Determination of the Insect Growth Regulator Methoprene in Wheat Grain and Milling Fractions Using an Enzyme Immunoassay

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An improved enzyme immunoassay was developed for the insect growth regulator methoprene, and the assay was used to determine methoprene in whole wheat grain and milling fractions, including flours, whole meal, bran, and wheat germ. Either methanol or acetonitrile could be used to extract methoprene from grain, flours, and bran; no cleanup of extracts was required. Methanol produced less matrix effects and could be tolerated at higher concentrations in the assay. However, it was a poor extractant of methoprene from wheat germ, where acetonitrile gave more reliable results. The improved assay had a sensitivity of 250 pg/mL, and 50% inhibition of antibody binding occurred at 3 ng/mL, corresponding to a maximum sensitivity of 60 ppb and 50% inhibition of antibody binding at 0.75 ppm in the wheat sample when the routine assay method was used. The latter value is in keeping with residue levels typically found in methoprene-treated stored grain. Good correlations were found between methoprene determined in wheat and grain fractions by using the enzyme immunoassay and by conventional HPLC analysis.

INTRODUCTION

Methoprene (isopropyl 11-methoxy-3,7,11-trimethyl-2E,4E-dodecadienoate) is different from conventional pesticides in that it is an analogue of insect growth regulator hormones based on a sesquiterpenoid structure (Wakabayashi and Waters, 1985). In contrast, most other insecticides act as stomach or cerebral poisons, commonly affecting neuromuscular transmission in insects. Methoprene instead has a juvenile hormone mimetic action, interfering with immature insect development, metamorphosis, and adult emergence. These morphological and developmental effects are often lethal to the target species (Staal, 1975). The low mammalian toxicity and lack of problems with residue persistence of methoprene (Miura and Takahashi, 1973) have made this compound an alternative adjunct to insect control strategies. Commercial use at present extends to tobacco, where it is used for cigarette beetle and tobacco moth, treatment of mosquito larvae (Quistad et al., 1974), and control of household fleas and insect pests of farm animals (Wright and Jones, 1976; Worthing, 1987). Methoprene has also been evaluated as a potential grain protectant, in some detail, in several countries including Australia, the United Kingdom, and the United States (McGregor and Kramer, 1975; Amos and Williams, 1977; Mian and Mulla, 1983; Edwards and Short, 1984; Hargreaves, 1985). This is because of its complementary mode of action to that of major insecticides such as organophosphates and synthetic pyrethroids and the significant activity of methoprene against stored product pests that are resistant to organophosphates, such as *Rhizopertha dominica*. Since methoprene does not prevent maturation of progeny of *Sitophilus* species at the doses used on other species, use of admixtures with compounds such as chlorpyrifos-methyl is required.

Residue analysis of methoprene is especially important for commodities such as grain, where it is intended that the compound be directly applied to the foodstuff (Snelson, 1987). A variety of methods have been used for methoprene analysis including gas chromatography of acetone extracts of tobacco, soil, meats, water, and forage (Miller et al., 1975; Dunham and Miller, 1978) or of petroleum ether extracts of grain (Mian and Mulla, 1983) or size exclusion high-performance liquid chromatography (HPLC) (Chamberlain, 1985). In the case of grain, hexane extracts have been used (Turnbull and Youssef, personal communication). While these methods provide reliable results in central, specialized laboratories, there is the need both for higher throughput and for lower cost methods for laboratory use, as well as for simpler methods that do not require dedicated chromatographic equipment and have the potential to be used in field situations. Enzyme immunoassay methods for agrochemical residues meet many of these requirements and have been developed for a variety of pesticides, herbicides, and fungicides (Bushway et al., 1988; Jung et al., 1989; Wratten and Feng, 1990).

Earlier, an enzyme-linked immunosorbent assay was developed for methoprene and applied to the quantitation of methoprene standards in aqueous phases (Mei et al., 1990). For application to matrices of agricultural importance, we have reconfigured this assay, removing one incubation step and also making the assay more sensitive. This paper describes the application of this new enzyme immunoassay (EIA) to the analysis of residues in wheat grain and grain fractions produced from commercial flour milling.

METHODS

Synthesis of Methoprene Immunogens and Peroxidase Conjugates. Methoprene was activated for conjugation to proteins according to the method of Mei et al. (1990), which uses a four-carbon spacer arm between methoprene and the protein. Briefly, the isopropyl ester of S-methoprene was hydrolyzed under basic conditions to yield S-methoprene acid (11-methoxy-3,7,11-trimethyl-2E,4E-dodecandienoate). This acid was esterified with the 2-trimethylsilyl ester of 4-hydroxybutanoic acid. The protected methoprene derivative was deprotected by using tetraethylammonium fluoride and esterified with N-hydroxysuccinimide prior to coupling to human serum albumin for use as

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Table I. Effects of Detergent, Protein, and Buffers on the Methoprene EIA

assay conditions		absorbance (450 nm)		
		control		blank
sample diluent	water solution	−solvent	+solvent[a]	+solvent
water	water	1.13	1.15	0.15
water	Tween–water	1.24	1.24	0.10
Tween–water	water	0.35	0.38	0.10
Tween–water	Tween–water	0.41	0.41	0.10
BSA–water	water	1.03	0.98	0.05
HSA[c]–water	water	0.98	0.96	0.06
Tween–PBS –BSA[b]	Tween–PBS	0.04	0.04	0.04

[a] Solvent used in assays is methanol (5% v/v, final). [b] BSA–Tween–PBS = 1% (w/v) bovine serum albumin–0.05% (v/v) Tween 20–50 mM sodium phosphate–0.9% NaCl, pH 7.2. [c] HSA, human serum albumin (1% w/v).

immunogen. Antibodies were produced in rabbits by three immunizations as described by Mei et al. (1990).

A methoprene–horseradish peroxidase (HRP) conjugate was prepared by conjugating the enzyme to a carboxylated derivative of *S*-methoprene (Mei et al., 1990) by use of *N*-hydroxysuccinimide. Sephadex G-25 was used to purify the conjugated materials from any unreacted methoprene. The stock conjugate was diluted with an equal volume of glycerol and stored at −10 °C.

Direct Competitive EIA for Methoprene. The method used in the current experiments was different from that initially published (Mei et al., 1990), which used a methoprene–human serum albumin conjugate coated to the solid phase, incubation of free methoprene simultaneously with the antiserum, and, after a washing step to remove unbound antibody, addition of enzyme-labeled goat anti-rabbit antibody. After a second wash, substrate was added; color development in the wells was inversely proportional to the concentration of methoprene in the sample. In the current studies, rabbit anti-methoprene antibody was coated directly to polystyrene microwells, and methoprene-containing sample was added simultaneously with peroxidase-labeled methoprene. This assay format had several advantages over the earlier format (see Results and Discussion).

In the modified EIA, antiserum to methoprene was diluted 1:40 000 in phosphate-buffered saline, pH 7.2, and used to coat Immulon II microtiter strips (Dynatech, Alexandria, VA). Working dilutions (1:35 000) of the conjugate were prepared in a pH 6.8 enzyme diluent buffer that was stabilized with an inert protein additive containing merthiolate; 80 μL of this conjugate was added to all wells except designated blanks. Samples, diluted 1/5 in solvent (see below) or standards, prepared in either methanol or acetonitrile, were further diluted 1/10 in water, and then 80 μL was added to the microwells. The well contents were mixed immediately by gentle manual rotation of the plate. The final concentrations of solvent in the assay were thus 5% (v/v) methanol or 2.5% (v/v) acetonitrile (chosen on the basis of preliminary experiments, see below). Plates were then incubated for 90 min at 20 °C and washed four times with water, and then 200 μL/well substrate–chromogen (3,3′5,5′-tetramethylbenzidine, in citrate buffer, pH 5.5–0.0006% hydrogen peroxide) was added and incubated for 45 min at 20 °C. Color development was terminated by addition of 2 M H₂SO₄, 50 μL/well. The absorbance of yellow product was measured at 450 nm by using a Bio-Rad (Hercules, CA) Model 2550 plate photometer.

Optimization of Methoprene EIA. The effects of Tween detergent, protein, and buffers on the methoprene EIA were studied by analysis of the color development in the presence and absence of solvent (5% methanol) and additionally in the absence of peroxidase-labeled methoprene. The effects of protein (1%) on Tween (0.05%) in the sample diluent were studied separately; in one experiment 1% (v/v) BSA–0.05% (v/v) Tween 20–50 mM sodium phosphate–0.9% NaCl, pH 7.2 (PBS) was used as the diluent. Wash solutions of water, Tween–water, and Tween–PBS were studied for each set of conditions (Table I).

In a separate series of experiments, using water as assay diluent and wash solution, the effects of methanol or acetonitrile (1–20% v/v) in the assay diluent were studied. EIA color
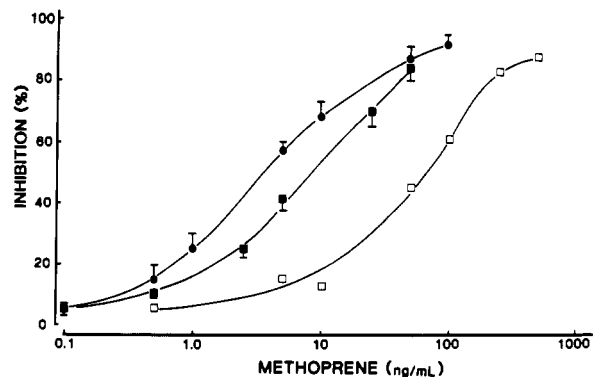

Figure 1. Comparison of solid-phase antibody (new format, solid symbols) and solution-phase antibody formats (Mei et al., 1990; open symbols) for methoprene standards in 5% methanol (●) or 2.5% acetonitrile (■).

development was expressed relative to absorbances obtained in the absence of organic solvent.

Sample Preparation. Either whole grain or ground grain (prepared by using a Udy cyclone mill, Udy Inc., Fort Collins, CO) was extracted by shaking in 2.5 volumes of solvent mechanically for 15 min, standing for 48 h at room temperature, and then shaking for a further 15 min before testing. In some cases, ground wheat samples were instead subjected to a 2-min high-frequency homogenization using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). Sample extracts were diluted 1/5 in solvent before further dilution in water. Milling fractions of flour (both straight run and last reduction flour, the latter from just under the bran layer and thus containing more pesticides), bran, and germ were obtained from commercial-scale milling trials of wheat that had been treated under field conditions with methoprene and stored for 3 months in a commercial grain elevator. These flours were extracted in 2.5 volumes of solvent, and the bran and germ were extracted in 5 volumes.

Instrumental Analysis of Methoprene. Grain or milling fractions were extracted for 48 h in hexane with intermittent agitation. Wheat was extracted in 1 volume of hexane, flour in 2.5 volumes, and bran or germ in 5 volumes. Methoprene was analyzed by R. Nelson and L. Swinden of the Australian Wheat Board (Melbourne, Victoria, Australia) using normal-phase high-performance liquid chromatography on a Resolve 5-μm spherical silica column (Waters, Milford, MA) column. The column was eluted with hexane containing 2% (v/v) tetrahydrofuran, and methoprene (both isomers eluting together) was monitored at 254 nm by using an ultraviolet detector.

RESULTS AND DISCUSSION

Optimization of Methoprene EIA. The previously described indirect enzyme immunoassay for methoprene (Mei et al., 1990) used a methoprene–human serum albumin conjugate adsorbed to the solid phase and addition of free methoprene and methoprene-specific antiserum, followed by enzyme-labeled anti-rabbit antibodies and substrate. This assay has now been simplified by precoating the microwells with methoprene-specific antibody (ImmunoSystems, Scarborough, ME), and, in a single incubation step, adding test sample (or methoprene standard) and HRP-labeled methoprene. In addition to simplifying the previous assay, antibody precoated microwells could be stored for up to 12 months (data not shown). The assay also became 10-fold more sensitive while remaining dynamic; that is, the steep concentration-response curve enabled discrimination of samples differing only moderately in methoprene content (Figure 1).

The antibody–antigen reaction was affected by small amounts of Tween 20, a detergent commonly used in EIA. The presence of 0.05% (v/v) Tween in the sample diluent led to much less color development in the assay (Table I).
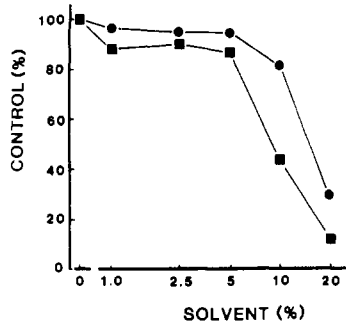
Figure 2. Inhibition of EIA color development by measuring concentrations of methanol (●) or acetonitrile (■). Control, color development in the absence of organic solvent. Data shown are means of three experiments; standard errors on individual data points did not exceed 10%.

Immunoassays for some other agrochemicals such as the pyrethroid permethrin (Stanker et al., 1989) have also exhibited sensitivity to Tween detergent. In contrast, a large number of immunoassays for agrochemicals, including other pyrethroids (Wing et al., 1978; Hill and Skerritt, unpublished data), function well in detergent-containing buffers. The use of either protein (bovine serum albumin or human serum albumin, the latter being the carrier protein used for raising methoprene antibodies) or phosphate buffer in the sample solvent did not change control or blank values. Similar results were obtained for assays using methanol (Table I) and acetonitrile (not shown) as solvents.

A variety of solvents have been used for the extraction of methoprene from grain and other plant materials, including acetone (Miller et al., 1975), ether (Rowlands, 1976), and petroleum ether (Mian and Mulla, 1983) as well as methanol, acetonitrile, or hexane (Turnbull and Youssef, unpublished data). Direct analysis of extracts by EIA requires use of solvents that are miscible with water and (at low concentrations) nondenaturing to proteins such as antibodies. Of the solvents listed above, methanol and acetonitrile were studied in detail. The assay was tolerant to small amounts of both solvents (Figure 2), although higher concentrations of methanol were able to be used: for example, 10% methanol inhibited the EIA color development by only 18%, while the same amount of acetonitrile inhibited color development by 56%. Accordingly, final concentrations of 5% methanol and 2.5% acetonitrile were used, both causing less than 10% inhibition of color development in a solvent-free control.

Comparison of Extractants. Determination of methoprene was 2.5–3 times more sensitive when the assay standard curve was performed in diluted methanol, compared with acetonitrile (Figure 2). When methanol was used, 50% inhibition of color development occurred at about 2.5 ng/mL methoprene and 10% inhibition at about 0.3 ng/mL. The assay was reasonably dynamic, a 5-fold difference in concentration at the near-linear position of the sigmoidal standard curve causing a 32% difference in inhibition.

In an attempt to understand the basis of the difference in sensitivity in the different diluents, two additional experiments were performed. In the first, methoprene, which has slight aqueous solubility (1.4 mg/L; Worthing, 1987), was diluted from a methanol stock, such that the final concentration of methanol at 100 ng/mL methoprene was 1%, at 10 ng/mL 0.1%, and at 1 ng/mL 0.01%, and so on. Despite decreases in the methanol concentration, the potency of methoprene was not altered, compared with the standard conditions of 5% methanol
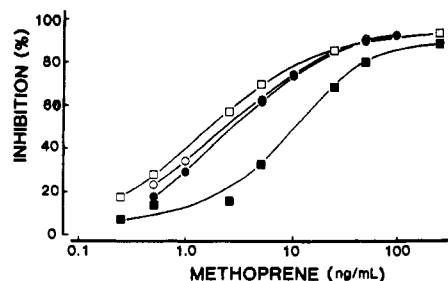

Figure 3. Effects of 1% human serum albumin (HSA) in diluent on standard curves for methoprene in 5% methanol [without HSA (●) and with HSA (O)] and in 2.5% acetonitrile [without HSA (■) and with HSA (□)].

throughout. Thus, the amounts of organic solvent (i.e., 5% methanol or 2.5% acetonitrile) routinely used do not affect the functional affinity of the antibody. The decreased inhibition of color development by methoprene in the presence of acetonitrile may be related to its greater disruption of the aqueous phase than methanol, since acetonitrile does not form hydrogen bonds.

In the second experiment, the effect of adding 1% protein [i.e., a 0.5% w/v final concentration of human serum albumin (HSA) or bovine serum albumin (BSA)] to the methoprene–methanol diluent was studied. While this addition did not change control absorbances (Table I) which were obtained in the absence of free methoprene, the ability to detect methoprene diluted in 2.5% acetonitrile was increased 5-fold by both HSA (Figure 3) and BSA (not shown). The methoprene in 5% methanol curve was not significantly affected. While HSA was the carrier protein used for raising the polyclonal antiserum, and its presence in the diluent would "block" antibodies to carrier protein, this phenomenon would seem unrelated to effects on potency since BSA had a similar effect on the acetonitrile–methoprene system. Neither HSA nor BSA affected the assay in the presence of methanol. Possibly the effect of these proteins was to shield the antibody–hapten complex from the denaturing effects of acetonitrile. Nonetheless, addition of BSA or HSA made the assay now sufficiently sensitive for acetonitrile to be used as an extractant for grain analysis. The addition may enable better analysis of leaf crops in which acetonitrile is the preferred extractant, such as tobacco (Miller et al., 1975).

Determination of Methoprene in Grain and Milling Fractions. Methanol was initially selected as the extractant for these studies, since earlier work with immunoassays for organophosphate and pyrethroid insecticides indicated that methanol extracts of grain provided most accurate results (Skerritt et al., 1991). In initial experiments, the effect of grain extract on the assay standard curve was studied by diluting methoprene standards in methanol grain extracts and then diluting further 1/5 and then 1/10 in methanol and water, respectively. Extracts of pesticide-free grain were prepared by using each of the three methods to be examined for extraction of methoprene from grain—standing of whole or ground grain for 48 h in solvent and high-frequency homogenization of ground grain for 2 min in solvent. The presence of each of these grain extracts did not affect the absorbances obtained in the absence of free methoprene but altered the potency of methoprene in the assay (Figure 4). However, in contrast to typical "matrix effects" seen in chromatographic as well as immunoassays for pesticides (Sharp et al., 1988; Wratten and Feng, 1990), the sensitivity of the methoprene EIA was actually increased, with the homogenized ground wheat extract causing the greatest
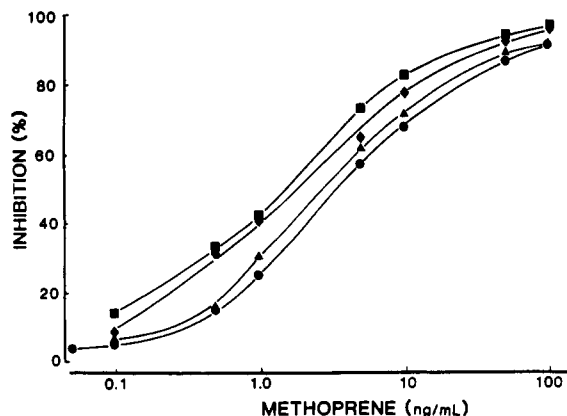
Figure 4. Effects of methanolic wheat extract on standard curve for methoprene: methanol only (●); whole wheat extract (◆); ground what extracted for 48 h (▲); homogenized ground wheat extract (■).

shift of the standard curve. One possibility is that co-extractives from the grain acted similarly to BSA or HSA (with acetonitrile) in shielding antibody from methanol solvent effects. Nonetheless, the simplest means to obtain reliable data in the presence of a matrix effect was to dilute methoprene standards in (untreated) wheat extracts. Accordingly, standard curves for analysis of grain fractions were prepared in this manner.

A series of ground wheat samples and milling fractions (flour, germ, and bran) were extracted either by 48 h of shaking or 2 min of homogenization in methanol. Results obtained with wheat grain, flour, and bran (taken together) correlated well with analyses by HPLC (Figure 5A,B). Values for flour were slightly low (11–21%) for both rapid and 48-h extraction but nonetheless correlated quite well with HPLC results: (1) rapid extraction, $n = 11$, r part (fit through zero) = 0.989, EIA result = 0.89 × HPLC result; (2) 48-h extraction, $n = 13$, r part = 0.927, EIA result = 1.05 × HPLC result. For bran and wheat, the EIA and HPLC results were close; there was too little spread in the values to perform individual regression analyses. The results obtained by rapid homogenization were slightly lower than those obtained by 48-h extraction of ground grain or flour (Figure 5C), although the difference (10%) was sufficiently low for either method to be used. However, for bran, 48-h extraction seemed necessary. Whole grain extracts prepared by 48-h extraction gave lower values (from regression, 9% lower) than those for similarly extracted ground grain, indicating incomplete extraction (regression: $r = 0.995$, 13 samples).

Methoprene values obtained in the EIA with methanol extracts of germ samples were somewhat lower (21% less for 48-h extraction, 29% less for 2-min extraction) than those obtained by HPLC analyses of hexane extracts of the same samples. While wheat germ is lipid rich and thus could give possible matrix (inhibition) effects in an EIA, the underestimations are most likely due to poor extraction of methoprene from germ with methanol, as shown directly by HPLC studies (Roubos, unpublished data). In fact, the more accurate results obtained when acetonitrile extracts were used in the assay ($n = 6$, $r = 0.994$, EIA value = 107% of HPLC value) indicated that poor extraction with methanol was indeed occurring.

GENERAL DISCUSSION

We have developed an improved enzyme immunoassay for methoprene, an insect growth regulator, and applied it to the analysis of methoprene in wheat grain and milling fractions. There was good agreement between the EIA
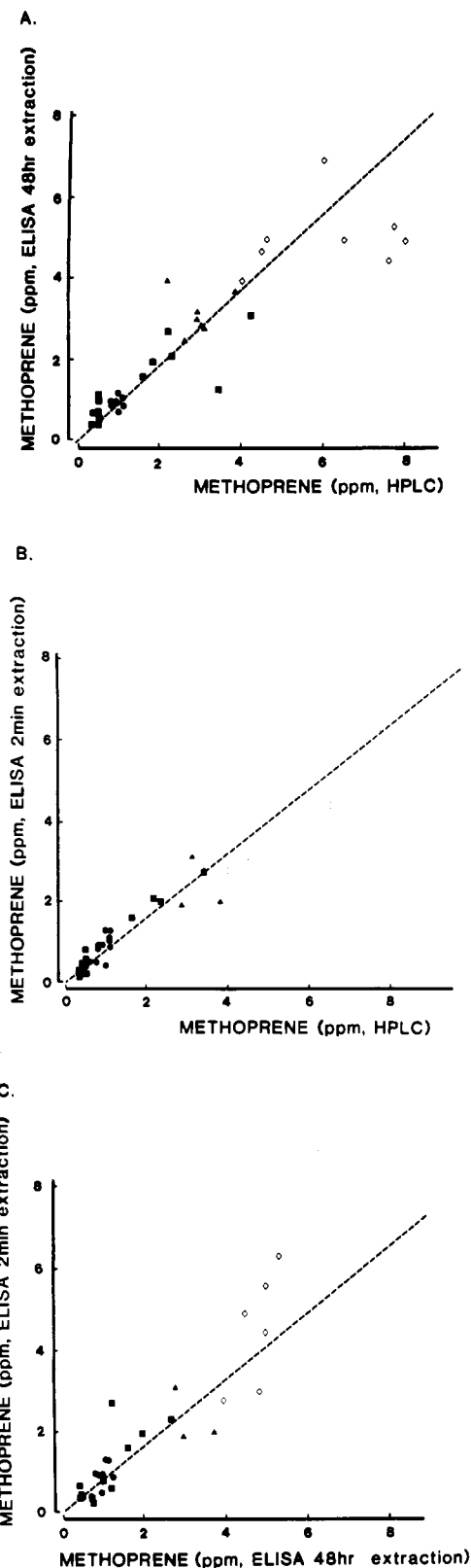

Figure 5. Relationships between HPLC and antibody determination of methoprene in ground wheat (●), flour (■), bran (▲), or germ (◇) using methanol extracts prepared as follows: (A) 48-h extraction; (B) 2-min homogenization; (C) comparison of 48-h extraction and 2-min homogenization. The lines indicated on each plot are for best fit (through zero) for wheat, flour, and bran only, since acetonitrile was the preferred extractant for germ. Equations: A, $n = 29$, $r = 0.954$, slope = 0.940; B, $n = 26$, $r = 0.966$, slope = 0.807; C, $n = 22$, $r = 0.934$, slope = 0.821. Equations for lines of best fit, not through zero, are as follows: A, $r = 0.853$, slope = 0.784, intercept = 0.38; B, $r = 0.909$, slope = 0.712, intercept = 0.19; C, $r = 0.788$, slope = 0.730, intercept = 0.18.

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and HPLC methods (Figure 5). Methoprene has been tested reasonably widely as a grain protectant but has not yet found extensive use on stored cereals. However, since it offers an alternative mechanism of insect control to the organophosphates, synthetic pyrethroids, and carbamates and also has very low toxicity to nonarthropodan species, methoprene has considerable potential. Since grain protectants are applied directly to commodities intended for human consumption, systematic analysis of residues in cereals (or cereal fractions from milling) before sale or consumption is critical to ensure that legal and customer specifications are met. The suitability of methanol as an extractant for the immunoassay of methoprene in most situations is a distinct advantage, since it is the extractant of choice for both conventional (Sharp et al., 1988) and antibody-based assays (Skerritt et al., 1990) of the major organophosphate and synthetic pyrethroid grain protectants. In common with some pyrethroid antibodies (Stanker et al., 1989), the methoprene antibody was sensitive to the presence of Tween detergents and to changes in solvent concentration. The presence of significant matrix effects also suggests that use of the antibody under standardized assay conditions (as in a kit) and identical dilution treatments of standards and sample extracts is critical for the accurate immunoassay of methoprene.

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