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IMMUNOASSAYS FOR THE DETECTION OF ORGANOPHOSPHORUS PESTICIDES ON STORED GRAIN: ASSESSMENT OF THREE COMMERCIALLY AVAILABLE KITS AND RECOMMENDATIONS FOR LABORATORY AND FIELD USE

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IMMUNOASSAYS FOR THE DETECTION OF ORGANOPHOSPHORUS PESTICIDES ON STORED GRAIN: ASSESSMENT OF THREE COMMERCIALLY AVAILABLE KITS AND RECOMMENDATIONS FOR LABORATORY AND FIELD USE

by

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Abstract

Rapid semi-quantitative assays for grain protectant pesticides based on immunological methods have been studied at the Central Science Laboratory (MAFF) in order to provide a comparison with established chromatographic methods. The latter can provide precise and reliable data but tend to be expensive, time consuming and require highly skilled analytical staff. Increased public awareness and the recent reduction of maximum residue levels (MRLs) will place greater demands on analytical laboratories. In many instances when grain is traded it is only necessary to know that a fixed limit, often the MRL, has not been exceeded and in this situation immunoassays may be able to provide a cost effective, rapid screening technique which will identify those samples requiring detailed analysis using conventional instrumental techniques.

Commercial tube and plate ELISA kits were obtained from Millipore Corp. (USA) for the detection of organophosphorus pesticides and their performance was assessed, initially using laboratory pesticide standards. A small change in the response was observed when the kits were tested in the presence of substances co-extracted from the grain matrix and it was shown that this effect could be partially corrected by using a blank which also contains grain matrix. The kits were also assessed for cross-reactivity with other organophosphorus pesticides and the common metabolites which are formed when these pesticides degrade. No cross-reactivity of practical importance was found. A direct comparison was made with the established analytical method, gas chromatography and between 88 and 98% correlation was obtained, indicating that the kits possessed a high degree of accuracy. The standard curves were found to be reproducible as shown by the low coefficients of variation.

The ease-of-use of the kits in both laboratory and field situations was appraised and it was found that in order to achieve optimum results, some degree of user training was required. The instructions supplied with the kits were not easy to follow and could lead to erroneous results, hence a protocol for practical use on stored grain was developed which will allow grain store keepers, millers and maltsters to obtain reliable estimates of pesticide residues.

i. Objectives

To assess the laboratory performance of three commercially produced immunoassay kits for the quantification of the organophosphorus pesticides: pirimiphos-methyl, chlorpyrifos-methyl and fenitrothion, present on stored grain.

The assessment addressed the problems of:-
– Response and sensitivity over the required concentration range.
– Selectivity for the analyte in the presence of chemically related compounds.
– The ability of the assay to function in a matrix which contains co-extractants from grain.
– The ease-of-use of the kits.

A direct comparison between ELISA and the established analytical method using gas chromatography was included to determine the reliability of the ELISA technique.

A rapid pesticide extraction method suitable for use outside the laboratory was developed and tested in field trials using tube format kits.

At the end of the assessment, recommendations were made about the suitability of the kits for use in analytical laboratories and their potential for use in the field, with working protocols for both laboratory and field use of the kits produced.

ii. Introduction

In most developed countries grain is stored in large quantities both on farms and in centralised stores. Storage of cereals provides the means to spread the surplus at harvest throughout the year, a way of stabilising grain price and a protection against shortage in the event of poor future harvests. Stored grain is an investment that must be protected from spoilage. The correct storage environment, requiring the control of temperature and moisture levels provides a degree of spoilage protection, however most stored grain is additionally treated with pesticide as a prophylactic measure to prevent insect infestation. Organophosphorus pesticides are widely used because of their low mammalian toxicity coupled with broad insecticidal activity (Cremlyn, 1978). In the United Kingdom (UK) six organophosphorus compounds are cleared for use on stored grain and the chemical structure of each compound is shown in Figure 1. However, fenitrothion and malathion are not commonly used in the UK at present. Recent surveys of farms (Olney and Garthwaite, 1993 and Prickett, 1987) and commercial grain stores

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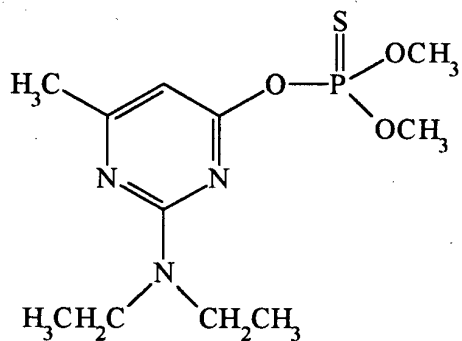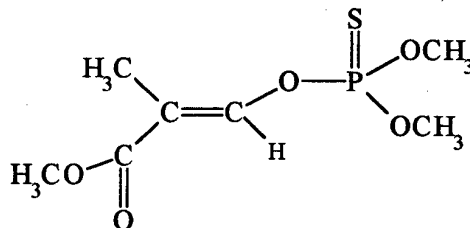
Pirimiphos-methyl

Methacrifos
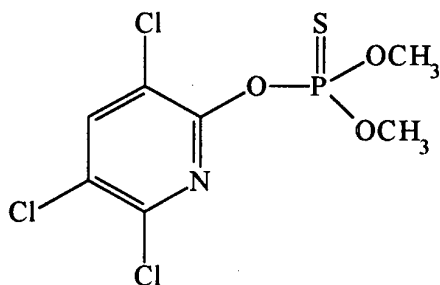
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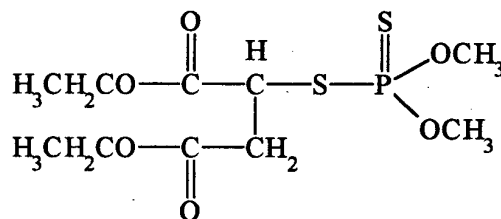
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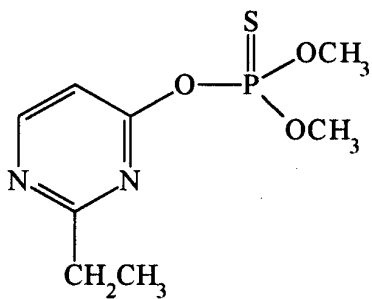
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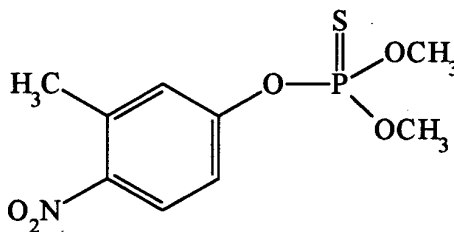
Fenitrothion

Figure 1: ORGANOPHOSPHORUS PESTICIDES

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(Prickett and Muggleton, 1991 and Garthwaite *et al*, 1987) show that pirimiphos-methyl is by far the most widely used organophosphorus grain protectant in the UK.

Increased public concern about food safety and new legislation (Table 1) that limits the levels of pesticide residues in food (Anon, 1991), is increasing the demand on analytical laboratories. Fast, accurate pesticide analysis must be performed at a reasonable cost. In analytical laboratories the current method of assay for organophosphorus pesticides is gas liquid chromatography (GLC), which is a reliable and established technique but requires a relatively large capital investment and highly trained and experienced analysts. The ability to determine whether or not grain has been treated or requires retreatment because of the decay of residues, would clearly be of direct commercial benefit to farmers, traders, storekeepers and processors.

Table 1: U.K. Maximum Residue Limits (MRLS) for Organophosphorus Pesticides on Stored Wheat

Pesticide	Manufacturers recommended application rate (mg/kg)	Old MRL (mg/kg)	New MRL (mg/kg)
Chlorpyrifos-methyl	4.5	10	5
Pirimiphos-methyl	4.0	10	5
Methacrifos	4.75	10	5
Etrimphos	4.2	10	5
Malathion	10	8	8
Fenitrothion	6	10	5

(Anon., 1993)

The EnzyTec kit for the detection of pesticides in the field has previously been tested for the Home-Grown Cereals Authority (Matthews and Price, 1992). This relied on the inhibition of the enzyme acetylcholinesterase by organophosphorus compounds which could, in theory, be used to assess the level of pesticide in the sample. The pesticide must be extracted into water for this assay to operate and, since most organophosphorus pesticides are only sparingly soluble in water, this extraction process is not very efficient. Coupled with this, the assay proved to be insensitive and the detection of low levels of pesticide was unreliable. Before the assay can be carried out the pesticide must be converted to the active oxon form (Figure 2). The activation of pirimiphos-methyl

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using the oxidising agents supplied with the kit was not possible, because of the electronic structure of the molecule, and thus residues of pirimiphos-methyl could not be detected. These factors seriously limited the assay's practical application and interest has since been focused on immunological techniques.

$$R—P\overset{\overset{\displaystyle S}{\|}}{\underset{OCH_3}{\diagdown}}{\diagup}^{OCH_3} \longrightarrow R—P\overset{\overset{\displaystyle O}{\|}}{\underset{OCH_3}{\diagdown}}{\diagup}^{OCH_3}$$
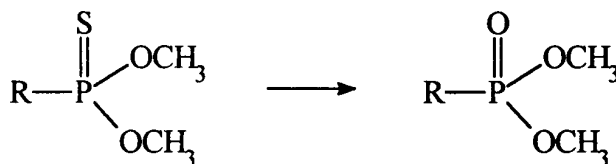
Figure 2: PESTICIDE ACTIVATION

One of the major hurdles in the realisation of immunological pesticide test kits has been the production of suitable antibodies. Pesticide molecules, with a molecular weight below 1000D are not immunogenic. The molecular weight of such substances, termed haptens, must be increased to induce an immune response. This is achieved by conjugating the pesticide to a protein carrier such as bovine serum albumin (Figure 3), usually via a spacer arm. The length of the spacer arm (between 2 and 6 carbon atoms), the coupling site (and hence orientation) and the number of pesticide molecules

PESTICIDE — CARBON SPACER ARM — PROTEIN
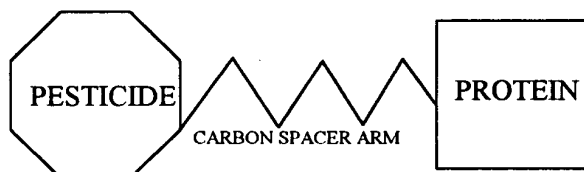
Figure 3: PESTICIDE PROTEIN CONJUGATE

conjugated to each protein molecule will determine both the degree of the immune response and the nature of the antibodies produced. The conjugate is injected into the animal, normally a rabbit if polyclonal antibodies are required, or a mouse for monoclonal antibodies. Polyclonal antibodies have been used in all the commercial kits which have been investigated.

The commercial kits which have been tested are presented in an antibody bound competitive enzyme-linked immunosorbant assay (cELISA) format. The kits contain microtitre well strips or coated tubes to which the antibody is bound, pesticide linked to an enzyme label (horseradish peroxidase) and a substrate/chromogen system to detect the enzyme label. Pesticide is extracted from the grain sample using methanol and once concentrated this can be added to the diluent buffer supplied and used in the assays with no additional clean-up (Figure 4). The pesticide-enzyme label (conjugate) is then added so that this and the free pesticide in the sample are in competition for the limited number

of antibody binding sites. For the plate kits this reaction reaches equilibrium after one hour when the wells are washed. The tube kits do not reach equilibrium and are washed after 10 minutes, hence they cannot be regarded as giving fully quantitative results.
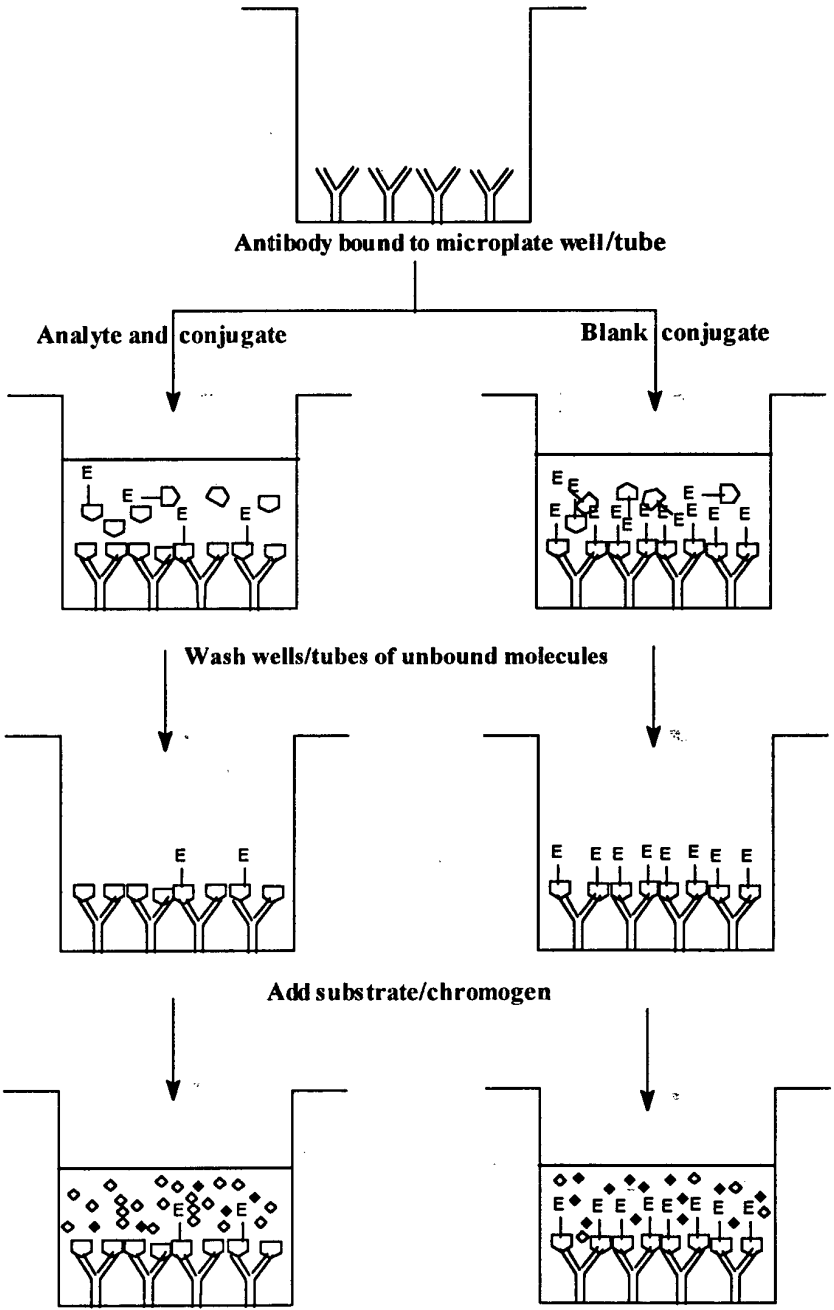
Figure 4: STAGES IN COMPETITIVE, BOUND ANTIBODY ELISA

After washing only bound molecules remain and a substrate/chromogen mixture is added to the wells or tubes, producing a colour change in the presence of the bound enzyme conjugate. This reaction is halted with acid, which changes the colour from blue to yellow and the absorbance may be measured spectrophotometrically at 450 nm. The colour change is inversely proportional to the logarithm of the concentration of the pesticide added, i.e. the more pesticide the greater the loss of colour.

The potential of immunoassay as a rapid sensitive screen for pesticide residues has been recognised for a number of years and the appropriate technology has long been used in the fields of clinical and veterinary diagnostics. Immunological methods for the analysis of pesticides in food stuffs have been reviewed in the literature (Newsome, 1986), (Hammock and Mumma, 1980) and the direct applicability of immunoassays to testing cereals has also been described (Skerritt et al, 1990). However, further extensive validation needs to be carried out before immunoassay can be accepted as a reliable method for pesticide analysis (Lesnik, 1994). It is recognised that the major problems likely to be encountered are those of cross-reactivity and non-specific interactions, both with the matrix and other chemicals that may be present in the sample (Masseyeff, 1993). Once the extent of any matrix effects has been determined the assay can be adapted to compensate for them. Immunoassays may also suffer from the production of false positive results for reasons which are as yet unknown, although attempts have been made to explain them using reaction kinetics (Notermans et al, 1991). This should not pose a practical problem as any positive results should be confirmed by intrumental analysis, but a tolerable limit for false positives of less than 25% has been set by the United States Environmental Protection Agency (USEPA) (Lesnik, 1994). Another problem that must be overcome if immunoassay methods are to be generally accepted in environmental monitoring is the lack of a standard method to assess their performance particularly in as far as determining limits of detection (LOD) and limits of quantification (LOQ) are concerned. Commercial ELISA kits can only test for a single analyte at a time and their effective concentration range is narrow when compared to chromatographic methods. The first of these limitations could be addressed by producing generic immunoassays, similar to those described for the s-triazine herbicides (Muldoon et al, 1993). The second limitation should not be a problem for this application, providing the assay can estimate pesticide concentration in a range around the reporting limit required by the user.

The commercial ELISA kits have low capital and operational costs and require little sample clean-up, which makes it possible for testing to be performed outside the specialist analytical laboratory environment. The suitability of commercially produced plate and tube format immunoassay kits for the quantification of grain protectant organophosphorus pesticides has been evaluated at the Central Science Laboratory for HGCA. This assessment has addressed the problems of response and sensitivity over the required concentration range, selectivity for the analyte in the presence of chemically related compounds and the ability to function in the presence of co-extracted compounds from the grain matrix. At each stage, direct comparison has been made with the established analytical method, gas chromatography. In addition to the analytical considerations, the ease-of-use of the kits has been appraised with regard to the requirements of the potential end users and the conditions they may be working in. A working protocol has therefore been developed to enable users of the kits to meet these requirements.

iii Materials and Methods

General Methods

Grain Treatment, Sampling and Milling

150g samples of wheat (variety: Tonic, moisture content 13.2%) were treated with pesticide standards using diethyl-ether as a solvent. Various pesticide concentrations were used to produce grain treated at between 0 and 10mg kg^{-1}. The pesticide was dissolved in 1ml of diethyl-ether and added dropwise to the stirred grain. The glassware was then rinsed with 0.5ml of diethyl-ether and this was also added to the grain. The grain was then tumbled for 1 hour in a sealed jar to ensure homogenous treatment as far as practicable (Adams, 1985) and was spread out on a foil covered tray in the air flow of a fume cupboard for 5 minutes to allow the diethyl-ether to evaporate. It was returned to the jar, resealed and tumbled for a further 2 hours. The grain was sampled immediately and the samples were frozen. The remaining treated grain was stored at 10-12°C for 9 weeks. Once stored the grain was tumbled for 1 hour then resampled. Sampling was achieved by taking 30g of the grain which was coarsely milled using a Tecator Cemotec 1090 sample mill (setting No. 7). This was then divided into three 10g subsamples which were sealed in vials and frozen until they were required for extraction.

Extraction Procedures

The extraction procedures described in the instructions for the kits were found to be inefficient in our hands (Matthews and Price, 1992) and introduced a dilution factor which was unnecessary. A conventional solvent extraction procedure (Anon, 1980) was therefore substituted in this laboratory study and a rapid extraction method was also developed which was suitable for the extraction of pesticide residues in the field.

Standard Methanol Pesticide Extraction

The 10g samples of milled grain were homogenised with 30 ml of methanol for 1 minute. The methanol extract was isolated using a sintered glass funnel under vacuum and this process was repeated, using the filter cake scraped from the funnel, with a further two 30 ml volumes of methanol. The last volume was additionally placed into an ultrasonication bath and sonicated for 1 minute prior to filtration. The solids were then discarded and the sintered glass funnel rinsed with a further 10 ml of methanol to give a total volume of 100 ml. This was concentrated to 10ml using a Büchi rotary film evaporator fitted with a cold finger and this extract was then used in both the GLC and ELISA analyses without clean up.

Rapid Extraction Procedure

10 g samples of whole grain were weighed into 100 ml screw-topped reagent jars. 10ml of methanol was added to each jar and the contents were swirled for 10 seconds. The jars were sonicated for 10 minutes. As much solvent as possible was decanted from the jar into a 10ml volumetric flask and made up to the mark with methanol. The extract was then diluted with the buffer in the tube ELISA tests.

Immunoassay Procedure

The procedures adopted for the individual plate and tube kits varied slightly, especially with regard to volumetric additions (Tables 2 and 3 respectively).

Plate Format Kits

Pesticide standards were prepared in methanol over the linear concentration range of each kit and were diluted, together with a methanol blank, firstly in methanol and then in the sample diluent buffer provided with the kit, according to the instructions supplied. Sample extracts were similarly diluted according to the instructions. 100µl of each diluted sample or standard was added to three microtitre wells. To each well was then

added 100μl of pesticide conjugate, which had been diluted with its buffer solution as described in the kit instructions. The contents of the wells were mixed with a gentle circular motion and covered with sealing film. After one hour the film was removed and the contents of the wells were vigorously shaken out into a sink. The wells were then washed out four times with buffer, shaking out as much of the buffer as possible between washes. Substrate and chromogen mixture in a volumetric ratio of 2:1 was added to the washed wells and then, after 30 minutes, acid stop solution was added to each. The absorbance of each well was measured at 450nm on a Bio-Rad 3550 plate reader. Values of %B/Bo (percentage of the blank absorbance) were calculated using:-

$$\%B/Bo = \frac{\text{Sample Absorbance}}{\text{Blank Absorbance}} \times 100$$

and these values were plotted against pesticide concentration on a logarithmic scale.

Table 2: Volumetric Additions Made at Various Stages in the Plate Immunoassays

Stage in assay	Pirimiphos-methyl	Fenitrothion	Chlorpyrifos-methyl
Dilution of the extract/ standard with methanol	100μl into 1900μl of methanol (100μl into 900μl in later kits)*	100μl into 400μl of methanol	100μl into 900μl of methanol
Addition of the diluted extract/standard to diluent buffer	100μl diluted extract /standard into 900μl of diluent buffer	200μl diluted extract /standard into 800μl of diluent buffer	100μl diluted extract /standard into 900μl of diluent buffer
Addition of diluted extract/standard and conjugate onto the plate	100μl of diluted extract/standard & 100μl of conjugate	100μl of diluted extract/standard & 100μl of conjugate	100μl of diluted extract/standard & 100μl of conjugate
Addition of substrate and chromogen mixture.	120μl of substrate/ chromogen mixture (150μl in later kits)*	120μl of substrate/ chromogen mixture	150μl of substrate/ chromogen mixture
Addition of stop solution	40μl of stop solution (50μl in later kits)*	40μl of stop solution	50μl of stop solution

* new kits used for the stored grain analysis

Table 3: Volumetric Additions Made at Various Stages in the Tube Immunoassays

Stage in assay	Pirimiphos-methyl	Chlorpyrifos-methyl
Dilution of the extract/ standard with diluent buffer	10µl of extract/standard into 1ml of diluent buffer	20µl of extract/standard into 1ml of diluent buffer
Addition of diluted extract/ standard and conjugate to tube	500µl of diluted extract/standard & 200µl of conjugate	500µl of diluted extract/standard & 200µl of conjugate
Addition of substrate	500µl of substrate	500µl of substrate
Addition of stop solution	500µl of stop solution	500µl of stop solution

Tube Format Kits

Pesticide standards over the linear concentration range of each kit were prepared in methanol and these were diluted, together with the sample extracts in the diluent buffer provided with the kit according to the instructions supplied. 500µl of each diluted sample or standard was added to each antibody coated tube. To each well was then added 200µl of pesticide conjugate and the contents of the tubes were mixed gently. After 10 minutes the tubes were vigorously shaken out into a sink and they were then washed out five times with buffer, shaking out as much of the buffer as possible between washes. 500µl of substrate solution was added to the washed tubes and then, after 5 minutes, 500µl of stop solution was added. The absorbance of each tube was measured at 450nm. Values of %B/Bo (percentage of the blank absorbance) were calculated and these values were plotted against pesticide concentration on a logarithmic scale.

Gas Liquid Chromatography Procedure

Triplicate injections of each sample and standards were made onto a 3 metre packed column in a Carlo-Erba Instruments HRGC 5300 Mega Series GLC under isothermal conditions at $220^{\circ}C$. The column was packed with 7.5% OV210 on Chromosorb WHP 80/100 mesh and the carrier gas was helium at a flow rate of 50ml min^{-1}. A nitrogen/phosphorus ion selective thermoionic detector with P-mode selected was used. Chromatographic reporting was on a Spectra Physics SP4270 integrator/plotter. Pesticide concentration of the extracted samples was found by interpolation of the calibration curve using regression analysis.

Packed column GLC was selected instead of the more sensitive capillary column GLC in order to avoid the extensive sample clean-up which is required prior to using these systems and thus allow a direct comparison between ELISA and GLC.

Kit Assessment Method

Characterising Assay Response Using Standard Solutions

Standard pesticide solutions were prepared in methanol and were tested using the kits as detailed above.   The resulting data was plotted to give a response curve of %B/Bo against concentration.

Assessing the Effect of a Grain Matrix Using Spiked Blank Grain Extract

10g samples of untreated grain were extracted as above to provide 100ml of concentrated blank grain extract.   This was used to produce matrix-matched standards and matrix blanks as required.

Estimating Pesticide in Freshly Treated and Stored Grain

For each pesticide, freshly treated and stored grain (9 weeks post-treatment) was sampled, milled and extracted as described above.   The plate ELISA and GLC estimation were carried out on the same samples in parallel so that a true comparison could be made.

Cross Reactivity Studies

A series of candidate cross-reactants were tested for each plate kit.   The structurally related pesticides at a concentration of 5.0 mg l^{-1} and the common metabolites of the target pesticide at 0.5 mg l^{-1} were tested.   The %B/Bo for potential cross reactants at these concentrations was determined.   The potential cross-reactants tested with each of the kits are shown in Table 4   Where significant, the cross-reactivity was quantified as a percentage of the target pesticide response at 50%B/Bo by analysing a series of concentrations of the cross-reactant.

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Field Trials

Field trials in which the tube format kits were used in a field situation without analytical laboratory facilities were carried out on two separate occasions. Both trials were conducted over two days. The first trial was carried out indoors at a barley maltings with basic facilities available. The second trial was conducted outdoors with no facilities and considerable variations in temperature. Treated grain samples were extracted using the rapid extraction method and analysed using the tube kit protocol detailed previously. The views and opinions of potential users were sought and this helped in the production of the simplified assay protocols.

iv. Results

Ease-Of-Use of the Kits

The plate assays were found to be more difficult to perform than the manufacturer's instructions suggested and therefore simplified protocols have been written to assist the user (Appendix). Although the three kits tested followed the same general protocol, there were differences between the methods used for each kit particularly with regard to the reagent addition (Tables 2 and 3). Hence each kit initially had a different set of instructions. The reagent addition volumes have changed between the batches of pirimiphos-methyl kits used for the early work and those used for the later stored grain analysis. The instructions for the latest kits have been standardised, which has increased their ease-of-use considerably. In our hands, the grain extraction protocol supplied with the kits was found to be inefficient and unreliable (Matthews and Price, 1992) and was replaced with the procedures detailed here. It was found that the calibration standards supplied were x2.5 more dilute than they were marked, presumably to remove the effect of the dilution produced by the extraction procedure. These were replaced in this study with analytical pesticide standards because of concerns regarding the stability of relatively dilute calibration standards stored at 4°C. The rest of the assays were performed as instructed.

A direct plot of absorbance (450nm) vs. pesticide concentration (log) as described in the kit instructions was not used as this does not allow direct comparison between separate test runs. %B/Bo was used instead of absorbance and as this is a ratio of the sample response to the blank response, variations between runs resulting in differences from

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reagents, plates or tubes and temperature should have less effect, thus generating more comparative data.

The tube kits were found to be much easier to use than the plate kits even under adverse conditions outside the laboratory. They had simpler protocols which require less reagent dilutions and had a much faster turn-around time. However only six tubes could be used at once and given that a blank and two standards must be analysed concurrently, this meant that only three samples could be assayed at any one time.

Efficiency of Rapid Extraction Procedure

In order for these tests to be applied in a field situation, a rapid extraction procedure was developed. Using this procedure it was possible to extract 76% of pirimiphos-methyl and 80% of chlorpyrifos-methyl from grain in 10 minutes. These recoveries compare favourably with those obtained using a standard extraction procedure where an average organophosphorus pesticide recovery of 80% was obtained from grain (Anon, 1980).

Plate Kits

Pirimiphos-methyl Plate Kit
The preliminary tests on this kit using laboratory standards produced a shallow sigmoid standard curve (Graph 1). The assay showed a poor dynamic range and over the pesticide concentration range tested (0.1 to 10 mg l^{-1}) the response produced by the assay was only 30% of Bo. This therefore limited the effective sensitivity and range of the kit. A close approximation to linearity was achieved over the pesticide concentration range 0.5 to 2 mg l^{-1} and the steepest slope was also observed over this range indicating the region of greatest sensitivity. The response from the assay did not change significantly with concentration above a level of 5mg l^{-1} or below a concentration of 0.2 mg l^{-1}. This linear range is somewhat narrower than that claimed for the kit of 0.2 to 10 mg l^{-1}.

Tests using grain extract spiked with pesticide and untreated grain extract as the blank produced a curve which showed a significant positive displacement from the standard curve (Graph 2). This was almost certainly due to non-specific interactions between the antibody and substances in the grain extract and was more apparent at zero or very low analyte concentrations.

Tests for possible cross reactivity showed that out of all the related pesticides and metabolites studied only pirimiphos-ethyl produced a positive response (Table 4). A range of pirimiphos-ethyl concentrations was investigated (Graph 5) and a response of 196% compared to pirimiphos-methyl was found at 50%B/Bo which indicates that the ELISA test is twice as sensitive to pirimiphos-ethyl than pirimiphos-methyl. Glyphosate produced a small negative effect on the assay giving 130%B/Bo at a concentration of 5mg l^{-1}.

Table 4:   Cross Reactivity with the Immunoassay Kits for the Chemicals Investigated
expressed as %B/Bo at 5 mg l^{-1} for pesticides and *0.5 mg l^{-1} for pesticide metabolites.

Chemical Kit:	Pirimiphos-methyl	Fenitrothion	Chlorpyrifos-methyl
Pirimiphos-methyl		95	80
Pirimiphos-ethyl	29	not tested	87
Chlorpyrifos-methyl	95	102	
Chlorpyrifos-ethyl	not tested	not tested	27 (at 3ppm)
Malathion	99	110	89
Methacrifos	93	107	95
Etrimfos	100	107	86
Dichlorvos	97	96	96
Fenitrothion	93		90
Glyphosate	130	not tested	74
Pirimiphos-methyl oxon	110	not tested	not tested
2-diethylamino-6-methylpyrimidin-4-ol	102.9	not tested	not tested
2-ethylamino-6-methylpyrimidin-4-ol	101.4	not tested	not tested
2-amino-6-methylpyrimidin-4-ol	101.4	not tested	not tested
Fenitrothion oxon	not tested	74	not tested
Chlorpyrifos-ethyl oxon	not tested	not tested	19
3,5,6-trichloro-2-pyridinol	not tested	not tested	34

Freshly treated and stored grain samples were assayed both by GLC and ELISA. Because of possible interference effects from the grain extract the ELISA tests were carried out using an untreated grain extract as a blank. The results from both methods were used to plot correlation graphs (Graphs 3 and 4). Below a pesticide concentration of 5 mg l^{-1} the correlation coefficient between the two methods was 88 % for freshly

treated and 94 % for stored grain extracts. Above this concentration the two methods could not be correlated, as the ELISA antibody binding sites were then saturated with pesticide molecules which produced a non-linear response from the assay. The freshly treated grain extracts gave an ELISA result that was very close to the concentration estimated by GLC. However, the stored grain samples were overestimated when analysed by immunoassay as compared to GLC (Graph 4).

Fenitrothion Plate Kit

The curve produced by this kit (Graph 1) when using laboratory standards over the concentration range tested (0.05 to 10 mg l^{-1}) produced a response from the assay of 50% of Bo. Linearity was achieved over the pesticide concentration range 0.5 to 3 mg l^{-1}, which was also the region of greatest sensitivity. Half the suggested lower limit of detection was achieved. Increase in response became negligible for pesticide concentrations above 6 mg l^{-1} which was thus the effective upper limit of this kit. Using an untreated grain extract as a blank with this kit also produced a curve which showed a positive displacement from the standard curve (Graph 6). Only fenitrothion oxon produced a positive response when tested for cross-reactivity with this kit (Table 4), however since this standard was supplied in ethyl acetate which even after dilution was found to interfere with the assay this result could not be confirmed or quantified.

Both freshly treated and stored grain samples have been extracted and assayed by GLC and ELISA and the results were used to plot correlation graphs (Graph 7 and 8). Below a pesticide concentration of 6 mg l^{-1} the correlation coefficient between the two methods was 92% and 96% respectively. The freshly treated grain extracts gave an ELISA result that was close to the concentration estimated by GLC. However, the stored grain samples were underestimated when analysed by immunoassay by approximately 50%.

Chlorpyrifos-Methyl Plate Kit

Tests using laboratory standards over the pesticide concentration range tested (0.01 to 10 mg l^{-1}) produced a response from the assay of 60% of Bo (Graph 1). Linearity was achieved over the pesticide concentration range 0.01 to 1 mg l^{-1}, which was also the region of greatest sensitivity although a significant increase in response for this kit was still observed between 1 and 5mg l^{-1}. This kit is about 10 times more sensitive than claimed by the manufacturer.

The addition of the matrix into the assay had the effect of positively displacing the response curve, this displacement was partially corrected by using an untreated grain extract as the blank (Graph 9). Cross-reactivity tests with other pesticides and metabolites (Table 4) produced only a very small response from the assay, with the exception of chlorpyrifos-ethyl, chlorpyrifos-ethyl oxon and 3,5,6-trichloro-2-pyridinol which produce measurable responses. A range of concentrations of chlorpyrifos-ethyl and 3,5,6-trichloro-2-pyridinol was investigated (Graph 10) and chlorpyrifos-ethyl gave a response of 159% compared to chlorpyrifos-methyl at 50%B/Bo which indicates that the ELISA test is 1.5 times more sensitive to chlorpyrifos-ethyl than chlorpyrifos-methyl. Despite increasing the concentration of 3,5,6-trichloro-2-pyridinol to 100 mg l^{-1} a 50%B/Bo was not obtained.

Both freshly treated and stored grain samples have been extracted and assayed by GLC and ELISA and the results were used to plot correlation graphs (Graph 11 and 12). Below a pesticide concentration of 6 mg l^{-1} the correlation coefficient between the two methods was 94% and 98% respectively. The freshly treated grain extracts gave an ELISA result that was reasonably close to the concentration estimated by GLC. However, the stored grain samples were overestimated when analysed by immunoassay compared to GLC.

Tube Kits

Cross-reactivity studies were not repeated for the tube kits as the same antibodies as in the plate kits were used in their production.

Pirimiphos-Methyl Tube Kit
Tests on this kit using laboratory standards produced a shallow sigmoid response curve (Graph 13). A close approximation to linearity was achieved over the pesticide concentration range 0.1 to 1.0 mg l^{-1} and the steepest slope was also observed over this range indicating the region of greatest sensitivity. The response from the assay did not change significantly with a pesticide concentration above 3 mg l^{-1}. This range is somewhat lower than that indicated for the kit of 0.2 to 4.0 mg l^{-1}.

Chlorpyrifos-Methyl Tube Kit
Tests on this kit using laboratory standards produced a shallow sigmoid response curve (Graph 14). Linearity was achieved over the pesticide concentration range 0.01 to 0.25 mg l^{-1} and the steepest slope was also observed over this range indicating the region of

greatest sensitivity.   This range is somewhat narrower than that indicated for the kit of 0.01 to 1 mg l^{-1}.

Field Trials

Both tube kits performed well out of the laboratory and were not adversely affected by external conditions (Graphs 15 and 16).   The presence of wheat or barley matrices had a small effect on the assay but did not impair the detection of pesticide within the range measured.   The standard curves were similar to those obtained under laboratory conditions.   It was found to be possible to use the test kits either as a purely qualitative means of pesticide detection, simply by matching the colour change by eye or as a semi-quantitative method using a field photometer.

v. Discussion

The standard curves obtained from the assays (Graph 1, 13, 14) could be divided into three sections; the lower and upper portions curved towards a zero response and the central portion approximated to linearity.   The non-linear parts of the response were associated with the active sites of the antibody being saturated with either pesticide or pesticide conjugate.   Only the linear section of the plot could be used quantitatively.   Concentrations both above and below this section could not be accurately determined and it was therefore more appropriate to define an analytical range for each assay rather than a limit of quantification (LOQ) which only defined the lower end of this range.   The limit of detection (LOD) lay outside the linear region of the graph and a concentration many orders of magnitude lower than the LOQ might produce a qualitative response, however any numerical limit would have to be associated with a measure of its statistical certainty.

The kits performed well over an analyte concentration range that fell inside their linear response range and samples above this range were diluted into the assay range so they could be analysed.   However, as this altered the concentration of the matrix, a suitably diluted blank had also to be used.   An indication of the reproducibility of the kits was given by the variance in the production of data points on the standard curves (Graph 1 & 13) as shown by the error-bars.   Within the linear response range the variance in the pirimiphos-methyl plate kit was generally larger than that for fenitrothion and chlorpyrifos-methyl.   Problems of repeatability may be addressed by simplifying the

protocols to reduce the number of dilution steps required as has been done here
(Appendix) or by using automated ELISA plate systems which reduce the errors
associated with repeated pipetting. The linear ranges of the kits determined
experimentally were different from those quoted by the manufacturer and in many cases
were nowhere near the stated values. (Table 5) The chlorpyrifos-methyl plate kit was
actually 10 times more sensitive than quoted, therefore samples had to be diluted into the
working range for analysis. The other kits all had a much narrower linear range in
practise than quoted and hence did not perform as widely as the manufacturer claimed.
Accurate linear range data is vital if the user is to be able to reliably use the kits for
screening as this will dictate the dilution factors that will need to be used. Ranges
required by potential users from the brewing and milling industries were determined by
discussion and are shown in Table 5.

Table 5: Linear Ranges of the ELISA Kits

Kit	Experimental Linear Range (mg l^{-1})	Manufacturers Indicated Range (mg l^{-1})	Range Required by Users (mg/kg grain)
Pirimiphos-methyl plate kit	0.5 - 2	0.2 - 10	0.1-1
Chlorpyrifos-methyl plate kit	0.01 - 1	0.1 - 10	0-1
Fenitrothion plate kit	0.3 - 3	0.025-10	0.1-1
Pirimiphos-methyl tube kit	0.1 - 1	0.2 - 4	0.1-1
Chlorpyrifos-methyl tube kit	0.01 - 0.25	0.01 - 1	0-1

* As the extraction methods use 1ml of solvent per 1 g grain, mg l^{-1} and mg/kg are equivalent for the above
comparison.

The only cross-reactions found using the plate kits were with closely structurally related
compounds that were able to bind to the antibody recognition site thus producing a false
positive result. Pirimiphos-ethyl was shown to cross-react with the pirimiphos-methyl
kit at significant levels, however this should not be a practical problem as it is not used as
a grain protectant in the UK. Glyphosate caused a small negative effect on the assay,
but as it is only applied in the field, residues are not likely to carry over into grain. The

only cross-reactant so far observed with the fenitrothion kit was the oxon metabolite, which was not used in the manufacturers tests. Measurable cross-reactivity was demonstrated for chlorpyrifos-ethyl, chlorpyrifos-ethyl oxon and 3,5,6-trichloro-2-pyridinol with the chlorpyrifos-methyl kit. The cross-reactivity of the oxon was not mentioned by the manufacturers and the kit was 17 times more sensitive to chlorpyrifos-ethyl than claimed.

The cross-reactivities of chlorpyrifos-ethyl and 3,5,6-trichloro-2-pyridinol were investigated in detail. Chlorpyrifos-ethyl gave a curve (Graph 10) of a similar shape to that produced with chlorpyrifos-methyl, because of the close similarities in the chemical structure of the two molecules. This should not prove to be a practical problem as chlorpyrifos-ethyl is not used as a grain protectant in the UK. The curve (Graph 10) produced by 3,5,6-trichloro-2-pyridinol showed a flat response of 70-75%B/Bo between concentrations of 0.005 to 100mg l^{-1} and this background negative signal may interfere with the assay. Other pesticides produced a small response to this assay (Table 4) but this response would be diluted out in practice. In general, cross reactivity should not prove to be a practical problem if the interfering agent is absent from the samples. If this is not the case then the hapten used to produce the antibody should be redesigned to exploit a unique moiety in the analyte thus improving the selectivity of the assay.
The presence of grain matrix in the sample extracts caused a displacement of the standard curves due to non-specific interactions between the antibody and co-extractants from the grain. The effect was more noticeable outside the linear range of the kits and could be partially resolved by using a matrix blank to calculate Bo. This enabled the tube kits to be used outside the laboratory satisfactorily as the results of the field trials show. When using plate assays in a laboratory situation, matrix effects could be fully overcome by using matrix-matched standards and blanks.

ELISA plate tests have been shown to produce a high degree of correlation with traditional chromatographic methods for the analysis of pesticides both from samples of freshly treated and stored grain (Graphs 3,4,7,8,11 and 12). The freshly treated grain extracts gave results that were close to those produced by GLC, but had a greater degree of spread where the pesticide concentrations were outside the linear range of the kits standard curve. However, after the treated grain had been stored the results produced by the two methods varied with the pirimiphos-methyl and chlorpyrifos-methyl kit overestimating residue levels and the fenitrothion kit underestimating them compared to the GLC analysis. Immunoassays can appear to overestimate analyte concentration when compared to conventional chromatographic methods, possibly due to compound

loss in phase transfer or adsorption to various surfaces during chromatography (Lucas *et al*, 1995). GLC has also been shown to underestimate chlorpyrifos-methyl concentration on wheat when compared to radiochemical methods. (Matthews, 1990) This discrepancy between ELISA and GLC can be corrected mathematically using the slope of the correlation plots, however parallel studies may be required for each matrix that is encountered. Despite this it has been demonstrated that the kits can function well in a complex matrix such as grain extract containing many potential interfering agents, thus removing the requirement for costly clean-up procedures.

The plate tests were relatively easy to perform compared with chromatographic methods although to be carried out on samples some experience and/or training is required to achieve optimum reproducibility. As well as an appropriate number of standards and blanks, approximately 24 triplicate samples may be analysed in parallel using a single plate kit, producing quantitative results in a turnaround time of a few hours. This study has shown that in their present form the plate kits can be used to screen samples reliably in an analytical laboratory environment, with positive samples then being analysed in more detail using traditional chromatographic methods.

The ELISA tube tests were easier and faster to perform than the plate assays and could be performed by non-specialist staff. Only six tubes could be used at once, therefore only three samples may be analysed in parallel along with two standards and a blank, in a turnaround time of approximately 40 minutes. Results may be semi-quantitative by reading the developed tubes in a spectrophotometer, or purely qualitative, by comparing the colour of the samples by eye to the two standards. The tube kits performed well outside the laboratory environment even under fairly adverse conditions and provided a reliable estimate of pesticide residues on grain in the field. To back up grain passports and observe due diligence, cereal processors such as grain store managers, millers and maltsters currently take samples, both on receipt and during processing, for pesticide residue analysis. Most of these samples are subsequently sent to contract laboratories for analysis. A rapid technique that would allow the user to screen samples at the weighbridge or the mill intake would therefore offer a considerable advantage. Tube ELISA kits would be ideal for this purpose as only a few samples would need to be analysed at a time. For applications which require simultaneous analysis of more than three samples, plate kits would be most suitable. To prevent disruption of a weighbridge operation, such tests must produce reliable results in 30-40 minutes, hence a rapid method of extracting pesticide from grain was developed which complemented the speed of the ELISA tests. The method was designed to be suitably rugged for field use,

give reproducible pesticide recoveries and have an overall extraction time of 10 minutes per sample. (Appendix) Samples that gave positive results using ELISA could then be confirmed by an instrumental method in an analytical laboratory.

With both formats of immunoassay kit from Millipore the protocols varied slightly, particularly with regard to reagent addition (Tables 2 & 3). Standardisation of these instructions would increase the ease with which the assays could be performed. The manufacturers have gone some way to addressing this problem with the latest batch of pirimiphos-methyl plate kits by adjusting the assay to make the volumes of the reagent additions the same as the chlorpyrifos-methyl plate test. Discussion with potential users revealed that the instructions supplied with the kits were not easy to follow which could lead to misuse of the kits and hence erroneous results. Therefore, simplified users protocols have been written to overcome this potential problem (Appendix).

This study has shown that ELISA tests can be used to reliably determine the presence of pesticide residues on stored grain. The tests will meet the requirements of the milling and brewing industries and simplified user protocols have been developed and tested to enable the results to be obtained dependably and reproducibly.

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GRAPH 1: Standard Curves Produced With Each Plate Kit
Showing the Dynamic Range of Each Assay
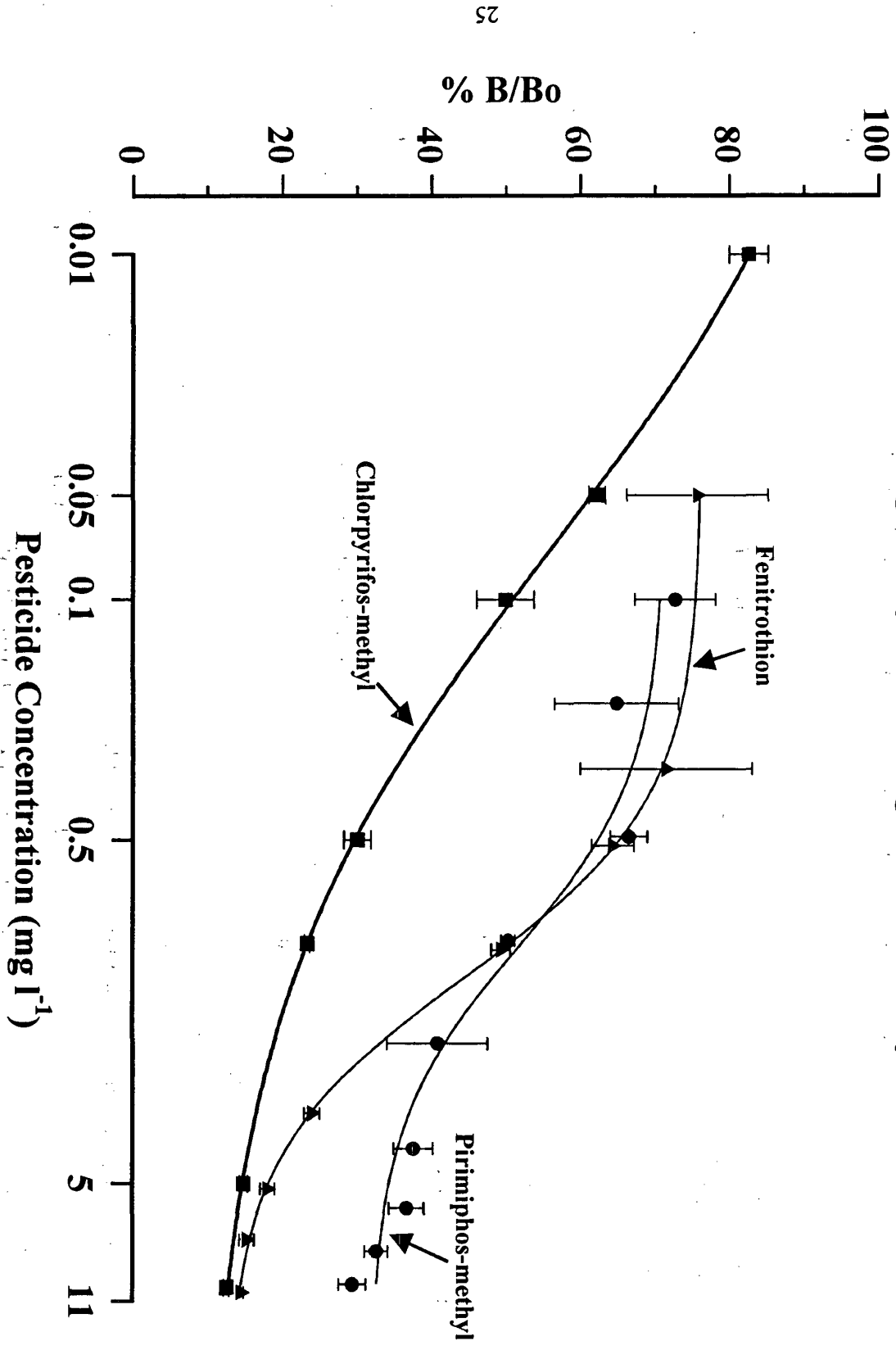
GRAPH 2: Plot Showing the Effect of a Grain Matrix on the Pirimiphos-methyl Plate Kit and Using a Blank Containing Matrices Affects the Test.
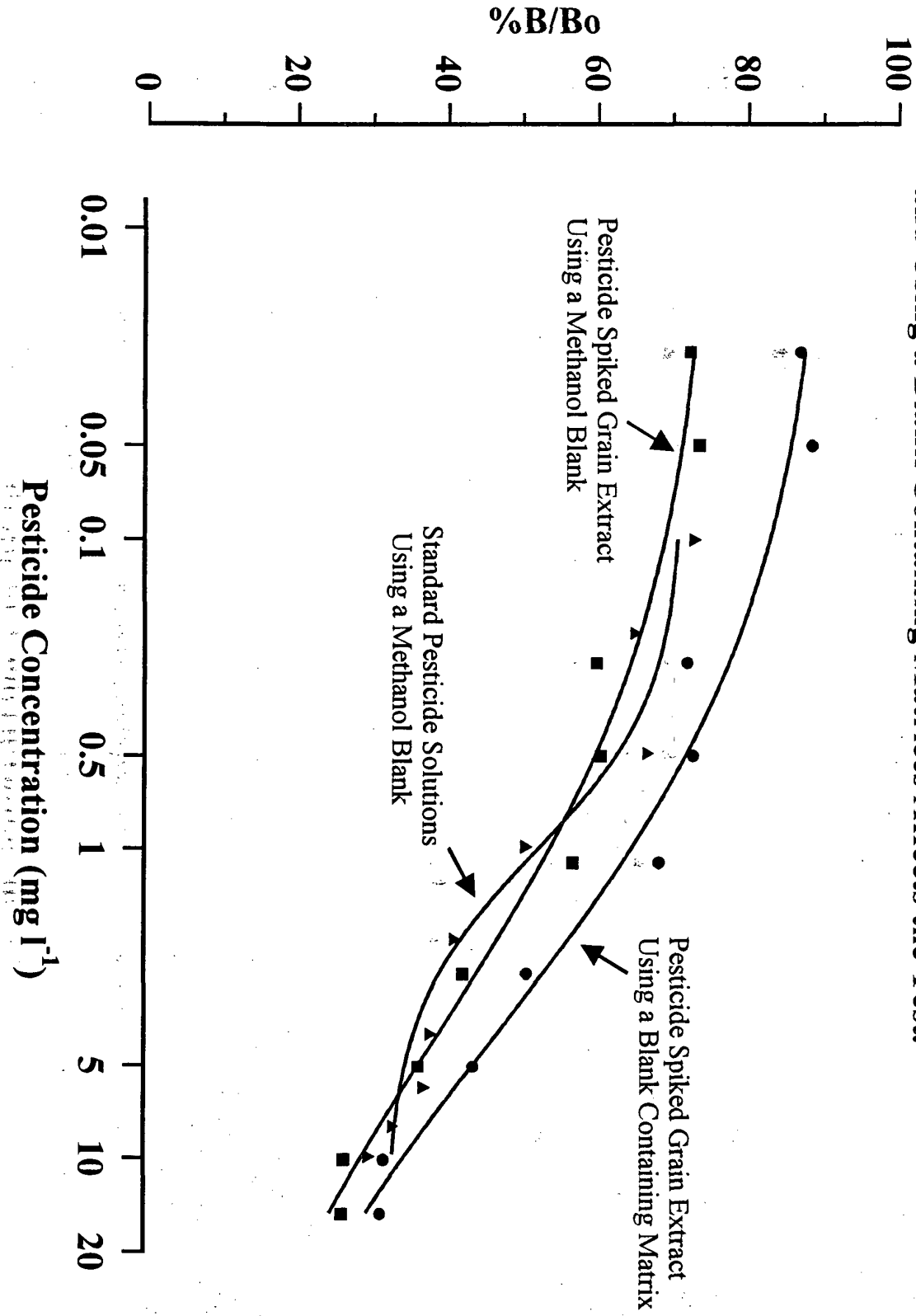
GRAPH 3: Comparison of Pirimiphos-methyl Concentrations Estimated
By Plate ELISA and GLC in Extracts of Freshly Treated Grain

Pesticide Concentration Estimated by GLC (mg l^{-1})

Pesticide Concentration Estimated by ELISA (mg l^{-1})

r = 0.88
Slope=1.06
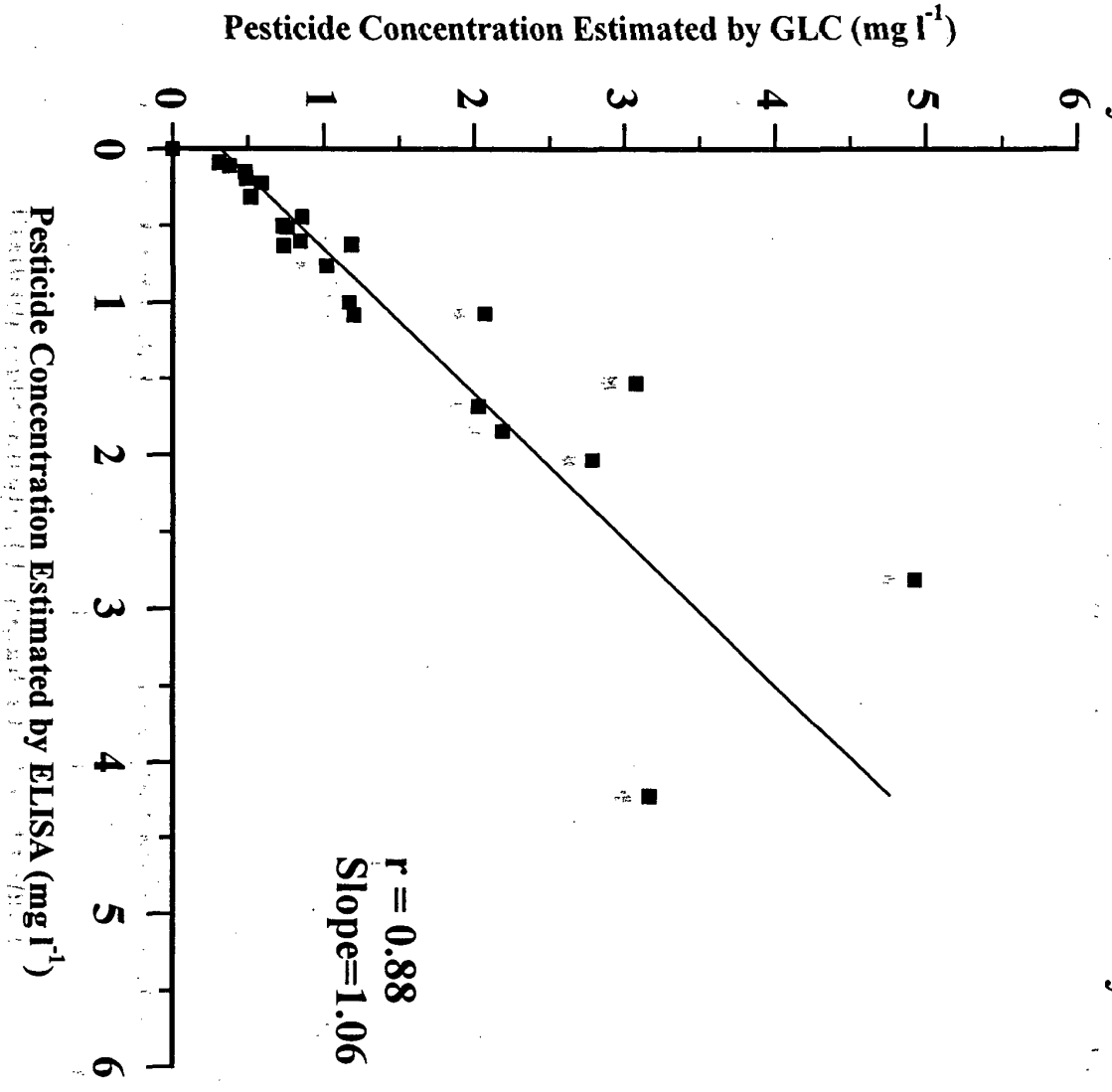
GRAPH 4: Comparison of Pirimiphos-methyl Concentrations Estimated By Plate ELISA and GLC in Extracts of Stored Treated Grain

r = 0.94
Slope=0.59

Pesticide Concentration Estimated by GLC (mg l⁻¹)

Pesticide Concentration Estimated by ELISA (mg l⁻¹)
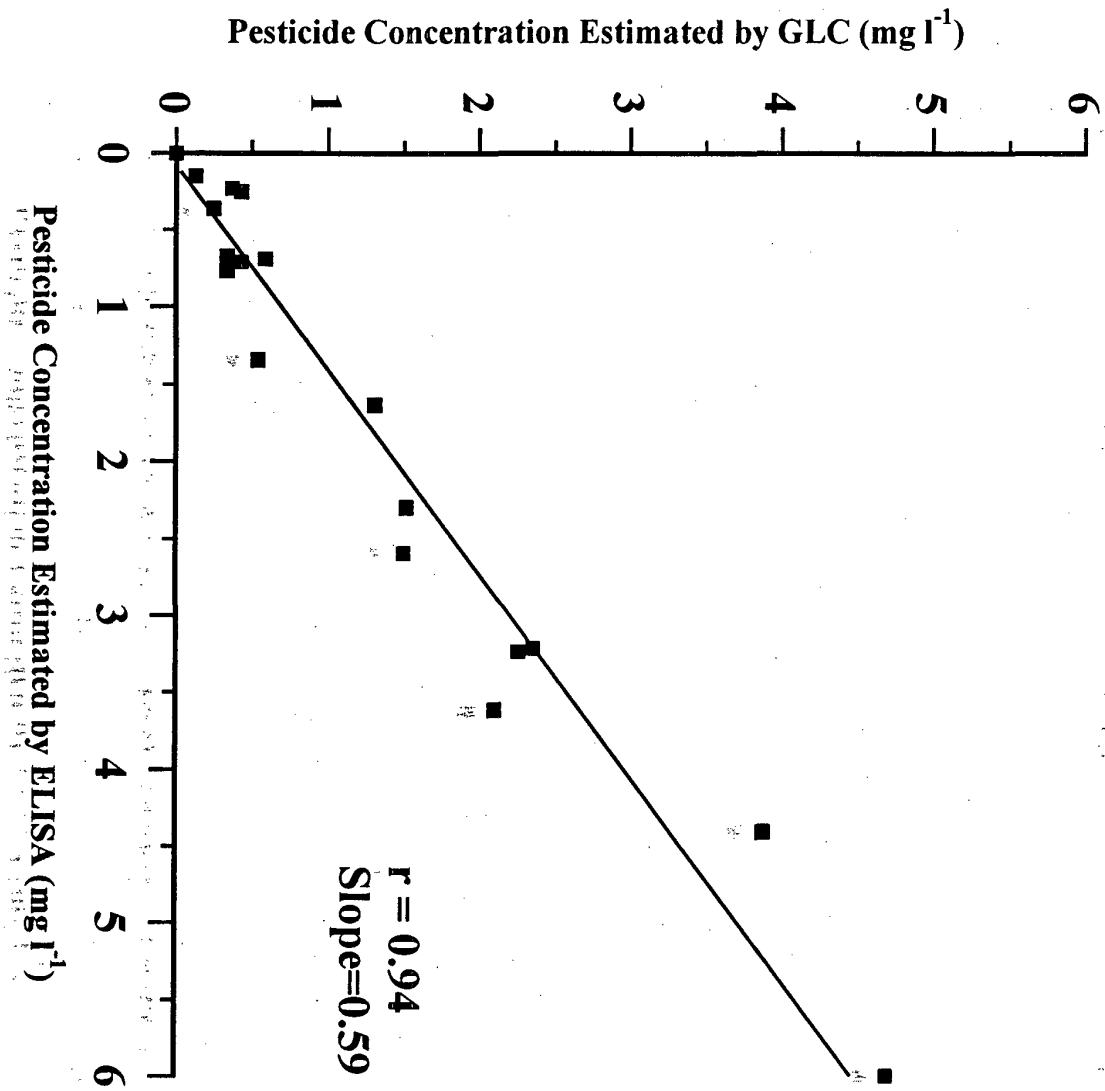
GRAPH 5: Standard Curves For Pirimiphos-methyl and Pirimiphos-ethyl Produced with the Pirimiphos-methyl Plate Kit

% B/Bo

Pesticide Concentration (mg l^{-1})

Pirimiphos-methyl

Pirimiphos-ethyl

GRAPH 6: Plot Showing the Effect of a Grain Matrix on the Fenitrothion Plate Kit and How a Blank Containing Matrix Partially Corrects the Effect

% B/Bo

Pesticide Spiked Grain Extract
Using a Blank Containing Matrix

Pesticide Spiked Grain Extract
Using a Methanol Blank

Standard Pesticide Solutions
Using a Methanol Blank

Pesticide Concentration (mg l⁻¹)

GRAPH 7: Comparison of Fenitrothion Concentrations Estimated By Plate ELISA and GLC in Extracts of Freshly Treated Grain

Pesticide Concentration Estimated by GLC (mg l^{-1})

Pesticide Concentration Estimated by ELISA (mg l^{-1})

r = 0.92
Slope=1.11

GRAPH 8: Comparison of Fenitrothion Concentrations Estimated
By Plate ELISA and GLC in Extracts of Stored Treated Grain

Pesticide Concentration estimated by GLC (mg l⁻¹)

Pesticide Concentration estimated by ELISA (mg l⁻¹)

r = 0.96
Slope = 2.09
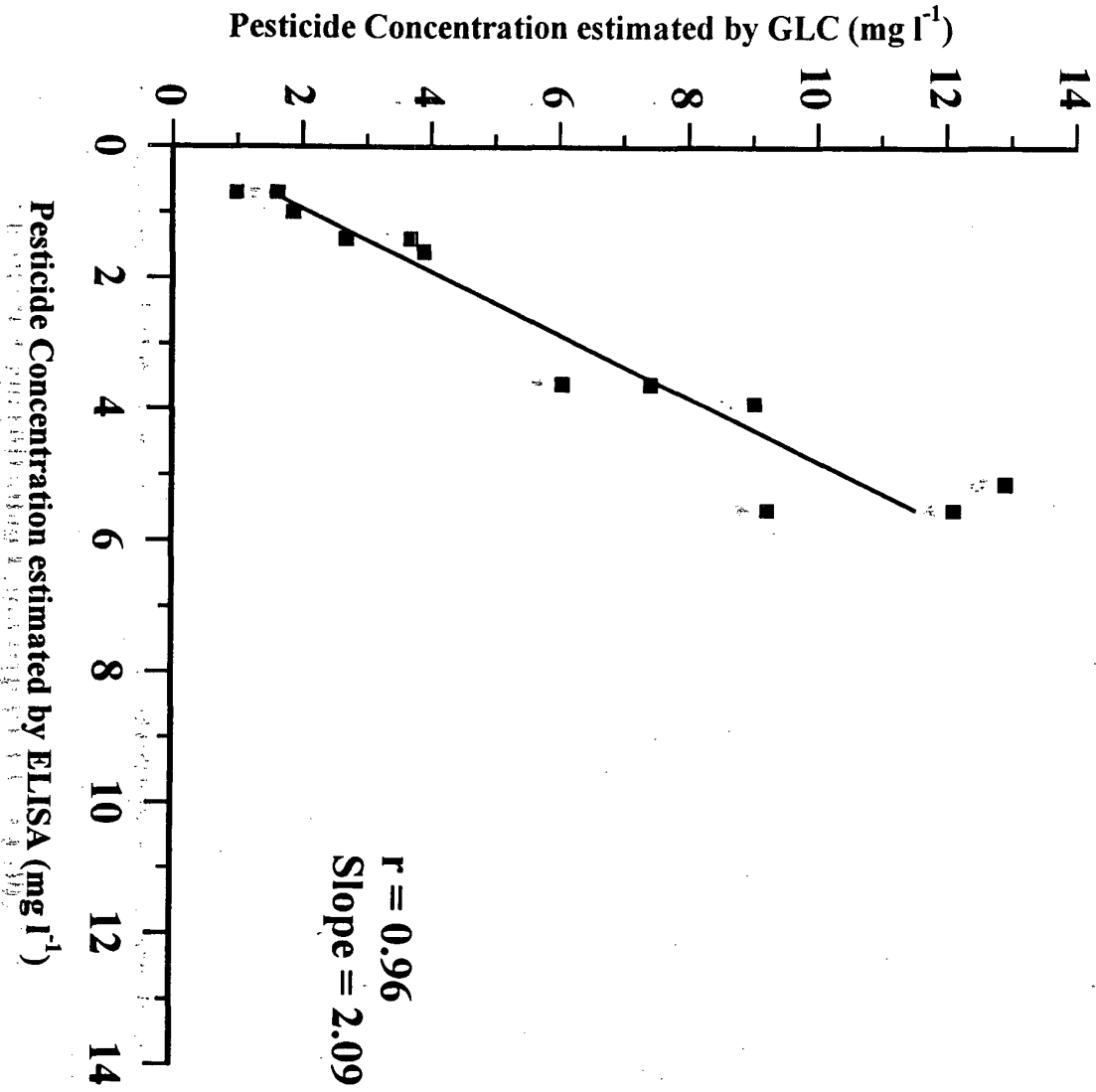
GRAPH 9: Plot Showing the Effect of a Grain Matrix on the Chlorpyrifos-methyl Plate Kit and How a Blank Containing Matrix Partially Corrects the Effect

% B/Bo

Standard Pesticide Solutions
Using a Methanol Blank

Pesticide Spiked Grain Extract
Using a Blank Containing Matrix

Pesticide Spiked Grain
Extract Using a Methanol Blank

Pesticide Concentration (mg l^{-1})
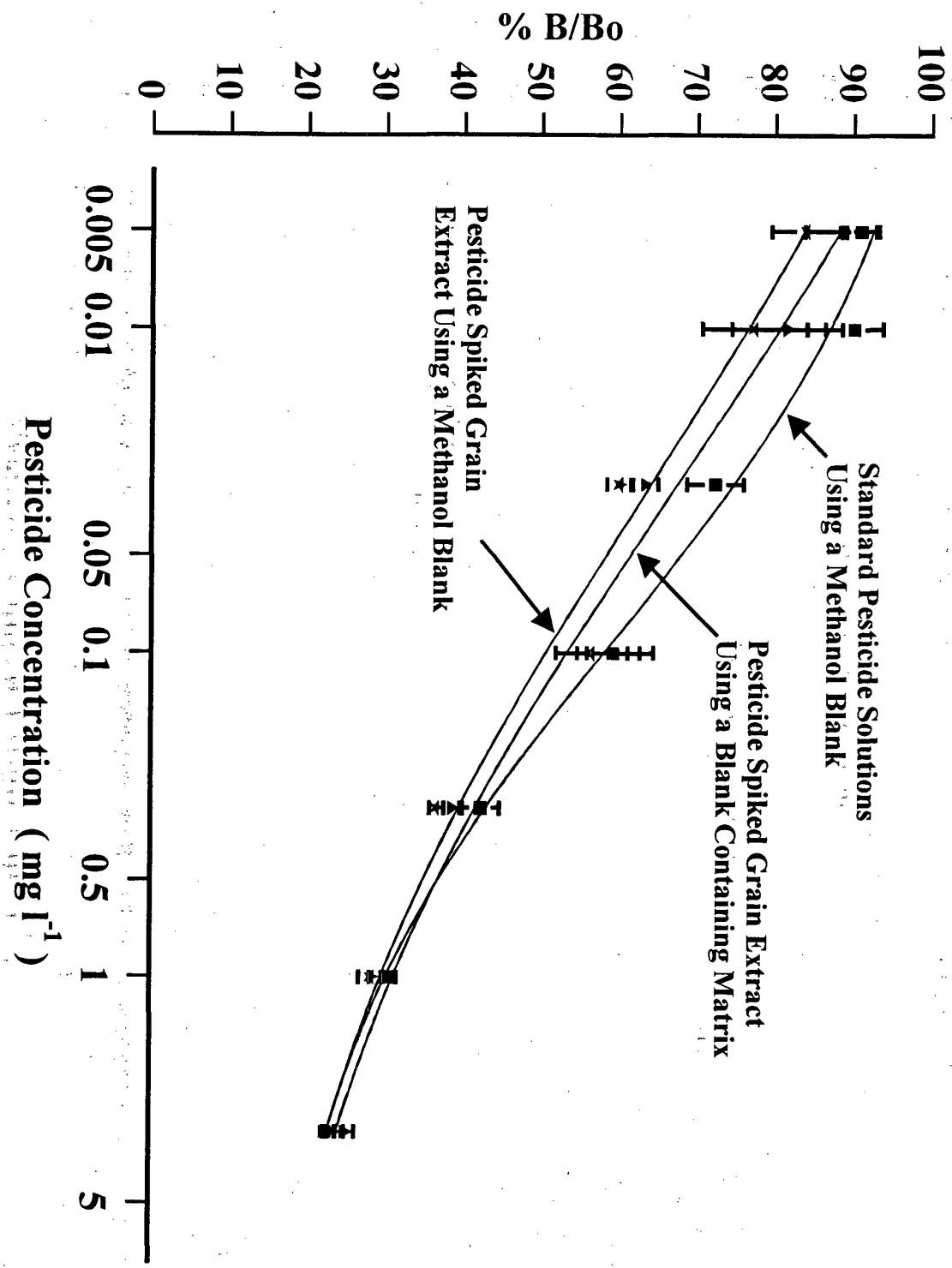
GRAPH 10: Standard Curves For Chlorpyrifos-methyl, Chlorpyrifos-ethyl and 3,5,6 Trichloro-2-pyridinol Produced with the Chlorpyrifos-methyl Plate Kit
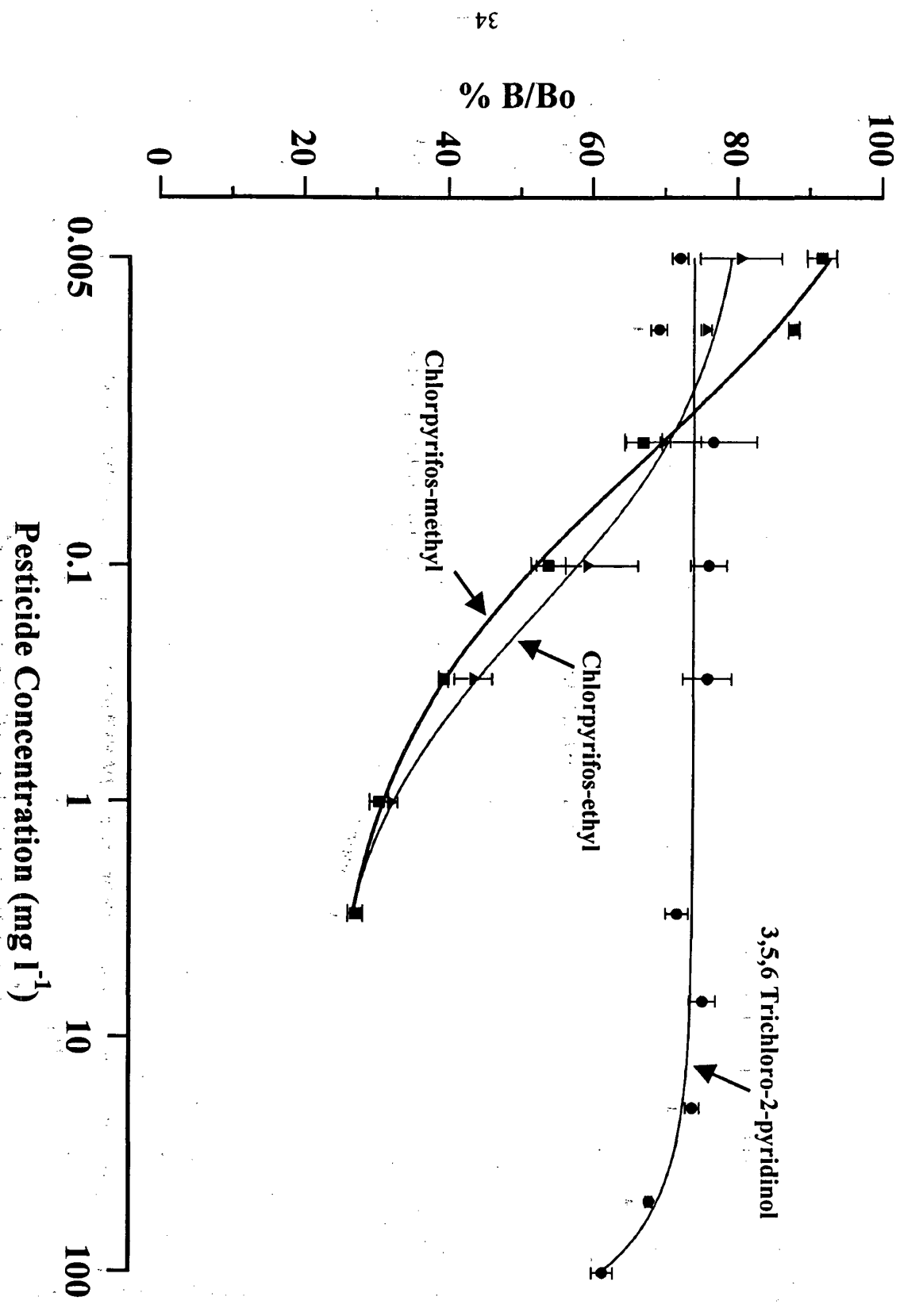
GRAPH 11: Comparison of Chlorpyrifos-methyl Concentrations Estimated By Plate ELISA and GLC in Extracts of Freshly Treated Grain
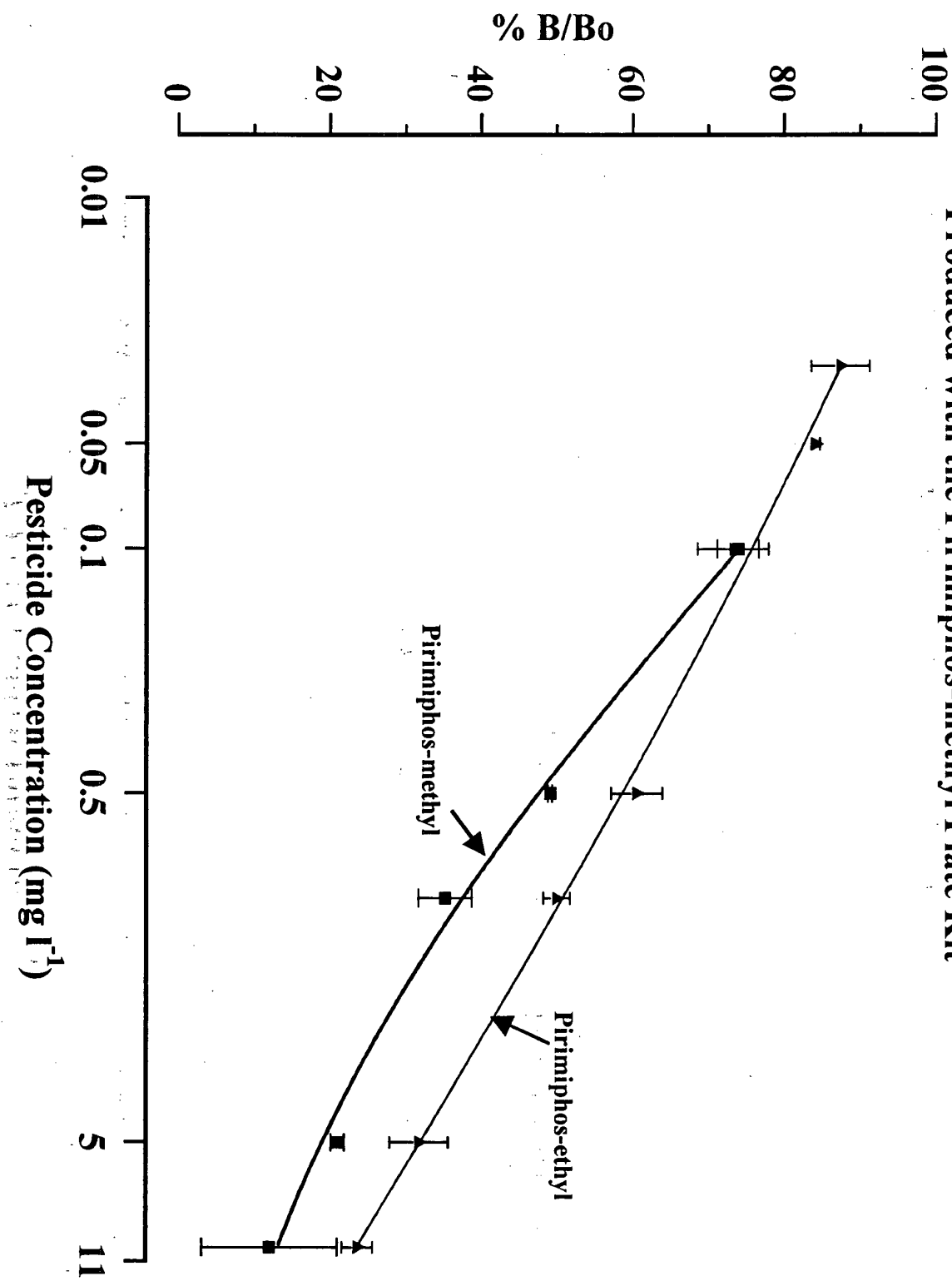
r = 0.94
Slope = 1.13

GRAPH 12: Comparision of Chlorpyrifos-methyl Concentrations Estimated By Plate ELISA and GLC in Extracts of Stored Treated Grain

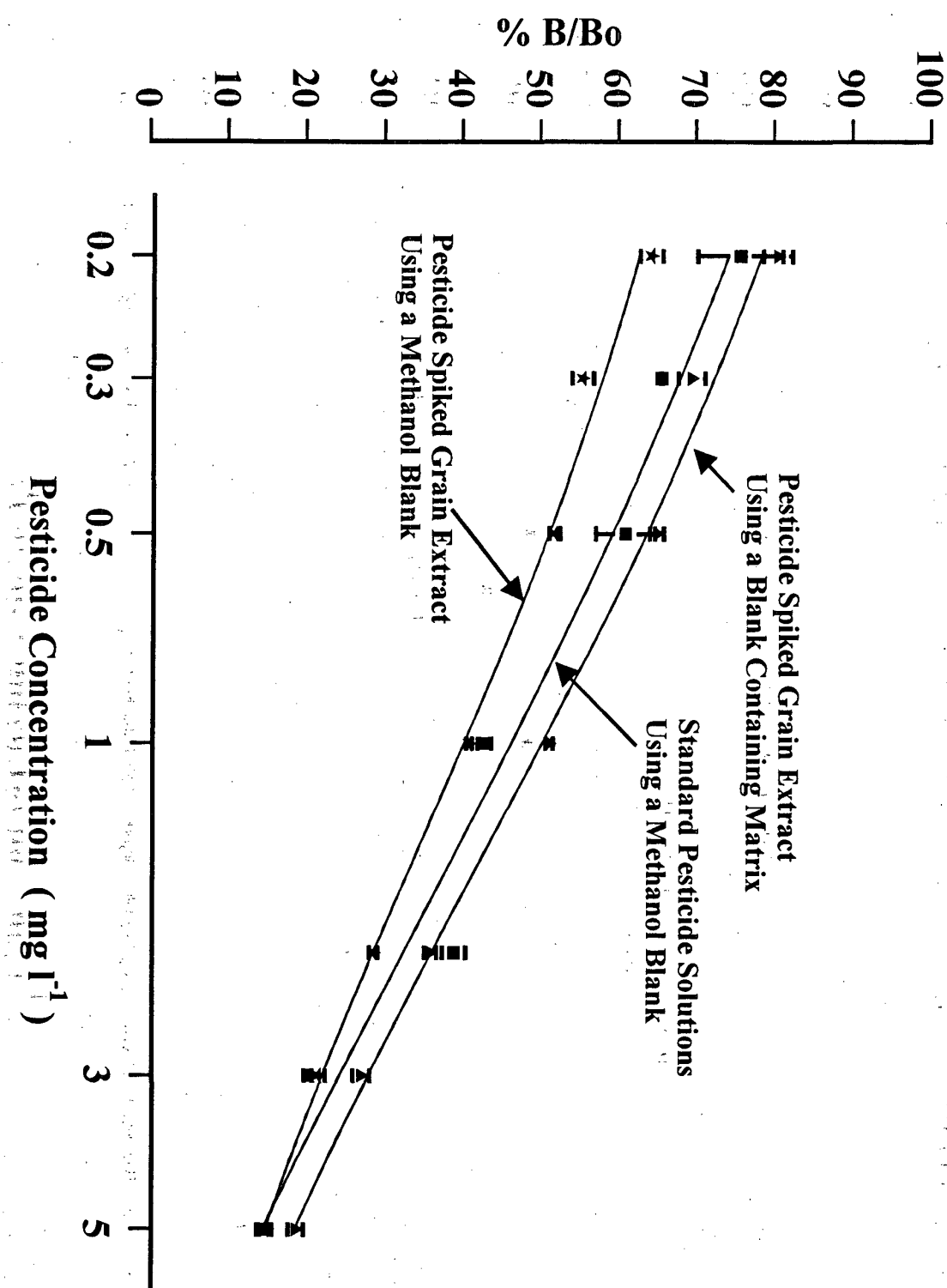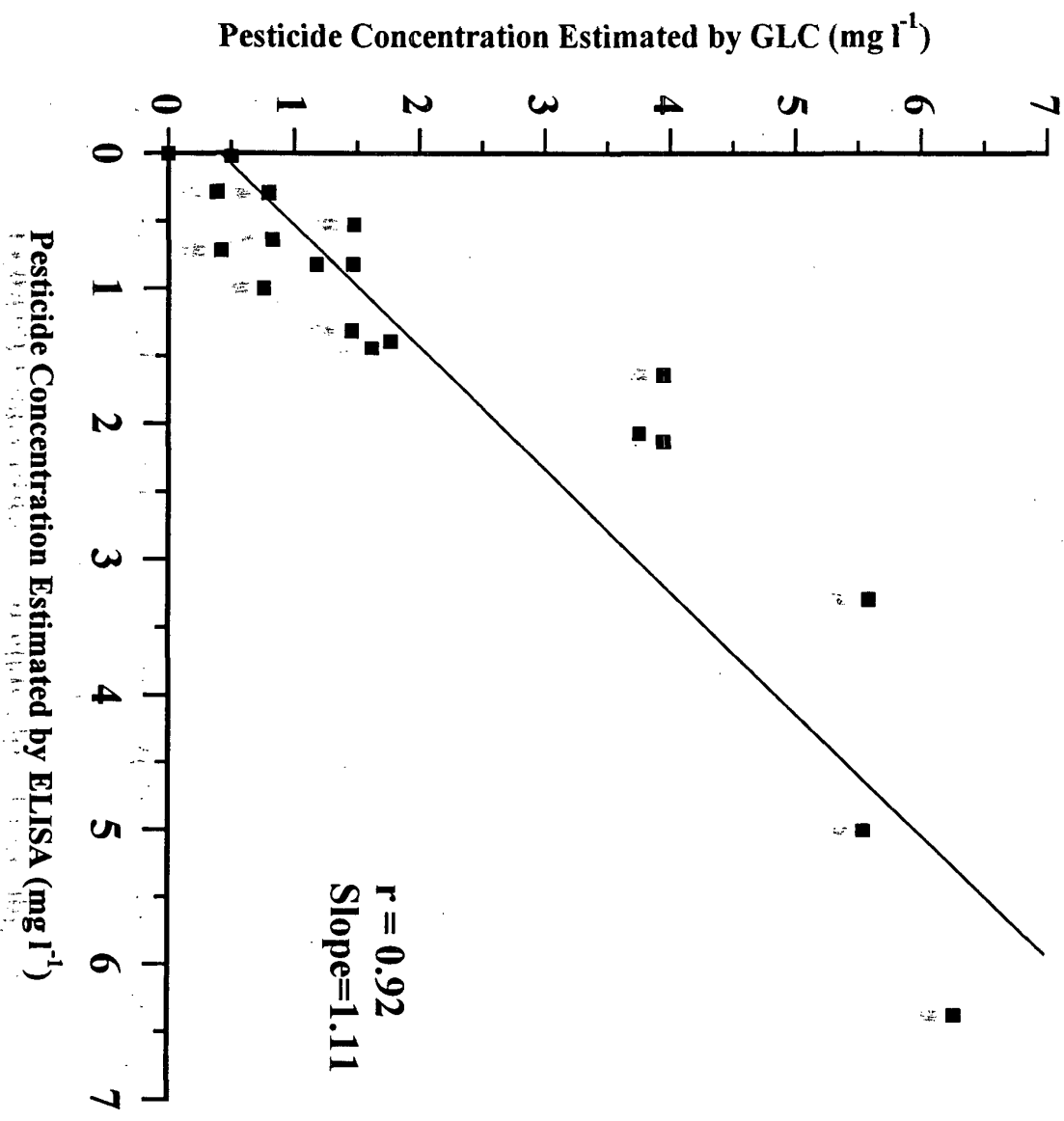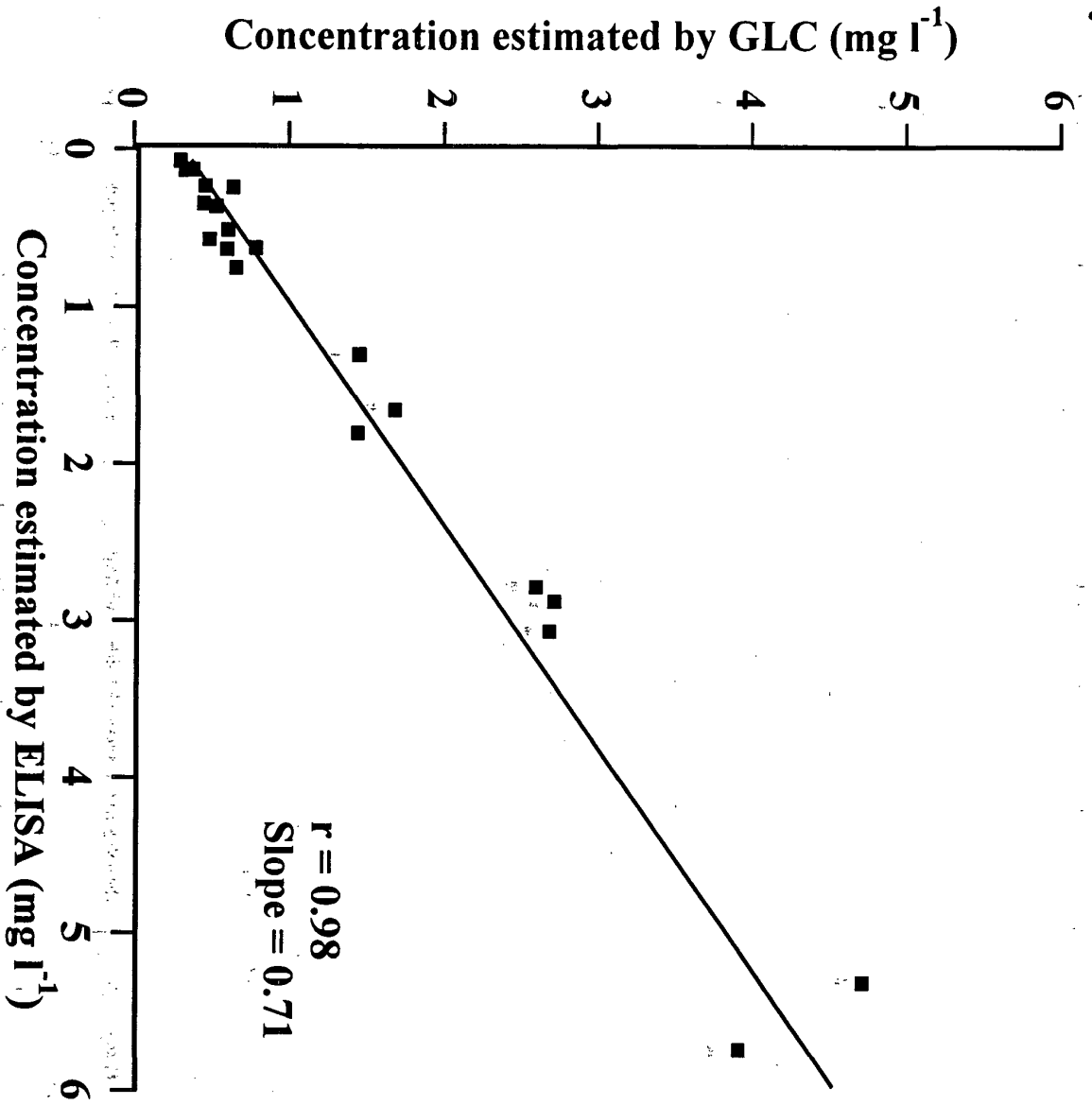GRAPH 13: Standard Curve for the Pirimiphos-methyl Tube kit
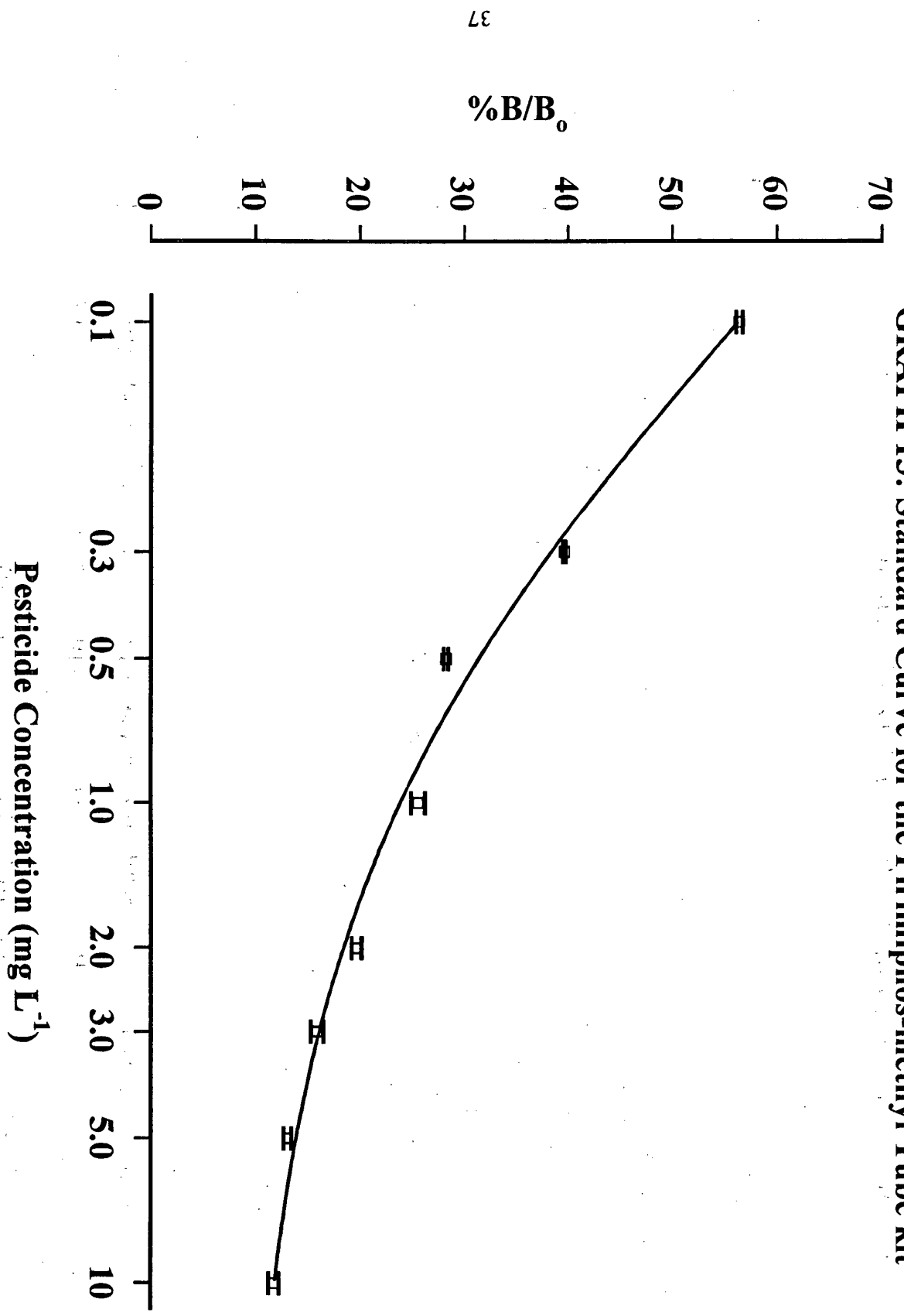
GRAPH 14: Standard Curve for the Chlorpyrifos-methyl Tube Kit
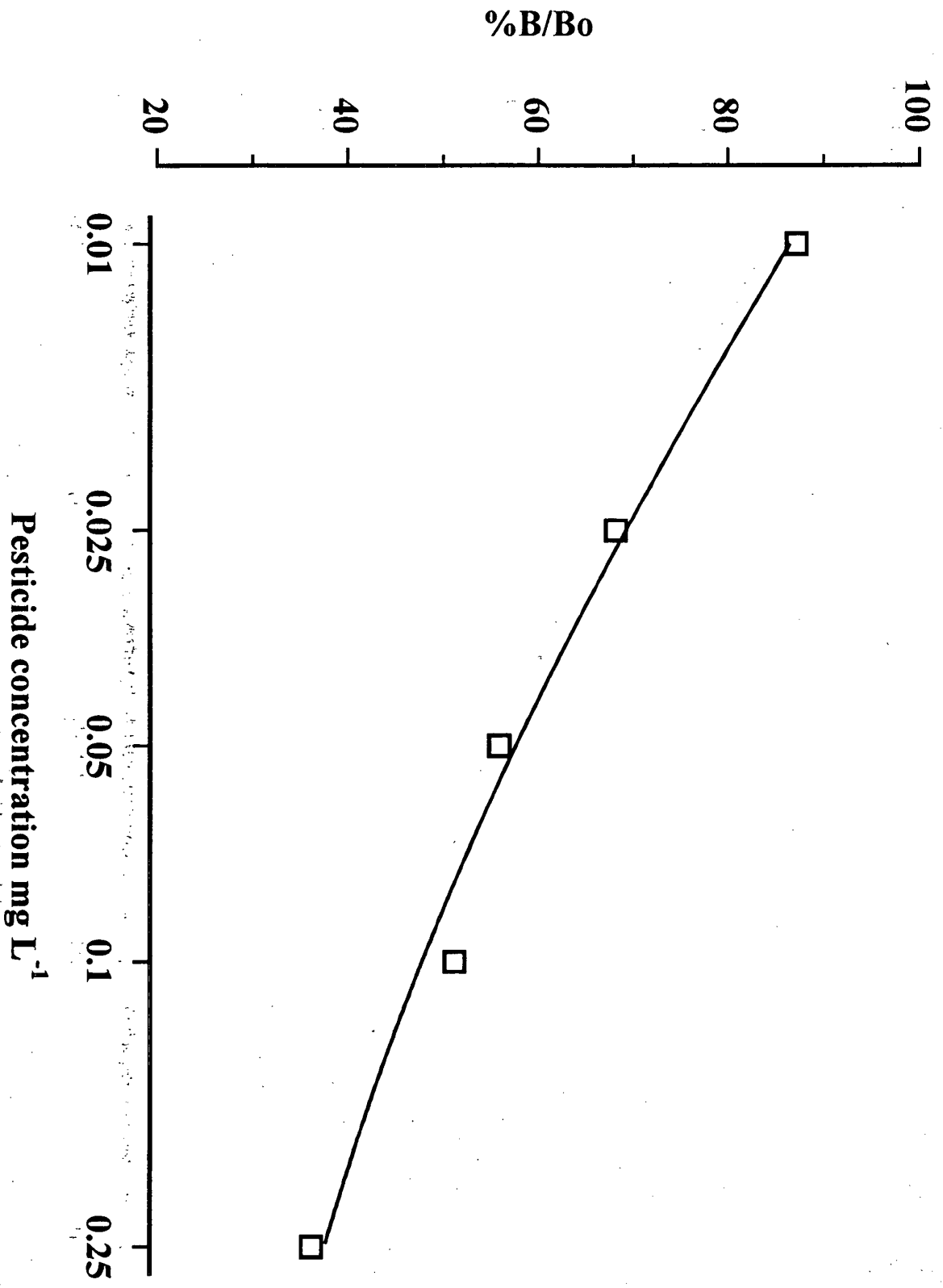
GRAPH 15: Pirimiphos-Methyl Tube ELISA Kit
Field Test 1

%B/Bo

Pesticide Concentration (mg l⁻¹)

r=0.99

Calibration standards + barley matrix
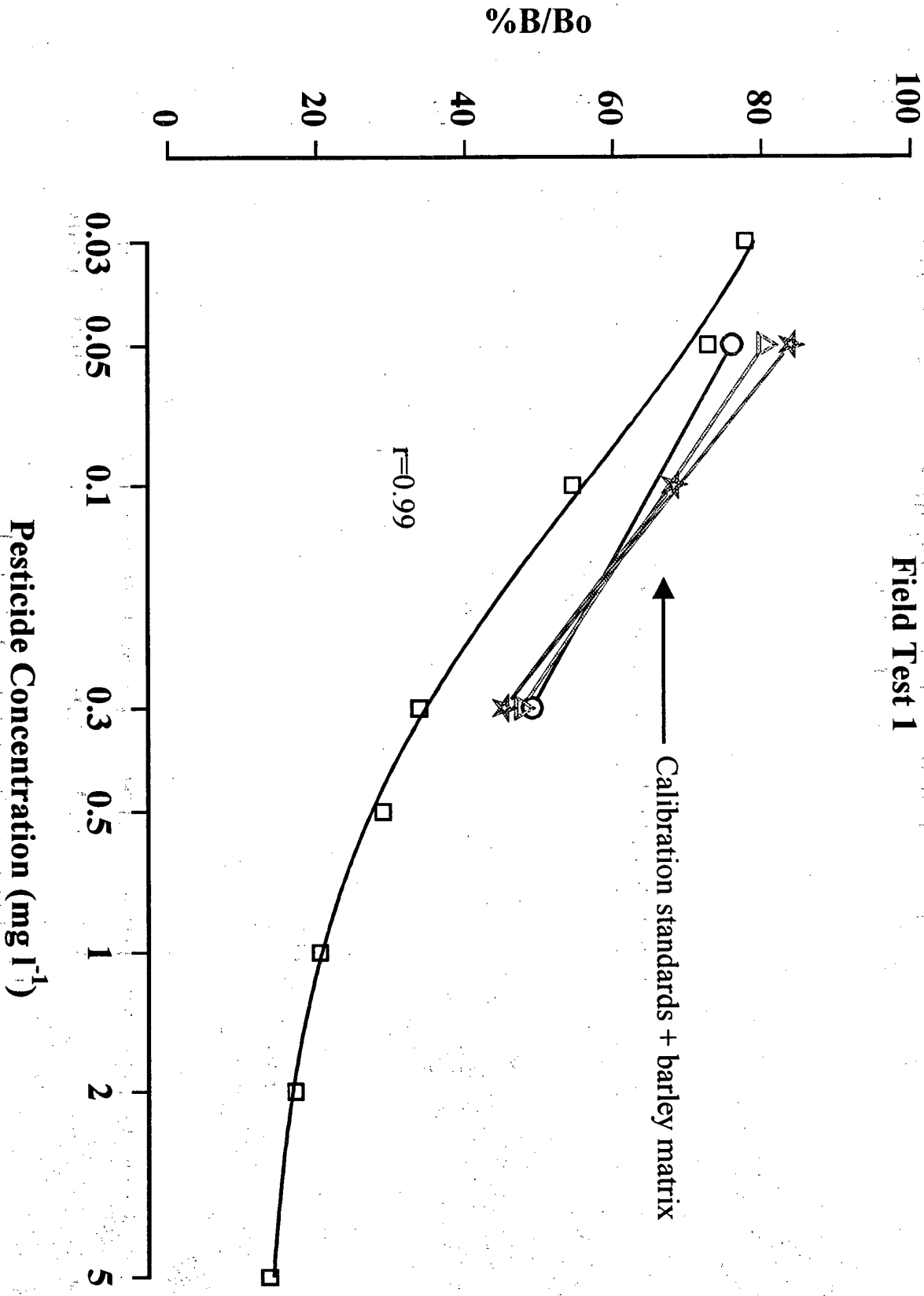
GRAPH 16: Chlorpyrifos-methyl tube ELISA kit
Field test 2

□ Standard dilutions in wheat matrix
Error bars for $S.D_{(n-1)}$ n=5
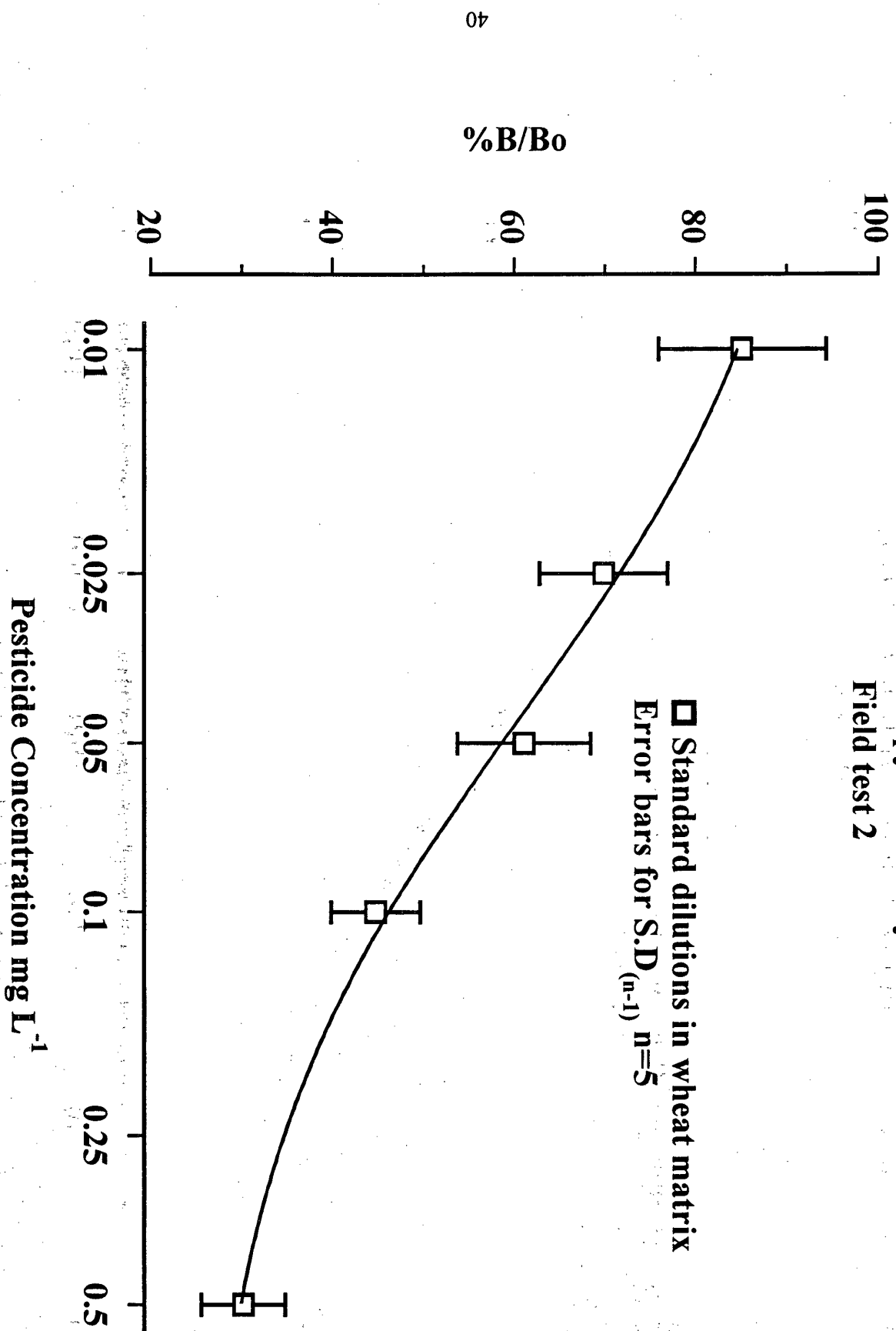
Appendix

User Protocol for the Determination of Pirimiphos-methyl, Chlorpyrifos-methyl, or Fenitrothion on Grain using plate and tube ELISA kits.

User Protocol for the Determination of Pirimiphos-methyl, Chlorpyrifos-methyl, or Fenitrothion on Grain using plate and tube ELISA kits.

Assay Principles

Immunoassays exploit the specific binding of an antigen (the analyte) to an antibody. Antibodies are selective to a single compound so one kit only determines one pesticide residue level. These antibodies are adsorbed onto the surface of the wells of a microtitre plate. During an competitive assay, labelled (the enzyme conjugate) and un-labelled antigen (blank, standard or sample) compete for binding sites on the antibody molecules. After a period of incubation, equilibrium is reached between the bound and unbound molecules. The unbound molecules are then washed off the plate, a proportion of the molecules which are bound to the plate will be labelled with enzyme, the remaining available binding sites will be occupied by the antigen. A substrate/chromogen mixture is then added which is converted to a blue product in the presence of the enzyme. Therefore amount of colour produced is inversely proportional to the concentration of the antigen in the sample. After an incubation (30 minutes) the enzyme reaction is halted by the addition of acid, this also changes the colour to yellow. The colour of the wells is measured using a microplate reader (set at λ-450nm). In order to overcome run to run variations (such as temperature changes) the sample/standard response is expressed as a proportion of the blank response, reported as a percentage (%B/Bo values) using:

$$\%B/Bo = \frac{\text{Sample Absorbance}}{\text{Blank Absorbance}} \quad x \quad 100$$

and these values are plotted against pesticide concentration on a logarithmic scale.

Precautions

- All reagents must be allowed to reach room temperature before commencing the assay.

- Pipette tips should be changed between different liquid handling stages to avoid cross-contamination.

- If the microtitre plate is in a strip format these should be labelled as they can fall out of their carrier during the wash steps of the assay.

- Initially it is important to use 5 to 7 standards to assess the range that the kit is required to operate over to ensure that the response is linear - do not use the kits outside their linear range.

Grain samples are extracted in solvent and analysed using either plate or tube format kits (Steps 1, 2a, 2b & 3)

Step 1: Extraction of Grain Using the Rapid Extraction Method

Weigh out **10g grain samples** into 100 ml screw-topped reagent jars.

↓

Add 10 ml of methanol

↓

Swirl for 10 seconds

↓

Place in a bath and sonicate for 10 minutes

↓

Decant all the liquid from the jar into a 10 ml volumetric flask

↓

Make the volume of liquid in the volumetric flask up to the line

↓

Extracts for use with plate or tube ELISA test kits
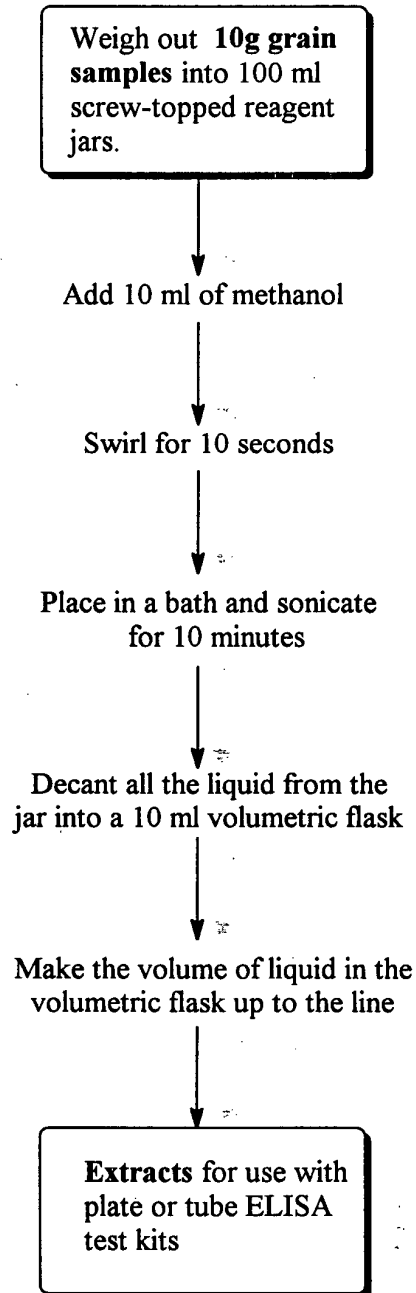
Step 2a: Experimental Steps When Performing a Plate ELISA

Sample extract or Standard in blank sample matrix

Add 100 µl

to 900 µl of methanol in a small test tube and mix.

Add 100 µl

to 900 µl of prepared *diluent buffer* in a small test tube and mix

Add 100 µl

to three wells in the microtitre strip

Add 800 µl of diluent buffer concentrate to 3200 µl of purified water for each microtitre strip to be used (4 blanks/standards/samples)

Repeat for a blank and each standard/ sample extract

Add 16 µl of enzyme conjugate stock solution to 1600 µl of conjugate diluent buffer for each microtitre strip to be used.

Add 100 µl of *enzyme conjugate* to each used microtitre strip well

Mix and cover with laboratory sealing film

WAIT: 1 hour

Uncover microtitre strips and shake out the contents of the wells into a sink

Repeat four times

Fill each well to overflowing with buffer

Pre-mix 1600 µl of substrate and 800 µl of chromogen for each microtitre strip to be used.

Add 150 µl of prepared *substrate/ chromogen* to each well

Mix and cover with laboratory sealing film

WAIT: 30 minutes

Add 50 µl of stop solution to each well

Read on a microplate reader at 450 nm

If blue colour fails to develop in the blank.

Discard used plates

Step 2b: Experimental Steps For Performing a Tube ELISA

Sample extract or Standard in blank sample matrix

Add | 10 µl (20 µl for the chlorpyrifos -methyl kit)

Repeat for a blank and each standard/ sample extract (Do not analyse more than 6 tubes at once)

to 1000 µl of *diluent buffer* in a diluent tube and mix.

Add | 500 µl

to each antibody coated tube

Add 500 µl of enzyme conjugate to each tube and swirl for 5 seconds

WAIT: 10 minutes

Shake out the contents of the tubes into a sink

Repeat four times

Fill the tubes with water

Add 500 µl of substrate to each tube

WAIT: 5 minutes

Add 500 µl of stop solution to each well

If blue colour fails to develop in the blank.

Read using a tubes at 450 nm

Discard used tubes

Step 3: Processing the Data

Absorbance values for the **standards**		Absorbance values for the **extracts**

Divide by absorbance values for the **blank**

Divide by absorbance values for the **blank**

Multiply by 100

Multiply by 100

%B/Bo values		%B/Bo values

Plot these values against the pesticide concentration on a logarithmic scale

Read these values off the graph to find the pesticide residue concentration of the extract

Pesticide Residue level (mg/kg)
provided the extract was not diluted in order to use the linear range of the kit