Analysis of the Grain-Protectant Pesticides Chlorpyrifos-Methyl and Methoprene with a 15-Min Immunoassay for Field or Elevator Use

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ABSTRACT

Antibody-based tests for field use have been developed to enable semiquantitative analysis of two grain protectants, chlorpyrifos-methyl and methoprene. In these tests, a pesticide-containing methanol extract of the grain sample and an enzyme-labeled analog of the pesticide are separately added dropwise to antibody-precoated test tubes containing an aqueous diluent. After a brief incubation period, the tubes are rinsed with tap water, and a substrate (chromogen) for the enzyme is added. The color developed is stabilized by acidification. The test result is read either by eye or in a portable field photometer. Pesticide could be extracted efficiently by blending either whole or ground grain for 1 min using a commercial blender or for 2 min using a probe homogenizer. The overall test time is under 15 min. The assays were sensitive to 0.05 ppm of residue in grain. Also, the results correlated well with those obtained using either laboratory immunoassay or chromatographic methods.

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One of the main advances in cereal technology over the last decade has been the development of methods that enable key analyses to be performed in nonlaboratory situations, such as at elevator receival of grain, on farms, or at shipping terminals. Examples include: near-infrared reflectance technology for determination of protein and quality characteristics such as hardness (Osborne and Fearn 1983); image analysis for rapid detection of quality type or varietal segregation (Myers and Edsall 1989); tristimulus color meter for screening red-grain type (Zounis et al 1992); and simple instruments to speed up the on-site determination of preharvest sprouting in grain (Ross et al 1987).

Another important aspect of grain quality is detection of residues of contaminants such as mycotoxins or grain-protectant pesticides. The main methods with potential for rapid analyses of these organic molecules are based on the specificity of the antigen-antibody reaction (Skerritt 1989). We have developed, for laboratory use, immunoassays to the major grain-protectant pesticides used internationally (Hill et al 1991, 1992; Skerritt et al 1992a,b). We reported earlier on the development of rapid assays for two organophosphate pesticides, fenitrothion and pirimiphos-methyl (Beasley et al, *in press*).

In this article, we describe simple field assays for another organophosphate, chlorpyrifos-methyl (RELDAN) and the isoprenoid insect growth regulator, methoprene (DIACON). Chlorpyrifos-methyl is one of the most widely used grain protectants in Europe, UK, United States, and Australia (Snelson 1987, Arthur et al 1992). Methoprene is currently used in admixture with chlorpyrifos-methyl as a very common pesticide treatment for wheat grain received into the central bulk-handling systems in eastern Australia. Its use as a grain protectant is new; widespread commercial use commenced only in the 1991–92 Australian harvest. It is likely that methoprene will find greater use internationally due to increasing insect resistance to alternative pesticides and the low human toxicity and application rates of methoprene (Snelson 1987).

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MATERIALS AND METHODS

Grain and Pesticide Samples

Wheat samples used for analyses were either commercially treated samples supplied by Australian state grain handling authorities or samples of pesticide-free wheat that was spiked in-house. In both cases, the samples had been stored at least one month at 20–30°C from the date of treatment to ensure distribution of the residues. Gas chromatographic (GC) analysis for chlorpyrifos-methyl was performed using flame-photometric detection with a phosphorus-selective filter (Desmarchelier et al 1977). Methoprene was determined by normal-phase high-performance liquid chromatography (HPLC) using a 30-cm × 3.9-mm μ-Porasil silica column (Waters, Milford, MA) eluted with 2% tetrahydrofuran in hexane (2 ml/min) with ultraviolet detection at 254 nm. The samples were stored at 4°C (temperature at which further residue decomposition is minimal) for up to four weeks (Desmarchelier and Bengston 1979) until analysis by immunoassay. Pesticide standards were obtained from Chem Service (West Chester, PA).

Extraction of Residues

Pesticide was extracted from grain using methanol, which is one of the most efficient extractants for both chlorpyrifos-methyl (Sharp et al 1988) and methoprene (Hill et al 1991). Standing whole grain, with only intermittent shaking in 2.5 volumes of methanol per gram for 48 hr gives near 100% recovery of chlorpyrifos-methyl (Desmarchelier et al 1981) and methoprene (J. H. Skerritt and S. L. Edward, *unpublished*). However, although this extraction procedure is simple, a more rapid extraction method is often required for use with a rapid test. A number of methods were compared using methanol (reagent grade) as the extractant.

1. Soaking of whole grain for 44–48 hr, with intermittent mechanical shaking (twice for 5 min) during the extraction period: (A) using 25 ml of methanol for 10 g of grain; (B) using 50 ml of methanol for 10 g of grain.

2. Shaking by hand for 2 min using 25 ml of methanol for 10 g of grain: (C) whole grain; (D) ground grain. Grain was ground using a Cyclone mill (Udy Corp., Fort Collins, CO).

3. A domestic 425-W blender (Breville Cyclonic Wizz food

processor): (E) single 2-min blend of ground grain, using 50 ml of methanol for 20 g of grain; (F) single 2-min blend of ground grain using 50 ml of methanol for 10 g of grain; (G) double blending, 20 g of ground grain plus 50 ml of methanol blended for 1.5 min in a domestic blender, the extract decanted and retained, a further 50 ml of methanol added, then blended for 1.5 min and both extracts combined.

4. Blending 1 min in a commercial 860-W blender (Waring model 8011S, Waring Products, New Hartford, CT) using 100 ml of methanol for 20 g of grain: (H) whole grain, (I) ground grain.

5. Use of a high-speed probe homogenizer (Ystral, Dottingen, Germany): (J) 2-min homogenization with 25 ml of methanol for 10 g of ground grain.

Enzyme-Immunoassay Method

The antibodies and pesticide conjugates used in these tests have been described earlier (Hill et al 1991). The rapid tests employed polystyrene test tubes (75 mm × 12 mm) coated overnight with rabbit antiserum to methoprene (1:10,000) or chlorpyrifos (1:70,000) using a proprietary process (ImmunoSystems Inc, Scarborough, ME). Once coated, the tubes were dried and stored at 4°C for up to one year before use. Pesticide calibrators were prepared by serial dilution of 0.1 mg per 1 ml of stock of either chlorpyrifos-methyl in methanol containing 0.1% (v/v) acetic acid or methoprene in neat methanol into methanol solvent.

The solution added to the antibody-coated tubes to initiate chlorpyrifos-methyl assay was 400 µl of 1% bovine serum albumin (BSA), 0.05% (v/v) Tween 20, 50 m*M* sodium phosphate, and 0.9% (w/v) sodium chloride. The solution added to initiate the methoprene assay was 400 µl of 1% BSA in water. The buffer and detergent components were omitted from the methoprene assay as they inhibited color development (Hill et al 1991). Next, 50 µl of grain extract and 50 µl of peroxidase-labeled pesticide (either 50 ng of chlorpyrifos-methyl-peroxidase or 350 ng of methoprene-peroxidase) were added. The tubes were mixed by gentle swirling for a few seconds and left to stand at room temperature for 7 min. For assessment of assay specificity, a range of concentrations of various pesticide standards (ChemService, West Chester, PA) prepared in methanol were added instead of grain extracts. At the end of this period, the contents of the tubes were tipped out, and the tubes were washed four times with cool tap water by repeatedly flooding and shaking out the tube contents. Then, 500 µl of enzyme substrate-chromogen mixture (3,3′,5,5′-tetramethylbenzidine-hydrogen peroxide) was added, and a blue color was developed for 3 min. Further development was arrested by adding 160 µl of 1.25*M* sulfuric acid, which turned the contents of the tubes yellow. Absorbance was read against water in a battery-powered photometer, using a 450-nm filter (DP model, Artel Products, Windham, ME).

RESULTS

Sensitivity of the Assays

In preliminary work, a range of immobilized antibody and conjugate concentrations was assessed for both assays. The conditions chosen gave both good color development under the assay conditions (absorbance values of 0.8–1.5 for pesticide-free controls) and appropriate sensitivity. Both the chlorpyrifos-methyl and the methoprene assays could tolerate a final concentration of 10% methanol in the tube without a significant decrease in color development. This degree of solvent tolerance was important: a couple of drops of methanol grain extract could be directly added to the tube, avoiding dilutions in the field.

Increasing pesticide concentration in the sample leads to decreases in color development in competition immunoassays for small molecules such as pesticides. The limit of detection is generally defined as the concentration of the analyte yielding 10% inhibition of color development from that produced in the absence of pesticide in the test sample. The chlorpyrifos-methyl tube assay (Fig. 1A) was sensitive to about 0.4 ppb, corresponding to about 0.02 ppm in the grain, after accounting for the extraction ratio of 5 ml methanol per gram of grain and 10-fold dilution upon addition of the methanol extract to buffer and conjugate in the tube during the assay. The concentration of chlorpyrifos-methyl causing 50% inhibition of color development (IC_{50}) was 5 ppb final concentration in the tube or 0.25 ppm in the grain. The methoprene assay was slightly less sensitive, with an IC_{50} of 15 ppb in the tube (Fig. 1B), corresponding to 0.75 ppm in the grain. The limit of detection was about 0.03 ppm in the grain.

The corresponding laboratory assays were three- to fivefold more sensitive in absolute terms. To compensate for the much shorter antibody and substrate-chromogen incubation periods used in the rapid assays, a higher concentration of pesticide-peroxidase conjugate was used (Fig. 1). Because the pesticide in the test sample competes with the labeled pesticide for a limited number of antibody-binding sites, it follows that when the

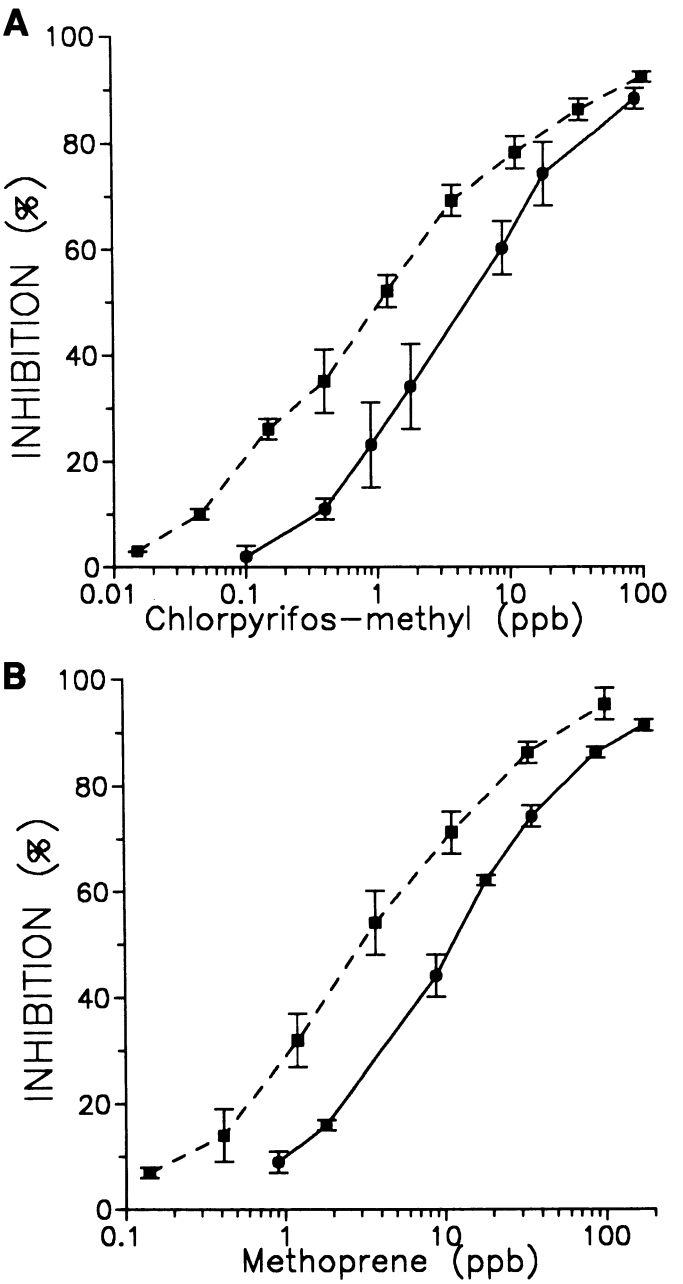

Fig. 1. Standard curves for detection of chlorpyrifos-methyl (**A**) and methoprene (**B**) using the rapid (●) and laboratory (■) immunoassays. Peroxidase conjugate concentrations: chlorpyrifos-methyl rapid assay 100 ng/ml, laboratory assay 25 ng/ml; methoprene rapid assay 700 ng/ml, laboratory assay 14.5 ng/ml. Data shown are means ± standard deviations of 10–12 assays and are percent inhibition of color development. Concentration data are final concentrations of pesticide in the assay tube or microwell.

concentration of the labeled component is increased, the sensitivity decreases (Johannsson 1991). However, both of the tube assays were sufficiently sensitive to detect chlorpyrifos-methyl and methoprene in grain over the ranges required, corresponding at the lower end to the reporting limit for pesticide-free grain and at the higher limit to the maximum permissible residue levels in grain trading.

Assay Specificity

Among the range of major grain protectants currently in use internationally, the methoprene assay was specific for methoprene, with no crossreaction from other grain protectant pesticides. These were tested at levels 20 times higher than the usual application rate of the target compound. No crossreaction was seen with 400-ppb (final concentration) fenitrothion, chlorpyrifos-methyl, pirimiphos-methyl, malathion, carbaryl, deltamethrin, and bioresmethrin; this would normally be equivalent to a 20-ppm residue in the grain, somewhat higher than the usual commercial application rates of these compounds (5–12 ppm for carbaryl and the organophosphates and 0.5–1 ppm for the pyrethroids).

None of the grain protectants tested at 2 ppm (corresponding to 100 ppm in grain), including fenitrothion, pirimiphos-methyl, etrimfos, malathion, carbaryl, methoprene, bioresmethrin, deltamethrin, or permethrin, were detected in the chlorpyrifos-methyl assay. This assay did detect some other organophosphates, with limits of detection as follows: chlorpyrifos-methyl (1.5 ppb final concentration in assay tube, 0.075 ppm in grain), bromophos and fenchlorphos (both 60 ppb final concentration, 3 ppm in grain). The structurally related herbicide, triclopyr, was also detected (limit of detection 0.8 ppb final concentration, 0.4 ppm in grain). However, none of these compounds are used as grain protectants, so the crossreaction is of little practical importance. Triclopyr is occasionally used for weed control in wheat crops, but negligible residues of herbicides persist into harvested grain after normal agronomic use (Bonafaccia and Cubadda 1991). No constituents from either whole or ground grain interfered with the assay.

Extraction of Residues

The efficiency of extraction was reported as the mean percentage recovery calculated for four or five wheat samples with a range of chlorpyrifos-methyl contents from 1.1–11.2 ppm and methoprene contents from 0.2–3.0 ppm (Table I). The proportion of residue extracted did not vary significantly at differing residue levels. In general, the treatments extracted either similar amounts of both pesticides or greater amounts of methoprene. Two-minute hand-shaking of whole grain extracted very little chlorpyrifos-methyl residue, although hand-shaking of ground grain was reasonably efficient, extracting over 75% of the residue. Similar extraction efficiencies could be obtained using a domestic blender. Using a double extraction cycle would be even more efficient. These trends agree with data obtained earlier for rapid extraction of fenitrothion and pirimiphos-methyl from grain. The most efficient rapid extraction methods (virtually quantitative) for both pesticides were methods I and J (i.e., blending ground grain in a commercial blender or extracting with a probe high-frequency homogenizer). Therefore, either of these methods is recommended. A domestic blender could be used for extraction for screening purposes, but caution is required to guard against possible explosion hazards in blending methanol slurries of grain. However, in many cases, such as surveys of residue distribution in elevators, a simple field method may be important but rapid results are not needed. In these situations, it may be preferable to extract residues by standing the grain for 44–48 hr, as this is a simple but very efficient method.

Accuracy and Precision of the Assay

The pesticide concentration-inhibition standard curves in the rapid assays were very reproducible, with standard deviation of inhibition values under 8% in all cases (Figs. 1A and B). In the chlorpyrifos-methyl tube assay, four separate whole wheat samples extracted for 48 hr in four separate assays gave mean data of 4.3, 4.0, 2.0, and 0.25 ppm with coefficients of variance (CV) of 22, 6, 13, and 20%, respectively.

The accuracy of results obtained using the field tests were investigated by comparison of results obtained with sets of grain samples containing 0–5 ppm of chlorpyrifos-methyl and 0–1 ppm of methoprene, and with either the standard method (48-hr extraction, GC or HPLC analysis) or the laboratory ELISA (48-hr extraction). Samples were read against curves of either chlorpyrifos-methyl or methoprene standards in methanol because no effects of pesticide-free grain extracts on the assays were seen. In each case, data obtained using the rapid tests exhibited good correlations (linear relationships) with the two laboratory methods, and with slopes of the regression lines near unity, indicating good recovery values. Several of these relationships are depicted for chlorpyrifos-methyl in Figure 2 (A–C). Correlations between GC data and data from the tube assays using the 48-hr extraction method (Fig. 2A) and the 2-min extraction method, respectively, were $r = 0.904$ and $r = 0.865$,

TABLE I
Extraction of Chlorpyrifos-Methyl and Methoprene[a] Using Various Techniques

Method	Description	Volume of Methanol (ml)	Weight of Grain (g)	Chlorpyrifos-Methyl Percent Recovery +/−SD[b]	Methoprene Percent Recovery +/−SD[b]
A	Soaking 48 hr (whole grain)	25	10	100[c]	100[c]
B	Soaking 48 hr (whole grain)	50	10	100[c]	100[c]
C	Shaking by hand-whole grain	25	10	13 ± 3	NT[d]
D	Shaking by hand-ground grain	25	10	76 ± 14	87 ± 5
	Domestic blender:				
E	Single blend (ground grain)	25	10	NT	84 ± 10
F	Single blend (ground grain)	50	10	60 ± 10	73 ± 4
G	Double blend (ground grain)	50	10	84 ± 9	78 ± 11
	Commercial blender:				
H	Whole grain	100	20	75 ± 4	77 ± 7
I	Ground grain	100	20	95 ± 9	90 ± 8
J	High-frequency homogenization (ground grain)	25	10	85 ± 10	104 ± 4

[a] Five samples per extraction method (except for methods H and I, which used four samples).
[b] Mean percent recovery = average of ppm of pesticide determined for chlorpyrifos-methyl and methoprene (methods H and I) by plate of enzyme-linked immunosorbent assay and for methoprene methods A–G and J by high-performance liquid chromatography expressed as a percentage of results obtained using the corresponding method for 48-hr extraction. SD = standard deviation of the mean.
[c] 48-hr extraction of whole grain taken as 100% extraction, for both (A) 25 ml methanol per 10 g of grain and (B) 50 ml methanol per 10 g of grain.
[d] Not tested.

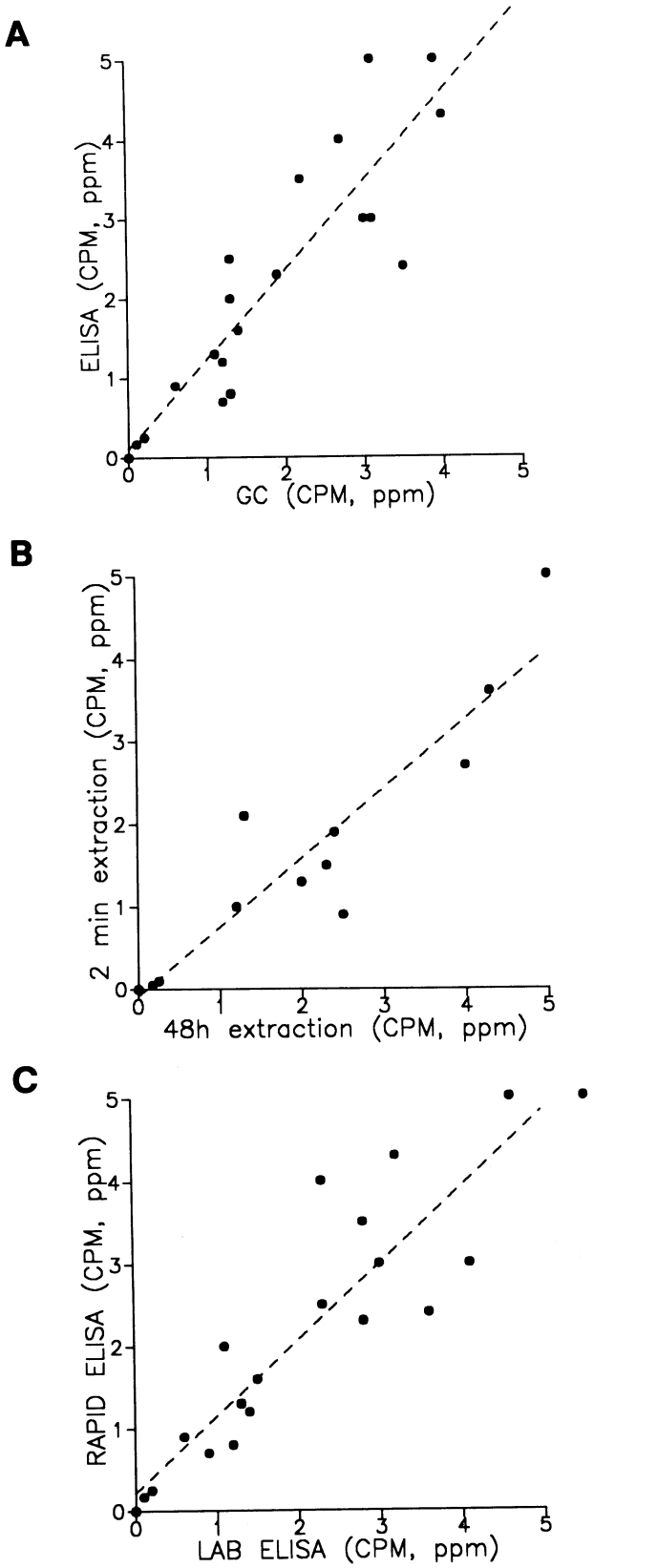

Fig. 2. Performance of the rapid tube immunoassay for chlorpyrifos-methyl (CPM). **A,** Relationship between results obtained in the rapid tube immunoassay (48-hr extraction) and gas chromatography (GC) analysis. ($n = 20$, $r = 0.904$, $P < 0.001$, slope = 1.13, intercept = 0.10). **B,** Relationship between results obtained in the tube immunoassay using 2-min homogenization of ground grain and 48-hr extraction of whole grain. ($n = 13$, $r = 0.929$, $P < 0.001$, slope = 0.84, intercept = −0.09). **C,** Relationship between results obtained in the rapid tube immunoassay (48-hr extraction) and laboratory immunoassay. ($n = 20$, $r = 0.912$, $P < 0.001$, slope = 0.90, intercept = 0.14). ELISA = enzyme-linked immunosorbent assay.

with regression slopes near unity (1.13 and 0.95). Data from the two extraction methods were also closely correlated ($r = 0.929$, Fig. 2B). Finally, the results obtained using the laboratory immunoassays correlated with the data from the tube assay using the samples extracted in the same manner: 48-hr extraction ($r = 0.912$, slope = 0.90, Fig. 2C) and 2-min extraction ($r = 0.828$, slope = 0.84).

With methoprene, data from the tube test correlated with HPLC analyses as: 48-hr extraction, $r = 0.959$ (recovery 87%; Fig. 3A); 2-min homogenization, $r = 0.976$ (recovery 93%; Fig. 3B). Comparing data from the 48-hr extraction in the laboratory assay with both the corresponding tube assay and HPLC analysis gave $r = 0.905$ and 0.979 respectively, and regression slopes of 1.26 and 1.24.

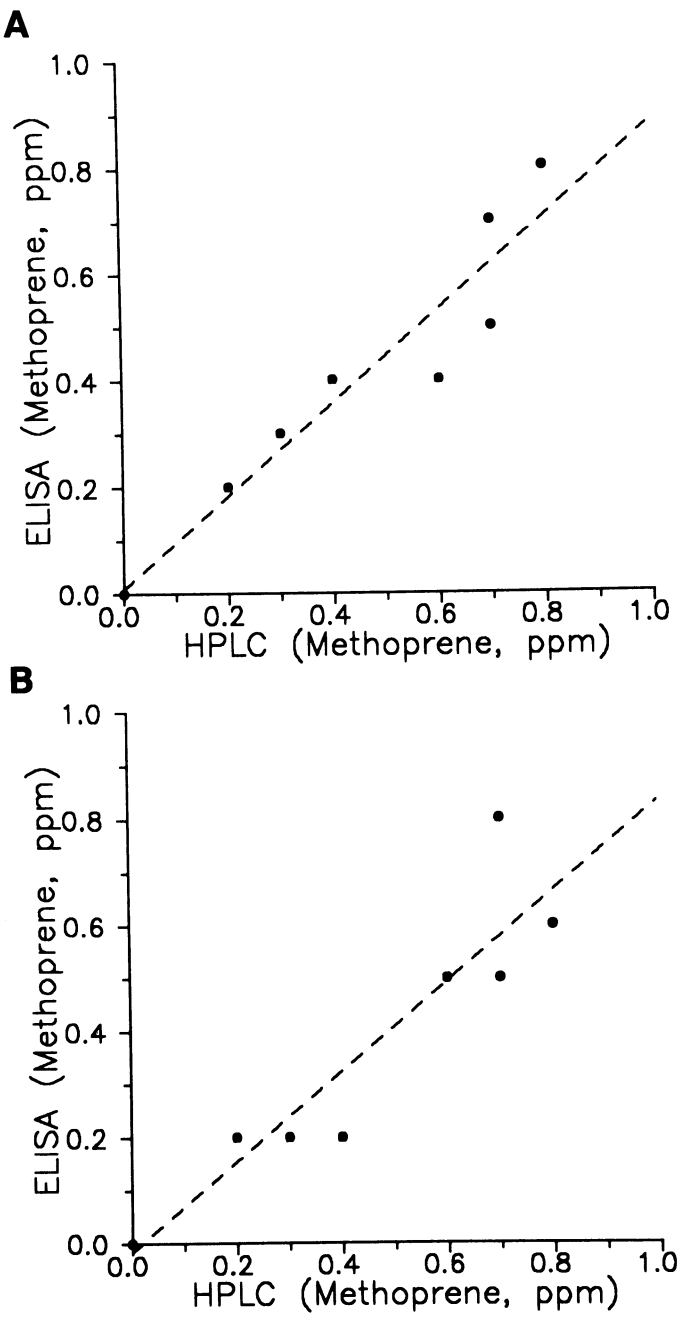

Fig. 3. Performance of the rapid tube immunoassay for methoprene. **A,** Relationship between results obtained in the rapid tube immunoassay (48-hr extraction) and high-performance liquid chromatography (HPLC) analysis (48-hr extraction; $n = 9$, $r = 0.959$, $P < 0.001$, slope = 0.87, intercept = 0.01). **B,** Relationship between results obtained in the rapid tube immunoassay (2-min homogenization of ground grain) and HPLC analysis (48-hr extraction; $n = 9$, $r = 0.976$, $P < 0.001$, slope = 0.93, intercept = −0.04).

DISCUSSION

These data indicate that the rapid tube-based immunoassay tests for two grain protectants, chlorpyrifos-methyl and methoprene, were useful semiquantitative methods for residue analysis in grain. A field test for residues in samples derived from elevators of bulk shipments need only provide data to a ±25% accuracy, as the levels of pesticide residues within an elevator often vary considerably after commercial application practices, even though they are intended to provide an even level of treatment (Minnett et al 1984, le Patourel 1992). These field tests for chlorpyrifos-methyl and methoprene complement field tests for two other organophosphates, fenitrothion and pirimiphos-methyl (Beasley et al, *in press*). There is a special need for the tests described here: chlorpyrifos-methyl has become one of the most widely used grain protectants worldwide, replacing malathion because of its insect resistance and because of the proposed elimination of the postharvest use of malathion in the United States (Abramson 1991). Simple methods to determine methoprene have also become important because of its widespread use on stored wheat in Australia since 1992 in admixture with chlorpyrifos-methyl. Admixtures provide a broader spectrum of activity against organophosphate-resistant species. Compared with the organophosphates, methoprene is relatively difficult to analyze by GC; it lacks elements such as nitrogen, phosphorus, or chlorine that facilitate specific detection. Its analysis by instrumental means is also complicated by the low residue levels that arise from application at relatively low rates (1 ppm).

These assays have now been formatted into compact test kits. The data described in this study were obtained using reagent additions made with pipets. However, in kits for field use, each of these additions would be made using dropper bottles. The assay can also employ prediluted calibrated standard solutions, corresponding to the pesticide levels that would be found in solvent extracts of grain containing 0, 0.5, 0.1, 0.5, 1, and 5 ppm (chlorpyrifos-methyl assay) or 0.1, 0.2, 0.5, 1, and 2 ppm (methoprene assay). The choice of calibrated standard solutions will depend on the range of residue levels of interest. Using the prediluted standard solutions permits semiquantitative analyses under field conditions, with relatively few calculation steps required in data analysis.

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