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Part 1 - Summary Project Details

Final Report

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Part 3 - Final Report Format

Plain English Summary

This project aimed to improve technical capability to study the transport off-farm in water and on sediments of pesticides such as endosulfan and members of the benzoyphenylurea (BPU) family. This was achieved mainly as the result of adaptation of existing enzyme-linked immunosorbent assays (ELISAs) for field studies, as well as the generation of new assays. These studies have indicated that endosulfan sulfate is formed as a biological product and that this the main reason for persistence of endosulfan residues in ponded runoff water. The project has also resulted in the availability of a new group-specific enzyme-linked immounosorbent assay (ELISA) for residue analysis of the whole family of BPU pesticides and of a compound-specific ELISA for flufenoxuron. Both of these assays were able to be applied in studying the dissipation of BPUs in water and soil. BPU residues in water could be quantitatively analysed directly without the need for cleanup, and soil samples required only a simple extraction with 90% methanol. Spike and recovery studies for five BPUs in soil and water indicated that these assays quantified BPUs with good recoveries. The assays were very specific for BPUs, and neither the environmental matrices nor other cotton agrochemicals interfered with the detection of BPUs. The presence of particulates and dissolved organic compounds in water and soil extracts did not interfere with the assays. An ELISA for the quantitative determination of pyrithiobac-sodium (Staple) developed in the USA was validated with Australian soils in this project. These studies confirm the utility of ELISA tests as a simple means of monitoring the environmental fate of cotton pesticides. The results of this project are published in four papers in recognised journals and nine papers given at conferences.

PART 3 – Final Report Format

Spatial Distribution of Chemicals used in Growing Cotton and the Potential for transport Off-farm

Background to the project

Despite the fact that a wide variety of insecticides have been registered for the control of insect pests in Australian cotton production system, current management is heavily dependent on insecticides from five classes; single representatives from the organochlorine class, endosulfan, and formamidine (amitraz) classes, and several from the carbamate, organophosphate, and pyrethroid classes. All of these are nerve poisons whose mode of action depends on binding at target sites in the insect's nervous system: endosulfan at the gamma-aminobutyric acid (GABA) gated chlorine channel, amitraz at the octopamine receptor, organophosphates and carbamates at the acetylcholinesterase and the pyrethroids at the voltage gated sodium channel. Heavy selection pressure on these relatively few target sites has contributed to problems of resistance. With the exception of formamidines, Australian field populations of insects have developed resistance to all of the above chemical groups (Forrester, 1994).

Genetic engineering has provided transgenic cotton expressing the *Bacillus thuringiensis* Cry 1Ac delta endotoxin, with insecticidal efficacy against *Helicoverpa* spp. However, even if this technology is exploited to its full potential under Australian conditions, additional, novel foliar insecticides for control of insects are needed to maintain an adequate range of choice for cotton farmers..

Based on above two reasons, new classes of insecticides are needed. These new insecticides should offer greater specificity to target pests, have greater intrinsic activity, achieving acceptable insect control at low field use rates. Such new toxicants also should be more environmentally acceptable than existing insecticide chemistry. Due to benzoylphenylurea pesticides' high selectivity and high biological activity, resulting in low application rates (Metcalf *et al.,* 1975), BPUs have only a slight effect on the natural enemies of various harmful insect species. These properties and low acute toxicity for mammals make them suitable for inclusion in integrated pest control programmes. Therefore, benzoylphenylureas were once under consideration for use in Australian cotton systems (Holloway and Forrester, 1998) while this project was in progress.

The trial with chlorfluazuron in cotton production systems (Helix, one member of BPUs) had shown promising results, but this compound was banned in 1994, resulting from the detection of residues accumulated in cattle fed with cotton trash. The withdraw of chlorfluazuron from Australian cotton represented a significant loss from the insecticide resistance management (IRM) strategy program and new, less persistent BPUs, such as diflubenzuron, flufenoxuron, lufenuron and teflubenzuron might be used for cotton instead. However, partly as a result of previous experience with chlorfluazuron, the National Registration Authority (NRA) and Environment Australia now require much more stringent examination on their environmental fate in the registration of new

chemicals, therefore an environmental fate study of this group of compounds is essential to minimise their environmental impact.

High performance liquid chromatography (HPLC) with UV detection or diode array detection is predominantly employed for the determination of BPUs (Tomsej and Hajslova, 1995; Tadeo, 1996; Hiemstra *et al.*, 1998) because they are thermally unstable and their GC properties are poor. The GC methods can also be used after derivatisation of the parent analyte or its hydrolytic products (Smith *et al.*, 1983; Stan and Klaffenbach, 1991). However, such methods, normally involving sample extraction followed by a multi-step clean up and concentration steps, are time consuming and laborious. As part of an efficacy program, a rapid and reliable immunoassay for such compounds was needed. Thus, ELISA methods were developed in this project for measuring BPU residues in environment. Such ELISAs may result in a sensitive and cost effective means of analysing environmental samples containing BPUs when compared to the HPLC methods.

Flufenoxuron was once regarded as a key candidate to be used to control *Heliothis* after banning of chlorfluazuron. In order to study more carefully on its environmental fate, a flufenoxuron-specific ELISA was developed in this study. Unfortunately, this compound was removed from trials in Australian cotton systems when it was found in field trials that the compound could not adequately control *Heliothis*.

When this project commenced, pyrithiobac-sodium was a relatively new herbicide being introduced to the cotton industry. Pyrithiobac-sodium is a post-emergence broad-leaf weed control herbicide for use in cotton. Post-emergence herbicides can reach the soil surface following application to foliage (Reddy *et al.*, 1994), where they would be subject to various transport, retention, and transformation processes. Thus there is a need to use a simple, fast and cost-effective method to follow its movement and dissipation in the environment. An ELISA method for this compound has been developed by DuPont, but it must be validated before being applied to environmental fate study. The validated ELISA would be able to be employed to monitor possible transport of this compound in Australian cotton production system. Therefore, a study was also conducted to characterise and validate the pyrithiobac-sodium immunoassay with Australian soils and the usefulness of this assay has been proven by employing it to study the environment fate of this compound in Australian cotton system.

The objectives and the extent to which these have been achieved

This project sought to:

- obtain information on the environmental fate of chemicals used in cotton growing, including endosulfan and insect growth regulators such as chlorfluazuron.
- develop analytical tools to allow the study of the environmental fate of benzoylphenylurea insecticides and herbicide pyrithiobac-sodium.

The objectives to be achieved in each year of the grant were:

Year one: Analysis of endosulfan and diuron in soil and water samples using

ELISAs previously developed and provided information regarding their transport off-farm.

Year two: Development of ELISA for benzoylphenylurea insecticides.

Year three:Development of ELISA for benzoylphenylurea insecticides and validation of pyrithiobac-sodium ELISA in Australian cotton soils.

The methodology and a justification for the methodology used

The experimental approach involved:

- collection of field soil and water samples from selected farms and sample analysis using ELISA.
- development of ELISA for BPUs including design and synthesis of haptens, conjugation of haptens to macromolecular carriers, antibody production and assay optimisation.

Detailed results including the statistical analysis of results. Discussion of results including an analysis of research outcomes compared with objectives

1. Studies on endosulfan and use of ELISAs for the Warren study on ponded runoff

When the project was planned, a study on the fate of chlorfluazuron was planned (see objectives above). However, the banning of Helix during the 1995-1996 cotton season negated these plans. It was decided to study other members of the benzoylphenylurea family instead.

In the early stages of this project, Sebastian Southan continued his basic research on dissipative mechanisms of endosulfan, determining relative rates of volatilisation, conversion to endosulfan sulfate and hydrolysis (Southan and Kennedy, 1996). These studies revealed that the α-isomer dissipated mainly by volatilisation and the β-isomer mainly by hydrolysis or conversion to the sulfate. Field trials conducted at Warren using ultraviolet shielding showed that ultra-violet radiation was not a significant factor in the rate of hydrolysis to the diol or oxidative conversion of the endosulfan isomers to endosulfan sulfate. Thus, the conversion to the sulfate is confirmed to be uniquely a biological process. The rate of conversion of the isomers to the sulfate in field soil was found to be strongly controlled by water content of soil. The wetter the soil and the sooner after irrigation, the greater the rate of conversion to endosulfan sulfate. This result, though explicable as a result of greater bioavailability in wetter soil, was partly unexpected. From these results, unless the field soil and beds are totally submerged, the restriction of oxygen seems unlikely to be sufficient to favour endosulfan diol formation.

An endosulfan ELISA developed in a previous CRDC project by N.A. Lee (US16C) was employed for a study conducted by S. Kimber at Auscott Warren, on the dissipation of endosulfan from an artificial pond filled with run-off. It was shown (Kimber *et al.*, 1996;

Kennedy *et al.* 2001) that 200 mg of endosulfan initially contained in the pond dissipated in a two-stage process with half-lives of 1.5 and 7.8 days. A rapid initial disappearance shown with GLC and ELISA methodology corresponded with the disappearance of the α- and β-isomers, while the second phase of dissipation was characterised by the presence of endosulfan sulphate remaining in runoff. Tota;l endosulfan on colloidal and suspended sediment (> 0.7 μm) decreased from 30 mg to 5 mg in that period. At the same time, a sedimentation process was observed to the floor of the pond, with pesticide residue levels in bottom sediment rising to about 100 mg, almost half of the initial load in runoff.

2. The development of the ELISAs for BPUs

The orientation of the hapten relative to the carrier protein is an important factor in determining the specificity of antibody-hapten binding. The region of the hapten which is distal to the point of attachment to the protein will be most exposed to the immune response, resulting in better recognition of this region (Landsteiner, 1945). This offers the potential to design hapten-protein conjugates for either production of a generic assay for detection of a range of analogues, or for a compound-specific assay with low levels of cross-reactivity with related compounds. In this study, this principle was applied to develop two types of immunoassays, class-specific assays (detecting five BPUs) and compound-selective assay (detecting flufenoxuron only).

The first objective was to design, develop, and optimise an ELISA for residue analysis of the whole family of benzoylphenylurea pesticides. Since all these five BPUs have a common aromatic-urea group, it was predicted that a broad-specificity assay for group of BPU pesticides would result from the synthesis of some haptens in which the common benzoyl urea motif of the BPUs was distal to the point of coupling. Three derivatives of diflubenzuron were synthesised as haptens, two of them with spacer arms attached at 4-position of aniline ring and one with a spacer arm bound to the urea nitrogen (Figure 2). They were conjugated to proteins and enzyme either by diazotisation of amine group, or by the active ester method using the acid group. Polyclonal antibodies against these immunogens were prepared in New Zealand white rabbits.

Direct competitive, solid-phase antibody enzyme-linked immunosorbent assays (ELISAs) were used in this study. Different combinations of antibody and enzyme conjugates were examined, indicating the heterologous assays were more sensitive than the homologous assays. With the same enzyme tracer, antibodies from hapten II-protein conjugate provided higher sensitivities to the five BPUs than to antibodies raised to the other hapten-protein conjugates, demonstrating the importance of an appropriate length for the bridge between hapten and protein to enhance the sensitivity of detection. A combination of antibody from hapten II-OA and enzyme from hapten I-DSS-HRP was selected and the optimised assay gave 50% inhibition of antibody binding (IC_{50}) at 0.6 ppb for diflubenzuron, 5 ppb for teflubenzuron, 10 ppb for flufenoxuron, 31 ppb for lufenuron and 45 ppb for chlorfluazuron, with detection limits in the range 0.05 - 2.3 ppb for these compounds. Since no cross-reaction with the structurally related aromatic urea herbicides and metabolites of diflubenzuron and teflubenzuron, it appears that both the

aromatic rings and the urea moiety in the BPU structure were critical for antibody binding, not as anticipated that only common benzoyl urea group was involved.

Although this assay can detect all five BPUs with a lower detection limit of less than 3 ppb, the detection of the larger BPU molecules was less sensitive. Molecular modelling studies were therefore employed to study why such a phenomenon occurred. The interaction of an antibody with a hapten is dependent on the conformational and electronic properties of both structures since these factors affect the van der Waals forces, hydrophobic interactions, hydrogen bonds and electrostatic bonds that determine binding affinity. In this study, we modelled the hapten in an attempt to correlate the structural and electronic properties of the hapten with antibody binding. Such a strategy has also been used by the group of Stanker (Elissalde *et al.*, 1995; Stanker et al., 1995; Beier *et al.*, 1996; Rose et al., 1996; Holtzapple *et al.*, 1997) to explain the immunoassay results they obtained.

Use of molecular models provided an explanation of this superior cross-reaction of diflubenzuron, indicating that

- the oxygen in the 4-position of aniline ring in chlorfluazuron, flufenoxuron and lufenuron makes the alkyl or aryl group almost completely out of plane, then sterically preventing their recognition by antibodies raised to haptens that had a coplanar group in that position.
- the electron-donating characters (with negative electrostatic potential isosurfaces) at the 4-position of aniline ring in chlorfluazuron, flufenoxuron and lufenuron may be less well recognised by antibodies raised to haptens that had an electron-withdrawing atoms (with positive electrostatic potential isosurfaces) in that position.
- the chlorine atom may be necessary for antibody binding because of its large size.

Since both aromatic rings and the urea moiety must be involved in antibody binding, any change in the aniline ring which results in major variation of geometric and electrostatic properties may have a significant effect on the binding activity.

An improved assay with more equal cross-reactivities to all five BPUs, especially to the larger BPUs, was therefore investigated using a new hapten synthesised according to the results of the molecular modelling study. Hapten IV (Figure 2) was made by introducing a spacer arm at the aniline moiety of the BPUs through an oxygen group at the 4-position, together with the inclusion of two chlorines at 3- and 5-positions, while keeping 2,6-difluorobenzoylurea moiety distal to the point of coupling. Compared to the previous assay, the cross-reactivities of this assay with the chlorfluazuron and teflubenzuron was improved greatly. The assay showed IC_{50} values of 1.3 ppb for diflubenzuron, 2.1 ppb for teflubenzuron, 9.3 ppb for chlorfluabenzuron, 35 ppb for lufenuron, and 44 ppb for flufenoxuron. This study confirms the importance of conformational and electronic properties of hapten in the design of pesticide immunoassay.

Although cross-reactivity of this second class-specific assay to all five BPUs was improved compared to the previous one, it was still less sensitive to larger BPUs. The degree of antibody specificity is determined by the extent of complementarity between

the hapten and amino acid sequence present in the antibody combining site. It is possible that the small size of diflubenzuron makes it more easy to fit into the antibody binding site for both of these assays.

The second objective was to develop a compound-specific ELISA for flufenoxuron, one member of BPUs. The approach in hapten synthesis was made into attaching spacer at the 2,6-difluorobenzoylurea moiety of flufenoxuron, thus preserving the unique characteristic 2-fluoro-4-(2-chloro-4-trifluoromethylphenoxy)aniline group of flufenoxuron. The resultant sensitive assay selectively detected flufenoxuron, with very low cross-reaction to the other four members of BPUs tested in this study (4000-fold less sensitive).

Based on the results on this research, two conclusions can be established regarding to the influence of hapten design and hapten heterology on the selectivity and sensitivity of the assay. First, the success of this research demonstrates that hapten design is critical to the development of a good immunoassay for small molecules. Different kinds of antibodies can be obtained by using different strategies of hapten design, the position of spacer arm attachment having a profound influence on the selectivity of the assay. By first having a clear idea of the goals of the assay and then carefully designing haptens with the aid of molecular modelling, it usually is possible to synthesise only a few haptens and obtain a group-specific or compound-specific assay, each having specific applications in residue analysis. As a second conclusion, the use of heterologous system proved to be a valuable approach to improve the sensitivity of immunoassays for pesticides. However, the tracer hapten should not be greatly different from the immunising hapten. Otherwise, such a tracer would not bind to the antibody with sufficient affinity to produce an adequate, analyte competitive assay signal.

Both class-specific and flufenoxuron-selective assays were able to be applied in studying the dissipation of BPUs in water and soil. BPU residues in water could be quantitatively analysed directly without the need for cleanup, and soil samples required only a simple extraction with 90% methanol. Spike and recovery studies for five BPUs in soil and water indicated that these developed assays quantified BPUs with good recoveries. The assays were very specific for BPUs, and neither the environmental matrices nor other cotton agrochemical interfered with the detection of BPUs. The presence of particulates and dissolved organic compounds in water and soil extracts did not interfere with the assays.

3. The validation of the pyrithiobac-sodium ELISA for Australian cotton soils:

An ELISA for the quantitative determination of pyrithiobac-sodium (Staple) was validated with Australian soils and its performance compared with that of gas chromatography-mass spectrometry (GC-MS). The detection limit for pyrithiobac-sodium is 4-5 ppt. Cross-reactivity of the assay with potential cocontaminants in cotton soil samples was examined, and none of them was found to interfere with the detection of this herbicide. Two extraction methods were compared: by shaking with PBS buffer and extraction with automated "accelerated solvent extraction" (ASE). This showed that ASE

extraction method was more efficient than the PBS extraction method. However, the PBS extraction method showed acceptable recoveries for one-week laboratory aged soils, except for one kind of soil which showed recoveries of less than 70% for the spiked levels of 6 -12 ppb. Thirty incurred field soil samples were analysed by ELISA and standard GC-MS methods. Samples were extracted for ELISA analyses by both PBS and ASE extraction methods, while extracted by ASE followed by cleanup for GC-MS analyses. Immunoassay results were in close agreement with the GC-MS results (R = 0.93 and 0.92, respectively for ASE and PBS extraction), demonstrating that ELISA can be used as the determinative step in pyrithiobac-sodium analysis. The usefulness of this ELISA was evident by the application of this validated assay to study the environmental fate of this compound in Australian cotton farming systems (Mitchell *et al.*, 1998).

It is considered that the objectives of this project were adequately met. The main role of the project's funding was the training of a PhD (Shuo Wang). Significant progress has also been made in improving our capacity to study the spatial distribution and environmental fate of several new chemicals used in pest control. In addition, four papers were published in international journals as well as nine other papers in the proceedings of conferences.

Assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian Cotton industry and future research needs

The ELISA methods developed in this project are useful in environmental studies, as well as in screening agricultural procedure. The methods developed in this project will also benefit other research projects, which are aimed at obtaining improved understanding the transport of pesticide residues from cotton farms. In conjunction with more sophisticated instrumental analytical methods, they would provide a powerful research tool for environmental studies of pesticides. In addition, developing field testing tubes coated with immobilised antibodies obtained from this thesis should also be valuable. Such field tube assay will enable residues of BPUs to be detected at field site and provide a means for protecting the quality of the environment as well as the quality of plant and animal produce. The advantages of pesticide immunoassay such as high throughput, simple, fast methods and cost effectiveness have been evidenced by the application of validated pyrithiobac-sodium ELISA in the environmental fate study of this herbicide in Australian cotton production system. This study assisted the registration process of pyrithiobac-Na with the National Registration Authority which is already benefiting the Australian cotton industry.

Description of the project technology (e.g. commercially significant developments, patents applied for or granted, licenses, etc)

No technology has been commercialised in this project, although this may occur in the future. The procedure involved would be to licence the technology, including immuno-

reagents, to commercial firms or partners for a set period of time. It is not normal to patent poly-clonal antibodies of the type generated in this project.

A technical summary of any other information developed as a part of the research project including discoveries in methodology, equipment design, etc.

The information developed in the project may be summarised in two areas. These are:

(i) the information on endosulfan generated in this project has been assimilated into the database of knowledge on the environmental fate of this chemical.

(ii) the ELISA methodology developed may be applied in future technology to be developed as a screening tool for produce or environmental samples..

Recommendations on the activities or other steps the may be taken to further develop, disseminate, or to Exploit the Project Technology

Before any attempt is made to exploit the technology there will be a need to further develop and further simplify the ELISAs in simple test kit form. At this stage, they should be considered as research tools applicable as laboratory techniques. In addition, extensive market surveys would be needed to ensure that any commercial development would be viable.

A list of publications arising from the research project

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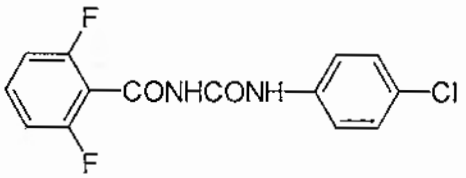
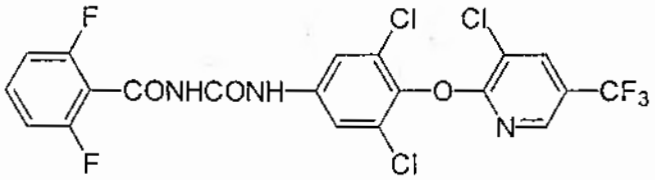


diflubenzuron



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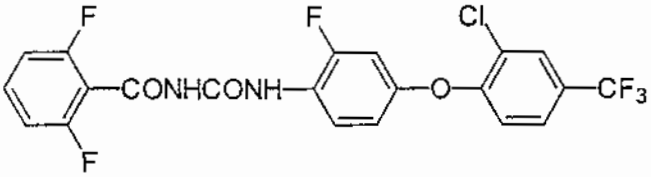

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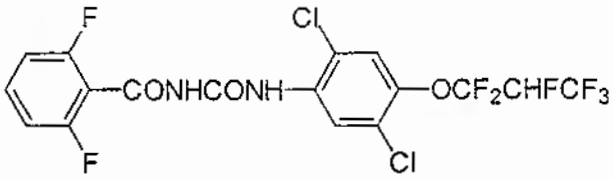

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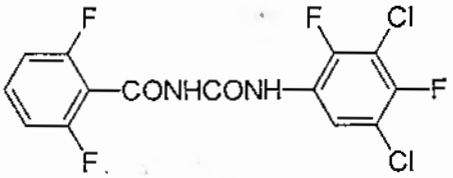
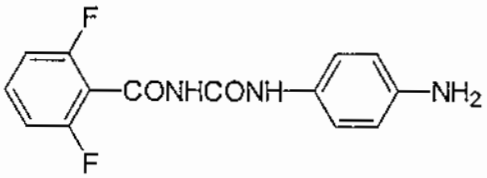
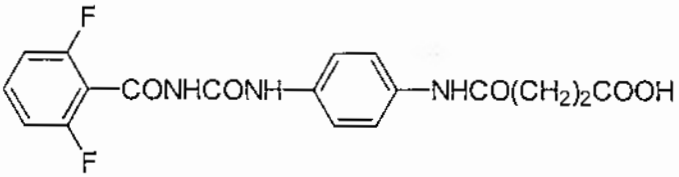


teflubenzuron

Figure 1 Chemical structures of five benzoylphenylureas.



Hapten I



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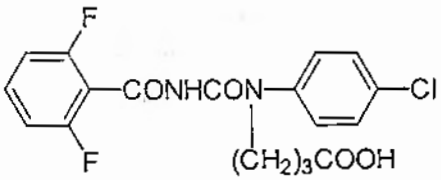

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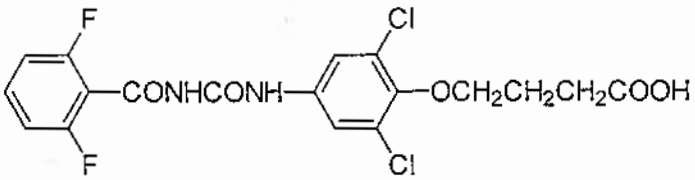

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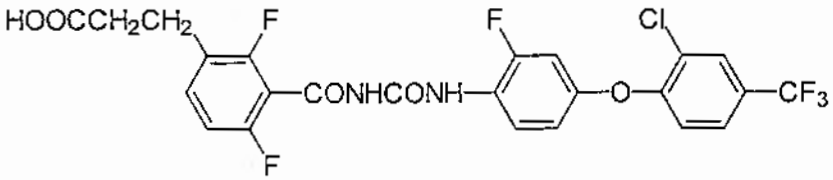

Hapten V

Figure 2 Chemical structures of five haptens.