Rapid testing for insecticide residues in stored products using immuno- and enzyme- assays

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Abstract

Accurate knowledge of the presence and levels of protectant residues in stored products is important for several reasons—adequate control of possible infestation, to ensure that levels do not exceed legal Maximum Residue Limits and to police specifications in sale/export contracts. While residue levels can be accurately determined by gas-chromatography, the large number of samples and sampling times required for thorough monitoring led us to develop an alternative method, suitable for on-site testing. Specific antibodies have been employed for the major grain protectants used in Australia and overseas: organophosphates (fenitrothion, chlorpyrifos-methyl, pirimiphos-methyl), carbaryl, methoprene and synthetic pyrethroids (bioresmethrin, permethrin, phenothrin). Tests for deltamethrin and some other pyrethroids are under development. In addition, other grain protectant compounds such as malathion, methacrifos, dichlorvos, bromophos and etrimfos can be detected using a novel cholinesterase inhibition test. The reagents needed to perform the tests have been packaged into compact test kits, some of which are now commercially available. All the immunoassay tests are performed in the same manner: grain or other commodity is extracted by blending in methanol, drops of the extract added to an antibody-coated microwell or tube followed by drops of an enzyme conjugate solution. After a few minutes, the microwell or tube is washed in water to remove unbound components then a colour developer added. Different levels of protectant in the sample produce graded differences in yellow colour. The cholinesterase test is performed similarly.

Tests have been tuned for detection in the 0.05/ 0.1 – 10 ppm residue range in the commodity. Kits designed for simultaneous testing of a large number of samples under laboratory conditions have also been developed and are suitable for analysis of malted, baked or noodle end-products as well as raw grain. Insecticidal protectants are also important in developing countries. In a new collaboration with Indian scientists, we are extending this work to some other plant-based commodities and organochlorines, fungicides and other organophosphates. Advantages of immunoassay kits here will be the low capital and per-test costs.

Introduction

Important elements in balancing possible residue problems arising from the use of agrochemicals with cheap and effective control of stored-product pests are the ability to adopt pest and postharvest management practices that lead to residue in foods for consumption being either undetectable or acceptably low, and the establishment of a comprehensive and reliable residue monitoring program (Snelson 1987). The major technique for residue monitoring is gas chromatography (GC) with either one of a variety of selective detectors, such as a nitrogen-phosphorus detector or a more general but less sensitive (and more expensive) detector such as a Mass Selective Detector (GC-MS). High-performance liquid chromatography, especially when coupled with variable wavelength or photodiode array detector is finding increasing use in residue analysis, especially of low volatility compounds.

Over the last few years, we have developed a range of new tests for pesticide residues in cereals and cereal-derived foods, based on the use of specific antibodies and pesticide-enzyme complexes. This has addressed a need in stored-product management, since most other groups working in the area of pesticide diagnostics have focused on environmental applications, such as determination of herbicides in water. Some of the tests have been formatted into complete kits, with separate kits intended for laboratory or field uses. In each case, the presence or absence of the particular pesticide in a food sample can be read by eye, as a simple colour change. The laboratory tests are designed for quantitative analysis of sets of samples, and require a blender, pipettes and a photometer. Field tests (which are also suitable for small laboratories) take as little as 5–10 minutes to perform and (for screening) require no equipment, only a source of running water. The tests are also designed to be use by minimally-trained individuals, and can be run in either qualitative or quantitative fashion (if a small photometer is used to measure the colour produced in the test). Despite being much simpler than GC or HPLC methods, determination using the kit methods is usually more sensitive and freer of matrix interference than instrumental methods.

Our emphasis to date has been on protectants used on grain and on analysis of grain matrices, as this represents a major use of postharvest agrochemicals in Australia. However, chemical use with other stored products is also important, especially in developing countries. More recently we have developed antibodies for some of these compounds of concern, such as parathion, organochlorines (cyclodienes, endosulfan, DDT) and synthetic pyrethroids. Work is under way, collaboratively with Indian scientists, to apply these new antibodies in assays for stored foods of plant origin. This paper reviews our achievements with stored grain and current studies on other plant-based foods.

Tests for Residues in Stored Grain

Harvested wheat and barley require protection during storage from damage by a range of insect species. Cereal grains are often treated with degradable pesticides, including mixtures of organophosphates, carbamates, synthetic pyrethroids or insect growth regulators to prevent insect infestation before processing and consumption. The identity and amounts of pesticide are important because: 1. small amounts of pesticide or pesticide metabolite may persist into the baked or brewed

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product, 2. pesticides used on some grains and in some countries may not be allowed in other situations, 3. an increasing proportion of customers specify 'residue-free' grain. A simple and economical means of grain protection involves the use of certain insecticides, including organophosphates, synthetic pyrethroids, carbaryl and methoprene. However, insecticide use brings with it the likelihood of residues appearing in foods derived from these grains. In response, we have developed antibody-based tests for the major pesticides used on stored grain, either using antibodies developed in-house, or obtained from collaborators, and have demonstrated good correlations between immunoassay data and data obtained using conventional instrumental methods. The assays have been applied to both raw wheat and barley grain, and to foods derived from these cereals.

To perform the immunoassays, samples (10–40 g) were extracted for 48 hours in neat methanol using 2.5 volumes (wheat, wholemeal, flours, noodles, barley, malt), 4 volumes (breads) or 10 volumes (pollard, bran, gluten) with intermittent shaking. The breads and noodles were first treated for 2 minutes in the methanol with a probe homogeniser. The immunoassay method was as follows:

1. Microwell plates were pre-coated with appropriate antibodies;
2. Methanol extracts of the grain products were allowed to settle, then were diluted 1/5–1/50 in 50 mm sodium phosphate - 0.9% NaCl (pH 7.2)- 0.05% Tween - 1% bovine serum albumin (BSA). In the case of methoprene, the BSA was deleted, and for permethrin, 0.005% Tween was used. Pre-dilution of extracts in methanol was performed such that all extracts had a final methanol concentration of 5%, except for fenitrothion and bioresmethrin (10%);
3. Diluted extracts or pesticide standards (100 µL) then pesticide-peroxidase conjugates (100 µL) were added to each well and incubated for 1 hour at 20°C;
4. The plate was washed, 160 µL substrate-3,3',5,5'-tetramethylbenzidine chromogen was added and incubated 30 minutes at 20°C;
5. Stopping reagent (40 µL 1.25 M sulfuric acid) was added and absorbency measured at 450 nm. In addition, methanol extracts of grain fractions containing fenitrothion, chlorpyrifos-methyl or pirimiphos-methyl were analysed by gas-chromatography. Methanol extracts of wheat products containing methoprene were dried then transferred to hexane for normal-phase HPLC.

Among grain protectants, each assay was specific for a single compound, except the permethrin antibody, which also detected 1R-phenothrin. Likely breakdown products of each pesticide were not detected by the assays. Assay sensitivity in whole grain was sufficient to detect down to the common reporting limits of each compound (Table 1). Methanol was selected because it has been shown to be an effective extractant of each of the pesticides under study from various grain matrices. The development of antibodies and performance of the various assays has been described in detail elsewhere:

- fenitrothion in wheat grain (Hill et al. 1992)
- chlorpyrifos-methyl in wheat and barley grain (Edward et al. 1993a,b)
- bioresmethrin in wheat and barley grain (Hill et al. 1993)
- methoprene in wheat grain and milling fractions (Hill et al. 1991)
- permethrin and phenothrin in wheat grain and milling fractions (Skerritt et al. 1992a)
- various organophosphates in wheat grain and milling fractions (Skerritt et al. 1992b)
- organophosphates, bioresmethrin and carbaryl in barley and malt (Edward et al. 1992)
- field tests for fenitrothion and pirimiphos-methyl (Beasley et al. 1993) and for chlorpyrifos-methyl and methoprene (Edward et al. 1993a)
- performance of assays with breads and noodles (Edward et al. 1993c)

Some of the relationships obtained between immunoassay and instrumental data in these studies are summarised in Table 2. The development of antibodies to carbaryl and methoprene has been described elsewhere (Mei et al. 1991; Marco et al. 1993).

Table 1. Sensitivity of laboratory immunoassays

Grain protectant	Sensitivity in grain	
	Limit of detection (sensitive assay)	Mid-range of standard curve (standard assay)
Organophosphates		
Fenitrothion	0.08 ppm	3.5 ppm
Chlorpyrifos-methyl	0.02 ppm	1.5 ppm
Pirimiphos-methyl	0.03 ppm	3 ppm
Synthetic pyrethroids		
Bioresmethrin	0.05 ppm	0.6 ppm
Permethrin	0.08 ppm	0.5 ppm
Methoprene	0.02 ppm	0.6 ppm
Carbaryl	0.05 ppm	0.6 ppm

Opportunities for Developing Countries

Strategies to reduce postharvest pesticide usage are as important to developing countries as they are to Australia. However, the higher cost of many of these strategies together with usual conditions of high humidity and temperature after harvest mean that high usage of persistent pesticides such as organochlorines will continue for some years to come. For example, in India in 1991, 65000 tonnes of insecticides were used, including 48000 tonnes of organochlorines. Widespread use, high application rates, the persistent nature of the major compounds used (HCH and DDT), and the difficulty of policing appropriate application and witholding times, lead to the regular finding of excessive and even unsafe residues in food commodities. The cost and logistic constraints to performing systematic surveys in most developing countries are daunting. However, the monitoring of pesticide residues in food is an important aspect to minimising potential hazards to human health. When unacceptable residues are found, steps can be taken to identify the cause and prevent violations recurring. Failure to adequately screen and control residues in food, especially of compounds such as organochlorines (to which Western countries strictly apply Maximum Residue Limits) is one factor that has restricted opportunities for many developing countries in the export market for tropical fruits, spices and other agricultural commodities. While Australian concerns about pesticide residue levels in foods are largely driven by our need to conform with export market requirements, in countries such as India, residues in food can pose regular health risks.

To monitor residues adequately, there is a need for simple, rapid and inexpensive methods for residue detection. Barriers to more-effective pesticide testing in developing nations include price of equipment, cost and slowness of each analysis, and problems with decentralisation of testing and in coping with a statistically correct proportion of samples. Immunoassay and related methods are appropriate technologies for developing countries as they are simple (little training

needed), inexpensive (few dollars/test) and do not require expensive equipment (Table 3). Immunoassays and related assays can be formatted into compact test kits which can be designed for either laboratory or field use.

A new project aims to develop, adapt and apply, in collaboration with the Central Food Technological Research Institute of India, a range of simple-to-use test kits for detection and quantification of residues of agrochemicals in food (predominantly plant-based foods, including fruit and vegetables, pulses, cereals and oilseeds, groundnuts and spices; Table 4). Because of major residue problems, some extension of the methods to drinking water and milk is also planned. Kits for both rapid and on-site (field) monitoring and laboratory use in residue quantitation will be developed, with emphases on major insecticides and fungicides used in Indian intensive and broadacre agriculture. Appropriate targets are major use compounds whose continued but more appropriate use is important for productivity of the agricultural industry and whose use is associated with risks of human exposure to unacceptable residues and damage to developing food export markets.

Enzyme Assays

One of the major shortcomings of immunoassays is that in many cases their specificity does not lend itself to broad screening for residue levels. Detection of members of a class of compounds could potentially be performed by use of the sites of action of the compounds in an *in vitro* biochemical assay for the compound. One such assay involves the use of cholinesterase enzymes, the site of action of many organophosphates and carbamates. Several cholinesterases have been described in the literature and some test kits for pesticide screening, using enzyme from electric eel, red blood cells or

Table 2. Relationships between immunoassay and instrumental data for grain protectants. Data obtained using whole grain, except for bioresmethrin determination on barley (ground grain)

Pesticide	Commodity	Assay	Recovery (%)	Correlation coefficient	Number of samples
Fenitrothion	Wheat	Laboratory	113	0.94	57
	Wheat	Field	102	0.93	25
	Barley	Laboratory	103	0.95	105
Chlorpyrifos-methyl	Wheat	Laboratory	101	0.97	35
	Wheat	Field	113	0.90	20
	Barley	Laboratory	111	0.98	7
Pirimiphos-methyl	Wheat	Laboratory	105	0.99	11
	Wheat	Field	99	0.99	9
Bioresmethrin	Wheat	Laboratory	96	0.94	15
	Barley	Laboratory	86	0.90	34
Permethrin	Wheat	Laboratory	81	0.99	6
1(R)-Phenothrin	Wheat	Laboratory	124	0.99	6
Methoprene	Wheat	Laboratory	94	0.95	29
	Wheat	Field	87	0.96	9
Carbaryl	Barley	Laboratory	72	0.91	20

Table 3. Enzyme-immunoassays reported in the literature for insecticides and fungicides[a]

Insecticides			
Organophosphates	Parathion	Fenitrothion	Chlorpyrifos
	Diazinon	Chlorpyrifos-methyl	Pirimiphos-methyl
Carbamates	Aldicarb	Carbaryl	Methomyl
Organochlorines	Cyclodienes	Endosulfan	DDT
Pyrethroids	S-Bioallethrin	Bioresmethrin	Cypermethrin
	Deltamethrin	Permethrin	Phenothrin
Insect growth regulators	Diflubenzuron	Methoprene	
Toxins	*Bacillus thuringiensis*		
Non-systemic fungicides			
Organosulfur	Captan		
Chlorinated aromatic	Chlorothalonil		
Dicarboximides	Iprodione	Vinclozolin	Procymidone
Systemic fungicides			
Benzimidazoles	Benomyl	Carbendazim	Thiabendazole
Morpholines	Fenpropimorph		
Triazoles	Triadimefon	Propiconazole	
Phenylamides	Metalaxyl		

[a]citations are listed in Gee et al. 1994

Table 4. Some specific pesticide/commodity combinations to be studied in Indian foods

Pesticide	Commodities
1. Organochlorines	
HCH	Wheat, maize, rice, coffee
DDT and HCH	Milk, butter, ghee, rice, peanut oil, peanuts, ginger
Endosulfan	Coffee, salad vegetables
Endrin	Rice, (shellfish)
2. Organophosphates	
Fenitrothion	Wheat, some fruit and vegetables
Pirimiphos-methyl	Wheat
Dimethoate	Cardamon, okra, potato, onion
Methyl-parathion	Cardamon, other spices
Malathion	Wheat
Carbaryl	Spices, tropical fruits
3. Fungicides	
Carbendazim	Mango, banana, litchi, apples, ginger
Thiram	Tropical fruit, grapes

serum based on cholinesterase, are on the market. However, many of these assays suffer from low sensitivity or are incompatible with organic solvents. We have overcome these problems by use of an enzyme prepared from fly head.

The assay is performed in a similar manner to an immunoassay, with some minor procedural changes. The microwells or tubes are uncoated, there is an oxidation and neutralisation step but the washing step is not required. The assays are extremely sensitive for most compounds. With wheat extracted in 2.5 volumes of methanol, and these extracts diluted 1/10 before analysis, the assay had lower limits of detection in grain as follows: fenitrothion—0.004 ppm, chlorpyrifos-methyl— 0.00001 ppm, pirimiphos—methyl—0.0001 ppm, malathion—0.0003 ppm, methacrifos—0.0002 ppm, carbaryl— 0.003 ppm. This compares with 'nil tolerance' values of 0.05–0.1 ppm for most of these compounds. Thus it would be preferred to dilute the extracts 1/100–1/1000 for assay. We aim to extend this work on broad-specificity screening for pesticides by evaluating other natural systems, such as enzymes and receptors which function as the sites of action for specific insecticides, fungicides and herbicides.

Conclusions

The major technology that 'competes' with pesticide immunoassay is conventional laboratory instrumental analysis of pesticides by gas-liquid chromatography or high-performance liquid chromatography. While this approach will remain the established methodology for some time to come, and has the advantage of being able to perform both multiresidue analysis and screening for unknown contaminants, disadvantages include cost, low sample throughput, limitation of analytical resources to a few central and specialised laboratories and inability to perform tests directly in the field (Skerritt 1994). The resultant delays mean that valuable time is lost in that immediate action cannot be taken to quarantine, destroy or at least trace-back contaminated food products.

There are advantages of immunoassay as a laboratory method (as well as a field method) for residue analysis. Before GC or HPLC analysis, sample extracts commonly require 'clean-up' to remove interfering components and to concentrate the agrochemical to enable detection to be sufficiently sensitive. This process is often very labour-intensive. Although sample handling and analysis can be automated, at extra cost, most instrumental methods can analyse only one sample at a time. Thus only 10–20 samples can be done per day, making systematic residue monitoring of food samples either very expensive or impractical. On the other hand, major disadvantages of immunoassay methods in pesticide analysis are as follows:

1. Need to develop specialised reagents for each analysis. Nevertheless, by the completion of this project, the reagents for detection of the major residues of concern in Asian countries will be available.
2. Immunoassays are best suited for analysis of samples where the nature of residues likely to be present is known. Given that there are usually only a limited number of chemicals permitted for use on a particular commodity, this is often the case. For example, the compounds of primary concern in India are DDT and HCH.
3. Pesticide immunoassays do not yet have 'Official Analytical Methods' status. However, with immunoassays for mycotoxins and protein contaminants in foods having obtained such status in recent years, this will almost certainly change.

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References

Beasley, H.L., Skerritt, J.H., Hill, A.S. and Desmarchelier, J.M. 1993. Rapid field tests for the organophosphates, fenitrothion and pirimiphos-methyl—reliable estimates of pesticide residues in stored grain. Journal of Stored Products Research, 29, 357–369.

Edward, S.L., Hill, A.S., Ashworth, P., Matt, J.L and Skerritt, J.H. 1993a. Analysis of the grain protectants, Chlorpyrifos-methyl and Methoprene with a 15-minute immunoassay for field or elevator use. Cereal Chemistry, 70, 748–752.

Edward, S.L., Skerritt, J.H., Hill, A.S. and McAdam, D.P. 1993b. An improved immunoassay for chlorpyrifos-methyl (Reldan(R)) in grain. Food and Agricultural Immunology, 5, 129–144.

Edward, S.L., Hill, A.S., Desmarchelier, J.M., Gore, P.J. and Skerritt, J.H. 1993c. Persistence of grain protectant residues into milling fractions, breads and noodles. In Wrigley, C.W., ed. Proceedings of the 43rd. Australian Cereal Chemistry Conference, Sydney, September 1992 (Royal Australian Chemical Institute, Melbourne) Pp 123–127.

Edward, S.L., Skerritt, J.H., Hill, A.S., Beasley, H.L and McAdam, D.P. 1992. Pesticide residues in barley and malt: new, simple and rapid test methods for breweries. In: Proceedings of the 22nd Convention of the Institute of Brewing, Australia and New Zealand Section, Melbourne, March 1992, pp 30–36. (Winetitles Publishers, Adelaide).

Gee, S.J., Hammock, B.D. and Skerritt, J.H. 1994. Diagnostics for Plant Agrochemicals, Chapter 8 In: Skerritt, J.H. and Appels, R., ed., New Diagnostics for the Crop Sciences, CAB Press, Cambridge, U.K. in press.

Hill, A.S., Beasley, H.L., McAdam, D.P. and Skerritt, J.H. 1992. Mono- and polyclonal antibodies to the organophosphate, fenitrothion. 2. Antibody specificity and assay performance. Journal of Agricultural and Food Chemistry, 40, 1471–1474.

Hill, A.S., Mc Adam, D.P., Edward, S.L. and Skerritt, J.H. 1993. Quantitation of bioresmethrin, a synthetic pyrethroid grain

protectant by enzyme-immunoassay. Journal of Agricultural and Food Chemistry, 41, 2011–2018.

Hill, A.S., Mei, J.V., Yin, C-M, Ferguson, B.S. and Skerritt, J.H. 1991. Determination of the insect growth regulator, methoprene, in wheat grain and milling fractions using enzyme-immunoassay. Journal of Agricultural and Food Chemistry 39, 1882–1886.

Marco, M-P., Gee, S.J., Cheng, H.M., Liang, Z.Y. and Hammock, B.D. 1993. Development of an enzyme-linked immunosorbent assay for carbaryl. Journal of Agricultural and Food Chemistry, 41, 423–430.

Mei, J.V., Yin, C.M. and Carpino, L.A. 1991. Hapten synthesis and competitive ELISA for methoprene. Journal of Agricultural and Food Chemistry, 39, 2083–2090.

Skerritt, J.H. 1994. Field tests for pesticide residues. In: Kurtz, D.A., Skerritt, J.H. and Stanker, L.H. eds., Agrochemical Immunoanalysis-94, AOAC International, Arlington, VA, USA, in press.

Skerritt, J.H., Hill, A.S., McAdam, D.P . and Stanker, L.H. 1992a. Analysis of the synthetic pyrethroids, permethrin and 1R-phenothrin in grain using a monoclonal antibody-based test. Journal of Agricultural and Food Chemistry, 40, 1287–1292.

Skerritt, J.H., Hill, A.S., Thwaites, H.L., Edward, S.L. and McAdam, D.P. 1992b Enzyme-immunoassay for quantitation of the organophosphate pesticides, fenitrothion, chlorpyrifos-methyl and pirimiphos-methyl in wheat grain and flour-milling fractions. Journal of the Association of Official Analytical Chemists International, 75, 519–528.

Snelson, J.T. 1987. Grain Protectants. Canberra, Australian Centre for International Agricultural Research, Monograph No. 3, 448p.