HGCA

PROJECT REPORT No. 44

STUDIES ON THE RAPID DETECTION OF PESTICIDES ON STORED GRAIN

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STUDIES ON THE RAPID DETECTION OF PESTICIDES ON STORED GRAIN

by

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Final report of a twelve month project at the MAFF Central Science Laboratory, London Road, Slough SL3 7HJ. The work commenced in June 1989 and was funded by a grant of £52,956 from the Home-Grown Cereals Authority (Project No. 0011/2/89).

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Summary

1. The EnzyTec Pesticide Detection Program was investigated as a rapid field test for the detection of pesticides on stored grain in the UK.

2. The test depends upon the formation of a blue colour. If pesticides which react with the enzyme acetylcholinesterase are present, ie organophosphates or carbamates, the enzyme becomes blocked and no colour develops. The organophosphorus pesticides used as grain protectants in the UK only react with the enzyme if present in an oxidised form. The kit therefore contains ampoules of an oxidising agent which is used before the test is carried out.

3. Equal volumes of the grain to be tested and water are shaken together for 1 minute and the test is carried out on 20 ml of the water extract.

4. In tests carried out at the Central Science Laboratory, Slough on grain treated with commercial formulations at the recommended doses, only methacrifos and malathion were detected; pirimiphos-methyl chlorpyrifos-methyl, etrimfos and fenitrothion were not. This is because these compounds are not very soluble in water and the concentrations present in the extracts were not high enough to react with the EnzyTec ticket.

5. A method was developed, using a detergent solution and multiple extractions, which enabled sufficient chlorpyrifos-methyl, malathion, methacrifos and etrimfos to be extracted from the grain to give a positive response at a dose on the grain of 5ppm. At doses below this the test was found to be unreliable.

6. Pirimiphos-methyl and fenitrothion did not react with the enzyme on the detector ticket at all. In the case of pirimiphos-methyl this was found to be because oxidation did not proceed. It did not prove possible to overcome this problem. Therefore, the EnzyTec kit, or any other based upon the use of the acetylcholinesterase enzyme, may not be suitable to detect pirimiphos-methyl. The problem with fenitrothion was not investigated.

7. The amount of pesticide extracted with water from grain decreased with time after the treatment, so much so that by 8 weeks after treatment a positive response with the ticket could not be relied upon.

8. The tickets purchased from EnzyTec were found to vary considerably in terms of performance. Tickets which would have given uneven responses were removed after visual inspection but replicate tests were required to obtain a reliable result, thus increasing the cost of the tests.

9. The performance of the tickets deteriorated four fold over a storage period of two years at room temperature. The best responses were obtained in the first 4 months.

10. It was concluded that the EnzyTec Pesticide Detection Program would only be useful for testing UK treated grain for the compounds chlorpyrifos-methyl, malathion, methacrifos or etrimfos at doses of 5ppm or more and if the testing was carried out within 8 weeks of the treatment.

1. Introduction

The widespread use of post-harvest treatments of cereals with pesticides, often as a prophylactic measure, has resulted in detectable levels of residues being found in at least 50% of cereal grain and cereal products in the UK (Anon,1989). There is increasing public concern over the presence of such residues. Thus, there is a need to know the treatment status of a consignment of grain as it passes from the farmer to the ultimate consumer. Chemical analysis of grain for pesticides is complex, time consuming and expensive and the availability of a rapid test kit for the presence of approved pesticides on grain would be invaluable in giving an on-the-spot indication of the presence of pesticides above a certain limit and go some way to reconciling the often conflicting requirements of the cereal industry. Such a test cannot replace chemical analysis but it would be a valuable addition to the technology of grain storage as an indicator of whether full chemical analysis was required and would assist in making decisions regarding re-treatment of grain.

The six insecticides currently cleared for use on stored cereals in the UK (pirimiphos-methyl, chlorpyrifos-methyl, methacrifos, etrimfos, fenitrothion and malathion) all have the same basic organophosphorus structure, Fig. 1. Consequently, they have a common mechanism of action which is to react, when converted to the oxidised form, Fig. 2, with the enzyme acetylcholinesterase resulting in the enzyme becoming permanently blocked. The oxidation of these organophosphorus compounds is normally accomplished within living organisms as a toxication process and results in the formation of the potent oxidised form which, after inhibiting the acetylcholinesterase enzyme ultimately causes death. Acetylcholinesterase is vital in the transmission of impulses in the nervous systems of both insects and vertebrates and its biochemistry has been intensively studied by researchers for many years. One of the first requirements in such investigations was a method for measuring the enzyme's activity and a number of colorimetric assays were developed. Such assays normally use alternative substrates from the natural acetylcholine and these substrates are designed to produce a coloured product when they react with the enzyme and measurement of this colour is thus a measure of the enzyme activity.

If a sufficient amount of an inhibitor such as an organophosphorus insecticide is present (in its oxidised form) then the enzyme is unable to react with the substrate and no colour is formed. This represents the extreme in an "all or nothing" situation and detailed spectrophotometric measurements of the intensity of the colour can determine all the intermediate situations.

Any detection kit based upon this principle must include an oxidation step to ensure that all the organophosphorus compounds are detected. This is usually accomplished by the use of dilute bromine water. Other requirements for a kit suitable for use in the field are an enzyme system which is stable enough to remain active after storage at ambient temperatures for long periods and cheap enough to make the test economically feasible. Also a suitable substrate/chromogenic reagent mixture must be devised to produce a distinctive colour change which can be measured against a chart for comparative purposes.

Given that these criteria can be satisfied, such a kit should in theory be the ideal system for detecting the presence of pesticides on grain in the UK, as it is specific for the six organophosphorus compounds which are likely to be present and there are few compounds (other than other insecticides) which would interfere with the system to give false positive results. Also, the enzyme has been shown to be extremely sensitive to the insecticides once they are in their oxidised form, although it will not distinguish between individual pesticides. Any kit based on the acetylcholinesterase enzyme will only be sensitive to organophosphorus and carbamate pesticides; it will not detect other classes of pesticides, such as pyrethroids and insect growth regulators.

In 1988 a pesticide detector kit based on acetylcholinesterase became available on the market in the USA. It is produced by EnzyTec Inc, a subsidiary of the Midwest Research Institute based in Kansas City. The Company intend to market the kit in the UK in the near future; aimed initially at the food and cereal sector. The kit consists of two cellulose discs attached to a plastic ticket. The acetylcholinesterase enzyme is immobilised on one disc and the substrate/chromogenic reagent mixture on the other. The ticket is stored in a sealed foil pouch containing a desiccant and the enzyme is thus stabilised until it comes into contact with water. The kit was originally developed for use by the US army to detect nerve

Fig 1. The generalised structure of an organophosphorus pesticide and its oxidised form.
R = group specific to each compound

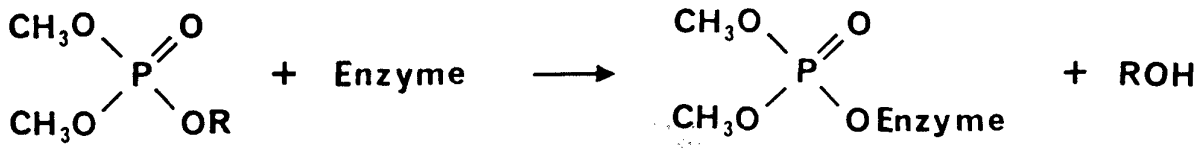


Fig 2. The reaction of the oxidised pesticide and the acetylcholinesterase enzyme

gases (potent acetylcholinesterase inhibitors) in water supplies and is marketed by EnzyTec for a wide variety of applications including testing of field samples of soil and water, detection of spills and leaks of pesticides and monitoring of pesticides on fruit, vegetables and other foodstuffs. Among the application methods supplied by the company is one for testing stored wheat - the Wheat Technique (Appendix 1). This formed the basis for the work carried out under the auspices of this research project.

The objectives of this work were:

i) To evaluate the EnzyTec pesticide detector kit for its suitability for use with stored grain in the UK. Initially this involved testing the methods supplied by EnzyTec in a manner as close as possible to the practical situation, i.e. the grain was treated at the recommended dose with commercial emulsifiable concentrates and was stored in ambient conditions. Possible modifications to the EnzyTec method were then investigated in order to try and improve and standardise the detection limits.

ii) To build up a library of validation data on the use of the EnzyTec ticket both with standard solutions of pesticides and grain treated in the laboratory.

iii) To assess and adapt one of the colourimetric assays currently used for the measurement of acetylcholinesterase as a possible alternative to the EnzyTec pesticide detector ticket.

2. Materials and General Methods

2.1 Materials

2.1.1 Detector Tickets

EnzyTec Pesticide Detector Tickets were purchased from:
EnzyTec Inc, 415 E 63rd St, Suite 104, Kansas City, Mo 64110-3361, USA.

2.1.2 Radio-labelled pesticides. These were purchased as follows:

Malathion, specific activity 4.6mCi/mmol from Amersham Plc
Pirimiphos-methyl, specific activity 57 mCi/mmol from ICI Tracerco Ltd
Chlorpyrifos-methyl, specific activity 9.18 mCi/mmol from Sigma Chemical Company

2.1.3 Analytical grade pesticides. These were obtained from the following suppliers:

Pirimiphos-methyl:	(97%) from Greyhound Chromatography Ltd
Chlorpyrifos-methyl:	(99.5%) from Dow Chemical Company Ltd
Etrimfos:	(97%) from Greyhound Chromatography Ltd
Fenitrothion:	(98%) from Greyhound Chromatography Ltd
Methacrifos:	(97%) from Greyhound Chromatography Ltd
Malathion:	(98%) from Greyhound Chromatography Ltd

2.1.4 Emulsifiable formulations of pesticides - were obtained from the following suppliers:

Pirimiphos-methyl:	Actellic 250g/L EC (ICI) from Berks, Bucks and Oxon Farm Supplies
Etrimfos:	Satisfar 525g/L EC (Sandoz) from Berks, Bucks and Oxon Farm Supplies
Fenitrothion:	Dicofen 500g/L EC (pbi) from Berks, Bucks and Oxon Farm Supplies
Malathion:	250g/L EC (Chimac-Agriphar) from FMC, Brussels
Methacrifos:	95% EC (Ciba-Geigy) from Northern Fumigation, Goole, Northumberland
Chlorpyrifos-methyl:	500g/L EC (Dow) from Berks, Bucks and Oxon Farm Supplies

2.2 Grain Treatment

2.2.1 Treatment of grain with analytical grade organophosphorus pesticides in diethyl ether

200 grams of winter wheat (variety Mission, moisture content 13.2%) were weighed into a 1 litre kilner jar. A 0.5 mg/ml treatment solution of pesticide was made up in Analar diethyl ether to achieve the required dose, usually 5ppm. It was applied dropwise to the wheat, while stirring constantly with a glass rod. The flask containing the treatment solution was rinsed twice, using 0.5 ml diethyl ether each time, and this was also applied to the wheat. The kilner jar was sealed with film and mechanically tumbled for 1 hour.

The treated wheat was spread in a single layer on an aluminium foil covered tray, and aerated in a fume cupboard draught for 6 minutes. The wheat was returned to the jar, sealed and tumbled mechanically for a further 2 hours. The wheat was then sampled and stored as required at 15^{0}C.

2.2.2 Treatment of grain with 14C radio-labelled pesticides in diethyl ether solutions

200 grams of wheat (variety Mission, 13.2% mc) were treated with a chemical dose of 5ppm and a radioactive dose of 20μCi/200g in each case. The treatment solutions were made up in 5ml of diethyl ether and were applied dropwise to the wheat while stirring constantly with a glass rod. The flask containing the treatment solution was rinsed twice with 200μl of diethyl ether and this was also applied to the wheat. The jar containing the grain was sealed and tumbled for 1 hour. The treated wheat was spread on a foil-covered tray and left in a stream of air for 30 minutes to allow the ether to evaporate from the grain. The wheat was returned to the jar which was sealed and tumbled for a further 2 hours. The wheat was then sampled and stored.

2.2.3 Treatment of grain with organophosphorus pesticides as emulsifiable concentrate formulations

150 grams of wheat (variety Mission, 13.2% mc) were weighed into a 1 litre kilner jar. A 1.5 mg/ml treatment solution of pesticide emulsifiable concentrate was made up with water to achieve the required dose as specified on the label. It was applied to the wheat dropwise, while stirring constantly with a glass rod. The flask containing the treatment solution was rinsed using 100μl of water, and this was also applied to the wheat. The jar was sealed with film and mechanically tumbled for 2 hours. The wheat was then sampled and stored as required at 15^{o}C.

2.3 Standard Extraction and Testing Procedures

2.3.1 Extraction and testing of pesticides from treated grain using water

Modifications to the EnzyTec method (Appendix I).

1. The treated grain was mechanically tumbled for 1 hour before sampling, to ensure that the samples were as homogeneous as possible.
2. A standard weight of wheat was used for each extraction - i.e. 25 grams plus 25 ml of water.
3. Tall jars were found to be more practical than wide mouth ones for carrying out extractions.
4. A 25 ml beaker MUST always be used for the ticket test - not a 50 ml one as there is not otherwise a sufficient depth of liquid in which to immerse the ticket.
5. 0.5 ml of a 0.72 mg/ml solution of bromine in water was used for each test as a direct equivalent to the activator ampoules supplied by EnzyTec. When using the extraction procedure for grain as described in section 2.3.3, it was necessary to use 1 ml of a 3.6 mg/ml solution of bromine in water. This is because of the presence of large amounts of co-extractives from the grain in the water which absorb much of the bromine added. In this case the solutions were allowed to stand for 50 minutes before being tested, to allow sufficient bromine to evaporate to prevent interference with the functioning of the ticket.
6. The bromine water was allowed to react for 4 minutes, rather than 2 as recommended, to ensure that most of the bromine had evaporated, before carrying out the test.
7. The enzyme disc on the ticket was exposed to the test extract for 2 minutes rather than 1 and was agitated in the solution during that time to improve the reproducibility of the results.
8. Tickets which had been stored in the fridge were allowed to attain room temperature before testing.

2.3.2 Extraction of pesticides from treated grain using a solvent

Modifications to the EnzyTec Method (Appendix II).

1. The treated grain was mechanically tumbled for 1 hour before sampling, to ensure the samples were as homogeneous as possible.
2. 200g of wheat were extracted with 200 ml HPLC grade methylene chloride.
3. Filtration was carried out using a Buchner funnel and a vacuum line.
4. 0.5 ml of a 0.72 mg/ml solution of bromine water was used in each test to replace the ampoules supplied by EnzyTec.

2.3.3 Extraction of pesticides from treated grain using an aqueous based detergent medium

The treated grain was tumbled mechanically for 1 hour, before sampling. Four equal samples of wheat (between 10-30 grams) were placed in large beakers. An equal volume, on a weight to volume basis, of 1mg/ml Triton X100 (a non-ionic detergent) was added to one grain sample (i.e. if 10 gram samples were used, 10 ml of 1 mg/ml Triton X100 was added). The grain was swirled in the detergent solution for 2 minutes. The grain extract was collected, its volume adjusted to the original level, and this was added to the next grain sample until each had been extracted in thisway.
The volume of the final extract was corrected to its original level. The extract was then tested either as described in Section 2.3.1 or using the spectrophotometric method described in Section 2.3.4.

2.3.4 Spectrophotometric pesticide detection in grain extracts using a standard cholinesterase test

This method is based on that of Ellman et al (1961). 2.5 ml of a 52 mM phosphate buffer pH 7.2 were placed in a 1 cm path length cuvette. 400μl of well mixed grain extract and 35μl of a 3.6 mg/ml bromine water solution were added. The mixture was stirred thoroughly, and left for 25 minutes for the bromine water to react and evaporate. 33μl of a 5 unit/ml solution of electric eel cholinesterase (in pH 7.4 citrate/phosphate buffer containing 0.5 mg/ml of Triton X100) were then added. The contents of the cuvette were stirred and left for 3 minutes for any reaction between the pesticide present and the enzyme to occur. 0.5 ml of 0.62 mg/ml dithiobis nitrobenzoic acid in phosphate buffer (as above) and 100μl of 45 mg/ml acetylthiocholine iodide (in water) were added to the cuvette and stirred thoroughly. The cuvette was transferred immediately to the spectrophotometer for assay at a wavelength of 405 nm and a bandwidth of 1 nm. The rate of reaction of the cholinesterase was recorded every 30 seconds for 6 minutes. The spectrophotometer went through a self test routine and had two control tests run through it on each occasion before readings were obtained as described above.

3. Detailed Methods and Results

3.1 Assessment of the performance of the EnzyTec ticket using the techniques recommended by the manufacturer

3.1.1 The performance of the EnzyTec ticket in conditions as close as possible to those found in practice in the field

Individual batches of grain were treated with commercial emulsifiable concentrate formulations of the six organophosphorus pesticides at doses of 5ppm using the method described in Section 2.2.3.

Each batch was then tested with EnzyTec tickets following the method described in the EnzyTec Custom System-Wheat Technique (Appendix I). If a negative response was found, i.e. the ticket could not detect the presence of pesticide, the grain was re-treated to give doses of 10, 15 or 20ppm until a positive response was obtained. The treated grain was then stored in sealed jars at 15°C and retested with the tickets at 4 and 8 weeks after storage.

The results obtained are shown in Table I. Pirimiphos-methyl and fenitrothion were not detected at all by the ticket at concentrations on the grain of up to 20ppm. After 8 weeks of storage only methacrifos at 5ppm and malathion at 10ppm could be detected. The other compounds no longer gave a positive response.

3.1.2 The performance of the EnzyTec ticket using methods recommended for use in testing for pesticide residues in the laboratory.

i) Individual batches of grain were treated with commercial emulsifiable concentrate formulations of malathion, chlorpyrifos-methyl and pirimiphos-methyl at doses of 5ppm using the methods described in section 2.2.3. The grain was stored at 15°C for 1 week and then extracted and tested using the method described in the EnzyTec Produce System - Total Insecticide Residue Technique (Appendix II).

The methylene chloride extracts of wheat obtained were in all cases brightly coloured yellow and this interfered with the colour change in the ticket so much so that no results either positive or negative could be obtained. Attempts to clean up the extracts using various purification cartridges were unsuccessful and the method was abandoned as a technique suitable for the assessment of pesticide residues on grain.

Table I. Detection of pesticide on grain treated with emulsifiable concentrates using the standard EnzyTec method.

GRAIN TREATMENT FROM WHICH EXTRACTIONS WERE MADE	DETECTION OF THE PRESENCE OF PESTICIDE TIME AFTER GRAIN TREATMENT		
	ZERO TIME	FOUR WEEKS	EIGHT WEEKS
5ppm METHACRIFOS	+	+	+
5ppm MALATHION	+	-	-
10ppm MALATHION	+	+	+
5ppm ETRIMFOS	+	-	NT
5ppm CHLORPYRIFOS-METHYL	-	NT	NT
10ppm CHLORPYRIFOS-METHYL	-	NT	NT
15ppm CHLORPYRIFOS-METHYL	+	+	-
5ppm PIRIMIPHOS-METHYL	-	NT	NT
10ppm PIRIMIPHOS-METHYL	-	NT	NT
15ppm PIRIMIPHOS-METHYL	-	NT	NT
20ppm PIRIMIPHOS-METHYL	-	NT	NT
5ppm FENITROTHION	-	NT	NT
10ppm FENITROTHION	-	NT	NT
15ppm FENITROTHION	-	NT	NT
20ppm FENITROTHION	-	NT	NT

NT = NOT TESTED, + POSITIVE RESULT, - NEGATIVE RESULT

The experiment was repeated using radio-labelled pesticides and methanol as the extraction solvent. Analysis of the extracts indicated that between 4 and 4.6ppm of the applied dose of 5ppm were extracted. Again the extracts were highly coloured and could not therefore be used with the ticket.

ii) Grain treated as in (i) above was also extracted and tested using the method described in the EnzyTec Solvent System - Dislodgeable Insecticide Residue Technique (Appendix III). Both methylene chloride and methanol were used as extraction solvents. The colour produced in the final concentrated sample was not as intense as in (i) above but was still sufficient to interfere with the ticket. In these cases the purification cartridges removed most of the colour but it was found (by repeating the experiment using radio-labelled pesticides) that a considerable amount of the extracted pesticide was also absorbed by the cartridges and therefore the technique was not considered further. Radio-chemical analysis of the extracts indicated that this extraction method removed between 1 and 3ppm of the 5ppm applied to the grain.

3.2 **Development of a standard extraction procedure**

The results obtained in Section 3.1 demonstrate that in our hands the EnzyTec pesticide detector kit is not suitable for use with cereal grains as it stands and modifications to the procedures for its use are required.

All the organophosphorus pesticides of interest in this study are at best of limited solubility in water and at worst virtually insoluble. Thus the use of water as the recommended extraction fluid results in insufficient insecticide being dissolved to give a positive result with the ticket. Therefore a number of different extraction procedures were investigated in an attempt to improve the sensitivity of the ticket in this situation.

Preliminary investigations demonstrated that water-based extractions of grain produced a large number of co-extractives which interfered with chromatographic analysis. Therefore, all the investigations described in this section were carried out using radio-chemical techniques. Other studies have shown that short term exposure of grain to the organophosphorus compounds under investigation results in little metabolic breakdown of the parent compound and therefore only total radio-label was measured.

Two methods of extraction were carried out:

i) Extraction of ground wheat with methanol. This is the extraction technique used in routine chemical analysis of grain and should therefore indicate the maximum amount of pesticide that can be recovered from treated grain.

   Wheat treated with radio-labelled insecticides was used. Triplicate 12g samples were ground in a laboratory mill. 10g of the ground wheat were homogenised with 3 x 30 mls of Analar methanol and the total volume made up to 100 ml. The radioactivity in 3 aliquots of the extract were determined by liquid scintillation counting. The recoveries were 92.1% (4.6ppm) for malathion, 82.6% (4.1ppm) for pirimiphos-methyl and 91.5% (4.6ppm) for chlorpyrifos-methyl of the 5ppm dose applied to wheat. It is not clear why the recovery of pirimiphos-methyl is lower than those for the other two compounds.

ii) Triplicate 10g samples of wheat treated with radio-labelled insecticide were shaken for 2 minutes in 10 mls of several different aqueous extraction media. The amount of radioactivity released was measured by liquid scintillation counting. The different extraction regimes used are shown in Table II together with the results obtained. The figures for water and methanol alone are included to indicate the range from the worst to the best possible case.

Table II. The recovery of insecticides (as radio-label) from freshly treated intact grains after 2 minutes shaking in various extractants.

	Amount recovered in extractant (ppm)*		
	Pirimiphos-methyl	Chlorpyrifos-methyl	Malathion
100% Methanol	1.25	0.75	2.9
10μg/ml Triton X100 in water	0.3	0.25	1.7
1mg/ml Triton X100 in water	0.5	0.8	1.8
10μg/ml Triton X45 in water	0.3	0.25	1.6
1mg/ml Triton X45 in water	0.55	0.5	1.9
10% Methanol in water	0.2	0.3	1.5
100% water	0.2	0.2	1.5

* Dose applied on grain = 5ppm

The recoveries using methanol from whole grains were considerably lower than those from ground, homogenised grain and when using water alone, as recommended by EnzyTec, the figures were reduced even further. Only in the case of malathion did this figure approach the limit of detection quoted for the kit by EnzyTec (2ppm for malathion, 1ppm for chlorpyrifos-methyl - see Appendix IV) and therefore it is unlikely that chlorpyrifos-methyl and pirimiphos-methyl would have produced a positive result at this dose level and this was found to be the case (section 3.1). The use of the Triton non-ionic detergents improved the situation somewhat and the best results were obtained with 1mg/ml solutions of Triton X100, which increased the recoveries to 0.5ppm for pirimiphos-methyl, 0.8ppm for chlorpyrifos-methyl and 1.8 ppm for malathion. However, these were still unlikely to be within the detection range of the kit.

Several other detergents were assessed for their suitability in this situation including Tween 80 (non-ionic detergent) and Aerosol-OT (anionic detergent). At concentrations of 1mg/ml these all performed only marginally better than water alone.

Assessements were also made of the effects all the detergents tested had on the tickets in case of any interference with the performance of the ticket. All of the detergents, with the exception of Triton X100,were found to interfere with the colour production of the ticket, affecting either the nature or the intensity of the colour produced. Thus Triton X100 was the detergent of choice to be used in any standard extraction procedure.

The inclusion of 10% methanol in water was found to have no effect on the extractability of the pesticides from grain and,

since tests had shown that this was the maximum amount that could be included in the extractant without affecting the enzyme, this procedure was not considered any further.

A number of other procedures were investigated as a means of increasing the amount of pesticide released from grain by aqueous extractants and these are detailed in Table III. Repetitive washing seemed to be the most effective and these procedures were investigated further using wheat treated with radio-labelled chlorpyrifos-methyl. Analysis of the extracts indicated that washing separate grain samples with the same extraction solution gave a much higher recovery of pesticide and this was therefore the method of choice. The cumulative amounts extracted from freshly treated grain are shown in Table IV and after 4 extractions the 5.7ppm present in solution should be more than sufficient to give a positive result with the ticket and this was found to be the case. Hence the standard extraction described in Section 2.3.3 was developed.

Table III. Extraction procedures used in an attempt to increase pesticide recovery.

Extraction method	Effect
Increase in shaking time in extraction medium	Slight increase in recovery
Increase in volume of extraction medium	Slight increase in recovery
Soaking grain overnight in 1mg/ml Triton X100	No effect
Extraction of ground grain with 1mg/ml Triton X100	Difficult to analyse due to formation of slurry
3 consecutive washes of grain sample with 1mg/ml Triton X100	Significant increase in recovery
Washing of 3 separate grain samples with the same solution of 1mg/ml Triton X100	Significant increase in recovery

Table IV. Recovery from grain freshly treated with chlorpyrifos-methyl using one volume of 1mg/ml Triton X100 to extract 4 grain samples.

	ppm recovered after each extraction	Cumulative total ppm
1st extraction	2.1	2.1
2nd extraction	1.4	3.5
3rd extraction	1.5	5.0
4th extraction	0.7	5.7

3.3 Sensitivity of the ticket

The ultimate sensitivity of the ticket to organophosphorus pesticides is dependent upon two separate reactions - the oxidation of the pesticide using bromine water and the reaction of the oxidised form with the enzyme. The work described in the previous sections has demonstrated that, provided sufficient bromine is present to saturate the system, the oxidation process does not affect the performance of the ticket. However the oxidised forms of the pesticides are not detected at the same concentrations by the ticket and therefore any procedure developed for use with grain must be geared to the positive responses obtained with the least sensitive compound. Thus limits of detection for the 6 organophosphorus compounds of interest were established using standard laboratory solutions of the pesticides in water and the testing method described in Section 2.3.1. Tests were also carried out using a 1mg/ml solution of Triton X100 as this was found to be the most suitable extractant for treated grain. The results are shown in Table V. Whilst conducting these tests it became apparent that pirimiphos-methyl and fenitrothion were not responding to the ticket at the concentrations used. Further tests using concentrations of 10, 20 and 50 ppm also failed to produce any positive response with the EnzyTec ticket. It therefore appeared that there was a problem with the use of the ticket with these compounds that required further investigation. The detection limits for the other compounds were all below 1.5ppm in water and the presence of Triton X100 did not affect these greatly in contrast to the other surfactants tested, as shown in Table V for Triton X45. Of the compounds responding, the ticket was most sensitive to methacrifos and least sensitive to chlorpyrifos-methyl.

Table V. The limits of detection of the EnzyTec ticket using laboratory solutions of pesticides.

	Concentration range in which detection limit lies ppm		
Pesticide Tested	Water	1mg/ml Triton X100	1mg/ml Triton X45
Chlorpyrifos-methyl	1.25 - 1.5	1.75 - 2.0	5.0 - 10.0
Malathion	0.31 - 0.62	0.62 - 1.25	0.62 - 1.25
Etrimfos	0.62 - 1.25	0.62 - 1.25	2.5 - 5.0
Fenitrothion	Not detected	Not detected	Not detected
Methacrifos	0.16 - 0.31	0.16 -0.31	0.16 - 0.31
Pirimiphos-methyl	Not detected	Not detected	Not detected

3.4 Investigations into the lack of response of the ticket to pirimiphos-methyl

The inability of the ticket to detect pirimiphos-methyl would render the EnzyTec kit of little value in the UK as pirimiphos-methyl is the market leader in so far as the treatment of grain is concerned. The investigation of the problem therefore concentrated on pirimiphos-methyl rather than fenitrothion as the latter is not widely used for grain treatments.

The first requirement was to establish whether the problem was associated with the oxidation step or the reaction with the enzyme on the ticket. In order to do this the ticket was tested directly with the oxidised form of pirimiphos-methyl. This compound is very unstable, particularly in aqueous solution, and therefore 20μl of a freshly made 11mg/ml solution in methanol was applied directly to the enzyme disc of the ticket. This represented one hundredth of the pesticide which would be present in solution in a standard ticket test using a 1ppm solution. The methanol was evaporated from the disc under a warm air stream and the disc was then wetted with three drops of water and allowed to react for 2 minutes. It was then developed in the usual way. In all cases the tickets responded positively to this concentration of pesticide indicating that the oxidised form of pirimiphos-methyl does react with the enzyme on the ticket. It therefore appears that the problem lies with the oxidation procedure.

TLC analysis was carried out on an oxidised solution of radio-labelled pirimiphos-methyl. Four compounds were found to be present in the solution, none of which corresponded to pirimiphos-methyl itself or its oxidised form, suggesting that the pesticide molecule breaks down in the presence of bromine as an oxidant. A study of the electronic structure of the pirimiphos-methyl molecule revealed

that, in the presence of bromine, the molecule would be more likely to hydrolyse at the -N-diethyl bond than be oxidised at the P=S bond. Reducing the pH of the solution might be a means of stabilising the molecule and the use of equimolar amounts of bromine might effect oxidation rather than hydrolysis.

Detailed investigations into the means of oxidising pirimiphos-methyl have been carried out and these are summarised in Appendix IV. Several methods of lowering the pH of the pesticide solution were investigated such as the use of different buffer solutions or weak acids such as peracetic acid. Unfortunately the enzyme on the ticket appeared to be very sensitive to acid conditions and was totally inactive in buffered control conditions at pHs below 4. Between pH 4 and 5 the enzyme was able to function in control conditions but did not perform reliably in the presence of malathion at 5ppm, (this being used as a positive control), until pH5 was reached. Therefore even if the oxidation of pirimiphos-methyl was achieved sucessfully in these conditions the enzyme was no longer able to react reproducibly and the ticket was not effective. Extensive testing using buffers between pH 4 and pH6, bromine concentrations between 80μg and 360μg and a 10ppm solution of pirimiphos-methyl did occasionally produce positive results but these were not reproducible. The use of peracetic acid was investigated as an alternative oxidising agent which would simultaneously create the acidic conditions required. The use of peracetic acid on an equimolar basis with the pirimiphos-methyl in solution resulted in very low pHs and the solutions were therefore neutralised before testing. However,the enzyme in the control test was found to be inactivated in these conditions and therefore no reaction could take place. A pH of 3.5 was achieved using a much smaller amount of peracetic acid and control tests with malathion indicated that the enzyme was active at this pH under these conditions. Testing of

solutions of pirimiphos-methyl between 2.5 and 16 ppm, using between 90 and 2160 μg of bromine, again produced some isolated positive results which were not reproducible.

Hydrogen peroxide was also tested as an alternative oxidising agent but this was found to inactivate the enzyme on the ticket even at very low concentrations. The enzyme catalase was therefore added to the test solution after the hydrogen peroxide had been allowed to react for 1 minute, as this enzyme is able to degrade hydrogen peroxide to water. The control tests indicated that the ticket was then able to respond but no results were obtained when using solutions of up to 10 ppm pirimiphos-methyl. It would seem that either the hydrogen peroxide failed to oxidise the pirimiphos-methyl or it was too strong an oxidising agent and caused the pesticide molecule to disintegrate.

Finally, the spectrophotometric method was used to investigate the problem of oxidation and detection of pirimiphos-methyl as this method has been shown to be more sensitive and reproducible than the ticket test,but the results were the same as those described above for the ticket test.

Therefore, it did not appear to be possible to use the EnzyTec ticket to detect pirimiphos-methyl.

3.5 **Tests using wheat treated in the laboratory**

A 1kg bulk of grain was treated with ^{14}C radio-labelled chlorpyrifos-methyl at 5ppm using the method described in Section 2.2.2. This was stored at 15^{o}C for 5 weeks and samples were taken at 0, 2 and 3 days and at 4 and 5 weeks. Four samples at each sampling time were extracted with the same 25ml volume of Triton X100 (1mg/ml) and were then tested with the ticket using the

standard method described in Section 2.3.1. Aliquots of the extracts were taken for radio-chemical analysis to determine the concentration of pesticide present. The results, shown in Table VI, indicate that the pesticide present on freshly treated grain is readily dislodged by a simple shaking technique. However, this process becomes increasingly more difficult with time, presumably as a result of the pesticide penetrating more deeply into the grain.

Nevertheless, the ticket is still able to detect the presence of pesticide four weeks after treatment when the amount recovered in the extract from 4 grain samples was 1.3ppm. This is in fact a lower limit of detection than that found using standard laboratory solutions of chlorpyrifos-methyl in Triton X100.

A series of tests was therefore carried out to determine the limit of detection of the ticket for chlorpyrifos-methyl in grain extracts prepared using the standard extraction procedure. The limit of detection in this situation was found to be between 1 and 1.25 ppm which was in close agreement with the results obtained in the storage experiment (Table VI). The presence of the co-extractives from the grain must therefore in some way be assisting the interaction between the pesticide and the ticket.

The effect of storage on the ability of the EnzyTec ticket to detect pesticide in extracts of grain was further investigated using grain which had been treated at 5ppm with unlabelled chlorpyrifos-methyl and stored at 15^{0}C for 13 weeks. Samples were taken at 1,2,3,4,5,6,8 and 13 weeks after treatment and extracted with Triton X100 solution as described above. The results of the tests using the EnzyTec tickets are shown in Table VII. There was positive

detection of pesticide in all samples except that taken at 8 weeks after treatment. This, coupled with the results from the previous experiment, indicate that the ticket was at its limit of detection at this stage and the difference between the negative response at 8 weeks and the positive one at 13 weeks was probably due to differences in sampling. It is therefore very marginal whether a 5ppm dose on a bulk of grain could still be detected by the ticket after more than 4 weeks of storage.

Table VI. The recovery of ^{14}C-chlorpyrifos-methyl from treated wheat and the response of the EnzyTec ticket.

Time after treatment	Cumulative recovery from 4 samples ppm	Response of the ticket (a)
0	5.8	+
2 Days	4.9	+
3 Days	4.8	+
4 Weeks	1.3	+
5 Weeks	1.2	-
Control	-	-

Table VII. The effect of ageing on the ability of the EnzyTec ticket to detect pesticide in grain extracts.

Time after treatment Weeks	Response of the ticket (a)
1	+
2	+
3	+
4	+
5	+
6	+
8	-
13	+
Control	-

(a) + pesticide detected
- pesticide not detected

3.6 An assessment of the quality of the tickets supplied by EnzyTec

Whilst carrying out the tests with the EnzyTec ticket described above it was noticed that the response obtained appeared to vary from batch to batch. The most obvious variable was the difference in colour of the substrate disc on the ticket which ranged from almost white to pink to brown. It was found that the tickets with the almost white and brown discs were frequently inactive and these were subsequently discarded. The numbers of such tickets varied considerably from batch to batch from the usual 10 to 15% to more than 25% in one case. Some discs, although having the normal pink colouration, were very patchy and these gave results which were very difficult to interpret, particularly if they were used with solutions which were near the limit of detection. In many cases these too were discarded before use. Several different aspects of the performance of the EnzyTec ticket were therefore investigated as described below. In all these experiments standard laboratory solutions of chlorpyrifos-methyl in water and the standard test method (section 2.3.1) were used.

3.6.1 The effect of storage on the performance of the tickets

Table VIII shows the effect on performance of storing the tickets at room temperature for periods of up to 2 years - the shelf life quoted by EnzyTec. There was a considerable deterioration in the performance of the ticket with time such that after 2 years the detection limit for chlorpyrifos-methyl had increased four fold. For best results the tickets should be used before they are four months old. The initial batches of tickets obtained from EnzyTec had expiry dates printed on each individual sachet but the more recent tickets did not have this. Therefore, if the kits were purchased from a commercial supplier there would be no means of knowing how old the tickets were.

Table VIII. The effect of storage time at room temperature on the performance of the EnzyTec ticket.

Storage time from date of arrival at CSL	Detection limit for chlorpyrifos-methyl ppm
1 day	0.62
2 months	0.62
4 months	1.25
6 months	1.25
2 years	2.5

Table IX. A comparison of different storage conditions on the performance of the EnzyTec ticket after 2 months.

Storage Conditions °C	Detection limit for chlorpyrifos-methyl ppm	
	Sealed storage of unopened tickets	Unsealed storage of unopened tickets
-30	1.25	0.62
4	1.25	1.25
12	0.62	0.62
25	1.25	1.25
46	1.25	1.25
Unstored tickets	0.62	0.62

It has been shown that different storage conditions did not unduly affect the performance of the ticket. Sealing the unopened sachets containing the tickets in jars also had little effect. These results were expected as the tickets are packaged in the sachets with a desiccant. The deterioration in the ticket performance with time suggests that even though the enzyme wasmaintained in a dry state there wasstill a slow degradation process occurring.

3.6.2 The reproducibility of the results obtained using the EnzyTec ticket

It had been noticed that when carrying out replicate tests in the previous experiments there was often a discrepancy in the results obtained on different tickets. This was most apparent when the limit of detection of the ticket was approached but was observed on other occasions as well. It seemed likely that the length of time for which the tickets had been stored might be one reason for this. Therefore, ten newly supplied tickets and ten which had been stored for four months at room temperature were tested using a 1.25ppm solution of chlorpyrifos-methyl. All of the newly supplied tickets responded positively whereas only 6 of the stored ones did so. This again emphasises the need for the storage time of the tickets to be kept to a minimum.

One of the principal advantages of the EnzyTec test is that it is able to provide a quick yes/no answer. However, with many of the tickets used in these investigations it was not possible to get such a clear cut answer as the enzyme disc was neither totally blue nor white at the end of the test. This problem was more apparent when the detection limit of the ticket was approached. The gradation in colour described in the EnzyTec procedure for

preparing a standard dose-reponse curve (Appendix VI) was rarely observed. It appeared that much of the lack of uniformity of colour in the results from the EnzyTec ticket tests was due to the enzyme either being present unevenly on the discs or reacting unevenly with the pesticide and/or the substrate. Tickets with an uneven distribution of substrate had already been discarded and could not, therefore, have contributed to this. In some cases it was clear that the enzyme was reacting unevenly as the disc was one colour in the middle and another round the outside, but the results on others were much more patchy. On several occasions tickets were cut in half and the two halves were tested together in standard solutions of 1.25ppm chlorpyrifos-methyl in water and only in 66% of the tickets did the two halves give the same result.

Testing of solutions using the EnzyTec ticket was, therefore, always carried out in triplicate to ensure that the correct result was obtained.

3.7 The use of a "wet" colourimetric technique as an alternative to the EnzyTec ticket

Since so many problems were encountered during this study which were associated with the EnzyTec ticket itself an assessment was made of the original spectrophotometric technique as a possible alternative to the ticket, albeit in a much simplified form. The spectrophotometric method used (see Section 2.3.4) was essentially that of Ellman et al (1961) with electric eel acetylcholinesterase as the enzyme source. This is the same enzyme as is present on the EnzyTec ticket and was chosen as it had been found by EnzyTec to be more sensitive than enzymes from other sources to the pesticides of interest and it can also function normally in solutions of Triton X100 of up to 1%.

A range of parameters were tested using this method and the detailed results are presented in Appendix VII. Using standard laboratory solutions of pesticide in water the spectrophotometric method was found to be more sensitive than the EnzyTec ticket test in that it could detect lower levels of pesticide readily. This was not only because this method is not restricted to a yes/no answer as is the ticket test but also because the amount of enzyme present could be varied easily. In most of these investigations an EnzyTec ticket test was carried out in parallel with the spectrophotometric test and the latter was found to be generally more reproducible, particularly when lower concentrations of pesticide were used. Many of the grain extracts made when testing wheat treated in the laboratory (Section 3.5) were also tested spectrophotometrically and the results demonstrated that the high level of co-extractives present did not interfere with this test. Also the small volume of the extract required for the test, 400μl as opposed to 20 ml for the EnzyTec test, meant that replication was more easily achieved. The necessary scaling up of the spectrophotometric method for any field testing procedure should, therefore, not be a problem.

Finally, the spectrophotometric method was used to address the problem of the oxidation of pirimiphos-methyl. In this situation the acetylcholinesterase enzyme was found to be more susceptible to changes in pH, probably because on the ticket its attachment to the cellulose provides some protection. Using this method it was much easier to assess the effects that modifying the aqueous medium was having on the enzyme and tests were only carried out when the enzyme was relatively unaffected. Nevertheless, no meaningful results were obtained when oxidation of pirimiphos-methyl was carried out using either bromine water or peracetic acid. Inhibition of the enzyme was observed on some occasions but a dose related response could not be achieved indicating that the problem lies with the process of oxidation of the pirimiphos-methyl. Until this problem is overcome no test based upon the use of the acetylcholinesterase enzyme would be likely to be able to detect this compound.

4. Discussion and Conclusions

The concept of the EnzyTec ticket as a quick yes/no dip test would appear to be ideal for the detection of organophosphorus and carbamate pesticides present on grain. However, the very non-specific nature of such a test can give rise to problems. It cannot identify the pesticide present other than as an organophosphorus or carbamate, and it is not quantitative. Positive result to a test carried out in the field indicates the presence of pesticide but gives no information about how much is present,and it is therefore vital that the means of interpreting such results are available if the EnzyTec test kit is to be viable. The work described here has attempted to determine the valid operating range of these kits for use with pesticides applied to stored grain in the UK.

The use of water as the extraction fluid in these tests, although ideal for field use, causes considerable problems in practice. All the pesticides likely to be present on grain in the UK have low solubilites in water and although they are applied in water in the form of emulsifiable concentrates, they cannot necessarily be removed with water. The EnzyTec kit detects only methacrifos and malathion on stored grain after a routine treatment with an emulsifiable concentrate. It is unfortunate that EnzyTec appear to have carried out all testing of their "Wheat Technique" using malathion, as this is one of the more soluble of the organophosphorus insecticides and is also applied at a higher dose than the other compounds. It therefore responds to the EnzyTec ticket more readily.

During the course of this work it was always borne in mind that any modifications made to the EnzyTec methods must be compatible with the use of the kit in the field and must also maintain its economic viability. Therefore the use of organic solvents to overcome the extraction problem, whilst in theory being able to be used with the ticket, was not

feasible and in practice the coloured co-extractives removed with organic solvents interferred with the operation of the ticket. Modification of the aqueous extraction medium was the most obvious solution and the use of a cheap, readily available detergent, Triton X100, greatly increased the extractability of the pesticides present on grain. This, together with the extraction of several samples of grain with the same extraction solution produced the standard extraction procedure which would enable a positive response to be achieved with the EnzyTec ticket after a single standard grain treatment. However, each pesticide responds to the EnzyTec ticket with a different sensitivity and therefore a field test would have to be geared to the lowest response of the least sensitive compound. Laboratory tests of standard solutions of pesticides showed this compound to be chlorpyrifos-methyl, given that pirimiphos-methyl and fenitrothion did not respond at all.

Extensive testing of grain treated with chlorpyrifos-methyl in the laboratory showed that the limit of detection of chlorpyrifos-methyl when extracted from grain using the standard extraction procedure described here, was at least as good as that obtained from standard solutions. The extractability of the pesticide decreased considerably with ageing of the treated grain. This considerably hampered attempts to correlate a positive response on the ticket with the amount of pesticide present on the grain. It was concluded that following a routine treatment of grain at 5ppm a positive response from the EnzyTec ticket could only be relied upon for up to 8 weeks after treatment.

Therefore,in order for the results of any field testing to be meaningful the age of the grain post treatment must be known. The detection limit The detection limit of the ticket for chlorpyrifos-methyl present on grain was 5ppm and as it is the least sensitive compound, this must also be taken as the limit of detection of the ticket when carrying out testing in the field. If the nature of the pesticide present on grain is not known, a positive result with an EnzyTec ticket would indicate that there may be as much as 5 ppm of malathion, chlorpyrifos-methyl, methacrifos or etrimfos present on the grain. Such a lack of sensitivity means that the Enzytec ticket is probably not going to be very useful in the practical situation for testing grain in the UK.

In the tests carried out here, the performance of the EnzyTec ticket was marred by the lack of reproducibility. This appeared to be due partly to poor quality control during the manufacturing process resulting in uneven application of test components to the cellulose discs and partly to deterioration of the response of the ticket during storage. It was therefore always necessary to repeat a test several times to be sure of a reliable result and in a field situation this would add considerably to the cost of the tests.

The original spectrophotometric method was therefore investigated as a possible means of overcoming such problems of reliability. The tests carried out in this laboratory demonstrated that this method is inherently more sensitive than the EnzyTec ticket and it was found to be highly reproducible. In its simplest form the end point of the reaction could be used as a yes/no type of test, similar to the EnzyTec ticket and such a kit is being currently marketed by Handsel Biotechnology Company in Taipei, Taiwan. With the use of the small portable spectrophotometers designed for use in the field and now on the market relatively cheaply, the test could be developed into a semi-quantitative analysis. No attempt was made to explore these avenues further as the biggest stumbling block in the use of any test method based upon acetylcholinesterase is the problem of the oxidation of pirimiphos-methyl. All efforts to carry out this reaction reproducibly in the aqueous environment required for the acetylcholinesterase enzyme to operate have so far failed so this method cannot be used to detect pirimiphos-methyl. Since pirimiphos-methyl is the grain protectant most widely used in the UK, the use of any acetylcholinesterase based test seems unlikely to provide an effective monitoring system which can be used in the field. In order to achieve this, a test based on an entirely different operating parameter will have to be developed. One approach would be to develop immunoassays for the individual pesticides. The use of a combination of these would in theory provide a specific and very sensitive test. However, in the field, any simple rapid test is going to have to

depend upon the use of water as the extraction agent as it is readily available and safe to handle. Also, nearly all biochemical tests, including immunoassays, require an aqueous medium. Therefore, the problems encountered here with the lack solubility of the organophosphorus pesticides applied to grain and the decrease in extractability from grain with storage time will still have to be overcome, whatever pesticide detection method is used.

5. References

Anon, 1989, Report of the Working Party on Pesticide Residues (1985 - 1988). Food surveillance Paper No. 25, HMSO, London.

Ellman, G L, Courtney, K D, Andres, V, Featherstone, R M, 1961. A new and rapid colorimetric determination of acetylcholinesterse activity. Biochem, Pharmacol, 1, 88-95.

Storage of data

The raw data relating to the work carried out under this project are held by Dr W A Matthews, Central Science Laboratory, Slough.

Custom System

Wheat Technique

The EnzyTec Program is setting industry standards in pesticide detection. The Program has eight self-contained systems that are inexpensive, easy-to-use, and yield results within minutes.

Each system has a variety of techniques, each designed to test a different kind of sample. The **Wheat Technique** is designed to be used in the field to determine if insecticides are present on wheat. The detection limit of this technique is low parts per million and should be used with water soluble insecticides.

An EnzyTec Pesticide Detection Custom System Kit Contains:

- Pesticide Detector Tickets • Activator Ampules
- 50 mL graduated beaker • Glass rod for breaking the Activator Ampules and stirring the sample

In addition, you will need:

- 6 oz. Wide Mouth Jar • Clean Water

APPLICATIONS:

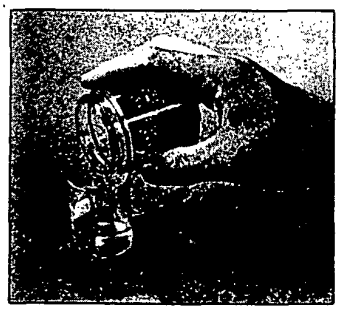
• Pesticide Manufacturers, Formulators and Distributors • Regulatory • Grain Treatment • Milling and Baking • Nurseries • Fish and Wildlife • Pesticide Container Disposal • Residue Levels

INSTRUCTIONS:

COLLECT • Collect representative wheat sample

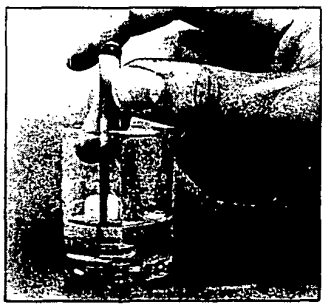
EXTRACT

- Add equal weights of wheat and water to a wide mouth jar
- Shake vigorously for 1 minute



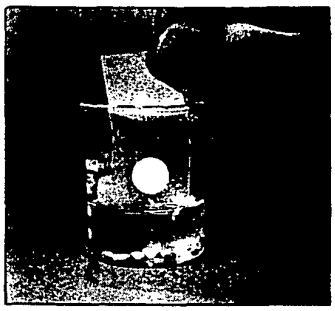
PREPARE

- Pour 20 mL of water extract into a 50 mL beaker



CONVERT

- Break activator ampule in solution
- Allow to react for 2 minutes

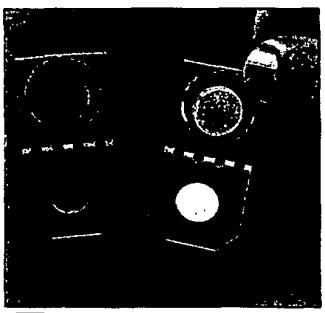


EXPOSE

- Remove ticket from packet
- Expose white disc only
- Dip white disc into beaker for 1 minute

DEVELOP

- Remove foil and expose second disc
- Fold ticket at perforation
- Press discs together
- Hold for 3 minutes



READ RESULTS

- Open ticket
- Blue color means pesticide is <u>not</u> detected
- White color means pesticide is present

Produce System

Total Insecticide Residue Technique

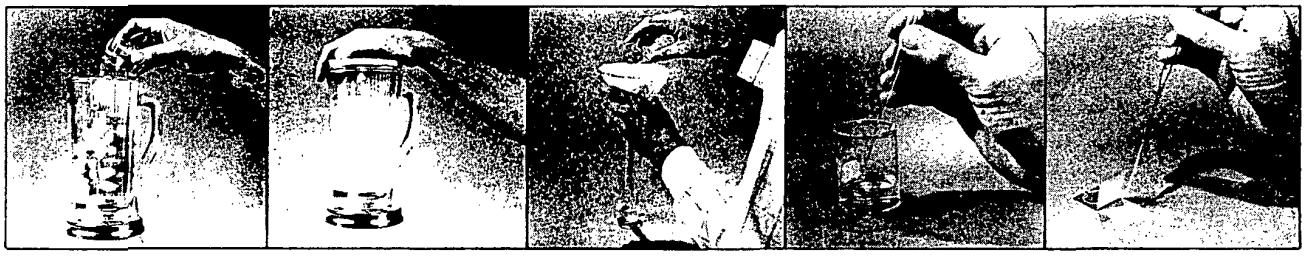
The EnzyTec Program is setting industry standards in pesticide detection. The Program has eight self-contained systems that are inexpensive, easy-to-use, and yield results within minutes.

Each system has a variety of techniques, each designed to test a different kind of sample. The **Total Insecticide Residue Technique** is designed to be used in a laboratory and gives results within several hours. The detection limit depends upon the amount of evaporation of the solvent used. This technique is used to find low parts per million.

An EnzyTec Pesticide Detection Produce System Kit Contains:	**APPLICATIONS:**
• Pesticide Detector Tickets • Activator Ampules • 50 mL graduated beaker • Glass rod for breaking the Activator Ampules and stirring the sample **In addition, you will need:** • Methylene Chloride • Blender • Filter Paper • Air Drying Equipment	• Processed Fruits, Vegetables and Nuts • Processed Wines and Juices • Poultry and Livestock • Pesticide Coverage and Effectiveness • Residue Levels

INSTRUCTIONS:

COLLECT
- Collect a representative sample of fruits or vegetables



EXTRACTION
- Add 200 mL of methylene chloride to 200 g of produce

GRIND
- Blend produce into fine particles

FILTER
- Filter the mixture

EVAPORATE
- Evaporate solvent with airstream

APPLICATION
- Apply solvent containing the insecticide to the disc

EVAPORATE
- Use heat gun to evaporate the solvent; the insecticide remains

CONVERT
- Break one ampule in 20 mL of clean water

- Place 3 drops of dilute fluid on disc and allow to react for 2 minutes

DEVELOP
- Remove foil and expose second disc
- Fold ticket at perforation
- Press discs together
- Hold for 3 minutes

READ RESULTS
- Open ticket
- Blue color means pesticide is not detected
- White color means pesticide is present

Solvent System

Dislodgeable Insecticide Residue Technique

The EnzyTec Program is setting industry standards in pesticide detection. The Program has eight self-contained systems that are inexpensive, easy-to-use, and yield results within minutes.

Each system has a variety of techniques, each designed to test a different kind of sample. The **Dislodgeable Insecticide Residue Technique** is designed to be used in laboratories and gives results in several hours. The detection limit depends on the evaporation of the solvent used in the extraction. This technique is used to find low parts per billion levels.

An EnzyTec Pesticide Detection Solvent System Kit Contains:

- Pesticide Detector Tickets • Activator Ampules
- 50 mL graduated beaker Glass rod for breaking the Activator Ampules and stirring the sample

In addition, you will need:

- Methylene Chloride • Air Drying Equipment

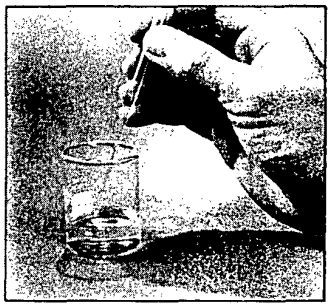
APPLICATIONS:

- Field Screening
- Laboratory Prescreening
- Residue Levels

INSTRUCTIONS:

COLLECT

- Collect insecticide by washing fruit with an organic solvent

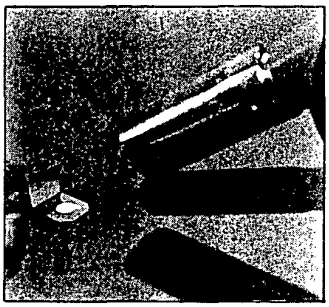


EVAPORATE

- Evaporate solvent with airstream

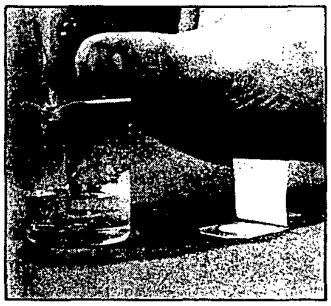
APPLICATION

- Apply solvent containing the insecticide to the disc



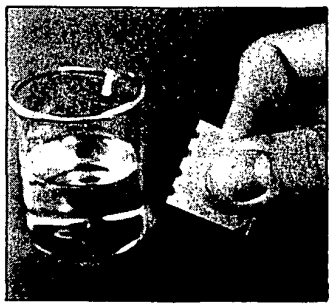
EVAPORATION

- Use heat gun to evaporate solvent, leaving insecticide



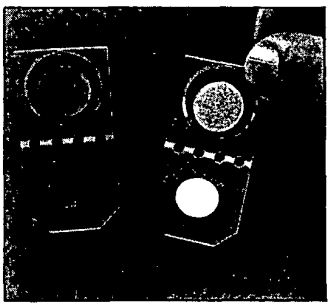
CONVERT

- Add 3 drops of activator solution to disc
- Allow the diluted activator solution to react on disc for 2 minutes



DEVELOP

- Remove foil and expose second disc
- Fold ticket at perforation
- Press discs together
- Hold for 3 minutes



READ RESULTS

- Open ticket
- Blue color means pesticide is <u>not</u> detected
- White color means pesticide is present

EnzyTec, inc.

PESTICIDE DETECTOR TICKET

Typical Pesticide Detection Limits

These detection limits are based on a one minute exposure of the Pesticide Detector Ticket in an aqueous insecticide soluton. EnzyTec has other techniques to lower these detection limits.

Insecticides

	Common Names	Trade Names	Detection Limit in Water (ppm)
Carbamates			
	Aldicarb	Temik®	0.2
	Carbaryl	Sevin®	7.0
	Carbofuran	Furadan®	0.1
	Mesurol	Methiocarb	5.0
	Methomyl	Lannate®	1.0
	MIPC	Isoprocarb	2.0
	Oxamyl	Vydate L®	1.0
	Propoxur	Baygon®	1.0
Organophosphates			
	DDVP	Vapona®	3.0
	Methamidophos	Monitor®	4.0
	Mevinphos	Phosdrin®	2.0
Thiophosphates [1]			
	Aspon	—	5.0
	Azinphos-Methyl	Guthion®	0.3
	Chlorpyrifos-Ethyl	Dursban® Lorsban®	0.7
	Chlorpyrifos-Methyl	Reldan®	1.0
	Diazinon	Spectracide® Dianon®	2.0
	EPN	—	0.2
	Fenitrothion	—	1.5
	Malathion	—	2.0
	Metasystox-R	—	20.0
	Methyl Parathion	—	4.0
	Parathion	—	2.0
	Phorate	Thimet®	3.0
	Phosmet	Prolate®	1.0
	Phosvel	Leptophos	0.8

[1] Requires conversion to oxygen analog for detection

Appendix V

INVESTIGATIONS INTO THE OXIDATION OF PIRIMIPHOS-METHYL

All the experiments described are modifications to the oxidation step of the standard extraction and test procedure, see Section 2.3.1.

All the pesticide solutions used were laboratory standard solutions. The buffers used in these investigations were 0.07M citrate/phosphate buffers.

Attempts were made to oxidize pirimiphos-methyl in the following ways.

i) Using between 105μg (ie equimolar to 20ml of 10ppm pirimiphos-methyl) and 1440μg of bromine water per test, to oxidize pirimiphos-methyl in aqueous solution at concentrations between 5ppm and 40ppm.

Solution of pirimiphos-methyl in water	Amount of Bromine water used		Detection of pesticide by the EnzyTec ticket
5ppm	52μg	*	-
5ppm	105μg		-
5ppm	210μg		-
10ppm	105μg	*	-
10ppm	210μg		-
10ppm	360μg		-
10ppm	720μg		-
20ppm	360μg		-
20ppm	720μg		-
40ppm	420μg		-
40ppm	1080μg		-
40ppm	1440μg		-
CONTROL	105μg		-
CONTROL	720μg		-
CONTROL	1440μg		-

\+ Pesticide was detected

\- No Pesticide was detected

* Equimolar quantities of bromine and pirimiphos-methyl present

The results showed that pirimphos-methyl could not be detected by the ticket i any of these tests, although the enzyme showed no inhibition in control tests. This indicated that pirimiphos-methyl could not be successfully oxidized by concentrations in excess of equimolar.

ii) Extensive tests were carried out on oxidation in buffered conditions between pH 4.0 and 6.0, using between 105μg and 380μg of bromine water to oxidize pirimiphos-methyl concentrations between 1.25ppm and 10ppm.

pH of 0.07M buffer	Amount of Bromine water used	Pesticide ppm	Detection of pesticide by the EnzyTec ticket		
			%+ve test	%-ve tests	% tests with no answer
6.0	105μg *	10ppm P.M.		100%	
	360μg	10ppm P.M.		100%	
	52μg *	5ppm P.M.		100%	
	360μg	5ppm P.M.		100%	
	360μg	CONTROL		100%	
5.5	105μg *	10ppm P.M.			100%
	110μg	10ppm P.M.			100%
	105μg	5ppm P.M.	50%	50%	
	110μg	5ppm P.M.	50%	50%	
	110μg	CONTROL		100%	
5.0	105μg *	10ppm P.M.		50%	50%
	105μg	5ppm P.M.	100%		
	105μg	2.5ppm P.M.		100%	
	210μg	10ppm P.M.			100%
	52μg *	5ppm P.M.		100%	
	52μg	2.5ppm P.M.			100%
	210μg	CONTROL		100%	
5.0	210μg	2.5ppm Mala		100%	
	210μg	0.62ppm Mala		100%	
	210μg	0.31ppm Mala		100%	
	210μg	0.16ppm Mala	100%		
4.8	210μg	10ppm P.M.		50%	50%
	210μg	5ppm P.M.		100%	
	210μg	2.5ppm P.M.		100%	
	210μg	CONTROL		100%	

ii) Continued..

pH of 0.07M buffer	Amount of Bromine water used	Pesticide ppm	Detection of pesticide by the Enzytec ticket		
			%+ve tests	%-ve tests	% tests with no answer
4.0	210µg	10ppm P.M.	33%		67%
	210µg	7ppm P.M.	50%		50%
	210µg	5ppm P.M.	20%	60%	20%
	210µg	4ppm P.M.	60%	20%	20%
	210µg	3.5ppm P.M.		100%	
	210µg	3ppm P.M.		75%	25%
	210µg	2.5ppm P.M.	40%	40%	20%
	210µg	1.25ppm P.M.		100%	
	210µg	CONTROL		90%	10%
4.0	380µg	10ppm P.M.	100%		
	380µg	5ppm P.M.			100%
	380µg	4ppm P.M.		100%	
	380µg	3.0ppm P.M.		100%	
	380µg	2.5ppm P.M.		100%	
	380µg	CONTROL		83%	17%

Key:

* Bromine at equimolar quantities with the concentration of pesticide in solution.

P.M. - Pirimiphos - methyl

Mala - Malathion

The results were not reproducible and showed no dose/response relationship in the detection of pirimiphos-methyl. The enzyme activity in the control tests was unreliable in buffered conditions below pH 5.0.

iii) The 4 minute period allowed for bromine water to evaporate from test solutions was eliminated, and the ticket was exposed to 10ppm and 5ppm pirimiphos-methyl at both pH 7.0 and 5.5 immediately after the addition of the oxidant.

No detection of pirimiphos-methyl was achieved with the ticket although the enzyme was uninhibited in the control tests. This suggests that the lack of detection was due to the pirimiphos-methyl not having been successfully oxidized, rather than the rapid decomposition of the oxidised form.

iv) Oxidation using hydrogen peroxide was investigated. Additions of between 3ml and 5µl of 30% hydrogen peroxide were made to 10ppm pirimiphos-methyl and controls and this was repeated in buffered conditions at pH 5.0.

Test solution	pH	Amount of hydrogen peroxide added	Quantity of catalase used	Enzyme status	Pesticide detected
Water	7.0	3ml of 30% soln	none	inhibited	
Water	7.0	1ml of 30% soln	none	inhibited	
Water	7.0	0.5ml of 30% soln	none	inhibited	
Water	7.0	200µl of 30% soln	none	inhibited	
Water	7.0	30µl of 30% soln	none	inhibited	
Water	7.0	5µl of 30% soln	none	inhibited	
Buffer	5.0	0.5ml of 30% soln	none	inhibited	
Buffer	5.0	200µl of 30% soln	none	inhibited	
Buffer	5.0	30µl of 30% soln	none	inhibited	
Water	7.0	2ml of 30% soln	3000µg	uninhibited	
Water	7.0	1ml of 30% soln	1000µg	uninhibited	
Buffer	5.0	1.5ml of 30% soln	2000µg	uninhibited	
10ppm P.M.	7.0	0.5ml of 30% soln	1000µg	uninhibited	-
10ppm P.M.	7.0	2ml of 30% soln	2000µg	uninhibited	-
10ppm P.M.	5.0	0.5ml of 30% soln	1000µg	uninhibited	-
10ppm P.M.	5.0	200µl of 30% soln	500µg	uninhibited	-

P.M. - pirimiphos-methyl

The results showed that the enzyme was inhibited in all the control tests where hydrogen peroxide was present. Between 500μg and 3000μg of catalase enzyme was added to the test solutions in order to breakdown the hydrogen peroxide, after allowing 1 minute for the oxidation reaction to occur. The results showed that although the enzyme was no longer inhibited in control tests the pesticide could not be detected. This indicates that either pirimiphos-methyl has not been oxidized by the hydrogen peroxide, or that hydrogen peroxide is too strong an oxidizing agent and causes the pesticide molecules to disintegrate.

(v) a) Peracetic acid was used as an oxidising agent on an equimolar basis with 20ml of 10ppm pirimiphos-methyl. This created a pH of 2.6, and the solution was neutralized before testing with sufficient sodium hydroxide, sodium carbonate, or sodium bircarbonate respectively to obtain pH 7.0.

(v) b) 70μl of a 4% peracetic acid solution was used to achieve a pH of 3.5 in test solutions containing between 2.5ppm and 16ppm pirimiphos-methyl. This quantity of peracetic acid would not be sufficient to oxidise the pesticide present and therefore between 90μg and 2160μg of bromine water were added to oxidize the pirimiphos-methyl.

(V)

Test solution	Peracetic acid added	Bromine water added	Neutral-izing agent	Testing pH	Enzyme activity in control tests	Pesticide detected
Water	560µl of 8% soln	None	Sodium Hydroxide	7.0	inhibited	
Water	560µl of 8% soln	None	Sodium Bicarbonate	7.0	inhibited	
Water	560µl of 8% soln	None	Sodium Carbonate	7.0	inhibited	
Water	70µl of 4% soln	1080µg	None	3.5	uninhibited	
Water	70µl of 4% soln	1620µg	None	3.5	uninhibited	
Water	70µl of 4% soln	2160µg	None	3.5	uninhibited	
10ppm P.M.	70µl of 4% soln	90µg	None	3.5	uninhibited	-
10ppm P.M.	70µl of 4% soln	360µg	None	3.5	uninhibited	-
10ppm P.M.	70µl of 4% soln	540µg	None	3.5	uninhibited	- / +
10ppm P.M.	70µl of 4% soln	900µg	None	3.5	uninhibited	- / +
10ppm P.M.	70µl of 4% soln	1080µg	None	3.5	uninhibited	+
5ppm P.M.	70µl of 4% soln	1080µg	None	3.5	uninhibited	-
5ppm P.M.	70µl of 4% soln	540µg	None	3.5	uninhibited	-
5ppm P.M.	70µl of 4% soln	360µg	None	3.5	uninhibited	-
14ppm P.M.	70µl of 4% soln	1080µg	None	3.5	uninhibited	-
14ppm P.M.	70µl of 4% soln	1440µg	None	3.5	uninhibited	- / +
14ppm P.M.	70µl of 4% soln	1800µg	None	3.5	uninhibited	-
14ppm P.M.	70µl of 4% soln	2160µg	None	3.5	uninhibited	-
16ppm P.M.	70µl of 4% soln	1080µg	None	3.5	uninhibited	-
16ppm P.M.	70µl of 4% soln	1440µg	None	3.5	uninhibited	- / +
16ppm P.M.	70µl of 4% soln	1620µg	None	3.5	uninhibited	- / +
16ppm P.M.	70µl of 4% soln	1800µg	None	3.5	uninhibited	-
16ppm P.M.	70µl of 4% soln	2160µg	None	3.5	uninhibited	-

+ Pesticide detected - No pesticide detected P.M. - pirimiphos-methyl

The use of equimolar quantities of peracetic acid even after neutralisation inactivated the enzyme on the ticket and much smaller quantities were therefore used to achieve a pH of 3.5. This did not appear to affect the enzyme activity and tests with pirimiphos-methyl were carried out. Once again the results were equivocal, there being no clear dose-response pattern.

(vi) Oxidation and detection of pirimiphos-methyl was attempted using the cholinesterase enzyme in solution using the methods detailed in Section 2.3.4. 5ppm and 10ppm solutions of pirimiphos-methyl were tested at various pHs using bromine water as the oxidising agent. Control results showed that the enzyme was active, but neither the 5 or 10ppm solutions caused any inhibition of the enzyme. Again, it would appear that the pirimiphos-methyl was not successfully oxidised.

Appendix VI

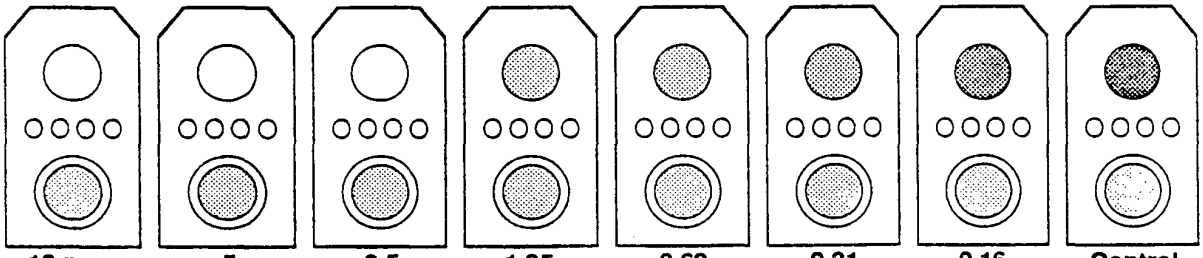
SAMPLE STANDARD CURVE

The use of the EnzyTec pesticide detector as a simple qualitative pesticide screen is well known. However, the detector may also be used as a simple quantitative tool by preparing a STANDARD CURVE of color test results against known concentrations of various pesticide solutions.

For example, a standard curve for Malathion® may be developed as follows:

1. A standard test solution of 10 ppm Malathion® was prepared and diluted with an equal volume of control water until corresponding test concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 ppm of Malathion® solutions were made.
2. Twenty (20) mLs of each solution were then treated with the standard 10 N activator water solution for 3 minutes. Since Malathion® is an organothiophosphate, activator water is used as an oxidizer to prepare the oxygen analogue which is more sensitive to the cholinesterase enzyme present on the detector.
3. A single detector ticket was immersed in each of the oxygenated test solutions for 1 minute to collect a sample. The collector of each ticket was then washed in water and contacted with the substrate disc for a 3-minute period to allow the inhibition reaction to occur. The degree of inhibition is directly related to the color change which occurs: a full blue color for NO inhibition, a complete white disc for TOTAL inhibition.
4. The results were recorded and illustrated as below.

In practice, a water sample from the suspected pesticide-contaminated material (water, soil, vegetable, air...) is collected, contacted with the detector and compared against the Standard Curve. Where possible, the water used to develop the Standard Curve should be taken from the same source used to extract the pesticide.



While this procedure has been prepared to the best of our abilities, it is not to be construed as a warranty or representation of expected performance for which we assume any legal responsibility.

For additional technical information contact: EnzyTec, Inc., 8805 Long, Lenexa, KS 66215, (913) 541-8585.

Appendix VII

Investigations using the spectrophotometric method to detect the presence of pesticide.

i) Sensitivity of the method

Tests were carried out using standard solutions of chlorpyrifos-methyl.

Test solution ppm chlorpyrifos-methyl	Enzyme activity % of control value	Pesticide detected using EnzyTec ticket
Control (water)	100	-
0.62	66	-
1.25	52	-
2.5	41	+
5	23	+
10	10	+

This method can readily detect chlorpyrifos-methyl at much lower concentrations than the EnzyTec ticket.

ii) Testing of extracts of aged grain prepared as described in Section 3.5.

Time after treatment weeks	Enzyme activity % of control value
1	48
2	61
3	35
4	46
5	62
6	56
8	73
13	77
17	78
Control	100

This clearly shows the decreasing inhibition of the enzyme as less pesticide is extracted from the grain as it ages. However, using this method the difference between the test sample and the control is still significant even after 17 weeks of storage when the extraction procedure is very inefficient.

ii) The effect of pH on the enzyme activity in solution.

0.07M citrate/phosphate buffers were used to modify the pH as before, and this was used to replace the phosphate buffer described in the method (Section 2.3.4).

Test solution	Enzyme activity % of control value
Control (pH 7.2)	100
pH 6.0	47
pH 5.5	23
pH 5.0	13
pH 4.0	4

This enzyme, when in solution, is very sensitive to pHs below 7 and it would be difficult to operate an inhibition test below pH6. On the ticket the enzyme appears not to be affected so much by changes in pH.