assay (ELISA)

The method was performed as previously described by Shan et al. [25]. Microplates were coated overnight at 4 °C with 100 μl per well of the appropriate coating antigen concentration in 0.1 M carbonate-bicarbonate buffer (pH 9.6). After the plate had been washed with washing solution (0.05% Tween 20 in distilled water), the surface of the wells were blocked with 200 μl of 0.5% BSA in 0.1 M PBS (8 g l^{-1} NaCl, 1.15 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} KCl) by incubation for 30 min at room temperature to minimize the non-specific binding in the plate. After another washing step, 100 μl of antiserum per well diluted in PBS (for titration experiments) or 50 μl per well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 μl per well of analyte solution were added and incubated for 1 h at room temperature. The standard analyte concentrations ranged from 0.05 to 5000 μg l^{-1}. Following a washing step, goat anti-rabbit IgG-HRP conjugate (diluted in 1:3000 in PBS with 0.05% Tween 20, 100 μl per well) was added and incubated for 1 h at room temperature. The plates were washed again, and 100 μl per well of substrate solution (3.3 μl of 30% H_2O_2, 400 μl of 0.6% TMB in DMSO per 25 ml of acetate buffer, pH 5.5) was added. The color development was stopped after 20 min with 50 μl per well of 2 M H_2SO_4. The absorbance was measured using a dual wavelength mode at 450 nm minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at no analyte present, B the curve slope at the inflection point, C the concentration of analyte giving 50% inhibition (IC_{50}), and D the minimum absorbance at infinite concentration.

2.2.5. Antibody characterization and assay optimisation

Antibodies and antigens were screened in a two-dimensional titration for the best dilution of coating antigen and antiserum. Then the competitive inhibition curves were measured for different antibody and antigen combinations. In this study, the combination of antibody and coating antigen with the lowest IC_{50} and more selectivity to cypermethrin (a type II pyrethroid over permethrin which is a type I analog) was selected for further assay development.

The effects of the solvents were tested by dissolving the analyte in PBS buffers containing various proportions of solvent (0, 10, 20, 40, 60, 80% solvent) and incubating these with antibody in PBSB on the coated plate. Methanol and DMSO were tested in this way.

In the experiment to evaluate pH effects, both the analyte and antiserum were dissolved in PBS buffer at the specified pH values of 5, 6, 7, and 8 and were tested in this incubation step with all other parameters of the assay fixed. Ionic strength effects were determined in the same manner as previously mentioned except that, instead of pH, PBS concentration was varied. PBS concentrations of 0.1, 0.2, 0.3, and 0.5 M were tested.

2.2.6. Cross-reactivity

The optimized assays were applied to cross-reactivity studies by using the standard solution of the analyte and other structurally related compounds. The cross-reactivity (CR) was determined by dividing the IC_{50} of the chemical, cypermethrin, assigned to be 100% by the IC_{50} of another compound and multiplying by 100 to obtain a percent figure.

2.2.7. Recovery

Industrial waters diluted 100 times with 20% methanolic PBS were spiked with cypermethrin standards (0.5, 1.0, 2.5, 5.0, and 10 μg l^{-1}) to estimate the recoveries from water samples.

3. Results and discussion

3.1. Hapten synthesis

The primary goal of this study was to develop a class specific immunoassay for type II pyrethroids. To design a hapten that has a common portion of type II pyrethroids and differs from type I pyrethroids is critical for such a purpose. Structurally, most pyrethroids (type I and II) are esters containing a phenoxybenzyl group and a dimethylcyclopropane. Type II pyrethroids can be distinguished from type I pyrethroids by having an α-cyano group in its alcohol moiety. The haptens were thus designed to contain the α-cyano phenoxybenzyl and dimethylcyclopropane. The carrier protein was to link through the dimethylcyclopropane end of the cypermethrin molecule. Since pyrethroids are highly lipophilic, a long side chain (handle) may allow the lipophilic hapten to fold into the hydrophobic interior of the protein and decrease the affinity of the resulting antibodies. Studied with dioxins, Sanborn et al. [26] demonstrated that a rigid or shorter side chain in an immunizing hapten is important for generation of sensitive and selective antibodies for a lipophilic target. A longer

Table 2
Summary of optical density of titration tests[a]

Ab/immunogen	**II**-BSA	**IV**-BSA	**IV**-OVA	**V**-BSA	**V**-OVA	**VI**-BSA	**VII**-BSA	**VIII**-BSA	**VIII**-OVA
3540/**II**-KLH	++++	++++	++++	++++	++++	++++	+	–	–
3541/**II**-KLH	–	+++	++++	+++	–	+++	–	–	–
3543/**II**-THY	–	–	–	–	–	+++	–	–	–
3545/**II**-THY	++++	++++	++++	++++	++++	++++	+	–	–
3546/**III**-THY	+++	–	++++	+++	+++	+	–	–	–
3547/**III**-THY	–	–	–	–	+++	+	–	–	–

Symbols refer to optical density ranges after 20 min of color development. (–) absorbance < 0.25; (+) absorbance = 0.25–0.50; (++) absorbance = 0.50–0.75; (+++) absorbance = 0.75–1.00; (++++) absorbance > 1.00.

[a] The data shown are at an antibody dilution of 1:16,000 and coating antigen concentration of 1 $\mu g\,ml^{-1}$.

linker that contained a double bond for rigidity was used for the development of a type I pyrethroid assay [17]. The hapten chosen for this study for immunization contains most of the cypermethrin molecule and has no side chain (Fig. 2). This approach differed from previous reports in which a long side chain was placed off the cyclopropane ring or attached at the α-cyano portion of pyrethroid [18]. In addition, the two haptens differed in the functional group used for coupling to carrier protein. This offered options for slightly different conditions for coupling, while not altering the actual linker.

3.2. Screening and selection of antisera

The antisera of six rabbits were screened against nine different coating antigens using a two-dimensional titration method with the coated antigen format. The objective was to find the coating antigen that has the highest affinity with tested antibody but could still be replaced by target analyte. The results of the titration experiments using the final bleeds are shown in Table 2. Animal to animal variability likely accounts for the highly different responses between rabbits 3543 and 3545 and 3546 and 3547 even though each pair was immunized with the same antigen. In all combinations, the titer of 3546 and 3547, which was generated from the immunogen **III**-THY, was much lower than those of 3540, 3541 and 3545, which were produced from the immunogens **II**-KLH and **II**-THY. Some of the difference in titer may be due to the difference in the coupling method used. The different methods used slightly different conditions that may have favored/hindered the optimal loading of the protein. Thus immunogens from hapten **III** may have had lower hapten loads than the immunogens from hapten **II** resulting in the lower titers for rabbit immunized with hapten **III**. The homologous assay, in which the same hapten was used in the coating antigen and immunogen, had a similar titer with the heterologous assay.

In general, good affinity was obtained with coating antigen haptens **II**, **IV**, **V** and **VI**. These haptens have in common, the exposed phenoxyphenyl moiety. On the other hand, little affinity was measured for coating antigen hapten **VII** and none measured for hapten **VIII** with all six antibodies. The antibodies appear to have a high degree of selectivity for the phenoxyphenyl ring and the presence of a methoxy group in hapten **VII** is likely the reason for the low binding to this antigen. Surprisingly hapten **VIII**, which is structurally similar to the immunizing haptens, is not bound by any of the antibodies. Pyrethroids are lipophilic molecules and the long linker may allow the highly recognized phenoxyphenyl moiety to fold back into the protein making it unavailable for binding to the antibody.

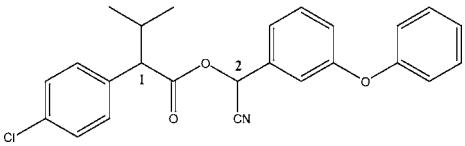
Interestingly, antibody 3546 shows a high titer for **IV**-OVA, but a low titer for **IV**-BSA. This same trend is true with antibody 3547 and coating antigen **V**-OVA and **V**-BSA. On the other hand, antibody 3541 shows a high titer for coating

Table 3
Selected competitive ELISA screening data against cypermethrin[a]

Immunogen	Ab/cAg	Cypermethrin				Permethrin			
		A_{max} (*A*)	Slope (*B*)	A_{min} (*D*)	IC_{50} ($\mu g\,l^{-1}$) (*C*)	A_{max} (*A*)	Slope (*B*)	A_{min} (*D*)	IC_{50} ($\mu g\,l^{-1}$) (*C*)
II-KLH	3540/**II**-BSA				>5000	0.63	1.21	0.21	589
II-THY	3545/**II**-BSA	0.36	1.05	0.12	165	0.32	1.00	0.10	137
II-KLH	3541/**IV**-BSA	0.48	0.40	0.04	56.8	0.48	0.67	0.11	87.5
II-KLH	3541/**IV**-OVA	0.72	1.31	0.12	598	0.82	1.29	0.07	562
II-THY	3545/**IV**-OVA	1.20	0.81	0.05	914	1.16	1.40	0.19	342
II-KLH	3541/**V**-BSA	0.69	1.13	0.13	278	0.75	0.81	0.06	282
II-THY	3545/**V**-BSA	1.64	0.89	0.20	596	1.57	0.95	0.15	515
III-THY	3546/**IV**-BSA	0.57	1.10	0.08	346	0.55	1.03	0.07	319
III-THY	3546/**IV**-OVA	0.42	0.69	0.06	792	0.43	1.25	0.06	459
III-THY	3546/**V**-BSA	1.13	1.25	0.42	467				>5000
III-THY	3546/**VI**-BSA	0.65	1.03	0.05	193				>5000
III-THY	3546/**VII**-BSA	0.53	0.702	0.07	958	0.55	0.84	0.01	603

[a] The cypermethrin analyte standards were prepared in a 30% methanol–PBS solution.

Table 4
Cross-reactivities of pyrethroids and their metabolites

Analyte	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,l^{-1}$)[c] (C)	R^2	Cross-reactivity (%)[a]
Cypermethrin	0.53	0.97	0.03	78.4 ± 11	0.99	100
Type I pyrethroid insecticide						
Permethrin						N.I.
cis-Permethrin						N.I.
trans-Permethrin						N.I.
Bifenthrin						N.I.
Phenothrin						N.I.
Bioresmethrin						N.I.
Resmethrin						N.I.
Type II pyrethroid insecticide						
Fenvalerate isomers						
1*S*,2*S* (esfenvalerate)	0.57	0.97	0.03	1086 ± 109	0.99	1386.2 ± 140
1*R*,2*S*	0.55	0.92	0.05	945 ± 77	0.99	1206 ± 98
1*S*,2*R*	0.52	0.74	0.03	56 ± 9	0.99	72.1 ± 11
1*R*,2*R*	0.52	0.68	0.04	16 ± 2	0.99	20.8 ± 3
1*R*,2*R*/*S*	0.58	0.94	0.06	1011 ± 86	0.99	1289.2 ± 110
1*S*,2*R*/*S*	0.50	0.70	0.07	50 ± 14	0.99	63.6 ± 18
1*R*/*S*,2*R*/*S* (fenvalerate)	0.56	0.90	0.06	772 ± 60	0.99	984.2 ± 76
Cyfluthrin	0.53	0.70	0.05	30 ± 4	0.99	38.2 ± 5
Cyhalothrin	0.50	0.74	0.04	51 ± 9	0.99	65.4 ± 12
Deltamethrin	0.55	0.89	0.07	486 ± 37	0.99	619.6 ± 47
Fluvalinate	0.51	0.83	0.06	50 ± 7	0.99	63.5 ± 9
Metabolites						
Phenoxybenzyl alcohol						N.I.
Phenoxybenzoic acid						N.I.
Fluorophenoxybenzoic acid						N.I.

[a] ELISA system 3540/**VI**-BSA was used in the cross-reactivity studies. Cypermethrin was assigned as 100%. Values are the means and standard deviations of three experiments.

antigen **V**-BSA, but not **V**-OVA. With these three examples, two have higher titers on OVA and one on BSA, thus it is not likely that one carrier protein is better than another. With other antibodies, no difference in titer is noted between BSA and OVA antigens ruling out likely differences due to hapten load. Because other antibodies do bind to these antigens, failed coupling is also ruled out. Thus this observation is most probably due to animal variability.

Competitive inhibition experiments were performed to optimize antiserum and coating antigen concentrations for high sensitivity. In addition, to distinguish the selectivity of the combinations of antisera and coating antigens to the type **II** pyrethroids, the competitive inhibition experiments to cypermethrin and permethrin were conducted simultaneously (Table 3). The IC_{50} of the homologous assay (3540 or 3545/**II**-BSA) showed that this combination was not selective for cypermethrin. Even though some combinations of antibody and coating antigen had lower IC_{50}s, they did not show good selectivity to cypermethrin. After preliminary screening, the combination of antiserum 3546 with coating antigen **VI**-BSA gave the lowest IC_{50} values for cypermethrin and no inhibition for permethrin despite the lower titer. This system was selected for cross-reactivity studies and was chosen for further assay development and optimization.

3.3. Cross-reactivity

Table 4 demonstrates the cross-reactivity of the ELISA system 3546/**VI**-BSA with different pyrethroids and pyrethroid metabolites. This system had the highest cross-reactivity with the type II pyrethroids such as esfenvalerate (1386%), followed by fenvalerate (984%), deltamethrin (620%), cyhalothrin (65%), fluvalinate (64%) and cyfluthrin (38%). Less or no cross-reactivity was measured for the type I pyrethroids such as bifenthrin, bioresmethrin, permethrin, phenothrin, resmethrin and permethrin metabolites in this system. Since our primary goal was to develop a class-selective immunoassay with no or low cross-reactivity to the type I pyrethroids, 3546/**VI**-BSA was selected for further optimization.

The hapten used in this study for generating antibodies contained a very short side chain that resulted in antibodies that were highly selective for type II pyrethroids. Short or rigid side chains are considered desirable for lipophilic hap-

Table 5
Effects of DMSO concentration on assay parameters[a]

DMSO (%)[b]	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,l^{-1}$)[c] (C)	A/D	R^2
0	0.59 ± 0.03	0.96	0.06	113.0 ± 6.22	10.70	0.99
10	0.28 ± 0.04	0.63	0.04	167.4 ± 7.78	7.65	0.99
20	0.11 ± 0.01	0.43	0.03	280.0 ± 16.09	3.27	0.99
40	0.10 ± 0.01	0.24	0.04	1041.3 ± 29.14	2.39	0.99

[a] ELISA conditions: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000).
[b] Concentration of DMSO in cypermethrin standard solution (PBS–DMSO).
[c] Mean value ± S.D. Each set of data represents the average of three experiments.

tens to prevent their folding back onto the protein surface. Such folding may result in less antibody selectivity and sensitivity. Although it is difficult to compare antibodies from one laboratory to another, the antibodies derived from longer chain, non-rigid haptens resulted in assays that were not as selective, but of similar sensitivity as the assay reported here [18].

3.4. Solvent, pH, and salt effects

Finding a proper co-solvent for an immunoassay is very important for the analysis of hydrophobic chemicals such as cypermethrin. The solvent (methanol or DMSO) effects on the ELISA system (3546/**VI**-BSA) were evaluated by preparing cypermethrin in PBS containing various amounts of solvent. DMSO strongly interferes with the assay sensitivity and maximum absorbance (Table 5). Using methanol as a cosolvent, absorbance was enhanced with higher methanol concentration (Fig. 3), which was also found by Shan et al. [25]. The IC_{50} value of the assay varied depending upon the different concentrations of the cosolvent methanol. The lowest IC_{50} was found at 20% methanol (70.2 $\mu g\,l^{-1}$), which is approximately two times and four times lower than that at 40% methanol (148 $\mu g\,l^{-1}$) and 60% methanol (300 $\mu g\,l^{-1}$) respectively. At concentrations up to 80% methanol, an incomplete inhibition curve was demonstrated. The co-solvent could be an important factor in assay performance, especially for hydrophobic compounds. A high concentration of co-solvent will help the solubility of hydrophobic cypermethrin in reaction solution, but it could also affect the interaction of antibody and antigen, especially the low affinity of antibody and antigen. On the basis of the IC_{50} values and the ratios of maximum and minimum absorbances for the cypermethrin standard curves, a methanol concentration of 20% was selected for subsequent experiments.

To address potential interferences from aqueous environmental samples, the effects of pH and ionic strength on ELISA performance were evaluated in this study. In system 3546/**VI**-BSA, when analyte was dissolved in buffer at various pH values, no significant effect upon the IC_{50} was detected, indicating that the assay could effectively detect cypermethrin at pH values ranging from 5.0 to 8.0 (Fig. 4). Ionic strength strongly influenced ELISA performance (Fig. 5). A higher salt concentration in the assay system resulted in lower absorbance and higher IC_{50} values. The absorbance values at salt concentrations of 0.3 and 0.5 M PBS decreased by approximately 53 and 68%, respectively, from the absorbance value at a salt concentration 0.1 M PBS. Therefore, the maintenance of a minimal ionic strength appears to be important.

The optimized cypermethrin ELISA used coating antigen **VI**-BSA at 0.25 $\mu g\,ml^{-1}$, antibody 3546 at a dilution of 1:5000, and cypermethrin in 20% methanol–PBS buffer. The IC_{50} value of this assay was 78.2 ± 5.0 $\mu g\,l^{-1}$ (Fig. 6).

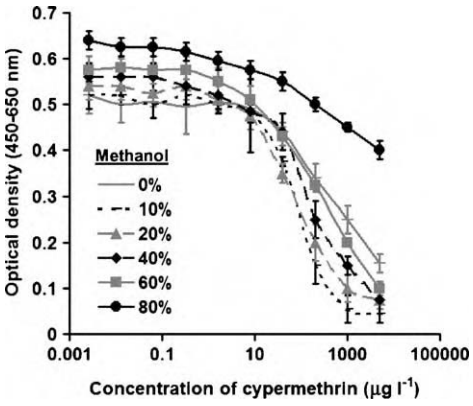


Fig. 3. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various concentrations of methanol. Reagent concentrations: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

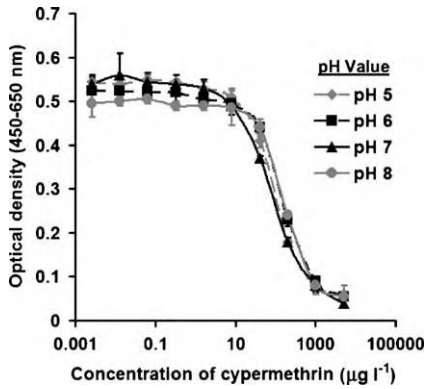


Fig. 4. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various pH values. Reagent concentrations: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

Table 6
Recovery test of cypermethrin in industrial water

Analyte	Spiked ($\mu g\,ml^{-1}$)[a]	Theoretical ($\mu g\,l^{-1}$)[b]	Measured ($\mu g\,l^{-1}$)	Recovery (%)[c]	Mean ± S.D.
Cypermethrin	1	20	18.4	92.0	92.7 ± 1.6
			18.9	92.5	
			18.3	91.5	
	4	80	87.0	108.8	107.0 ± 3.6
			87.5	109.4	
			82.3	102.9	
	10	200	201.2	100.6	101.5 ± 2.2
			198.2	99.0	
			210.0	105.0	

[a] Industrial water was spiked with an appropriate concentration of cypermethrin.
[b] Fifty times dilution with 20% methanolic PBS.
[c] Percent recovery was calculated as the measured spiked concentration of cypermethrin divided by the theoretical spiked concentration of cypermethrin × 100.
Three spiked samples were used in each study.

Table 7
Recovery test of the type II pyrethroids

Analyte	Spiked ($\mu g\,ml^{-1}$)[a]	Theoretical ($\mu g\,l^{-1}$)[b]	Cypermethrin equivalent ($\mu g\,l^{-1}$)	Relative recovery (%)	Mean ± S.D. (%)	Actual recovery (%)
Cyfluthrin	4	80	22.4	28.0	31.6 ± 4 3	82.7 ± 11.2
			29.1	36.4		
			24.3	30.4		
	10	200	70.4	35.2	37.6 ± 2.7	98.4 ± 7.2
			74.0	37.0		
			80.2	40.6		
Cyhalothrin	4	80	47.2	59.0	59.4 ± 6.0	90.9 ± 9.2
			42.9	53.6		
			52.5	65.6		
	10	200	128.6	64.3	67.6 ± 2.9	103.3 ± 4.4
			137.6	68.8		
			139.2	69.6		
Deltamethrin[c]	1	5	39.1	782.0	697.3 ± 2.9	112.5 ± 16.2
			29.3	586.0		
			36.2	724.0		
	4	20	113.1	565.5	546.2 ± 17.6	88.1 ± 2.8
			106.2	531.0		
			108.4	542.0		
Esfenvalerate[d]	1	2.5	40.2	1608.0	1440 ± 257.1	103.9 ± 18.5
			39.2	1568.0		
			28.6	1144.0		
	4	10	132.8	1328.0	1407.3 ± 134.0	101.5 ± 9.7
			156.2	1562.0		
			133.2	1332.0		
Fluvalinate	4	80	43.2	54.0	58.7 ± 5.0	92.4 ± 7.9
			51.6	64.0		
			46.5	58.1		
	10	200	107.2	53.6	54.8 ± 2.4	86.2 ± 3.8
			115.1	57.6		
			106.2	53.1		

[a] Industrial water was spiked with an appropriate concentration of cypermethrin.
[b] Fifty times dilution with 20% methanolic PBS.
[c] Two hundred times dilution with 20% methanolic PBS.
[d] Four hundred times dilution with 20% methanolic PBS.

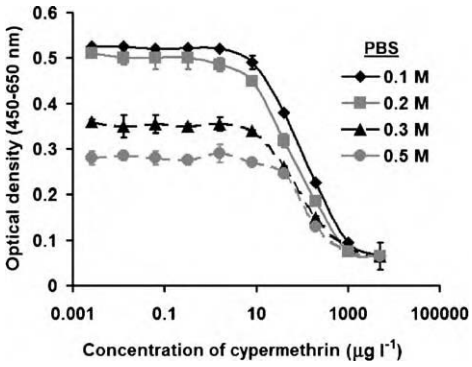


Fig. 5. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various concentrations of ionic strength. Reagent concentrations: coating antigen **IV**-BSA (0.25 μg ml^{-1}); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

3.5. Recovery

To estimate reliability, a recovery test was performed using an industrial water sample. When the standard curve was plotted with industrial water spiked with cypermethrin, the absorbance was significantly increased. There are many metal ions and other potential interferences in industrial water that could account for this enhanced response. It was necessary to dilute the samples with PBS prior to analysis. Although the matrix effect can be largely eliminated by solid phase extraction, a simple pretreatment without cleanup is important for real water analysis to enhance the speed of analysis. The effect of dilution factors on sensitivity was tested as shown in Fig. 7. The results indicate that the matrix effect was eliminated with more than 10-fold dilution with 20% methanolic PBS. Table 6 shows the recovery of cypermethrin from industrial water. Industrial waters, diluted 50-fold with 20% methanolic PBS, were tested in the linear range from 20 to 200 μg l^{-1}. The recoveries of cypermethrin (∼100 or >100%) were satisfactory. In addition, the recoveries of other type II

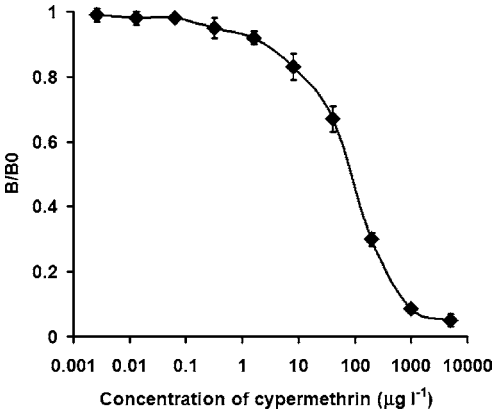


Fig. 6. ELISA inhibition curves of cypermethrin. Reagent concentrations: coating antigen **IV**-BSA (0.25 μg ml^{-1}); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Standard curve represent the average of 16 curves.

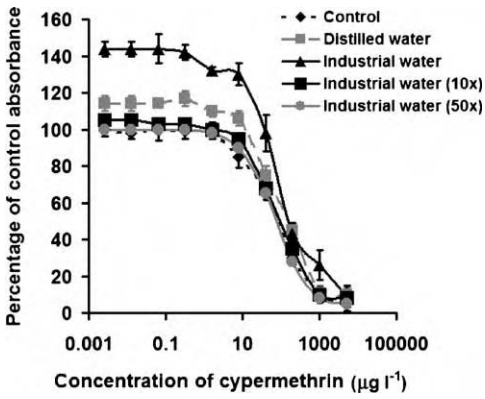


Fig. 7. Effects of the dilution of the matrix on the reliability of ELISA. Samples were diluted with 20% methanolic PBS.

pyrethroids using a cypermethrin standard curve were tested. Tap waters were spiked with 1, 4 and 10 μg l^{-1} cyfluthrin, cyhalothrin, deltamethrin, esfenvalerate, and fluvalinate (dissolved in methanol), and were diluted in an appropriate dilution factor with 20% methanolic PBS (Table 7). The results indicate that this assay will be a useful screening test for type II pyrethroids.

In order to estimate the recoveries of different kinds of type II pyrethroids, the relative cross-reactivity factor (shown in Table 4) could be used as reference values. Along with assays for parent compounds it may be possible to estimate the amount of different type II pyrethroids using mathematical approaches for multianalyte analysis similar to those developed by Wortberg et al. [27] for the triazine herbicides.

4. Conclusions

A general and broad selective immunoassay for the type II pyrethroid insecticides has been developed by using **III**-THY as the immunizing hapten and **VI**-BSA as the coating antigen. ELISA 3546/**VI**-BSA had a relatively lower IC_{50} and also demonstrated no or little inhibition to the type I pyrethroids. Also, this system exhibited good performance characteristics at various pH values and medium solvent (20% methanol) levels. Although the assay is sensitive to high ionic strength, with the implementation of dilution, interferences can be minimized for field sample measurement. This assay is useful for screening type II pyrethroids and the use of such a general assay has several advantages. One such advantage is to screen out samples containing non-detectable residues thus enhancing efficiency of further instrumental analysis such as GC–MS.

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Development of Sensitive Immunoassays for the Detection of the Glucuronide Conjugate of 3-Phenoxybenzyl Alcohol, a Putative Human Urinary Biomarker for Pyrethroid Exposure

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Pyrethroids are widely used in agriculture as insecticides. This study describes a sensitive enzyme-linked immunosorbent assay for the detection of the glucuronide conjugate of 3-phenoxybenzyl alcohol, a putative pyrethroid metabolite that may be used as a biomarker of exposure to pyrethroids. Four antisera were elicited against two different immunizing haptens. Antisera were characterized in combination with several coating haptens. The lowest IC_{50} value (0.5 ng/mL) was obtained with antiserum 1891 and 3-phenoxybenzoic acid−BSA conjugate as the coating antigen. Antiserum 1891 was highly selective for the target compound with an overall cross-reactivity of <0.3% to structurally related compounds. The assay sensitivity was negligibly affected by pH 4−9. A 5-fold improvement in IC_{50} was observed using a 10-fold concentrated phosphate-fuffered saline as the assay buffer. Compared to assays conducted in normal phosphate-fuffered saline, the maximal absorbance was almost identical. A good correlation ($r^2 = 0.99$ and 0.97 for urine samples A and B, respectively) was observed between spiked levels and the levels detected by the immunoassay.

KEYWORDS: ELISA; pyrethroid; glucuronide conjugate; human exposure; hapten

INTRODUCTION

Pyrethroids exert neurotoxic effects on the axons of the nervous system by interacting with sodium channels in insects and mammals (*1*). High potency in controlling a wide spectrum of insects and low toxicity to birds and mammals have made it widely accepted for application in agriculture, forestry, homes, horticulture, and public health around the world (*2*−*4*). Although pyrethroids are considered to be safe for humans, there have been concerns arising from their potential effects on human health from long-term exposure or high exposure of children. Environmental concerns include accumulation and leaching into the surface water and groundwater (*5*−*8*). Some research indicates that high exposure to pyrethroids might cause endocrine disruption, suppressive effects on the immune system, lymph node and splenic damage, and carcinogenesis (*9*−*11*). Pyrethroids are also known to be highly toxic to some aquatic species and beneficial insects. Pyrethroids have been found in various agricultural and commercial products such as vegetables, fruits, and shampoo and surface water and groundwater (*12*). Therefore, it is important to develop a rapid, sensitive, and efficient analytical method for both toxicological and epidemiological monitoring.

Most commercial pyrethroids are esters. These pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage to *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA) and 3-phenoxybenzyl alcohol (**Figure 1**). The 3-phenoxybenzyl alcohol is further processed to 3-phenoxybenzoic acid (PBA). DCCA and both the benzyl alcohol and the benzoic acid may undergo further metabolism to glucuronide, glycine, taurine, and sulfate conjugates (*13*−*18*). The cyanobenzyl alcohol from compounds such as cypermethrin and fenvalerate is expected to rearrange chemically to the corresponding benzoic acid (PBA), and thus the biomarker developed here will not be useful for type II pyrethroids. However, the glucuronide conjugate of 3-phenoxybenzyl alcohol (3-PBAlc−Gluc) should be a useful biomarker for exposure to type I pyrethroids such as phenothrin and permethrin that contain a 3-phenoxybenzyl alcohol moiety.

Currently HPLC and GC-MS are the primary methods used to analyze urinary metabolites of pyrethroids (*19*−*22*). Although these methods provide accurate and reliable results, somewhat complicated sample preparation cannot be avoided. Immunoassay has proven to be a sensitive analytical method for clinical diagnostics, agriculture, environmental monitoring, and food quality assessment. Previously we reported several immunoassays for the detection of pyrethroid parent compounds (*23*−*27*), their primary metabolites, and various conjugates (*28*−*30*). Although no study has specifically determined the exact

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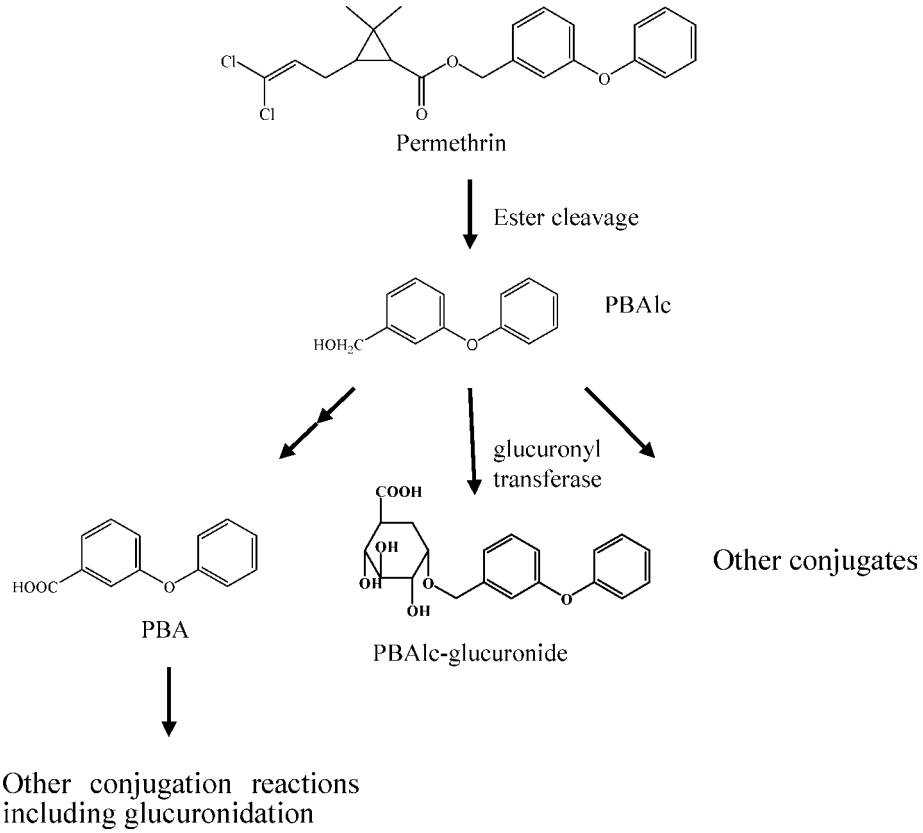


Figure 1. Mammalian metabolism of the type I pyrethroid permethrin. Conjugates vary by species, but the relative abundance of various conjugates for humans has not been determined.

conjugates of pyrethroid metabolites in humans, the development of immunoassays for all types of metabolites enhances the capability of biological monitoring for an exposure to pyrethroids because the pyrethroid metabolite profile will vary from individual to individual, and analysis for a single metabolite will not give a complete picture. In this study, we report a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the detection of the putative urinary biomarker of pyrethroid exposure, 3-PBAlc−Gluc. We describe the synthesis of the glucuronide target analyte, hapten preparation using heterobifunctional cross-linkers, antibody production against the target conjugate, assay development, and validation with urine samples.

MATERIALS AND METHODS

Chemicals. Organic chemicals for the syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany). Column chromatographic separations were carried out using Baker silica gel (40 μm average particle size) using the indicated solvents. Heterobifunctional cross-linking reagents, (3-[2-aminoethyl]-dithio)propionic acid·HCl (AEDP) and (*N*-[ϵ-trifluoroacetylcaproyloxy]-succinic ester (TFCS), were purchased from Pierce (Rockford, IL). Other coupling reagents were purchased from Aldrich. Bovine serum albumin (BSA), thyroglobulin (Thyr), goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR−HRP), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO).

Instruments. ^{1}H nuclear magnetic resonance (NMR) spectra of compounds synthesized were obtained on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA) using tetramethylsilane as an internal standard. Electrospray mass spectra in the positive (MS-ESI$^+$) or negative (MS-ESI$^-$) mode were recorded on a Micromass Quattro Ultima triple-quadrupole tandem mass spectrometer (Micromass, Manchester, U.K.). Melting points were determined on a Thomas-Hoover Uni-Melt apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. ELISAs were performed on 96 well microtiter plates (Nunc-Immunoplate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Menlo Park, CA) in dual wavelength mode (450−650 nm).

Synthesis of 3-Phenoxylbenzyl β-D-Glucuronide (3-PBAlc−Gluc) (4; Figure 2). Methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranouronate (**1**; 7 g, 18.6 mmol) prepared from glucuronolactone by methylation with sodium methoxide and acid-catalyzed acetylation with acetic anhydride was dissolved in dry CH_2Cl_2 (100 mL), and 45% hydrobromic acid in acetic acid (20 mL, 111.6 mmol) was added at ice temperatures. After stirring at room temperature for 3 h, the mixture was diluted with $CHCl_3$ and successively washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl. The organic layer was filtered through activated charcoal to remove the color. After evaporation, the residue was recrystallized with ethyl ether to afford methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate (**2**; 5.6 g, 76%). For the Koenigs−Knorr reaction, to a solution of **2** (500 mg, 1.3 mmol) in dry CH_2Cl_2 (10 mL) were added 3-phenoxybenzyl alcohol (350 mg, 1.8 mmol), dry silver trifluoromethanesulfonate (400 mg, 1.6 mmol), and tetramethylurea (180 mg, 1.6 mmol), and the mixture was stirred under an argon atmosphere at −20 °C for 12 h. The reaction was filtered and diluted with ethyl acetate (EtOAc). The organic layer was washed with saturated aqueous $NaHCO_3$ and applied to flash chromatography (stepwise elution with hexane/EtOAc; 20, 30, and 40% EtOAc) to afford 3-phenoxybenzyl methyl 2,3,4-tri-*O*-acetyl-D-glucopyranouronate (**3**; 350 mg, 55%). Compound **3** (250 mg, 0.48 mmol) was dissolved in methanol (MeOH, 5 mL), and a 1 M sodium methoxide solution (10 mL) was added. The mixture was stirred at room temperature for 24 h, and an excess of $Ba(OH)_2$ was added. The reaction mixture was stirred at room temperature for 24 h. After neutralization with Dowex-50 (H^+-form), the mixture was filtered and evaporated in vacuo. The residue was purified with silica gel column chromatography (stepwise elution with $CHCl_3$/MeOH; 10, 20, and 30% MeOH) and with ODS column

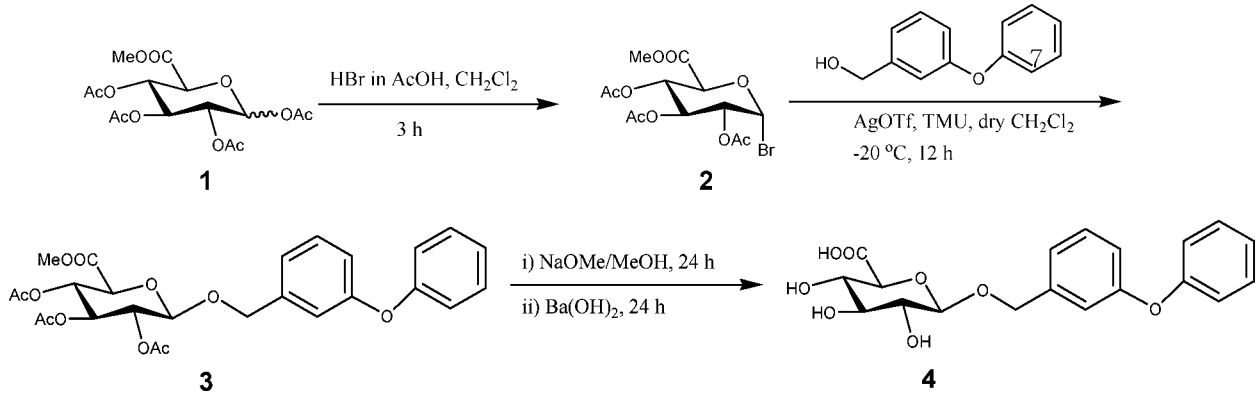


Figure 2. Scheme for synthesis of 3-PBAlc–glucuronide.

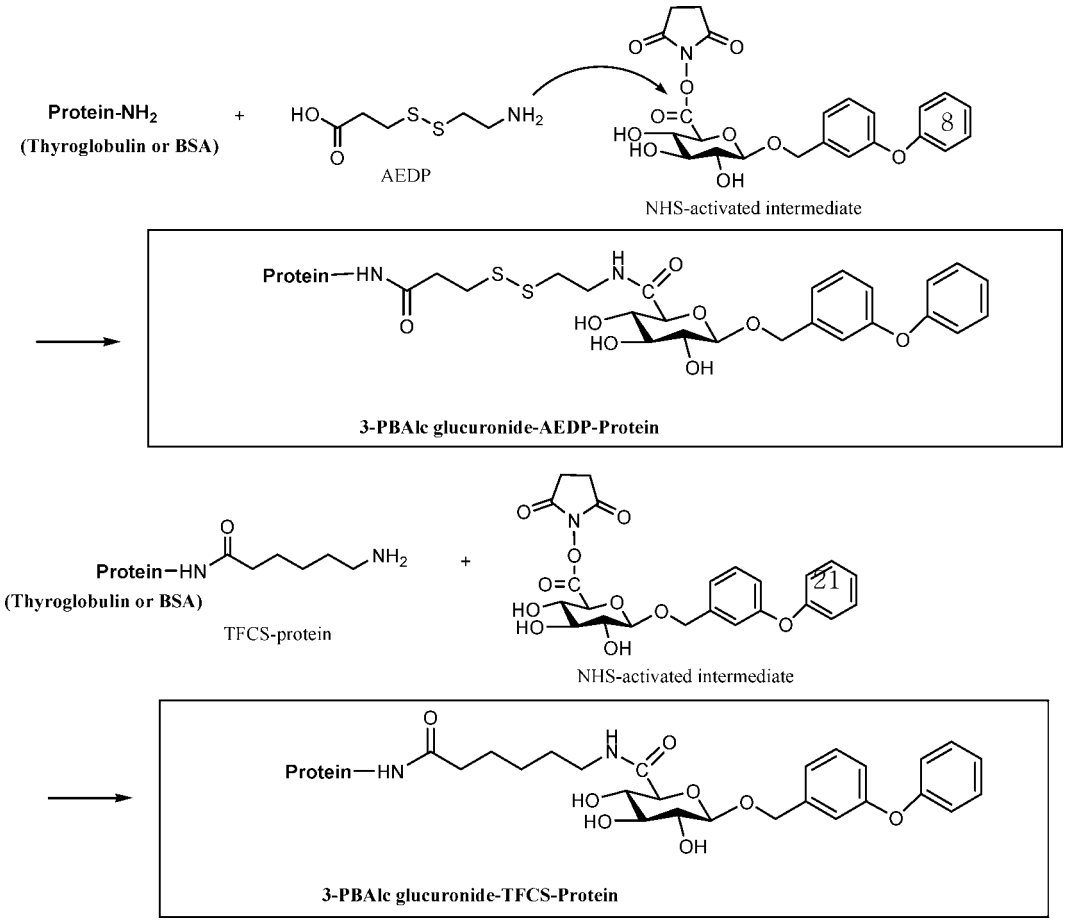


Figure 3. Conjugation route of 3-PBAlc–glucuronide to the protein (thyroglobulin or BSA) through the heterologous bifunctional linkers

chromatography (stepwise elution with acetonitrile/H_2O; 20, 50, and 80% acetonitrile). Freeze-drying gave analytically pure 3-phenoxybenzyl β-D-glucuronide (**4**) as a white powder (70 mg, 40%; total yield from **1**, 17%): ^1H NMR_H (CD_3OD), δ 3.26−3.63 (2H, Gluc H-4 and 5), 3.64 (1H, dd, J = 7.8 and 6.3 Hz, Gluc H-3), 4.41 (1H, dd, J = 7.8 and 6.3 Hz, Gluc H-2), 4.67 (1H, dd, J = 11.7 and 5.1 Hz, $OCH\underline{H}_a$-Ph), 4.87 (1H, d, J = 7.8, Gluc H-1), 5.01 (1H, dd, J = 11.7 and 5.1 Hz, $OC\underline{H}_bHPh$), 6.92−7.40 (9H, −Ph−O−Ph); HR-neg-MS, m/z [M − H]$^+$ calcd for $C_{19}H_{19}O_8$, 375.1080; found, 375.1052.

Preparation of Immunogens and Coating Antigens. 3-PBAlc−Gluc was coupled to proteins to yield hapten−protein conjugates for immunizing and coating antigens using two heterobifunctional coupling reagents. Each heterobifunctional cross-linker possesses two different reactive groups that allow for sequential conjugations with specific functional groups of proteins. The resulting conjugates were similar except that the acid moiety of 3-PBAlc-Gluc was linked through either a $-NHCH_2SS(CH_2)_2CO-$ or $-NH(CH_2)_5CO-$ spacer by using AEDP or TFCS, respectively (**Figure 3**). 3-PBAlc−Gluc−AEDP or 3-PBAlc−Gluc−TFCS was conjugated to Thyr for use as immunogen. For plate coating antigens, both haptens (cAg01, cAg02) and PBA (cAg05) were coupled to BSA.

Cross-Linking of 3-PBAlc−Gluc to Protein by AEDP. *N*-Hydroxysuccinimide (NHS) (0.06 mmol) and dicyclohexylcarbodiimide (DCC) (0.05 mmol) were added to 3-PBAlc−Gluc (0.04 mmol) dissolved in 0.2 mL of dry dimethylformamide (DMF). After the mixture had been stirred overnight at 4 °C under a N_2 atmosphere, the precipitated dicyclohexylurea was removed by filtration. To the activated ester solution (about 0.2 mL) were added AEDP (0.04 mmol) and triethylamine (0.04 mmol). The mixture was allowed to react at 4 °C overnight. After 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.08 mmol) in 0.5 mL DMF was added to the mixture and reacted at 4 °C overnight, the reaction mixture was added slowly to the protein solution (25 mg of protein in 5 mL of 0.05 M borate buffer at pH 8) with vigorous stirring and then allowed to stir gently at 4 °C for 24 h to complete the conjugation. This was followed by exhaustive dialysis against normal strength phosphate-buffered saline.

Cross-Linking of 3-PBAlc−Gluc to Protein by TFCS. TFCS (0.04 mmol) dissolved in 0.5 mL of DMF was added to the protein solution (25 mg in 5 mL of 100 mM sodium phosphate, 0.15 M NaCl, pH 7.2). After the mixture had been stirred at 4 °C for 2 h, the pH was adjusted to pH 7.8−8.1 with diluted 1 N NaOH and allowed to incubate for 2 h to remove the trifluoroacetyl protecting group of the cross-linker. The NHS-activated ester of 3-PBAlc−Gluc (0.04 mmol) was slowly added to the linker-attached protein solution. The reaction mixture was purified as described above.

Conjugation of PBA to Protein. NHS (0.06 mmol) and DCC (0.05 mmol) were added to PBA (0.04 mmol) dissolved in 0.2 mL of dry DMF. The activated ester of PBA was added to a BSA solution (25 mg in 5 mL of 0.05 M borate buffer at pH 8) as described above.

Hapten Density Analyses. For the determination of the amount of hapten (3-PBAlc−Gluc−linker) conjugated to protein, the BSA conjugate was dialyzed against distilled water for 24 h to remove salts, and then a powder was obtained by lyophilization. Hapten densities of the BSA conjugates were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard BSA to that of the conjugates. MALDI spectra were obtained by mixing 1 μL of matrix (*E*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg/mL) and 1 μL of a solution of the conjugates (5 mg/mL in 5% formic acid)

Immunization and Antiserum Preparation. Two female New Zealand white rabbits were immunized for each immunogen. Each immunogen (3-PBAlc−Gluc−AEDP−Thyr or 3-PBAlc−Gluc−TFCS−Thyr, 100 μg) in 0.5 mL of 0.85% saline was emulsified with an equal volume of Freund's complete adjuvant, and then the emulsion was injected subcutaneously. After 3 weeks, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1, v/v). The boosts were given every 3 weeks, and blood samples were drawn 7 days after each boost to check the titers of antisera. The final antisera were collected 5 months following the first immunization. The blood was collected into a Vacutainer tube with a serum separation gel. The antisera were obtained by centrifugation and stored at −80 °C. The antiserum was used without further purification.

ELISA Buffer Solutions. Normal strength PBS (1× PBS; 8 g/L of NaCl, 0.2 g/L Na_2HPO_4, and 0.2 g/L of KCl, pH 7.5), PBST (PBS containing 0.05% Tween 20), carbonate−bicarbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.6), and 0.05M citrate−acetate buffer (14.71 g/L $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 5.5) were used for immunoassay.

ELISA. Indirect competitive ELISAs were performed. The 96-well microtiter plates were coated overnight at 4 °C with 100 μL/well of the appropriate concentration of coating antigen (cAg) in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After it had been washed five times with PBST, the plate was incubated with 200 μL/well of a 1.0% BSA solution in PBS for 1 h at room temperature. After another washing step, 100 μL/well of antiserum diluted in PBST per well (for titration experiment) or 50 μL/well of antiserum diluted in PBST and 50 μL/well of standard analyte or sample solution were added and incubated for 1 h at room temperature. After the plate had been washed, 100 μL/well of the secondary GAR−HRP (1:6000 in PBST) was added and incubated for 1 h at room temperature. The plate was washed again, and 100 μL/well of a substrate solution [0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% of TMB in dimethyl sulfoxide (DMSO) added to 25 mL of citrate−acetate buffer, pH 5.5] was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL/well of 4 N H_2SO_4. The absorbance was measured using a dual-wavelength mode at 450 minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which was fit to a four-parameter logistic equation.

Assay Optimization. The assay conditions were optimized in such a way that the IC_{50} values were minimized. This goal was achieved by screening antibodies and antigens in a two-dimensional titration for optimal dilution of cAg and antiserum. Then, competitive inhibition curves were obtained for different antiserum and cAg combinations, and the combination with the lowest IC_{50} was selected for further assay development.

Cross-Reactivity (CR). The optimized assay was assessed for cross-reactivity by using standard solutions of the analytes and other structurally related compounds (listed in **Table 3**). The CR was obtained by comparing the IC_{50} values of the 3-PBAlc−Gluc standard to the tested compounds, where %CR = (IC_{50} of 3-PBAlc−Gluc/IC_{50} of tested compound) × 100.

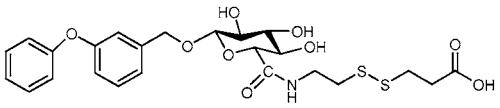
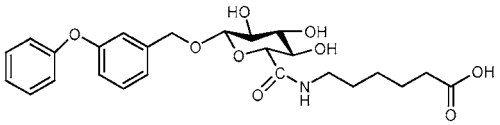
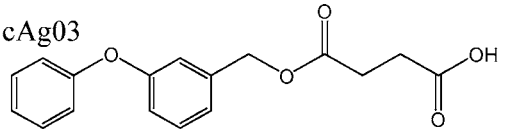
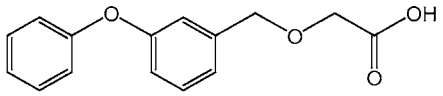
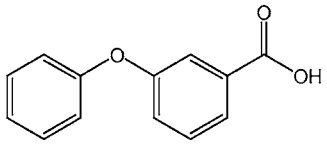
RESULTS AND DISCUSSION

Synthesis of Target Analyte and Hapten. It is known that the main detoxification reaction of carboxylic acid containing xenobiotics is conjugation either with an amino acid to form a peptide or with glucuronic acid to form a glucoside. In this respect, although the predominant type of conjugation for metabolites of pyrethroids has not been elucidated, 3-phenoxybenzoic acid is known as a main metabolite of pyrethroids and has been found in the general population at levels around 3 ppb (*31*). This metabolite arises from α-cyanopyrethroids by rearrangement of the corresponding cyanohydrin. It also can arise from PBAlc-containing pyrethroids by a two-step oxidation of PBAlc to PBA (**Figure 1**). PBA can be conjugated as a glucuronide, but acid glucuronides are less stable than alcohol glucuronides and also tend to transesterify around the glucuronide ring. We targeted the glucuronide of PBAlc as a possible biomarker which could distinguish among pyrethroids that hydrolyze primarily to PBAlc, PBA (ones with α-cyano moieties), or other alcohol moieties. Alcohol glucuronides are generally stable conjugates in the absence of glucuronidases. Thus, the development of a specific assay for each possible conjugate may be ideal for exposure monitoring.

The primary goals of this study were to synthesize the glucuronide target compound and develop an immunoassay specific to the 3-PBAlc−Gluc conjugate. The target compound was first enzymatically synthesized with uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) and 3-PBAlc as the substrate for glycosyl-*S*-transferase in mouse microsomes. Because the yield was low from the enzymatic synthesis, a chemical synthetic approach was taken. The chemical synthesis was based on the methods of Bulgianesi and Shen (*32*) and Sone and Misaki (*33*). The target analyte, 3-phenoxybenzyl β-D-glucuronide, was synthesized as shown in **Figure 2**. The Koenigs−Knorr reaction was used in the glucuronidation between the O-protected methyl D-bromoglucopyronurate and 3-phenoxybenzyl alcohol via its silver salt. For the Koenigs−Knorr reaction, 1-bromo derivatives of the glucuronic ester are necessary as common intermediates.

To generate a specific antiserum for an analyte, the site for the linker attachment must be selected to maintain the unique target structure as distal as possible from the linker site while minimizing structural conformation changes from the modification. The phenyl ring is a potential site for linker attachment. However, attachment at this site may result in antibodies with high CR to other urinary phenolic glucuronides. Because the phenoxybenzyl ring is the unique structural feature of the main metabolite and because the high polarity of the glucuronide may elicit a strong interaction for antibody recognition, the carboxylic acid on the glucuronide was finally selected for the linker introduction. Direct conjugation of the carrier proteins to the carboxylic acid may not allow the glucuronide to be easily accessible for antibody recognition, possibly due to steric hindrance caused by bulky carrier proteins. This hypothesis is supported by our observation that the antisera against *p*-aminophenyl-β-D-glucuronide−Thyr without a linker space between the hapten and carrier protein had very high IC_{50} values (240−400 ng/mL) (*34*). Therefore, we used two types of commercial cross-linkers, AEDP and TFCS, to provide space between the hapten and carrier proteins (**Figure 3**). These cross-linkers

Table 1. Titration Summary for Anti-3-PBAlc–Gluc Antisera

Coating Antigens	Antisera			
	1890	1891	1892	1893
cAg01 	++++	++++	+	+
cAg02 	++++	++++	++++	++++
cAg03 	+++	+++	-	-
cAg04 	+++	+++	-	-
cAg05 	++	++	-	-

[a] The data shown are at a cAg concentration of 1 μg/mL and an antiserum dilu ion of 1 20000; –, absorbance < 0.2; +, absorbance = 0.2–0.5; ++, absorbance = 0.5–0.7; +++, absorbance = 0.7–1.0; ++++, absorbance > 1.0. Antisera 1890 and 1891 are derived from 3-PBAlc–Gluc–AEDP–Thyr and an isera 1892 and 1983 from 3-PBAlc–Gluc–TFCS–Thyr. Coating antigens are derived from conjugation of the hapten shown to BSA.

contain a primary amine functional group that reacts with the carboxylic acid in the target molecule and a carboxylic acid group that reacts with the lysine residues in the protein. We used an active ester method to generate the amide linkage between the hapten and the protein because of the precise control and high yield of the reaction and to avoid raising antibodies to protein−urea complexes, which sometimes result from the use of water-soluble carbodiimides.

The MALDI-TOF-MS results showed the successful conjugation of 3-PBAlc−Gluc to BSA with the two linkers resulting in molar ratios of hapten to BSA of 23:1 and 15:1 for 3-PBAlc−Gluc−AEDP and 3-PBAlc−Gluc−TFCS, respectively.

Titers and Screening of the Antisera. Four antisera were obtained against two immunizing haptens (sera 1890 and 1891 from 3-PBAlc−Gluc−AEDP and sera 1892 and 1893 from 3-PBAlc−Gluc−TFCS). A checkerboard titration method was used to screen different combinations of those antisera with the various coating antigens (cAgs). As shown in **Table 1**, homologous combinations (cAg01 and cAg02 with antisera) showed higher titers than heterologous combinations (cAg03, cAg04, and cAg05 with antisera) due to the structural homology of cAgs with the immunizing antigens. All antisera showed high titers for cAg02, whereas antisera 1892 and 1893 showed very low binding recognition for cAg01. For other tested cAgs with the phenoxy moiety, antisera 1890 and 1891 showed measurable recognition. However, the other two sera had negligible binding. This result indicates that antisera appear to mainly bind the phenoxy moiety of the compounds. Thus, antisera 1890 and 1891 were selected for further screening. Competitive binding of antisera 1890 and 1891 against the free target analyte was evaluated with each individual cAg. Although there was no statistically significant difference in IC_{50} values between sera

Table 2. Selected Competitive ELISA Results

antiserum/cAg	ABS_{max} (A)	slope (B)	IC_{50} (C)[a] (ng/mL)	ABS_{min} (D)
1891/cAg01	0.5	0.7	5.0	0.08
1891/cAg02	0.6	0.6	9.5	0.07
1891/cAg03	0.8	0.8	2.0	0.3
1891/cAg04	0.8	0.6	1.7	0.2
1891/cAg05	0.5	0.8	1.3	0.04
1890/cAg01	0.4	0.7	25.6	0.1
1890/cAg02	0.4	0.7	24.6	0.08
1890/cAg03	0.6	0.8	2.0	0.4
1890/cAg04	0.6	0.6	4.4	0.3
1890cAg05	0.8	0.6	6.2	0.01

[a] Each value represents the mean value of four replicates.

Table 3. Summary of CR of 3-PBAlc–Gluc Assay

chemical	IC_{50} (ng/mL)	CR (%)
3-PBAlc–glucuronide	1.5	100
cypermethrin	>2500	<0.06
permethrin	>2500	<0.06
esfenvalerate	>2500	<0.06
cyfluthrin	>2500	<0.06
trans-DCCA	>2500	<0.06
cis-DCCA	>2500	<0.06
trans-DCCA glycine	>2500	<0.06
p-nitrophenyl glucuronide	>2500	<0.06
PBA	934	0.16
4-hydroxybenzoic acid	>2500	<0.06
PBA–glycine	705	0.21

1890 and 1891 for all cAgs, in general, the trend showed that heterologous assays had relatively lower IC_{50} values than homologous systems. More sensitive assays were obtained with antiserum 1891 than with antiserum 1890. Among the five tested cAgs, the most sensitive assay was obtained using cAg05 and antiserum 1891 (IC_{50} of 1.3 ng/mL). For both antisera, cAg03 and cAg04 showed good sensitivity but also a high background signal. Thus, cAg05 was finally selected for coating (**Table 2**).

CR. CR was evaluated with four parent pyrethroids (cypermethrin, permethrin, esfenvalerate, and cyfluthrin), their metabolites (*trans*-DCCA, *cis*-DCCA, *trans*-DCCA−glycine, PBA, and PBA−glycine), and structurally related compounds (*p*-nitrophenyl glucuronide and 4-hydroxybenzoic acid). Antiserum 1891 is highly selective for the target compound with negligible recognition of the other tested compounds (CR < 0.3%). Although the phenoxybenzyl group appears to be necessary for antibody recognition, an extremely low CR for PBA and *p*-nitrophenyl glucuronide indicates that the phenoxybenzyl and glucuronide are both required for a high binding of antiserum 1891 (**Table 3**).

Matrix Effects. High tolerance of an assay to the changes in pH and ionic strength is desirable because this assay is developed for direct detection of the metabolite in urine. Assay performance under various pH and ionic strength conditions was determined (**Figure 4A**). pH had little affect on the assay sensitivity. Compared to pH 7.0, an approximately 10% suppression of antibody binding was observed at pH 4, 5, and 6. No changes in maximal absorbance were observed at pH 8 and 9. Although a 2-fold lower IC_{50} value was observed at pH 7.0, this was not statistically significantly different. This indicates that changes of pH in the tested range would not affect the accuracy for the quantitation of the target compound. The assay was highly tolerant to changes in ionic strength (**Figure 4B**). In fact, as the ionic strength increased the IC_{50} decreased compared to the IC_{50} (2.5 ng/mL) at 1× PBS. Sensitivity was

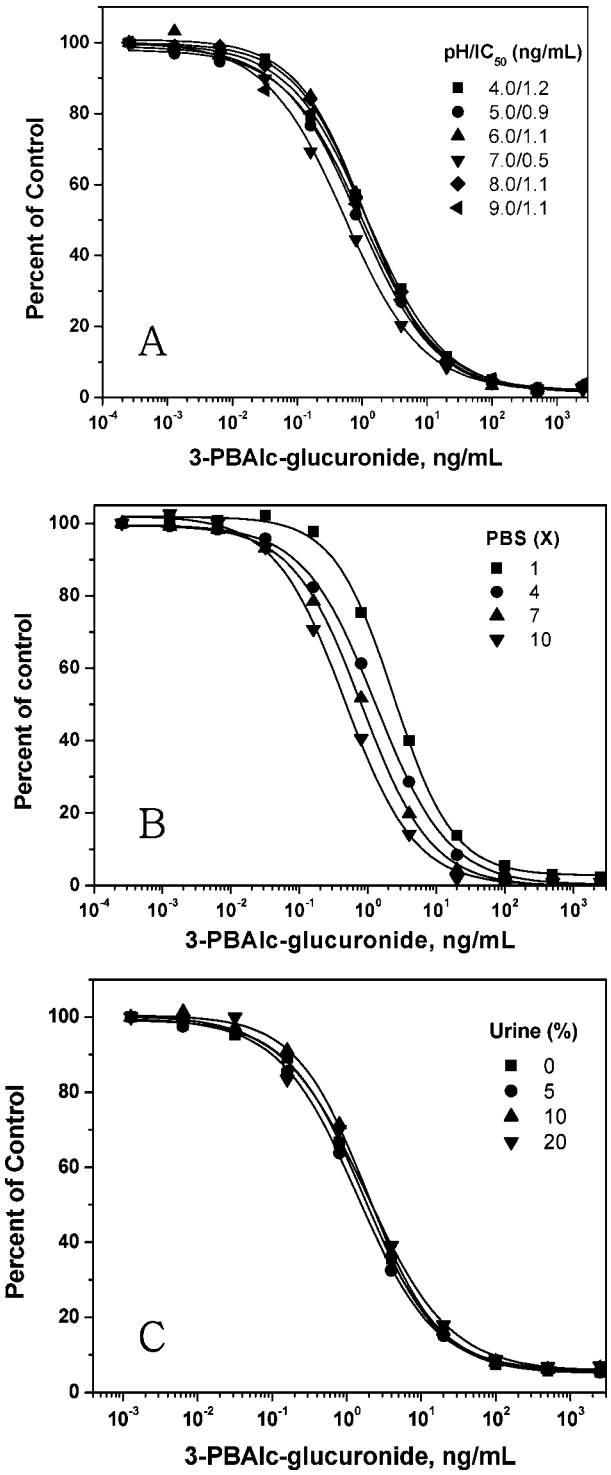


Figure 4. ELISA competition curves of 3-PBAlc–Gluc prepared at various (**A**) pH values, (**B**) ionic strengths, and (**C**) percentages of urine. Reagent concentrations were as follows: cAg, 0.25 μg/well of PBA-BSA; 1:4000 final dilution, Ab 1891; and GAR–HRP, 1:6000.

5-fold lower at 10× PBS. The antibody maintained 85% binding capability so that thereafter assays were performed with 10× PBS containing 0.05% Tween 20. This effect of ionic strength could be due to a better buffering capacity of 10× PBS.

The effect of urine as a matrix was evaluated (**Figure 4C**). In tests with four different urine samples, the assay parameters were unchanged at 5% urine, but the antibody binding was slightly suppressed at dilutions of 10 and 20%. Nevertheless, the IC_{50} values were almost identical. Thus, to use urine samples

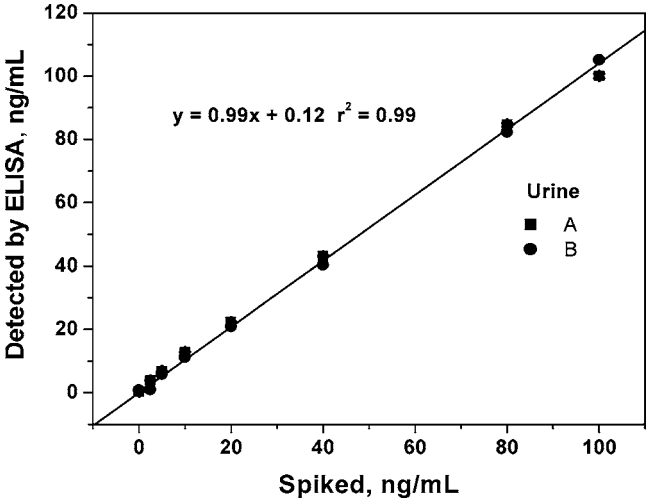


Figure 5. Relationship between analyte levels spiked into urine and measured by ELISA. Two urine samples from the two persons without known exposure to pyrethroids were used and are indicated in solid squares and solid circles.

at a 10 or 20% dilution, use of a control urine sample for the construction of standard curves may enable us to accurately detect analyte without further sample preparation or concentration.

Although a 5-fold lower IC_{50} value was observed with 10× PBS, this does not indicate that the assay with 10× buffer is more tolerant to the urine matrix. We compared IC_{50} values with 1× and 10× PBS buffer containing different percentages of urine (data not shown). Very similar matrix effects were observed, but the assay with 10× PBS buffer showed consistently lower IC_{50} values for each urine percent. Thus, the assays were carried out with 10× PBS. The competition curve obtained with 10× PBS buffer for the target compound showed that the calibration range is approximately 0.1−5.0 ng/mL with IC_{50} of 0.5 ng/mL.

Assay Validation. The assay validation was performed in a blind fashion by direct dilution of urine samples, which were spiked with PBAlc−Gluc concentrations ranging from 0 to 100 ng/mL (**Figure 5**). The linear regression analysis of ELISA results showed a good correlation ($r^2 = 0.99$) between spiked and detected levels. All recoveries were >86% of the spiked values. These results demonstrated that this assay is able to accurately detect pyrethroid metabolites at trace levels in urine samples.

Conclusion. A sensitive and selective immunoassay for a putative urinary metabolite of some type I pyrethroids, 3-PBAlc−Gluc has been developed. Heterologous ELISA with the use of cAg05 as a coating antigen and serum 1891 resulted in the lowest IC_{50} of 2.5 ng/mL. The assay run in 10× PBS showed 5-fold lower IC_{50} values than that with 1× PBS. The assay was remarkably robust, tolerating pH values between 4 and 7 and ionic strengths up to 10× PBS. The assay could tolerate up to 5% urine concentration. The maximal signals were slightly suppressed at 10 and 20% urine concentrations. However, IC_{50} values remained almost identical. The optimized assay had an IC_{50} value of 0.5 ± 0.03 ng/mL with a lower detection limit of 0.1 ng/mL. This ELISA was successfully applied to quantitate low parts per billion of analyte in spiked urine.

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High-Throughput Automated Luminescent Magnetic Particle-Based Immunoassay to Monitor Human Exposure to Pyrethroid Insecticides

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We have developed a sensitive, automated, competitive chemiluminescent immunoassay for the detection of 3-phenoxybenzoic acid (3-PBA), a metabolite common to many pyrethroid insecticides. The system uses a competitive hapten−protein conjugate that has been labeled with an acridinium ester as the chemiluminescent probe and secondary antibody-coated paramagnetic particles for the separation. After the immunoassay reagents and samples are combined for the competitive incubation step, a fully automated system is used to load the postincubation mixture into a delivery cuvette, facilitating the subsequent magnetic separation of the immunocomplex and the measurement of chemiluminescent signal for quantification. The immunoassay format described here supports the requirement for high throughput necessary for monitoring large numbers of samples in population-based studies. The optimized immunoassay was more sensitive than the conventional enzyme immunoassay in buffer (IC_{50} = 0.1 and 2 μg/L, respectively). The mixed-mode solid-phase extraction used for sample preparation to reduce possible urinary matrix effects allowed the accurate measurement of 3-PBA levels as low as 1 μg/L. The automated chemiluminescent immunoassay described here is sensitive, simple to use, and more rapid than the previously reported standard microplate assay.

High-throughput screening (HTS) technologies provide speed and efficiency, allowing many measurements to be made in a short period of time, thus facilitating the acquisition of large quantities of high-quality data. Automation is a critical contributor to HTS methodologies. Specialized robotics are often used for much of the assay process, from loading samples through final data analysis. An HTS robot can usually prepare and analyze many HTS plates simultaneously, further speeding the data collection process. HTS robots that can test up to 100 000 compounds per day currently exist.[1] Zweigenbaum and Henion[2] demonstrated that an HTS-enabled tandem LC/MS (liquid chromatography-mass spectrometry) instrument equipped with an autosampler, automated sample preparation, and data analysis software could significantly reduce the time and labor required for chromatographic separation and detection of multiple analytes in human plasma samples.

Recently, immunoassay automation has emerged as an innovative area for research and development in the clinical diagnostic industry. In this case, automation can reduce labor requirements, costs, and turnaround times. Automation has also been demonstrated to result in improved assay performance based on individual improvements in assay precision, sensitivity, dynamic range, as well as the elimination of sample handling/processing and data-reduction errors resulting from manual operations.[3] Multiplexed fluorescence-based immunoassay technology has recently been introduced as an automatic high-throughput format for multianalyte screening. It has been shown that fluorescent flow-cytometric assays combined with the use of differently sized or fluorescently labeled polystyrene microbeads can be used to perform specific and quantitative immunoassays for multiple biomarker analytes,[4] e.g., cytokines as indicators of inflammation,[5−8] and environmental analytes, e.g., glyphosate, atrazine, and the mercapturate of metolachlor[9] in urine of exposed farm workers. Although, according to one manufacturer, it is theoretically possible to expand this technique to up to 100 analytes, it must be considered that cross-reacting analytes can complicate the interpretation of results and the selection of multiple analytes will be limited by the sample preparation methods and assay conditions required for each analyte.

Unlike the multiplexed bead immunoassays, automated chemiluminescent (CL) immunoassay is used to measure a single analyte per sample. Nevertheless, CL-based immunoassays are

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emerging as an attractive option. CL immunoassays do not require an enzymatic substrate, an inhibitor to stop the reaction, or an excitation source for a fluorescent dye. Sensitive assay methods can be developed and optimized due to the high quantum yield and the rate for the CL reaction and the efficiency of the light detector as an alternative to the colorimetric detection of enzyme-conjugated immunoassays (ELISA).[10,11] In place of the chromogenic substrate used in the conventional ELISA, examples of CL signal include luminol oxidation by hydrogen peroxide (H_2O_2) and the enzyme horseradish peroxidase (HRP)[12] and the enzyme-independent oxidation of acridinium ester in dilute alkaline hydrogen peroxide.[13] The reaction rate of CL-based signal generation is fast compared to colorimetric ELISA methods with a signal lifetime of 1−2 min.

Automated CL immunoassays, performed by the ACS:180 analyzer used for the study reported here have been performed for diagnostic clinical chemistry applications such as the assays related to anemia, cardiac, oncology, reproductive, thyroid, and therapeutic drug monitoring.[14] The ACS:180 analyzer automatically performs up to 180 analyses per hour using paramagnetic particles for the automatic separation of the immunocomplex from the assay supernatant in a heterogeneous assay format. Direct chemiluminescence from an acridinium ester-labeled probe is used for high assay sensitivity. The ACS system is attractive for the rapid measurement of single analytes of interest and has been used for the sulfonylurea herbicide triasulfuron in soil samples (IC_{50} = 0.1 μg/L).[15]

Pyrethroid insecticides are widely used in agricultural, industrial, and residential applications to control insects. Since the organophosphate insecticides chlorpyrifos and diazinon have been phased out for residential applications due to suspected human toxicities, the use of pyrethroids has increased as a safer alternative class of insecticides. Although little is presently known about the hazardous effects of human exposure, animal studies have demonstrated that pyrethroid exposure may affect neurological development,[16,17] induce a cancer,[18] suppress the immune system,[19] and disrupt the endocrine systems.[20−22]

Recently, the Center for Disease Control and Prevention[23] reported that 3.3 μg/L was the 95th percentile urine concentration of 3-phenoxybenzoic acid (3-PBA), a general urinary metabolite and biomarker of exposure to some pyrethroid insecticides, found in much of the U.S. population. This value is similar to the 2 μg/L reference value set by the German Federal Environmental Agency,[24] indicating wide-ranging exposure. 3-PBA levels as low as 0.1 μg/L have been measured together with urinary biomarkers that indicate specific pyrethroid exposure (*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), 4-fluoro-3-phenoxybenzoic acid (F-PBA), and *cis*-3-(2,2-dibromovinyl-2,2-dimethylcyclopropane carboxylic acid (DBCA)) using an improved high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method.[25] Instrumental methods such as gas chromatography (GC) or HPLC combined with mass spectrometry (MS) are currently the methods of choice for large scale biomonitoring studies that screen for multiple analytes[25,26] and environmental monitoring.[27] Although these chromatographic MS techniques are well documented and give accurate results, they are prohibitive because of their requirements for personnel and budget necessary for the operation and maintenance of complex instrumentation, expensive isotopic standards, and complex sample preparation methods including extraction, cleanup, and possibly chemoselective derivatization.

As an alternative, immunoassays have been proven to be rapid, sensitive, relatively simple, and cost-effective analytical tools for routine monitoring applications.[28] Immunoassays can be formatted to use only a small volume of sample and prepared with relatively simple methods, as compared to those needed for subsequent instrumental analyses. Immunoassays are readily adaptable to high-throughput methods using the 96- or 384-microwell plates or cuvette-based autoanalyzer systems.[3]

The goal of this study was to adapt an immunoassay for a urinary biomarker of pesticide exposure to the well-established ACS:180 analyzer system currently used to measure many indicators of clinical effect. Thus, a researcher could use a single platform technology to measure both markers of exposure and effect. Such a strategy could be useful in geographic locations where economics limits technology or technology infrastructure.

EXPERIMENTAL SECTION

Chemicals and Instruments. Horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin (OVA), *N*-hydroxysuccinimide (NHS), and 1,3-dicyclohexylcarbodiimide (DCC), 3-phenoxybenzoic acid (3-PBA), Tween 20, acetonitrile (ACN), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF),

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casein, and NaN_3 were purchased from Sigma-Aldrich (St. Louis, MO). Acridinium C2 NHS ester (4-(2-succinimidyl-oxycarbonyl-ethyl) phenyl-10-acridinium-9-carboxylate trifluoromethyl sulfonate) was obtained from Assay Designs, Inc. (Ann Arbor, MI). Trigger solutions no. 1 and no. 2 to generate chemiluminescent signal were obtained from Bayer/Siemens (Los Angeles, CA). Goat antirabbit IgG-coated paramagnetic particles were obtained from Qiagen (San Diego, CA). Econo-Pac 10DG gel filtration columns were obtained from Biorad (Hercules, CA). The C18 solid-phase extraction (SPE) columns (500 mg/10 mL) were obtained from Varian (Harbor City, CA), and the Strata Screen-A mixed-mode SPE columns (200 mg/3 mL) were obtained from Phenomenex (Torrance, CA). A vacuum manifold having 20 ports was used for the cleanup of urine samples prior to immunoassay. The automated CL immunoassay was run on the ACS:180 bench top immunoassay analyzer (Bayer, Emeryville, CA). For the cross-reactivity test, 4-hydroxy-3-phenoxybenzoic acid, 4-fluoro-3-phenoxybenzoic acid, a glycine conjugate of 3-phenoxybenzoic acid, and a glucuronide conjugate of 3-phenoxybenzyl alcohol were synthesized in this laboratory.

Immunoreagents and Buffers. The specific antibody and competing heterologous hapten for the target analyte (3-PBA) were previously described by Shan et al.[29] Briefly, the polyclonal antibody (Ab 294) was produced against a conjugate of 3-((2-oxoethoxy)ethoxy)phenoxybenzoic acid and thyroglobulin. Potassium phosphate casein buffer (14.04 g/L $K_2HPO_4 \cdot 3H_2O$, 5.24 g/L KH_2PO_4, 1 g/L NaN_3, and 5 g/L casein, pH 7.0), normal strength PBS buffer (1× PBS; 8 g/L NaCl, 0.2 g/L KH_2PO_4, 1.2 g/L Na_2HPO_4, and 0.2 g/L KCl, pH 7.5), PBST (1× PBS containing 0.05% (v/v) Tween 20, pH 7.5), PBSB (1× PBS containing 0.5% (w/v) BSA, pH 7.5), and 0.05 M borate buffer (19.1 g/L $Na_2B_4O_7 \cdot 10H_2O$, pH 8) were used for the immunoassay.

Preparation of Acridinium Ester-Labeled Hapten−Protein Conjugate. The preparation of the 3-PBA−protein−acridinium ester (3-PBA−protein−A^+) conjugate is illustrated in Figure 1. Briefly, 3-PBA (6.5 mg, 0.03 mmol) was dissolved in 0.4 mL of dry DMF with NHS (5.2 mg, 0.045 mmol) and DCC (9.2 mg, 0.045 mmol). After 5 h of stirring at room temperature, the precipitated dicyclohexylurea was removed through filtration, and the amount (20-fold molar excess over protein) of the resulting activated ester solution of 3-PBA was added slowly to a solution of either HRP, OVA, or BSA in 1 mL of 0.05M borate buffer (pH 8.5). The mixture was stirred gently at 4 °C for 24 h to complete the conjugation, followed by dialysis against normal strength phosphate-buffered saline (1× PBS, pH 7.5) which was changed twice a day for 3 days to remove unreacted small molecules.

The acridinium C2 NHS ester (A^+, 1.1 mg) was dissolved in 0.55 mL of dry DMF. A 120 μL aliquot (5-fold molar excess of the acridinium ester over protein) of the total ester solution was slowly added with vigorous stirring into the 3-PBA−protein solution that had been adjusted to pH 8 with drops of 1 N NaOH. The mixture was stirred at room temperature. After 20 min, 100 μL of 10% lysine in the PBS (pH 8) was added and stirred for 15 min at room temperature to quench unreacted label. The resulting mixture was then transferred to an Econo-Pac 10DG gel filtration column, which had been washed and equilibrated with the PBS buffer, 1 mL fractions were collected, and the chemiluminescence activity of each fraction was measured. Fractions collected between 4 and 6 mL elution volumes were found to contain 3-PBA−protein−A^+ conjugate and were combined with 3 mL of 50% glycerin in water, aliquoted, and stored at less than −70 °C until use.

Analysis of the Densities of Hapten and Acridinium Tracer Labeled on BSA. For the determination of the degree of 3-PBA conjugation to BSA and of the degree of acridinium tracer conjugated to 3-PBA−protein conjugate, 3-PBA−BSA conjugate and the 3-PBA−BSA−A^+ conjugate were dialyzed against distilled water for 24 h to remove salts, and then a powder of each was obtained by lyophilization. The density of hapten conjugated to protein and that of acridinium tracer conjugated to 3-PBA−BSA conjugate was determined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparing the molecular weight of the standard BSA with that of the conjugates. MALDI spectra were obtained by mixing 0.5 μL of matrix (sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 5 mg/mL in 0.1% trifluoroacetic acid (TFA) in 65% ACN/water) and 0.5 μL of a solution of the conjugates (5 mg/mL in 5% formic acid) and spotting 0.6 μL to the MALDI target.

Automated Competitive Chemiluminescent Immunoassay for 3-PBA. The overall scheme for the 3-PBA assay formatted for the ACS:180 is illustrated in Figure 1. The assay was divided into the off-line competition step and, thereafter, to the on-line steps for immunocomplex separation and CL signal measurement. Briefly, 150 μL each of 3-PBA−protein−A^+ diluted in PBS containing 0.05% Tween 20 and polyclonal antibody diluted in the buffer was mixed in a 5 mL disposable plastic tube and preincubated for 30 min at room temperature. Equal volumes of 3-PBA standards or urine samples diluted in the buffer were added by pipet and incubated for at least 30 min at room temperature for the competition step. For the automatic, on-line steps, 200 μL aliquots of the mixture in each assay tube were added to each cuvette. After 7 min of incubation at 37 °C in the instrument, a 250 μL aliquot of goat antirabbit paramagnetic particles (GRPP) diluted 1:4 with buffer was added to each cuvette, and the resultant mixture was incubated for a further 5 min at 37 °C. The particle-adsorbed immunocomplexes in each cuvette were then automatically washed twice with 250 μL of deionized water by magnetic separation, and the CL signal was determined by adding a 250 μL aliquot of the trigger solution no. 1 to each cuvette with an equal volume of the trigger solution no. 2. The CL emission was then integrated over 5 s and expressed as photon counts. Finally, the signal of the buffer only (blank) was subtracted, and the data were plotted using a four-parameter logistic curve fitting algorithm.

Cross-Reactivity. The optimized assay was used to run cross-reactivity (CR) studies by using a standard solution of the permethrin metabolites and other structurally related compounds in MeOH stock solutions. The test compounds are listed in Table 1. The CR was calculated as a percentage with the following formula: (IC_{50} of the target analyte/IC_{50} of the tested compound) ×100%.

Urine Sample Preparation. *Cleanup with C18 Solid-Phase Extraction (C18 SPE).* Urine samples were kept at −20 °C without any treatment. After thawing, each sample was completely

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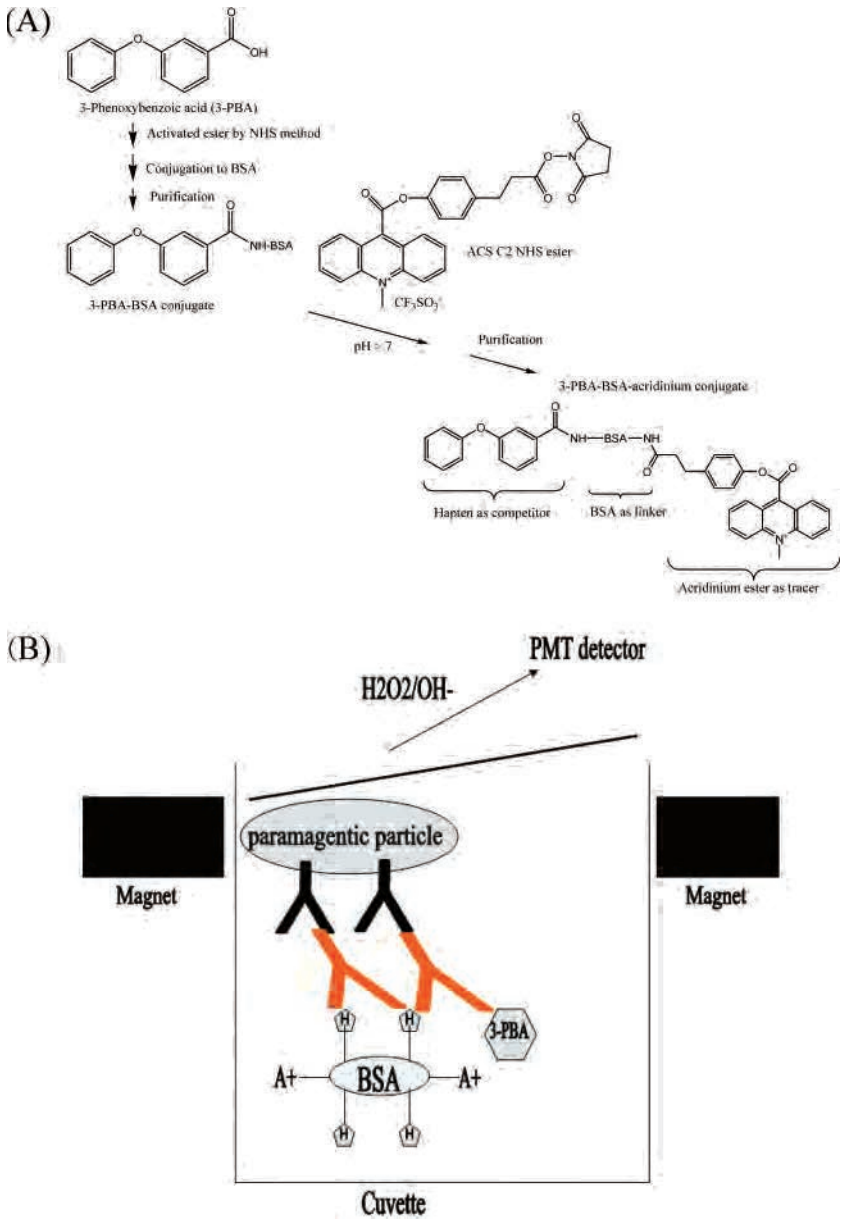


Figure 1. Scheme of the preparation of 3-PBA−BSA−A^+ conjugate (A) and the assay format (B). H, competing hapten; A^+, acridinium ester; Y, primary polyclonal antibody specific to 3-PBA; paramagnetic particle-Y, secondary antirabbit IgG-coated paramagnetic particle; PMT, photomultiplier tube, after magnetic separation and washing (solid line), chemical reaction generates light followed by measurement by PMT.

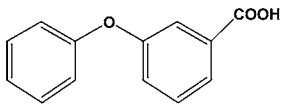
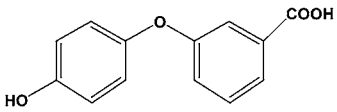
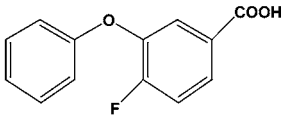
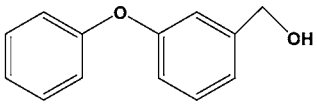
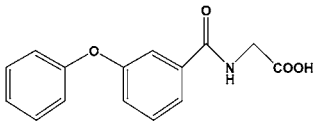
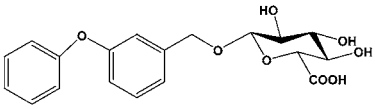
homogenized. Each C18 column was preconditioned with 3 mL of MeOH, 3 mL of 0.1% TFA in 1:1 MeOH/deionized water, and 3 mL of 0.1% TFA in deionized water. Each urine sample (0.5 mL) was then loaded onto the column and eluted with 2−4 mL/min flow rates. Columns were subsequently washed with 10 mL of 0.1% TFA in water and 3 mL of 60:40 MeOH/water with 0.1% TFA. After drying under high vacuum for 15 min, each column was finally eluted with 3 mL of MeOH or ACN (for further cleanup with liquid−liquid extraction). The eluate was evaporated to dryness under a stream of nitrogen gas, dissolved in 0.5 mL of 10% MeOH in PBS, and diluted up to 50 times with PBST prior to assay.

Further Cleanup with Liquid−Liquid Extraction (LLE) of the Acetonitrile Eluate. For further cleanup, the ACN eluate was washed twice with 2 mL of hexane to remove possible lipid coextractants. The ACN layer was then evaporated to dryness under a gentle stream of nitrogen gas, dissolved in 0.5 mL of 10% MeOH in PBS, and then diluted up to 20 times prior to assay.

Cleanup with Mixed-Mode SPE (C8 + Strong Anion Exchange, 200 mg Screen-A Tube). Each mixed-mode column was preconditioned with 1 mL each of MeOH, water, and sodium acetate buffer (pH 4). Urine sample (0.5 mL) was loaded onto the column, and an equal volume of sodium acetate buffer was added to it. Each column was subsequently washed with 2 mL of deionized water and 2 mL of MeOH. After drying under high vacuum (10 inches Hg) for 10 min, each column was finally eluted with 3 mL of 1% acetic acid in a 70:30 hexane/ethyl acetate mixture. The eluate was evaporated to dryness as described above, dissolved in 0.5 mL of 10% MeOH in PBS, and then diluted up to 5 times with PBST prior to assay.

Data Analysis. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which

Table 1. CRs (%) of Possible Metabolites with a Phenoxybenzyl Moiety in the 3-PBA Immunoassay

Compound	Chemical structure	CR (%)
3-Phenoxybenzoic acid (3-PBA)		100
4′-Hydroxy-3-phenoxybenzoic acid (4′-OH-3-PBA)		126
4-Fluoro-3-phenoxybenzoic acid (F-3-PBA)		72
3-Phenoxybenzyl alcohol (3-PBAlcohol)		0.8
Glycine conjugate of 3-PBA (3-PBA-Glycine)		2.4
Glucuronide conjugate of 3-PBAlcohol (3-PBAlc-glucuronic acid)		0.2

were fitted to a four-parameter logistic equation, $y = (A - D)/[1 + (x/C)^B] + D$, where A is the maximum signal at no analyte, B is the curve slope at the inflection point, C is the IC_{50}, and D is the minimum signal at infinite concentration. The IC_{50} value was expressed as the sensitivity of the immunoassay. Signal-to-noise (S/N) ratio was calculated from maximum signal (A)/minimum signal (D) from the above equation.

RESULTS AND DISCUSSION

Preparation of Acridinium Ester-Labeled Hapten−Protein (3-PBA−BSA−A^+) Conjugates and Their Comparison for the Immunoassay. Three proteins, BSA, HRP, and OVA, were evaluated as protein linkers between 3-PBA and the chemiluminescent acridinium ester. Neither 3-PBA−HRP−A^+ nor 3-PBA−OVA−A^+ conjugates produced standard curves with a low nonspecific binding. In addition, neither of these conjugates resulted in an assay more sensitive than the previously reported ELISA (data not shown). However, the 3-PBA−BSA−A^+ conjugate functioned well and its use generated a calibration curve with a S/N for required sensitivity and an optimal sigmoidal curve shape for the required dynamic assay range (Figure 2). The MALDI-TOF-MS results showed the successful conjugation of 3-PBA to BSA and acridinium ester tracer to the 3-PBA−BSA conjugate with a molar ratio of 16:1:5 3-PBA/BSA/A^+. BSA provides a convenient conjugation linker with multiple primary amines that can be used as conjugation points for NHS-esters of acridinium and 3-PBA and facilitates the easy separation of conjugate from unreacted chemiluminescent label/3-PBA with size exclusion methods. HRP and OVA may also be suitable linkers, but further studies would be needed to determine the cause of the high background.

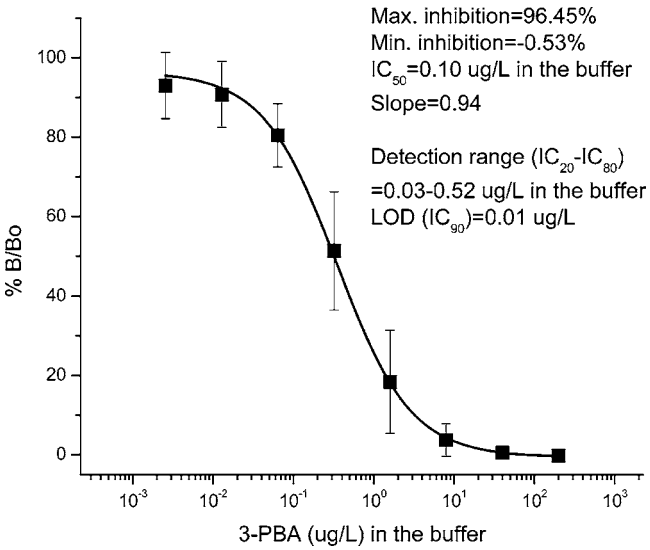


Figure 2. Chemiluminescent immunoassay (CL) inhibition curves for 3-PBA. Immunoreagent concentrations: antiserum no. 294, 1/48 000 (final dilution in cuvette); 3-PBA−BSA−A^+ conjugate, 1/72 000 (final dilution in cuvette). Calbration standards were prepared in PBST. The standard curve represents the average of 10 curves (±standard deviation) plotted from data obtained for 3 months. LOD, limit of detection.

Assay Optimization for the Immunoassay. *Optimal Concentration of Immunoreagent.* In order to determine the optimum concentrations of the 3-PBA−BSA−A^+ conjugate and Ab 294 for the assay, the effects of different concentrations of the immunoreagents on the assay were evaluated. Although 3-PBA−BSA−A^+ conjugate (5 mg/mL) showed the best sensitivity at a maximum 1 152 000-fold dilution under the conditions of certain concentrations of antibody, the dilution of 1:72 000 provided the highest S/N ratio of 8 and so was selected for further study (Supporting Information Figure 1A). As a maximum concentration, the 48 000-fold diluted antibody provided a S/N ratio of 16 as well as good sensitivity and was selected for use in the assay (Supporting Information Figure 1B).

Assay Buffer and Incubation Conditions. The buffer conditions were originally optimized for a 3-PBA ELISA format based on a hapten-coated microplate, so it was necessary to determine the optimal assay buffer conditions specifically suited for the CL format described in this report. Increasing the amounts of solvents (MeOH and DMSO) in the PBST decreased assay sensitivity and suppressed assay signal (Supporting Information Figure 1, parts C and D). However, acceptable assay sensitivity was observed when the proportion of added solvents was less than 5% of the total volume.

Buffers containing different protein "blockers" such as casein and BSA or a surfactant (Tween 20) were evaluated for their abilities to minimize the nonspecific binding. The standard buffer used for automated CL by our group, potassium phosphate with 0.5% casein, was again demonstrated to produce calibration curves with a S/N ratio of 9 (Supporting Information Figure 1E). On the other hand, a hook effect was often observed with this assay buffer resulting in a markedly reduced signal level when the 3-PBA concentration was zero (Supporting Information Figure 2). This hook effect could be caused by decreased saturation of the antibody binding sites with competing 3-PBA−BSA conjugate, thus inhibiting the formation of the immunocomplex and making this buffer a poor selection for the assay described here. After continued evaluation, PBS added with Tween 20 was observed to eliminate the "hook effect" and provided a S/N of 160. The optimum assay buffer was determined to be 0.15 M PBS, pH 7.5 plus 0.05% Tween 20.

Assay sensitivity was further enhanced by preincubating the antibody and 3-PBA−BSA−A^+ prior to adding the 3-PBA returning the IC_{50} value to that observed when PBS containing casein was used without the "hook effect" (Supporting Information Figure 3). Optimum incubation conditions were observed to be as follows: 30 min preincubation of 3-PBA−BSA−A^+ and Ab 294 followed by a 30 min incubation with 3-PBA in sample or calibrator. Although assay IC_{50} values were not affected by higher ionic strength (Supporting Information Figure 1F), an absorbance reduction was observed for 2× PBS (0.3 M) that was likely the result of suppressed antibody−antigen binding interaction. This result suggests that the assay may be sensitive to the ionic strength changes in urine samples; therefore, a dilution or analyte purification/enrichment method (SPE, LLE, etc.) is recommended, the latter being preferable to minimize the dilution of analyte. Finally, the effect of varying the time for the competition incubation on assay sensitivity was evaluated with 0.5, 1, and 3 h and overnight time points. While assay sensitivity was not significantly different for the competition times tested, the maximum luminescent signal increased with increasing incubation times (Supporting Information Figure 1G). The optimized competition incubation time was observed to be between 0.5 and 1 h for the assay described here. The optimized competitive immunoassay had a linear range (IC_{20-80}) of 0.03−0.52 μg/L 3-PBA and an IC_{50} value of 0.1 μg/L 3-PBA. The LOD in buffer was calculated as 0.01 μg/L, the IC_{10} value (Figure 2).

The optimized chemiluminescent paramagnetic particle-based immunoassay used 3-PBA−BSA−A^+ conjugate at a dilution of 1/72 000, Ab 294 at a dilution of 1/48 000, and antirabbit IgG paramagnetic particles at a dilution of 1/4.

Cross-Reactivity (CR). All reported possible metabolites derived from the phenoxybenzyl moiety of pyrethroids were evaluated for CR in this report (Table 1). Since the antibody was generated against a hapten, 3-(3-(2-oxoethoxy)phenoxy)benzoic acid that was synthesized to preserve the carboxylic acid (−COOH) group of 3-PBA, this immunoassay showed broad specificity for 3-PBA (100%), 4′-OH-3-PBA (121%; which is further metabolized from 3-PBA), and F-3-PBA (72%) which is derived from cyfluthrin. As expected, little CR was observed for 3-PBA-glycine, 3-phenoxybenzyl alcohol, and 3-phenoxybenzyl alcohol−glucuronide conjugate where the −COOH group is no longer exposed. Unlike chromatographic separation and detection methods that can provide nonspecific detection of the separated analyte, this immunoassay can be used to monitor 4-OH-3-PBA and F-3-PBA as well as 3-PBA. As expected, since there is no −COOH group exposed in the parent compounds, the recognition of the antibody for the native pyrethroid insecticides is negligible.[29] The IC_{50}s of the parent pyrethroid insecticides, esfenvalerate, cypermethrin, permethrin, deltamethrin, or cyfluthrin were over 10 000 μg/L, and the corresponding CRs are close to 0%.

Sample Cleanup of Urine. Because simple 50-fold dilution of the urine sample showed various signal differences at low concentration from individual urine samples (Supporting Information Figure 4), it was concluded that a sample cleanup method that reduced problems associated with urine matrix prior to immunoassay was needed. Although the automated CL immunoassay results coupled to C18 SPE sample cleanup facilitated acceptable recoveries with a 1:50 dilution of eluate (calculated on the basis of the original urine volume loaded onto SPE) (Table 2), the assay was limited to quantify those urine samples with concentrations >5 μg/L. The additional LLE step with hexane to remove possible lipid coextractants reduced the matrix-associated signal at a 1:20 dilution to approximately 1.5 μg/L. Sample preparation with mixed-mode SPE strong cation exchange gave excellent sensitivity and good average recovery of 91.7% for all tested concentrations with a 1:5 dilution prior to immunoassay (Table 2), and mixed-mode (C8 + strong anion exchange) solid-phase extraction of samples facilitated measurements of 3-PBA levels as low as 1 μg/L in urine with the immunoassay described here.

Assay Comparisons. With the spiked samples prepared by the mixed-mode SPE, the automatic luminescent paramagnetic particle-based immunoassay showed a tendency for underprediction of 3-PBA (Figure 3A, slope = 0.73) compared to the ELISA (Figure 3A, slope = 1.05). This might result from unresolved matrix effects or an unidentified systematic error. The assay

Table 2. Recovery of 3-PBA from Spiked Urine Samples, Prepared by C18 SPE and Mixed-Mode SPE (C8 + Strong Anion Exchange)

	C18 SPE			mixed-mode SPE
3-PBA spiked (μg/L)	urine no. 1 SPE/50-fold diln.	urine no. 2 SPE/50-fold diln.	urine no. 2 SPE/LLE with ACN and hexane/ 20-fold diln.	urine no. 2 SPE/5-fold diln.
0	3.17 ± 0.26	3.87 ± 0.03	1.39 ± 0.18	0.65 ± 0.10
1	*a*	*a*	*a*	1.21 ± 0.18 (121% ± 17.43%)
3	*a*	*a*	*a*	2.71 ± 0.24 (90% ± 7.99%)
5	4.55 ± 0.38 (91% ± 7.61%)	5.75 ± 0.93 (115% ± 18.73%)	5.25 ± 0.39 (105% ± 7.76%)	3.85 ± 0.18 (77% ± 3.62%)
10	*a*	*a*	*a*	7.85 ± 0.23 (79% ± 2.12%)
15	14.39 ± 1.29 (96% ± 8.62%)	13.81 ± 1.97 (92% ± 13.15%)	11.08 ± 0.93 (74% ± 6.25%)	*a*

[a] Not tested.

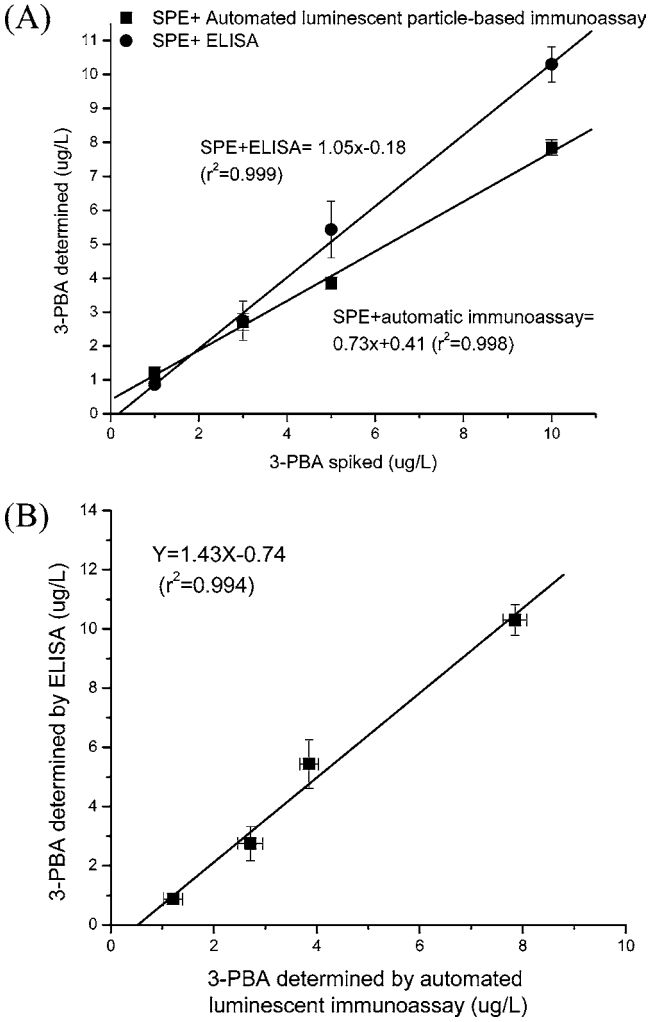


Figure 3. Correlations between the spiked and measured concentrations of 3-PBA for ELISA and automated immunoassay coupled with SPE (A) and between the concentrations measured by the automated immunoassay and ELISA (B). The ELISA was performed according to the method reported previously (ref 29) after the mixed-mode SPE for sample preparation.

comparison between the automated immunoassay and ELISA was achieved using the mixed-mode SPE method to minimize the matrix effect of urine samples which were spiked with 0−10 μg/L 3-PBA. Linear regression results showed a good correlation (r^2 = 0.994) with a slope of 1.43 (Figure 3B). These results demonstrate that the assay is appropriate for the detection of 3-PBA at low levels commonly found in urine samples.

CONCLUSION

The present report describes the successful development of a sensitive, automated CL immunoassay for use as a rapid high-throughput tool for the detection of 3-PBA in the urine of exposed human populations. This assay featured improved sensitivity with a low IC_{50} value of 0.1 μg/L for 3-PBA in buffer and an improved dynamic range of 0.03−0.52 μg/L, when compared to the previously reported microplate-based ELISA. In this case, the improved sensitivity likely results from the high affinity of antibody paired with a sensitive chemiluminescent probe and a reliable automated assay platform for liquid handling, magnetic separation, and data collection. Preincubation of the CL probe with antibody and the fact that binding events are occurring in solution rather than at a surface also likely contributed to the sensitivity of the assay. On the basis of the observed cross-reactivities, the assay can broadly monitor F-PBA and 4-OH-3-PBA as well as 3-PBA for studies monitoring human exposure to pyrethroid insecticides. The simple coupling method to prepare the conjugate containing both a competitive hapten and a signal reporter and the assay format using the automatic analyzer can be applied to other urinary biomarkers such as DCCA, the specific metabolite of some pyrethroids such as cypermethrin, permethrin, and cyfluthrin, to provide more information on human exposure to pesticides of interest.[30] This collection of urinary biomarkers together with appropriate meta data can provide information to further identity whether original exposures were to parent compounds or breakdown products themselves.

For optimum immunoassay performance, a sample preparation method was needed to reduce the urinary matrix effects. A mixed-mode (both C8 and strong anion exchange) SPE was observed to facilitate measurements as low as 1 μg/L of 3-PBA in urine. In comparison with conventional coating antigen-immobilized

(30) Ahn, K. C.; Ma, S.-J.; Tsai, H.-J.; Gee, S. J.; Hammock, B. D. *Anal. Bioanal. Chem.* **2006**, *384*, 713−722.

Table 3. Analytical Comparison between the Conventional ELISA and Automated Chemiluminescent Magnetic Particle-Based Immunoassay for 3-PBA

	indirect coating antigen-immobilized ELISA	automated immunoassay
immunoreagents:		
polyclonal antibody (dilution)	1:10000 (in the well)	1:48000 (in the cuvette)
competitive hapten−BSA conjugate (dilution)	1:5600 (from 2.8 mg/mL of 3-PBA−BSA conjugate)	1:72000 (from 5 mg/mL of 3-PBA−BSA−A^+ conjugate, in the cuvette)
secondary antibody (dilution)	1:3000 (from 1.1 mg/mL of IgG−HRP conjugate)	1:4 (0.1 mg IgG/mg magnetic particles/mL of antibody-coated paramagnetic particles)
total volume (μL) of the mixture of immunoreagents	100 μL in the well (sample and antibody)	450 μL in the cuvette (competitive hapten, sample, and antibody)
analytical sensitivity:		
IC_{50} (μg/L)	1.65	0.10
IC_{20-80} (μg/L)	0.20−5.0	0.03−0.52
LOD (μg/mL)	0.1	0.01
competition time between hapten and analyte to bind to antibody	1 h (the mixture of Ab and sample in 3-PBA−BSA coated well)	1 h (30 min for premixing Ab and sample, follow by 30 min of incubation after addition of 3-PBA−BSA−A^+)
analytical time after a competition step	96 results in a 96-well microplate in approximately 2 h with incubation times for the secondary antibody and color development	130 results in 1 h with the first result available in 15 min and subsequent results every 20 s
analytical procedure	hand-operated	automatic

microplate-based ELISA developed for 3-PBA (Table 3), the automated chemiluminescent 3-PBA immunoassay confers the following advantages: the sensitive chemiluminescent probe and specific antibody allow for sufficient reagent dilution to allow for assays approximately 20-fold more sensitive than the previous 3-PBA ELISA, thus allowing for the great economy of scale necessary for large studies; the enhanced sensitivity is advantageous as samples may be diluted more to decrease matrix interferences, while still maintaining the desired limit of quantitation; the ACS:180 provides the first result within 15 min of run time and produces up to 130 results per hour. As such, employing a fully automated analyzer after a competitive incubation step supports a high-throughput assay for population-based human studies which require the analyses of many samples by broadly applicable methods such as that described in this report. In addition, this classical platform is used with other markers such as hormones indicative of reproductive health.[31] With the ability to measure pesticide metabolites, a single platform can be used to measure both exposure and effect.

(31) Lohstroh, P.; Dong, H.; Chen, J.; Gee, N.; Xu, X.; Lasley, B. *Biol. Reprod.* **2006**, *75*, 24−33.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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APPENDIX II – Abstracts of Meeting Presentations

Development of a Sensitive Enzyme-Linked Immunosorbent Assay for the Detection of a Human Urinary Biomarker, 3-PBA

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Pyrethroids are widely used in agriculture as insecticides. In this study, we describe a sensitive immunoassay for the detection of PBA-glucuronide conjugate, a putative pyrethroid metabolite that may be used as a biomarker of exposure to pyrethroids. Four antisera were elicited against two different immunizing haptens. Antisera were characterized with several coating haptens. The best result was obtained with antiserum 1891 and PBA-BSA conjugate for coating giving an IC_{50} of 0.5 ng/mL. Antiserum 1891 was highly specific to target compound with overall CR <0.3% with tested compounds. Assay sensitivity was negligibly affected by a pH of 4-9. A five-fold improvement in IC_{50} was observed at 10X PBS with maximal absorbance almost same as that of 1X PBS. Good recovery and correlation were observed between spike and detection levels.

H.-J. Kim, K.C. Ahn, S.J. Ma, S.J. Gee, B.D. Hammock. ”Development of a sensitive enzyme-linked immunosorbent assay for the detection of a human urinary biomarker, 3-PBA” presented at the 232nd National Meeting of the American Chemical Society, September 10-14, 2006, San Francisco, CA (poster presentation).

An Immunoassay for a Human Urinary Metabolite of the Pyrethroid Insecticide Permethrin

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Permethrin is the most popular synthetic pyrethroid insecticide in agriculture and public health. For assessment of human exposure to permethrin, a competitive indirect enzyme-linked immunosorbent assay based on a polyclonal antibody for the detection of the glycine conjugate (DCCA-glycine) of a major metabolite, *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), of permethrin was developed. Various *cis*- and *trans*-haptens mimicking the target analyte were synthesized and conjugated with bovine serum albumin (BSA) and thyroglobulin by the active ester and diazotization methods. Sixteen polyclonal antibodies generated against hapten-thyroglobulin conjugates as immunogens and ten hapten-BSA conjugates as coating antigens were screened and homologous and heterologous ELISA systems were selected. The antibodies with highest sensitivity were optimized and characterized. The IC_{50} values for *trans*- and *cis*-target isomers of DCCA-glycine, in the assay buffer were 1-2 µg/L and 0.4-1 µg/L, respectively. These assay showed high selectivity for each isomer. Little or no cross reactivity for the parent compound and other free metabolites or glycine conjugates was measured. The immunoassay combined with solid phase extraction (SPE) which was used to reduce the matrix effect from human urine samples provided the analysis of total *cis/trans*-DCCA-glycine at ppb levels in the samples. This assay may be suitable for monitoring human exposure to permethrin.

Ahn, K.C., Watanabe, T., Gee, S.J., and Hammock, B.D. An Immunoassay for a human urinary metabolite of the pyrethroid insecticide permethrin. NIEHS Superfund Quad-University/EPA Region 9 Conference, Berkeley, CA October 8-10, 2004. “Emerging Scientific Issues for Superfund”

Development of Immunoassays to Evaluate Human Exposure to Permethrin

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The insecticide permethrin is one of the most commonly employed pyrethroids in agriculture and public health. For assessment of human exposure to permethrin, we have developed competitive indirect enzyme-linked immunosorbent assays (ELISAs) based on polyclonal antibodies for the detection of its possible major human urinary metabolites. The target analytes, 3-phenoxybenzoic acid (3-PBA), *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), glycine conjugates of 3-PBA and DCCA and a glucuronide conjugate of 3-phenoxybenzyl alcohol (3-PBalc) are proposed as permethrin biomarkers in human urine. The ELISAs developed with the processes of synthesis of haptens, production of antibodies, screening of antibodies against haptens in the ELISA development, and optimization and validation of ELISA showed good sensitivity, with the low IC_{50} values of 1.65 µg/L for 3-PBA, 0.40 µg/L for 3-PBA-glycine and 2.06 µg/L for DCCA-glycine, respectively. Currently, ELISAs for DCCA and 3-PBalc-glucuronide conjugate are being developed. The immunoassays with simple dilution and combined with solid phase extraction (SPE) which were used to reduce the matrix effect from human urine samples provided the analysis of each target analyte at ppb levels in the samples. This assay may be a useful monitoring tool for evaluating human exposure to the insecticide in terms of rapidity, sensitivity and practicality.

Ahn, K.C., Ma, S.-J., Gee, S.J. and Hammock, B.D. Development of immunoassays to evaluate human exposure to permethrin. 227th National Meeting of the American Chemical Society, Anaheim, CA, March 28-April 1, 2004.

Monitoring Human Exposure to Pesticides Using Immunoassay

Shirley J. Gee[1], Marja Koivunen[1], Ki Chang Ahn[1], Mikaela Nichkova-Doseva[1], Dosi Dosev[2], Ian M. Kennedy[2] and Bruce D. Hammock[1]

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To assess risk from exposure to pesticides it is imperative to measure the internal exposure and to determine the relationship between the internal exposure and potential health effects. One method for exposure assessment is to measure the amount of pesticide or pesticide metabolites in body fluids such as blood, urine and saliva. Comprehensive studies involving hundreds of samples result in high cost and time consuming analysis. One alternative analytical method for biomonitoring studies is immunoassay. Used extensively in clinical diagnostics, immunoassays are sensitive, selective and well suited to measurement in biological fluids. Recently conducted biomonitoring studies for paraquat and atrazine will be used to illustrate the strengths and weaknesses of the immunochemical method. Higher throughput and greater sensitivity are goals toward improving the utility of immunoassays for large-scale monitoring studies. Lanthanide oxide nanoparticles are promising fluorophores in biochemistry because of their large Stokes shift, sharp emission spectra, long lifetime and lack of photobleaching. The use of these nanoparticles in a multiplexed assay and for visualizing protein micropatterns as well as other strategies such as the adaptation of pesticide metabolite assays to an autoanalyzer using chemiluminescent acridinium labels and development of flow-through systems will also be presented.

Gee, S.J., Koivunen, M., Ahn, K.C., Nichkova-Doseva, M., Dosev, D., Kennedy, I.M. and Hammock, B.D. Monitoring human exposure to pesticides using immunoassay. American Chemical Society, San Diego, CA, March 28-April 1, 2005

Development of Immunoassays for Urine Biomarkers to Study Human Exposure to the Pyrethroid Insecticide Permethrin

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Department of Entomology, University of California Davis

The insecticide permethrin is one of the most commonly used pyrethroids in agriculture and public health. For risk assessment of human exposure to permethrin, we have developed competitive indirect immunoassays based on polyclonal antibodies for the detection of possible major human urinary metabolites of permethrin. The target analytes, 3-phenoxybenzoic acid (3-PBA), *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), glycine conjugates of 3-PBA and DCCA and a glucuronide conjugate (3-PBAlcG) of 3-phenoxybenzyl alcohol (3-PBAlc) are proposed as permethrin biomarkers in human urine. ELISAs had IC_{50} values of 2 µg/L for 3-PBAlcG and 11-22 µg/L for *cis/trans*-DCCA, respectively.
An improved automatic immunoassay using a chemiluminescent reporter and magnetic separation technique showed an IC_{50} value of 0.3 µg/L for 3-PBA in the buffer. These assays are being improved to provide a useful monitoring tool for evaluating human exposure to the insecticide in terms of rapidity, sensitivity and practicality.

Ahn, K.C., Kim, H.J., Gee, S.J. and Hammock, B.D. Development of immunoassays for urine biomarkers to study human exposure to the pyrethroid insecticide permethrin. American Chemical Society Meeting, Washington, DC, August 28-September 1, 2005.

Application of Immunoassays in Human and Environmental Monitoring

Ki Chang Ahn, Shirley J. Gee, Andres Gonzalez, Hee Joo Kim, Marja Koivunen, Mikaela Nichkova-Dosev, Eun-Kee Park, Donald Stoutamire, Bruce D. Hammock

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In environmental chemistry and monitoring of human health, the apparent strength of immunoassays is in processing large numbers of samples with minimal clean up and with a high degree of accuracy and precision. Enzyme linked immunosorbent assays (ELISAs) have dominated the field due to their ease of automation. Several examples will be given demonstrating the power and limitations of ELISA assays. In most cases, we develop immunoassays for the detection of parent toxin as illustrated by assays to TCDD, the herbicides atrazine and paraquat, and the pyrethroid insecticides. In addition, we normally develop immunoassays for key metabolites. This procedure is well illustrated by atrazine where little if any of the parent molecule is detected in urine but the mercapturate of atrazine is a dominant metabolite in urine. Recently we demonstrated that the mercapturate was still a dominant metabolite and thus a useful biomarker even at very low exposures by monitoring human metabolism of atrazine using accelerator mass spectrometry. In contrast, we were able to use immunoassays for the parent paraquat in human urine in support of a large epidemiological effort in Costa Rica. The pyrethroid insecticides are now the dominant insecticides in the world with a variety of active ingredients. To address human exposure with this group of compounds we have developed assays for metabolites that are class selective and compound selective. Although ELISA has proven to be a reliable technique, methods of greater sensitivity and higher sample throughput are needed. Several approaches are being taken in this laboratory and in collaboration with other groups to improve immunoassays by using specific peptides as coating antigens, developing biosensor formats, and using new labels to report antibody binding.

Ahn, K.C., Gee, S.J., Gonzalez, A., Kim, H.J., Koivunen, M.E., Nichkova-Dosev, M., Park, E.K., Stoutamire, D.W. and Hammock, B.D. Application of immunoassays in human and environmental monitoring. Society of Toxicology, San Diego, CA, March 5-9, 2006.