

Rapid on-site immunoassay for diflubenzuron in grains

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Abstract

An antibody-based test for field use has been developed to enable a screening test for diflubenzuron in animal feed grain. In this test, a pesticide containing methanol extract of the grain sample and an HRP-labeled diflubenzuron derivative are separately added to the antibody-precoated 8-well strip. After a brief incubation period, the strip is washed and a substrate for the enzyme is added. The color development is stopped by acidification, and the test result can be read either by plate reader or a portable field photometer. Diflubenzuron could be extracted efficiently by blending ground grain for 2 min using methanol. The overall test time is around 15 min. The test had a limit of detection of 3 ppb (0.75 ppm in grain) and gave quantitative estimates in the range of 0.75–25 ppm in the grain. No significant matrix effect was observed after methanol extraction of the residue was diluted 1/50 with 1% BSA–PBS. The results correlated well with those obtained using either laboratory immunoassay or HPLC method.

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1. Introduction

Benzoylphenylureas (BPU) are promising and effective insecticides, used for the control of insects attacking a wide range of crops. These compounds are generally recognized as insect growth regulators that interfere with chitin synthesis in target pests, causing death or abortive development [1,2]. Diflubenzuron, 1-(2,6-difluorobenzoyl)-3-(4-chlorophenyl) urea (Fig. 1) is the most widely used compound in this group.

Diflubenzuron is currently undergoing evaluation as a grain protectant in Australia, with AWB Ltd. sponsoring milling trials. However, in view of the previous experience involving serious contamination

of cotton waste and beefstock with chlorfluazuron [3], regular monitoring of this group of compounds is necessary. The instrumental analysis of diflubenzuron by HPLC is expensive and laborious. Rapid monitoring of a large numbers of samples would benefit from a more cost-effective analytical method.

Earlier, we developed ELISA tests for laboratory analysis of diflubenzuron [4,5]. The tests can be used by both small and large laboratories without the requirement for specialized equipment or highly trained staff. However, any laboratory method can also be expensive in terms of time involved in transporting the sample to the laboratory and in collating and sending back the residue result, especially when results are urgently required.

In the Australian feed industry there is a need for simple and rapid screening tests for pesticides in animal feed grain, which is fully portable and can

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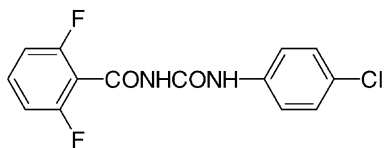


Fig. 1. Chemical structure of diflubenzuron.

be performed under local conditions, but still give reliable results.

This paper describes the modification of the previous laboratory assays for diflubenzuron to provide 15 min tests. Critical modifications included alterations of the coating and changes in the concentrations of antibodies and the conjugates containing the detecting enzyme. The method produced analytical data that correlated well with both the corresponding laboratory immunoassay methods and the standard instrumental technique. A rapid method for the extraction of the pesticide from grains was also developed.

Before analyses of diflubenzuron can be carried out, it must be extracted from the grains. Of water-miscible solvents, methanol was most effective for this purpose. However, this organic solvent is less suitable for health and safety reasons for more widespread use of the ELISA. Several detergents were tested for their ability to remove diflubenzuron from grain samples, in case these could replace methanol.

2. Experimental

2.1. Grain samples

Grain samples (barley, sorghum, and wheat) used in this study were supplied by CSIRO Livestock Industries at Long Pocket Queensland. The samples were free of pesticides. For a spiking study, 500 g of grain sample in a glass jar was spiked with a known concentration of diflubenzuron dissolved in methanol. The grain was mixed thoroughly overnight using a roller and then allowed to stand at room temperature for 48 h to allow the methanol to evaporate and diflubenzuron to equilibrate with the grain samples.

2.2. Extraction of residues

Diflubenzuron was extracted from grain using methanol, which was earlier found to be an efficient

extractant for this compound in soil [4]. The following detergents (0.1–20% in water) were tested: Tween 20, sodium dodecylsulfate (SDS), methyl- β -cyclodextrin (MC), and β -hydroxypropyl-cyclodextrin (HC). Also, different extraction methods were compared:

1. Whole grain samples were shaken with five volumes of methanol or detergent in water overnight.
2. Ten grams of sample was ground in an electric coffee grinder and blended with 50 ml of methanol or detergent for 2 min using a Waring blender.
3. Ten grams of ground sample in 50 ml of methanol was shaken by hand for 2 min.

2.3. Enzyme-immunoassay method

Pesticide calibrators were prepared by serial dilution of the diflubenzuron from a 100 μ g/ml stock in neat methanol. However, in a field kit form, the assay can employ pre-diluted calibrators, corresponding to diflubenzuron levels that would be found in solvent extracts of grain samples. The choice of calibrated standard solutions will depend on the range of residue levels of interest.

The antibodies and enzyme conjugates used in this study have been described earlier [4,5]. The rapid tests employed 8-well strips coated overnight with purified anti-benzoylphenylurea IgG (1 μ g per well in 100 μ l 50 mM carbonate buffer, pH 9.6). The next day, coated plates were washed three times with PBST washing solution [PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2) with 0.05% (v/v) Tween 20] and then nonspecific antibody binding blocked with 150 μ l of 1% BSA/PBS per well for 1 h. The assay was performed by the addition of 50 μ l of pesticide standard in 1% (w/v) BSA in PBS solution and 50 ml of enzyme conjugate solution diluted in the 0.1% FG-PBS to each well and incubation for 10 min. After being washed with washing solution, 150 μ l of 3,3',5,5'-tetramethylbenzidine-peroxide based substrate solution was added to each well. Color development was stopped after 5 min by adding 50 μ l of 2.5 N H₂SO₄, and absorbances were read.

2.4. Instrumental analysis

Instrumental analysis was performed with Waters 600 high-performance liquid chromatography (HPLC)

using a Waters Symmetry 5 μm C₈ column (150 mm \times 4.6 mm) with Waters 717plus auto sampler and Waters 996 photodiode array detector. The detection wavelength was 260 nm, and mobile phase was a mixture of methanol and water (11:9). A flow rate at 0.7 ml/min was used with injection volume of 20 μl .

3. Results and discussion

3.1. Assay performance

In preliminary work, a range of immobilized antibody and conjugate concentration was assessed for the assay. The conditions chosen gave color development of absorbance value of 0.8–1.2 for pesticide-free control.

Two of the antibodies prepared in our earlier work were checked, and it was found that one of these was not suitable for the rapid format, even where a higher antibody concentration for coating was used. The color development was very low and the sensitivity of this antibody for the target compound was too low. Because of the short incubation time for rapid assay, antibody–antigen reactions may not be at equilibrium, and only small portion of the available antibody may react. The individual kinetics of this antibody were deemed too slow for the test. However, the other antibody available proved good and the sensitivity was shown in Table 1. Fig. 2 shows the chemical structures of haptens which were used to raise antibody and conjugate to the HRP as enzyme tracer.

Compared with conventional ELISA (60 min incubation and 30 min color development), such rapid assay needs more reagents and the sensitivity was reduced. A coating concentration of 1 μg per well of IgG was chosen, as higher coating concentrations did not increase sensitivity.

Table 1
Comparison of conventional and rapid assays

Assay format	IC ₅₀ (ppb)	IC ₁₅ (ppb)	Optimal enzyme conjugate dilution ^a
Conventional	2 \pm 0.2	0.3 \pm 0.07	1/30000
Rapid	16 \pm 3	2.7 \pm 0.8	1/500

^a Enzyme conjugate dilution factors were determined as those yielding an absorbance of 0.8–1.1 units.

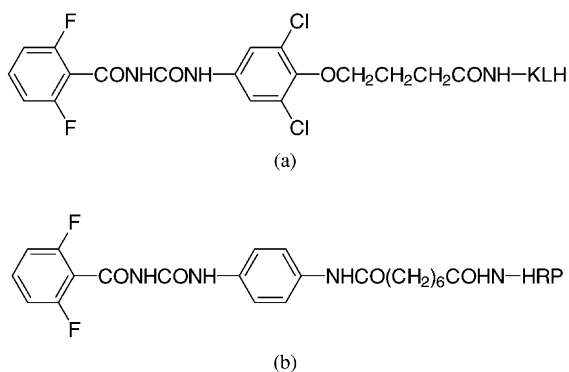


Fig. 2. Chemical structures of (a) immunizing antigen and (b) enzyme conjugate.

The corresponding laboratory assays were about eight-fold 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 required to provide adequate color development within the shorter assay time (Table 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 concentration of the labeled component is increased, the sensitivity decreases. However, this rapid assay was sufficiently sensitive to detect diflubenzuron in grains over the ranges required, corresponding at the lower end to the reporting limit for pesticide-free grain (0 ppm) and at the higher limit to the maximum permissible residue (2 ppm) levels in grain trading.

3.2. Extraction of residues

The efficiency of extraction was reported as the mean percentage recovery calculated for four barley, sorghum and wheat samples with a range of diflubenzuron contents with 10, 20, 50% maximum residue level (MRL) (Table 2). The proportion of residue extracted did not vary significantly at different residue levels.

The toxic nature of organic solvents has prompted researchers to find some alternative to extract pesticides. Surface-active molecules, such as detergents, are attractive for this purpose, because such molecules have a “dual nature”—hydroxyl functional groups on the exterior of the torus and a hydrophobic organic

Table 2
Extraction of diflubenzuron using various techniques^a

Method	Extractant	Barley	Sorghum	Wheat
Shaking 24 h (whole grain)	Methanol	90 ± 14	93 ± 12	90 ± 22
	2% Tween 20	60 ± 18	65 ± 16	59 ± 16
	2% SDS	21 ± 11	NT	NT
	20% MC	41 ± 7	35 ± 8	45 ± 7
	20% HC	48 ± 9	40 ± 12	41 ± 8
2 min blend (ground grain)	Methanol	65 ± 12	71 ± 17	63 ± 15
	2% Tween 20	31 ± 7	21 ± 12	38 ± 9
	2% SDS	17 ± 7	NT	NT
	20% MC	45 ± 4	41 ± 7	51 ± 7
	20% HC	50 ± 11	44 ± 10	47 ± 9
2 min shaking (ground grain)	Methanol	17 ± 4	15 ± 5	14 ± 7

^a SDS: sodium dodecylsulfate; MC: methyl- β -cyclodextrin; HC: β -hydroxypropyl-cyclodextrin.

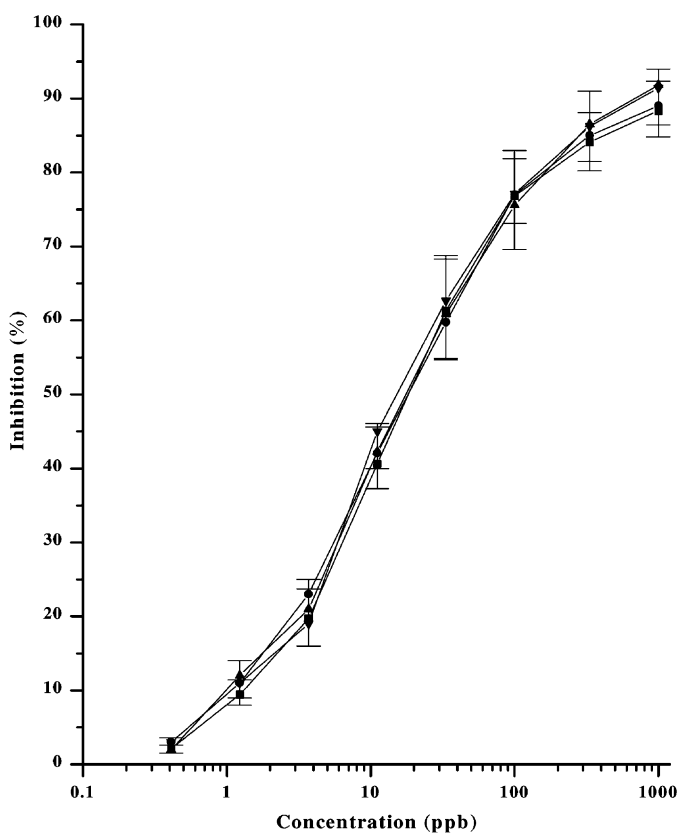


Fig. 3. Standard curves of diflubenzuron in 1% BSA-PBS (■), barley extract (●), sorghum extract (▲), and wheat extract (▼).

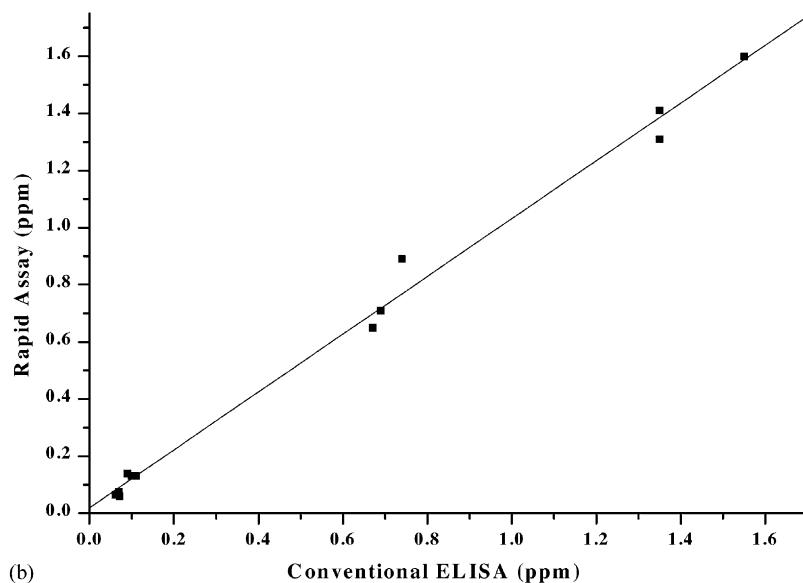
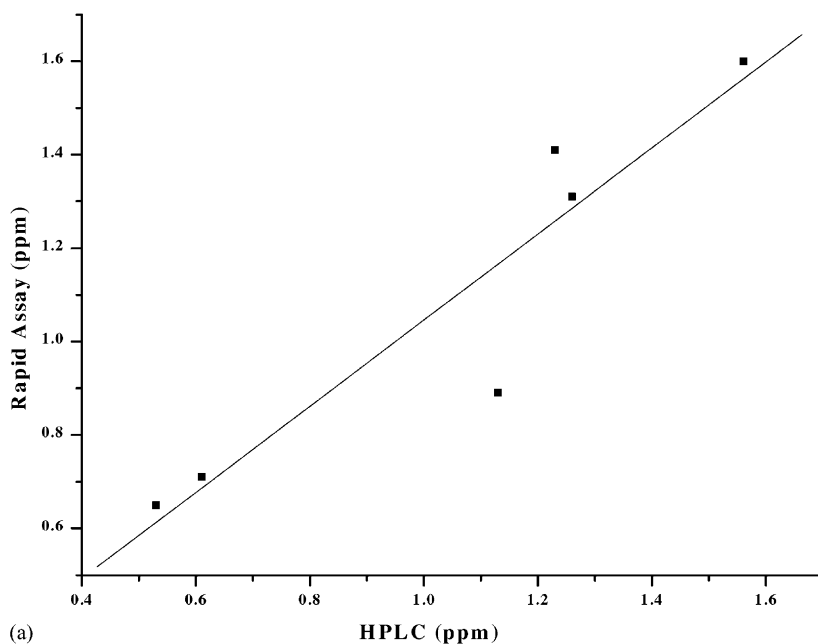


Fig. 4. Relationship between results obtained by rapid assay and HPLC (a) and laboratory conventional immunoassay (b). The regression details of the lines of best fit are as follows: (a) rapid assay vs. HPLC ($n = 6$, $r = 0.93$, slope = 0.92, intercept = 0.12); (b) rapid assay vs. conventional ELISA ($n = 12$, $r = 0.99$, slope = 1.01, intercept = 0.02).

cavity in the interior. Cyclodextrins (CD) are also such molecules, and have been reported to be used to extract pesticides from soil [6]. However, Table 2 shows that the tested chemicals are not effective to remove the

diflubenzuron from grains. It seems that the affinity of detergents for diflubenzuron is not sufficient enough. As a result, the methanol was chosen for extracting diflubenzuron.

Shaking the whole grain with five volume of methanol for 24 h gives nearly 100% recovery of diflubenzuron. However, although this extraction procedure is simple; a more rapid extraction method is often required for use with a rapid test. Two-minute hand-shaking of ground grain extracted very little diflubenzuron residue. Two-minute blending of ground grain was reasonable efficient, extracting over 60% of the residue. Although this method is not efficient compared to 24 h shaking, it is reproducible enough to be used for extraction for screening purposes. However, the relative lower recoveries (60%) indicate the rapid extraction method may have a negative bias in the analysis of grain samples.

3.3. Matrix effects

Table 2 shows that detergent-based extraction method did not provide efficient recoveries for the diflubenzuron from grains in this study. So methanol has to be used for the extraction. Dilution of methanol grain extract in PBS alone could not reduce matrix interference from extract even then diluted 1/100. Addition of Teleostean fish skin gelatin, BSA and Tween 20 to diluent of methanol extract was examined to reduce nonspecific interactions, and 1% BSA–PBS was found to be the best as diluent because matrix interference can be overcome after 1/50 dilution with this diluent. Fig. 3 shows the result obtained using diflubenzuron standard curves prepared in methanol extracts of barley, sorghum and wheat samples and 1% BSA–PBS buffers as control. The superimposition of the standard curves suggested that there were no significant matrix effects grain extract after this extract was diluted 1–50 into 1% BSA–PBS. The color development was affected if dilution was less than 1/50, however the sensitivity was not affected with these less dilutions. Although filtration and column cleanup have been found to reduce the matrix effect (data not shown), however, these will defect the advantage of this rapid assay.

3.4. Assay specificity

Assay specificity was evaluated using several structurally-related aromatic urea herbicides (chlorobromuron, diuron, fluometuron, monolinuron, metobromuron, metoxuron, neburon, tebuthiuron, 3-(3,4-dichlorophenyl)-1-methylurea, 3,4-dichlorophenyl-

urea), metabolites of diflubenzuron (4-chloroaniline, 2,6-difluorobenzamide, 2,6-difluorobenzoic acid) and some structurally dissimilar compounds that may reasonably be expected to be found in the Australian grain industry (bifenthrin, carbaryl, chlorpyrifos-methyl, cypermethrin, DDE, DDT, deltamethrin, dichlorvos, dieldrin, fenitrothion, methoprene, and pirimiphos-methyl). The IC_{50} values for each of these compounds were above 10,000 ppb, suggesting that the assay was specific to diflubenzuron.

3.5. Accuracy and precision of the assay

The pesticide concentration-inhibition standard curve in the rapid assay was reproducible, with standard deviation of inhibition values under 15% in all cases. The accuracy of results obtained using the rapid test (2 min blending extraction method) was investigated by comparison of results obtained with HPLC method and laboratory conventional ELISA method. Fig. 4 shows that this rapid test gave good correlation (linear relationship).

4. Conclusion

A rapid, simple test for use in field situations has been developed for diflubenzuron. To achieve this an effective rapid extraction technique was developed using ground grain and methanol. The rapid test produced analytical data that correlated well with both the corresponding laboratory immunoassay methods and the standard instrumental technique. This test could be a valuable method enabling on-site residue analysis and a model for other pesticides of concern in feed grains.

This assay has now been formatted into prototype test kit. The data described in this study were obtained using reagent additions made with micropipets. However, in kits for field use, each of these additions would be made using disposable plastic pipets or dropper bottles.

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