Analysis of the Synthetic Pyrethroids, Permethrin and 1(*R*)-Phenothrin, in Grain Using a Monoclonal Antibody-Based Test

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A monoclonal antibody generated to the synthetic pyrethroid-related hapten, (3-phenoxybenzyl)-2,2-dimethylcyclopropane-1,3-dicarboxylate–protein conjugate, was used to develop assays for determination of permethrin and 1(*R*)-phenothrin in wheat grain and flour milling fractions. The earlier 3-h assay was simplified using two approaches. The antibody was directly conjugated to the enzyme horseradish peroxidase (HRP), which removes a separate incubation and washing step from the assay. Also, an assay has been developed using microwell-bound monoclonal antibody and a HRP-labeled 3-phenoxybenzoic acid derivative. These assay formats have advantages in increased sensitivity and, in the case of the latter assay, accuracy with grain and flour samples. The most sensitive assay format could detect 1.5 ng/mL permethrin; 50% inhibition of antibody binding occurred at 10 ng/mL. These values corresponded to 75 and 500 ppb, respectively, in the original wheat sample. Methanol was the most effective pyrethroid extractant. Use of a simple cleanup procedure for ground grain extracts improved ELISA accuracy but could be omitted for screening purposes.

INTRODUCTION

A variety of pesticides (grain protectants) is applied to wheat and barley, to prevent infestation with insects during storage. Some compounds may also be applied at grain export terminals or by farmers storing grain on their farms. While considerable progress has been made in chemical-free storage of grain under controlled atmospheres or by grain chilling, these approaches will remain economical in only a minority of cases for some time to come. Modern grain protectants are designed to decompose appreciably before the grain is processed for consumption by humans.

One aim of our work is to enable the decentralization of residue testing in grain and cereal products by the development of simpler, faster, and less expensive test methods. This should enable the potential of load-by-load screening of export shipments at central grain terminals and of grain receivals from farmers and mills. Both semiquantitative screening methods (to check that residue levels in grain are below legally set maximum residue limits) and quantitative tests for use in regional or mill laboratories are being developed (Skerritt et al., 1990). In addition, tests are needed to ensure that grain has been adequately and evenly treated with protectants so that subsequent infestation will not occur. Conventional instrumental analytical techniques maintain their importance to confirm results for samples found to be violative in "field" screening tests.

The two main classes of chemical grain protectants most widely used include organophosphates and synthetic pyrethroids. Of the synthetic pyrethroids, bioresmethrin, permethrin, 1(*R*)-phenothrin, deltamethrin, and fenvalerate have been used in a number of countries. The synthetic pyrethroid of choice is most commonly used in conjunction with an organophosphate and the pyrethroid synergist piperonyl butoxide.

In Australia, these combinations are used because of the activity of pyrethroids against the lesser grain borer, *Rhyzopertha dominica*, for which most organophosphates are not effective. 1(*R*)-Phenothrin has been assessed in intensive field trials in a number of countries (Bengston et al., 1983; Desmarchelier et al., 1987). Although it is not as potent as bioresmethrin and tends to produce higher residue levels in flour and bread (Nambu et al., 1981), it is somewhat less expensive and shows better persistence on grain stored for long periods. Other applications of this compound include disinfection of aircraft and household surface insecticide sprays. Permethrin is similar to 1(*R*)-phenothrin in its spectrum of activity and stability on grain (Halls, 1981).

Due to the lower levels of application of permethrin and phenothrin, as compared to those of organophosphates such as fenitrothion, and their relative lack of volatility, many pyrethroids have been shown to be more difficult to determine by the conventional means of high-performance liquid chromatography and gas chromatography (Mestres et al., 1979; Bolygo and Zakar, 1983; Sharp et al., 1988). We have used the monoclonal antibody raised to a (3-phenoxybenzoyl)-2,2-dimethylcyclopropane-1,3-dicarboxylate hapten in three different assay formats to determine permethrin and phenothrin residues in stored grain products. Different extraction methods have been evaluated, and the effectiveness of cleanup procedures on ground grain will also be discussed.

EXPERIMENTAL METHODS

Materials. Monoclonal antibody Py-1 was produced in ascites fluids as described earlier (Stanker et al., 1989) and purified using affinity chromatography on protein G–Sepharose. Purified antibody was conjugated to horseradish peroxidase (HRP) using the method of Nakane and Kawoi (1974).

3-Phenoxybenzoic acid (3-Pba; Pierce, Rockford, IL) was coupled to bovine serum albumin (BSA) using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide as described earlier (Stanker et al., 1989). 3-Pba or 3-phenoxybenzyl alcohol was also coupled to HRP in two ways: directly using *N*-hydroxysuccinimide and

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Table I. Specificity of Pyrethroid Monoclonal Antibody in Different Assay Formats

	assay format			
	direct competitive (immobilized antigen)		immobilized antibody	
compound	acetonitrile	methanol	acetonitrile	methanol
1(*R*)-phenothrin (90 cis:10 trans)	10	10	18	10
1(*R*)-phenothrin (50 cis:50 trans)	NT[b]	NT	12	7
permethrin (cis)	15	10	18	10
permethrin (40 cis:60 trans)	NT	NT	40	25
deltamethrin	1000	750	1000	800
fenvalerate	2500	2500	2500	3000
cypermethrin	2500	2500	1500	2000
cyhalothrin	NT	NT	1000	1500
bioresmethrin	–[c]	–	–	–
tetramethrin	–	–	–	–
allethrin	–	–	–	–
bio(*d-trans*)allethrin	–	–	–	–
S-bioallethrin	–	–	–	–
chrysanthemum monocarboxylic acid	NT	NT	–	–
chrysanthemum monocarboxylic acid ethyl ester	NT	NT	–	–
piperonyl butoxide	–	–	–	–
fenitrothion	–	–	–	–

[a] Data shown are concentrations in ng/mL yielding 50% inhibition of antibody binding. [b] NT, not tested. [c] No inhibition at 1000 ng/mL.

with this reagent following amide formation with β-alanine. The latter HRP conjugate therefore had a three-carbon spacer arm.

Details of these syntheses are as follows: Succinimidyl 3-phenoxybenzoate (I) was prepared by addition of 3 mmol of *N*-hydroxysuccinimide to 2.5 mmol of 3-phenoxybenzoic acid in 10 mL of dichloromethane. Following addition of 3 mmol of dicyclohexylcarbodiimide, the mixture was stirred for 30 min at 0 °C and then for 5 h at 25 °C. The mixture was filtered and the product (in the filtrate) found to be homogeneous by thin-layer chromatography (in methanol/chloroform 5:95) and proton NMR spectroscopy. Yield was 95%.

(3-Phenoxybenzyl)succinimide succinate (II) was prepared using 3-phenoxybenzyl alcohol (5 mmol) reacted with succinic anhydride (10 mmol) in 5 mL of pyridine for 16 h at 20 °C. The product (67% yield) was isolated by column chromatography on silica gel, eluting with chloroform and then methanol/chloroform (5:95). Proton NMR spectroscopy data: δ 7.2 (m, 9 H, Ar), 5.10 (s, Ar CH_2O), 2.67 (s, 2 × $COCH_2$). This product was reacted with *N*-hydroxysuccinimide, as for 3-phenoxybenzoic acid, to yield II.

Dimethylformamide solutions of I or II (15 mg/mL, 50 μL) were added to 5 mg of HRP in 2 mL of 0.2 M potassium phosphate, pH 9.1 and mixed for 16 h at 4 °C. The HRP conjugates were desalted on Sephadex G-25 (Pharmacia, Uppsala, Sweden) before use.

Grain and Flour Samples. Permethrin-containing wheat was obtained from elevator storages of several tons of wheat treated at differing levels of permethrin in conjunction with fenitrothion. Experiments on antibody specificity (Table I) showed that fenitrothion did not interfere with permethrin determination. Representative samples of these were milled commercially to yield flour, bran, and germ fractions. In initial experiments, 500-g samples of pesticide-free wheat were spiked with a small volume (0.5–2 mL) of ether solutions of permethrin (a mixture of cis and trans isomers, 40:60 ratio) and 1(*R*)-phenothrin (a mixture of cis and trans isomers, 1:1 ratio) and, following evaporation of the solvent, were stored for 3 months in the dark at 20 °C before analysis. Residues of spiked pesticide aged in this way have earlier been shown to have extraction behavior identical to that of pyrethroids in commercially treated grain (*Sharp et al., 1988*).

Extraction of Pyrethroids. Extraction methods involve standing the grain (whole or ground) in 2.5 volumes of either methanol, acetonitrile, hexane, or acetone for 24–48 h with intermittent (3 × 5 min at 100 rpm) shaking. Whole meal and flour samples were extracted with 2.5 volumes of methanol, while germ was extracted using 5 volumes and bran using 10 volumes of methanol. A small volume (300 μL) of the hexane and acetone extracts was transferred to glass tubes and the solvent evaporated using a heating block (65 °C). An equal volume (300 μL) of methanol was then added to the samples prior to analysis. The methanol and acetonitrile extracts of ground grain underwent alumina cleanup. Small alumina columns were made by packing 1 g of basic alumina (Ajax, Sydney, Australia) into a glass pipet. The extracts were passed through the column, which retained the coextractives such as fatty acids and acidic proteins.

Instrumental Analysis of Permethrin and 1(*R*)-Phenothrin. These were analyzed by the Australian Wheat Board (Melbourne, VIC, Australia) using gas chromatography with electron capture detection for permethrin and high-performance liquid chromatography with ultraviolet detection for 1(*R*)-phenothrin. Grain or milling fractions were extracted for 48 h in hexane using intermittent agitation. Wheat was extracted in 1 volume of hexane, flour in 2.5 volumes, and bran or germ in 5 volumes. Milling fraction extracts were run through a C18 Sep-Pak column (Waters, Milford, MA) to remove coextractives prior to analysis.

ELISA Assay Formats. *1. Two-Step Indirect Competitive Assay.* 3-Pba–BSA, 500 ng in 100 μL of water, was added to wells of Nunc Maxisorp polystyrene plates (Roskilde, Denmark), and well contents were evaporated at 37 °C overnight. Following three washes in 50 mM sodium phosphate (pH 7.2)–150 mM NaCl (PBS)–0.001% Tween 20, unreacted sites were blocked using 0.1% fish skin gelatin (Sigma, St. Louis, MO)–PBS for 1 h at 20 °C. Blocking solution was removed, and then 50 μL of pyrethroid standards or cereal extracts (in 10% methanol in 1% BSA–PBS–0.001% Tween 20) was added, followed by 20 ng of Py-1 in 50 μL of BSA–PBS–Tween. The microwells were mixed and incubated 1 h at 20 °C. Following three washes, 100 μL of HRP-labeled rabbit-antimouse antibodies (DAKO, Glostrup, Denmark) diluted in BSA–PBS–Tween was incubated 30 min at 20 °C. After final washes, 150 μL of substrate–chromogen [3-ethylbenzothiazoline-6-sulfonic acid (Sigma) 4.5 mM in 100 mM sodium citrate buffer (pH 4.5) plus 0.003% hydrogen peroxide] was added, and color development was terminated after 15 min by addition of 50 μL of 3% (w/v) oxalic acid, giving a final volume of 200 μL. Absorbance was determined at 414 nm.

2. One-Step Direct Competitive Assay. After coating and blocking of the microwells as described above, 50 μL of pyrethroid standards or cereal extracts (in 10% methanol in 1% BSA–PBS–0.001% Tween 20) was added followed by 50 μL of a 1 ng/mL solution of HRP-labeled Py-1. The microwells were mixed and incubated at room temperature for 60 min. Following three washes, 150 μL of substrate–chromogen solution was added and color development was terminated after 10 min.

3. One-Step Assay with Immobilized Antibody. Py-1, 1 μg in 100 μL of 0.1 M carbonate buffer (pH 9.6), was added to wells of Nunc Maxisorp polystyrene plates and incubated for 1 h at 20 °C. Following three washes in PBS–0.001% Tween 20, unreacted sites were blocked using 0.1% *Teleostean* fish skin gelatin–PBS for 1 h at 20 °C. Blocking solution was removed, and then 50 μL of pyrethroid standards or cereal extracts (in 10% methanol in 1% BSA–PBS–0.001% Tween 20) was added, followed by 50 μL of a 0.8 ng/mL solution of HRP–Pba diluted in 1% BSA–PBS–0.001% Tween 20. The microwells were mixed and incubated for 30 min. After final washes, 150 μL of substrate–chromogen was incubated and color development was terminated after 10 min as described above.

Test Compounds. Pesticide standards of permethrin (technical grade, cis:trans 40:60 ratio) and phenothrin (technical grade, cis:trans 1:1) were obtained from Wellcome Australia, Cabarita, NSW. The compounds used in the cross-reaction study (Table I) were purchased from Chem Service (West Chester, PA).

RESULTS AND DISCUSSION

Assay Development. Earlier studies had shown that more sensitive ELISA results were obtained if protein–Pba conjugates were used, as solid-phase antigen, compared with pyrethroid–protein conjugates in the indirect ELISA. Initially, the antigen (3-Pba–BSA) was diluted to 5 μg/mL in 0.1 M carbonate buffer (pH 9.6), and 100 μL was added to each well and left to incubate overnight

at room temperature. As expected, we found the degree of inhibition was affected by antibody concentration; however, more notable was the significant increase in antibody activity (maximal absorbance) and improved inhibition when the antigen was diluted in distilled water and evaporated at 37 °C onto the plate as described by Stanker et al. (1989). Further experiments suggested that it was the evaporation process rather than the actual composition of the buffer that is important (results not shown).

We have successfully labeled 3-Pba with HRP either directly via the acid group or through a three-carbon spacer arm, which has allowed us to develop a faster and more sensitive competitive ELISA. In this assay, the antibody is immobilized on the microwell. The HRP–Pba coupled via the three-carbon spacer arm enabled an extremely sensitive assay to be developed, which used a 30-min sample incubation and a 10-min substrate step. In contrast, HRP conjugated directly to Pba was enzymically active but bound very weakly to immobilized antibody. Since nanogram quantities of the HRP–3 carbon spacer arm Pba conjugate bound well, the reduced binding of the former conjugate was probably due to steric effects. Free Pba did not exhibit any inhibition in the assay format, whereas free phenoxybenzyl alcohol exhibited potency similar to permethrin. This was probably due to the fact that the Pba is charged, whereas the alcohol is not.

Relative Sensitivity of Assay Formats. The limits of detection of the assays for permethrin when dissolved in methanol were as follows: (1) two-step indirect competitive assay, 5 ng/mL; (2) one-step direct competitive assay, 2 ng/mL; and (3) one-step assay with immobilized antibody, 1.5 ng/mL.

The permethrin concentration–inhibition plots for the three assay formats were plotted using a computer-assisted four-parameter logistic plot (Figure 1A). The plots were sigmoidal, with a virtually linear portion between 25% and 75% of maximal absorbance (i.e., wells with no competitor present). A steep standard curve is important for accurate quantitation of pesticide in a test sample—all three formats were similar in standard curve steepness (i.e., degree of change in inhibition with change in permethrin concentration); a 5-fold change in permethrin concentration resulted in a 30–35% change in inhibition on the assay standard curves.

Phenothrin exhibited a similar potency in each format (Figure 1B) with limits of detection values as follows: (1) 6 ng/mL; (2) 5 ng/mL; and (3) 2 ng/mL. The curves were sigmoidal and showed similar steepness to permethrin.

Specificity of Pyrethroid Monoclonal Antibody. A study of the cross-reaction of a range of pyrethroids and related compounds in both the one-step direct competitive assay the and one-step assay with immobilized antibody was conducted using both methanol and acetonitrile solvents for each compound. I_{50} values (concentration of pesticide that resulted in a 50% inhibition of the control absorbance) are shown in Table I. Whereas permethrin and 1(*R*)-phenothrin are detected to levels lower than 10 ng/mL, cypermethrin, deltamethrin, cyhalothrin, and fenvalerate are over 100-fold less potent. Pesticides in methanol solutions were slightly more potent than those in acetonitrile solutions.

Solvent Effects. The amounts of different solvents that the antibody could tolerate were studied in both of the one-step assay formats. Using acetonitrile, the final concentration of solvent could not exceed 5% for both immobilized and solution-phase antibody assays: even at this concentration there was a 15% reduction in absorbance compared to control absorbance. When methanol was used, the final concentration could be increased to

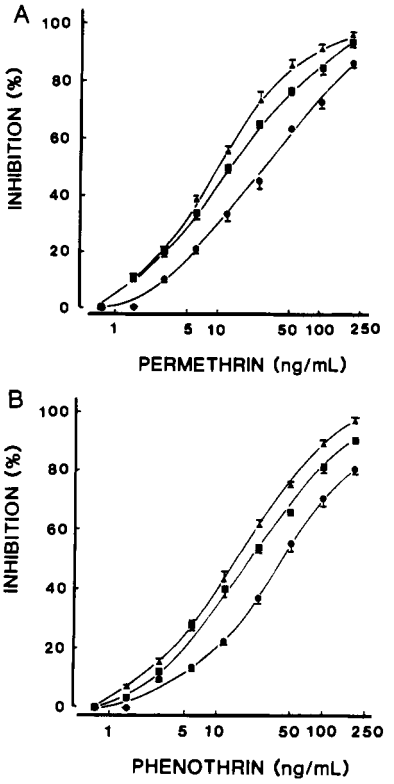

Figure 1. Inhibition of antibody binding by synthetic pyrethroids; comparison of assay formats: two-step indirect competitive assay (●); one-step direct competitive assay (■); and one-step assay with immobilized antibody (▲). (A) Permethrin standards (40 cis-: 60 trans) in 5% methanol; (B) phenothrin standards (90 cis-: 10 trans) in 5% methanol. Results are means of three to five determinations.

Table II. Effect of Solvent Concentration on ELISA Color Development and Inhibition by Permethrin in Solution

	assay format			
	direct competitive (immobilized antigen)		immobilized antibody	
solvent	A_{414}	I_{50}[a]	A_{414}	I_{50}[a]
methanol				
0%	1.29	NA[b]	1.25	NA
2.5%	1.29	15	1.18	8
10%	1.09	15	1.10	10
25%	0.73	62	0.49	40
50%	0.49	200	0	–[c]
acetonitrile				
0%	1.44	NA	1.34	NA
2.5%	1.14	25	1.20	10
10%	0.59	35	0.63	20
25%	0.09	–	0.09	–
50%	0	–	0	–

[a] I_{50}, concentration (ng/mL) yielding 50% inhibition of antibody binding. [b] NA, not applicable. [c] Not able to determine I_{50}.

10% without affecting the antibody or reducing the sensitivity of the assay (Table II). This result is important for analysis of wheat samples dosed with very low amounts of permethrin, since the extracts required less dilution before analysis.

Matrix Effects. All three assay formats exhibited a matrix effect to some degree with pesticide-free wheat, especially with extracts of ground, rather than whole, wheat. Using the incubation conditions of Stanker et al. (1989), the matrix effect was manifest as both a decrease in absorbance in the control sample (i.e., in the absence of pyrethroid) and a loss of potency of the pyrethroid when prepared in a pesticide-free wheat extract compared with solvent. The extent of the matrix effect was evaluated by comparing standard curves of permethrin and phenothrin

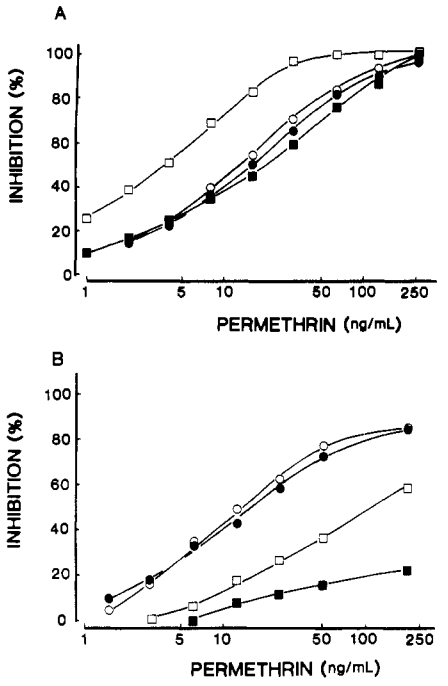

Figure 2. Reduction of matrix effects in permethrin ELISA by addition of bovine serum albumin to assay diluent buffer: PBS–Tween diluent permethrin standards (□); permethrin in wheat extract (■); PBS–Tween–BSA diluent permethrin standards (○); permethrin in wheat extract (●). (A) One-step direct competitive assay; (B) one-step assay with immobilized antibody. Results are means of 5 determinations.

in solvent and a pesticide-free wheat extract. Attempts to reduce this effect included (1) study of a range of extractants and (2) comparison of a number of different diluents containing various protein "quenchers" which may prevent or decrease pesticide binding to wheat component. The addition of 1% BSA to the PBS–0.001% Tween 20 diluent for these extracts markedly decreased the matrix effect in each assay format (Figure 2). However, in the indirect ELISA (immobilized 3-Pba–BSA) there was a 3–5-fold decrease in the potency of pyrethroid following addition of BSA to the diluent; however, the sensitivity of pyrethroid in extracts of pesticide-free wheat was increased. In the assay format using an antibody immobilized onto the microwells, the addition of BSA led to an increase in ELISA color development, which was greater than 30%.

The effect of BSA was even more significant when using immobilized Py-1 on the solid phase (Figure 2B). When there was no BSA present, there was a significant decrease in potency of permethrin. We also noted significant reduction in the overall absorbance values. The HRP–Pba required additional protein in the diluent buffer to enable binding to the immobilized antibody.

Analysis of Permethrin-Containing Wheats Using Different Solvents and Cleanup Procedures. Preliminary studies indicated that methanol may not quantitatively extract pyrethroid from the grain. A series of whole grain samples were spiked with known amounts of permethrin and, after aging and grinding, extracted with either methanol, acetonitrile, hexane, or acetone. Whole grain samples were also included for comparison. With the hexane- and acetone-extracted samples, the solvents were evaporated from the extracts and methanol was added to the residue prior to analysis in the immobilized antibody assay. In addition, an aliquot of the methanol and acetonitrile extracts of ground grain was run through an alumina column as a cleanup procedure prior to analysis.

Using methanol and acetonitrile extracts, results obtained with whole grain gave better correlations with the

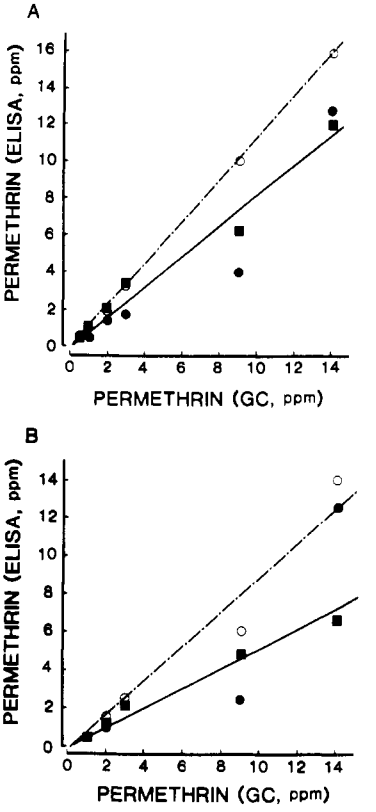

Figure 3. Relationship between gas chromatographic and antibody determination of permethrin using the one-step assay with immobilized antibody: whole grain (■); ground grain (●); ground grain after alumina cleanup (○). Regression data as follows, fitted through zero: (A) methanol extracts [whole grain [n = 6, r = 0.993, b (slope) = 0.812], ground grain (n = 5, r = 0.964, b = 0.768), cleaned up (n = 6, r = 0.999, b = 1.128)]; (B) acetonitrile extracts [whole grain (n = 6, r = 0.995, b = 0.499), ground grain (n = 5, r = 0.930, b = 0.709), cleaned up (n = 6, r = 0.986, b = 0.898)].

application rates than ground grain data. In general, methanol extracted more permethrin than acetonitrile—for whole grain, 80% of application rate compared with 50% for acetonitrile (Figure 3). With methanol, ground grain gave lower results than whole grain, but acetonitrile extracts gave 20% higher values than whole grain. However, after the alumina cleanup, results correlated well with the amounts of pesticide applied for each extraction solvent. The potency of permethrin diluted in an extract of pesticide-free grain before and after alumina cleanup did not change significantly; however, overall accuracy obtained with permethrin-containing wheat samples improved, indicating that interfering substances that possibly complex with permethrin during storage of the grain had been removed by the alumina cleanup (Figure 4).

Acetone and hexane were not as effective extractants as either methanol or acetonitrile, giving significant underestimates for both whole and ground grain. With whole grain (six samples), ELISA recoveries averaged 47 and 42% with acetone and hexane, respectively, while with ground grain (six samples) ELISA recoveries averaged 59 and 48%, respectively.

Analysis of Permethrin Milling Fractions. A series of ground wheat samples containing permethrin applied at elevator storage and milling fractions from these wheats (flour, germ, bran) were extracted in methanol by either 24-h standing (with intermittent mixing) or 2-min homogenization. There was no cleanup procedure performed on these samples. Both of the immobilized 3-Pba–BSA competition formats gave significant underestimates (averaging 45%) for the milling fractions. When analyzed

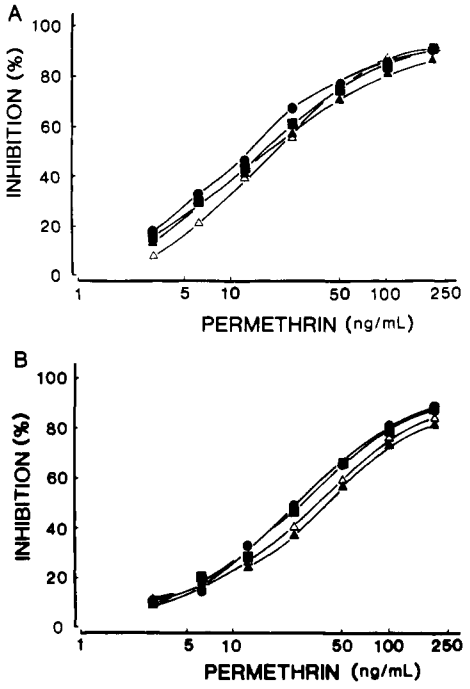

Figure 4. Effects of pesticide-free wheat extract on standard curve for permethrin: solvent only (●); whole wheat extract (■); homogenized wheat extract (▲); ground wheat extract after alumina cleanup (▲). (A) Methanol wheat extract; (B) acetonitrile wheat extract.

separately, flours and whole grain gave reasonably good correlation with gas chromatographic data (10–20% lower values), whereas bran and germ consistently gave lower results (50% lower).

The format utilizing immobilized antibody on the microwells showed significantly better correlation with gas chromatographic data (from regression, $n = 15$, $r = 0.974$) and, when all of the milling fractions were taken together, gave slightly lower (15%) results (Figure 5). A similar underestimate was seen with whole grain. This could be attributed to incomplete extraction by methanol or the presence of coextractives in the sample extract (which were not cleaned up in this case), especially with bran and germ samples which have a high lipid content. Bran and germ results gave significantly better correlation with this format (ELISA results 15–20% lower than gas chromatographic analyses).

Analysis of 1(*R*)-Phenothrin-Containing Wheats Using Immobilized Antibody Format. Whole grain samples were extracted in 2.5 volumes of methanol by 24-h standing with intermittent shaking. Results obtained by ELISA gave slight overestimates (20%) compared with HPLC data (Figure 6).

GENERAL DISCUSSION

Synthetic pyrethroids are effective against most household and food storage insect pests. Permethrin and 1(*R*)-phenothrin are extremely effective against some organophosphate-resistant insects and also exhibit lower mammalian toxicity than the organophosphates. Deposits on grain have been shown to be particularly stable (Simpson, 1979; Bengston et al., 1980; Noble et al., 1982), indicating the potential of these compounds as future grain protectants. Although their mammalian toxicity is lower than that for the organophosphates, a greater proportion of pyrethroid residues is carried through the milling and processing steps; therefore, a reliable analytical method is necessary for accurate determination of residue levels. In general, analytical methods for synthetic pyrethroids

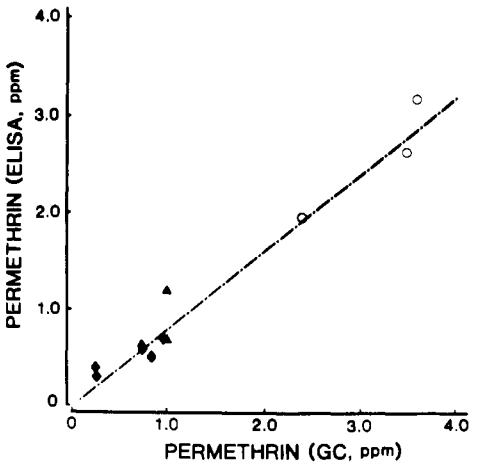

Figure 5. Relationship between permethrin residues determined by gas chromatography and ELISA for milling fractions, using the one-step assay with immobilized antibody. Fractions: flour (◆); bran (○); germ (▲). Regression data for combined set, fitted through zero: $n = 15$, $r = 0.974$; slope (b) = 0.857.

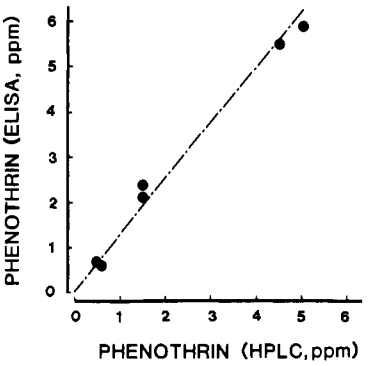

Figure 6. Relationship between high-performance liquid chromatography and ELISA data for 1(*R*)-phenothrin-containing wheats, using the one-step assay with immobilized antibody. Regression data, fitted through zero: $n = 6$; $r = 0.994$; slope (b) = 1.24.

are more complex than those for the organophosphates due to their lower application rates, relative lack of volatility, and extensive cleanup procedures (Mestres et al., 1979; Braun and Stanek, 1982; Papadopoulous-Mourkidow, 1983; Bottomley and Baker, 1984); however, immunoassays have been developed for a small range of pyrethroids (Wing et al., 1978; Wraith et al., 1986; Stanker et al., 1989).

We have modified a two-step competitive ELISA using immobilized hapten–protein conjugate for the analysis of permethrin and phenothrin in grain and milling fractions. By directly labeling the antibody with HRP, we have decreased incubation times as well as increased sensitivity. By coupling HRP to the Pba hapten, we have developed an assay using antibody immobilized on the microwell, which gave better accuracy with methanol extracts of grain. The immobilized antibody format exhibited the greatest sensitivity (1.5 ng/mL); the solid-phase antigen format using HRP–Pba had a sensitivity of 2 ng/mL, while the format similar to that published earlier (Stanker et al., 1989) had a sensitivity of 8 ng/mL. Phenothrin was of similar potency in each of the three formats. Closely related compounds such as cypermethrin, deltamethrin, and cyhalothrin bound more weakly to the antibody, due to steric hindrance by the cyano group α to the phenoxybenzoic ester. Fenvalerate also bound only weakly, supporting the earlier finding that the cyclopropane ring is involved with antibody binding (Stanker et al., 1989).

Solvent concentration was found to be an important factor with all three formats. As the concentration of

methanol increased, the control absorbance values (i.e., where no competing pyrethroid was present) decreased, indicating the solvent had affected antibody activity. The relative potency of free pesticide also decreased; the higher amounts of solvent probably inhibited the interaction of antibody with free pesticide. The antibody exhibited a lower tolerance to acetonitrile than to methanol. Since permethrin and phenothrin are routinely applied at 1–2 ppm (Bengston et al., 1983), the greater tolerance for methanol by the antibody is important for analyzing wheat samples treated with these pesticides. Components in the wheat extract itself inhibited antibody–antigen binding only very weakly; however, the inhibition potency of free pesticide was reduced significantly. This could be overcome by the addition of bovine serum albumin to the diluent buffer and was found to be essential for optimal activity of the HRP–Pba conjugate in the immobilized antibody format.

When a number of wheats and milling fractions were analyzed using all three formats, the immobilized antibody format gave the most accurate results. Since ELISA values were lower than data obtained by instrumental analysis, further studies were carried out on extraction procedures. It has been reported that methanol tends to solubilize protein and lipid coextractives which could interfere with pyrethroid determination (Sharp et al., 1988). We found that both methanol and acetonitrile extracts of ground wheat consistently gave lower results than expected; however, after extracts were cleaned up on an alumina column, the ELISA data correlated well with the instrumental data. Whole grain extracts gave good correlation with instrumental data, when methanol was used, indicating that pesticide permeation into the grain, reported to occur for both permethrin and 1(*R*)-phenothrin (Bengston et al., 1983), did not pose a problem with these extraction procedures. Acetonitrile extracts of whole grain gave lower than expected values, suggesting this solvent was not as effective for extracting permethrin from grain surface. The results obtained with phenothrin-dosed wheats were slightly greater than expected; however, the analysis by HPLC was based on hexane extraction, which is not as effective an extractant as methanol.

We have found that accurate results for permethrin- and phenothrin-containing wheat samples were obtained when the antibody was immobilized on the microwell. The inherent problems of extracting these pesticides effectively from the grain without other interfering substances have been successfully overcome. Applying a simple cleanup procedure on ground grain and milling fractions should improve the reliability of results obtained with this method in situations when high accuracy is required. However, for screening purposes, cleanup may be omitted.

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