



☒ Description : paged ..1.... to paged ..21..
☒ Claim(s) : paged ..22.. to paged ..24..
☒ Drawing(s) : paged ..119.. to paged ..919..
☒ Abstract : paged ^{one page}..... to paged

ASSAY METHOD**FIELD OF THE INVENTION**

The invention relates to a method for assaying enzyme modulators, particularly inhibitors, using immobilized enzymes.

5

BACKGROUND OF THE INVENTION

Intensive farming leads to the frequent use of pesticides, such as insecticides. It is therefore extremely important that pesticide residues be monitored closely to ensure that every vegetable consignment is fit for consumption. This problem is prevalent particularly in South East Asia where methamidophos (meth) is one of the most frequently used pesticides.

10

Gas chromatography is a sensitive and reliable method for pesticide residue detection. However, the instrument is expensive, and requires highly skilled personnel and sophisticated laboratories for its effective operation. It uses expensive reagents, and the organic solvents used are often toxic, resulting in additional expenses in waste disposal. There is therefore the need to design cheaper methods that can handle large numbers of samples in a short period of time. Ideally, the method should be fast, simple and robust, and can be used in whole sale markets with simple laboratory equipment for mass screening.

15

20

25

Enzyme immobilization onto various matrices using various cross-linkers is well-known. In general, enzymes may be immobilized onto gels, membranes, electrode surfaces, magnetic particles, nylon net and glass beads.

30

For example, Marty et al describe in Electroanalysis, 4(1992)



249-252, a biosensor for the detection of organophosphate and carbamate insecticides. This article primarily relates to the immobilization of choline oxidase and acetylcholinesterase on cellulose nitrate membranes so as to determine by amperometry inhibition by paraoxon. This approach is described in further detail in Biosensors and Bioelectronics 8(1993) 273-280 in relation to other organophosphorous and carbamate pesticides.

Shekhovtsova and Chernetskaya report in Analytical Letters 27(15) (1994), 2883 to 2898 a method for determining mercury using "immobilized horse radish peroxidase". These authors describe a complexation or "immobilization" of horse radish peroxidase with chitozan. Detection of enzyme activity appears to be performed after the chitozan is dissolved into solution in a microtiter plate or on paper.

Rogers et al report in Fundamental and Applied Toxicology, 16:810 (1991) a flow cell method for determining the effect of insecticides on acetylcholinesterase. A biosensor is reported in which acetylcholinesterase is immobilized on a quartz fibre within the flow cell, to which is tagged a fluorescein isothiocyanate (FITC). Inhibition studies were performed in which the quartz fibre operates as a fibre-optic evanescent fluorosensor integral with the flow cell. The fluorophore emission from the FITC is detected by the fluorosensor. Methodology of this type involves expensive materials and specialized equipment.

A sophisticated approach is also taken by Kindervarten et al in Analytica Chimica Acta 234(1990) 113-117 and by Gunther and Biliteswki in Analytica Chimica Acta 300(1995) 117-125. In these



papers the authors describe a method for assaying various pesticides using a magnetic reactor in a stopped flow apparatus. Acetylcholinesterase was bound to magnetic particles positioned in a magnetic reactor in which the enzyme reaction is performed.

5 After a fixed incubation period, the reaction mixture is passed into a detector for photometric or electrochemical detection.

A difficulty with this methodology is that small fluctuations in the assay conditions give rise to fluctuations in the results obtained. In order to overcome these difficulties, it is

10 necessary to automate the flow injection system using computer-controlled pumps and injection valves. Equipment of the type necessary to make these prior art methods sufficiently reliable can therefore be costly or not easily portable.

15 SUMMARY OF THE INVENTION

The present invention seeks to overcome the disadvantages of the prior art by providing a simpler method for assaying an enzyme modulator. The method comprises:

(a) immobilizing an enzyme on an interior wall of a reaction
20 vessel to form an immobilized enzyme;

(b) conducting in the reaction vessel in the presence of the immobilized enzyme a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel;

25 (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modulator so as to form an immobilized modulated enzyme;

(d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining
30 spectroscopically the initial rate of the modulated reaction (V_1) in the reaction vessel; and



(e) comparing V_1 with V_0 to determine the effect of the enzyme modulator.

By conducting each reaction in the same reaction vessel, each vessel therefore serves as its own control, effectively eliminating variability from one reaction vessel to the next. Because the initial rate of reaction is determined, the enzyme reaction is not required to be stopped at a certain end point and no stopping solution is needed. As a result, the enzyme is not destroyed and the same well can be used for the modulator assay. Moreover, if the modulator is found to be absent, the well can actually be recycled for another round of detection with another sample.

On a general level, the interior of the reaction vessel may be made from any material which does not interfere with the reaction and which is capable of acting as a substrate for immobilizing enzymes. Suitable materials include plastics such as polystyrene, or glass. Microtitre wells, particularly of plastics such as polystyrene, are especially useful as reaction vessels in the present invention. Microtitre wells are conveniently supplied in microtitre plates such as those commonly used for ELISA assays having an 8x12 format. As described in further detail below, a microtitre plate reader can be used in measurements on the microtitre plates so as to measure the initial rate of reaction in a large number of samples.

Cuvettes made of glass or plastic are also useful reaction vessels. These are convenient because they are portable and because a simple device can be constructed from them using a filter and light-emitting diode in combination with a photodiode



for the purpose of monitoring the desired wavelength.

Various products are commercially available for the purpose of immobilizing enzymes, such as Hybond-N+ membrane from Amersham, Cellulose acetate membrane from Sartorius, or Nylon mesh from Cole-Palmer. Typically, cross-linkers are employed to couple the enzymes to the interior wall. For example, nylon membranes can be used in conjunction with glutaraldehyde as the cross-linker. Alternatively, photo-cross-linkable polymers such as polymers of polyvinyl alcohol bearing styrylpyridinium groups can be cross-linked to entrap the enzyme. The latter photo-cross-linkable polymers are available from Toyo Gosei Co., Tokyo, Japan. It is also possible to immobilize enzymes onto quartz, although this can be expensive.

The enzyme modulator may be any compound or molecule which alters the activity of the enzyme. Thus, the modulator may be an inhibitor or, alternatively, a potentiator of enzyme activity. The modulator may act on the enzyme either reversibly or irreversibly and the method according to the present invention can be tailored to assay the action of the modulator as described in further detail below. An important class of modulators are the pesticides, such as organophosphorous or carbamate pesticides, including methamidophos, triazophos, parathion, chlorpyrifos, profenofos, prothiophos and carbofuran. Proteins can also act as modulators. In the case of protease enzymes such as trypsin, irreversible inhibition of the enzyme can take place by proteolytic degradation. Another modulator is phosphate, which is a competitive inhibitor of alkaline phosphatase. Further examples of modulators are heavy metal ions and other ions and salts.



A broad range of enzymes is useable in the present invention and includes any biomolecules which catalyse biological or chemical reactions, particularly proteins. The reactions in question can be any reactions which are capable of being assayed directly or indirectly, including redox reactions. It is therefore possible to use as the enzyme in the present invention all types of enzymes including electron transport proteins. The enzyme activities may be assayed directly by measuring the disappearance of reactant or the appearance of product or they may be measured indirectly by the use of a secondary reaction linked to the disappearance of reactant or appearance of product from the first reaction. A good example of the enzyme and its assay is acetylcholinesterase, which catalyses the production of thiocholine from acetylthiocholine iodide. This reaction is described in further detail below. The enzyme is assayed by making use of the secondary reaction of thiocholine with 5,5"-dithio-bis-2-nitrobenzoic acid (DTNB) to form a product which can be monitored photometrically at 405nm. Acetylcholinesterase activity is found to be inhibited in the presence of various important pesticides. As a further example, horse radish peroxidase can be inhibited by mercury or other heavy metal ions.

The initial rates of reaction in the absence and presence of the modulator are preferably determined by measuring the progress of each reaction at a plurality of time points or continuously with time. Provided that the measurements are made in the linear portion of the kinetic reaction as is observed, for example, in the presence of excess substrate, the initial rate of reaction is simply the gradient of a plot of the appearance of product (disappearance of substrate) against time. It is advantageous to determine the initial rate in this manner because the accuracy



of the determination of the rate is unaffected by the starting point of the reaction.

The initial rates of reaction may be determined by any convenient method of spectroscopy. Photometry, fluorometry and luminescence are preferred on account of their ready application to commonly-used reaction vessels such as microtitre wells, plates and cuvettes. Enzyme assay systems can be chosen, as discussed above, so that the appearance of product or disappearance of substrate is measured by one of these techniques. For example, the appearance of TNB may be monitored photometrically at 405nm. Advantageously, the initial rates of reaction are determined spectroscopically using detection means remote of the reaction vessel. In this way, there is no need for detection means to be used which is dedicated to the reaction vessel. This enables the assay to be performed in commonly-available reaction vessels such as cuvette or microtitre wells in conjunction with pre-existing standard detection means such as a spectrophotometer or spectrofluorimeter, for example in conjunction with a standard microtitre plate reader.

The assay method may be conducted discontinuously involving standard apparatus of the type described. For example, each of steps (b), (c) and (d) may be conducted by pipetting appropriate solutions into the reaction vessel. This has the advantage of simplicity in that no special apparatus needs to be used for performance of the assay.

One or more washing steps may be incorporated into the method, depending on the type of modulator being assayed. Advantageously, between steps (b) and (c) a further step of



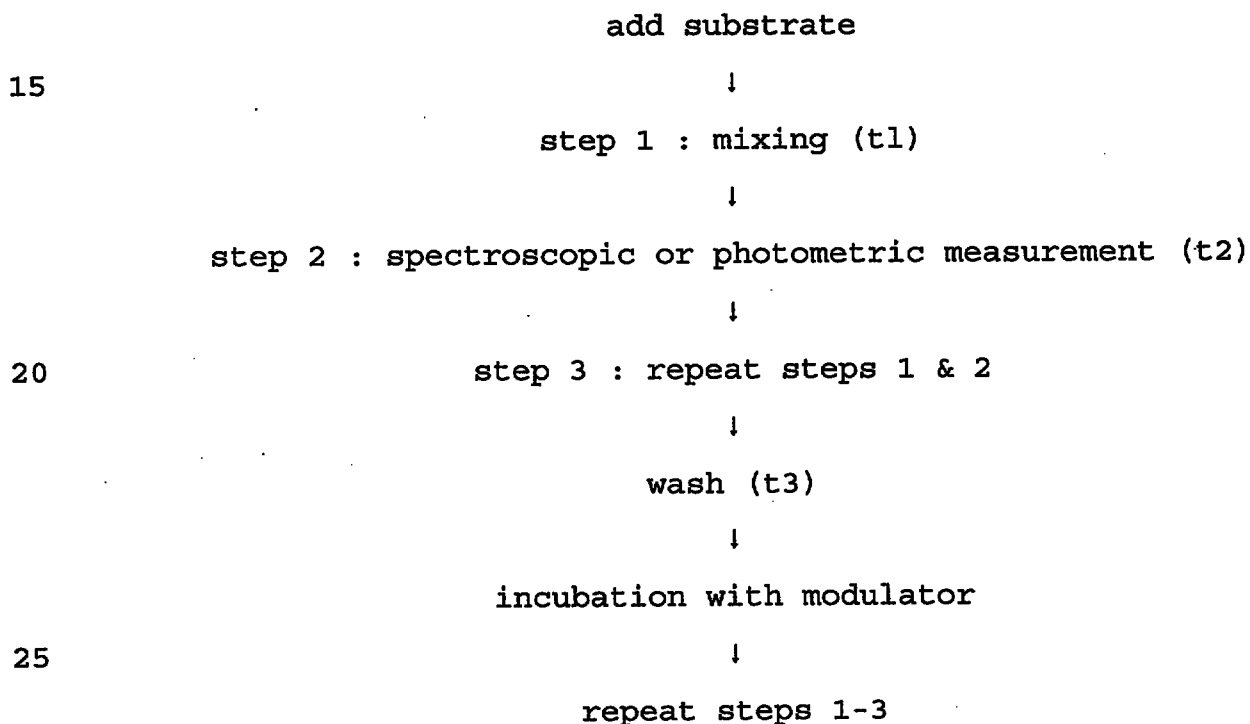
washing the immobilized enzyme may be performed so as to remove any reaction mixture from the reaction vessel prior to incubation of the enzyme modulator with the immobilized enzyme. Where the enzyme modulator acts irreversibly to form the immobilized enzyme-modulator complex, after incubation with the enzyme modulator, a further washing step may be performed to remove from the reaction vessel all of the test sample. This has the advantage that substances in the test sample which might interfere with the assay are washed away. Because the immobilized enzyme-modulator complex is formed irreversibly, little or no bound modulator would be expected to be washed away with the sample.

Where the action of the modulator is reversible, the second wash step is omitted so that the modulated enzyme activity assay is performed in the presence of the modulator. Although the presence of the test sample may interfere with the enzyme assay, this generally results only in an increase in the base line of the assay (absolute absorbance in photometry) but does not affect the slope of the reaction with respect to time. Thus, the initial rate of reaction is still readily determinable.

The temperature at which the reaction is conducted will depend, to some extent, upon the organism from which the enzyme is purified. For many enzymes it is possible to conduct the reaction at ambient outdoor temperatures from near freezing to as high as 45°C, typically in the temperature range 10 to 40°C. However, for enzymes purified from thermophilic organisms, it is possible to conduct the reaction at more extreme temperatures of up to, say, 95°C.



For a typical assay there are three different sets of timings to consider; measurement time, mixing time and washing time. Additionally, there is the length of incubation time of the enzyme with the modulator. Referring to the flow diagram below, in the case of a microtitre assay, step (1) has a mixing step to allow efficient mass transfer of the substrate to the immobilized enzyme and this is followed by an almost instantaneous spectroscopic or photometric measurement in step (2). This would yield the first point of a curve from which the rate of reaction will subsequently be generated. This cycle would be repeated at least one more time to generate at least two points for the slope determination:



The total time necessary for one assay with n number of points for determining the slope of the reaction rate would be $t_t = n(t_1 + t_2)$. Typically, n can be as little as 2 or as much as 10 or more, preferably 3 or 4. If the average rate is calculated by



a microtitre plate reader or spectrophotometer automatically, it would be necessary to ensure that t_c is within the linear range of the enzyme reaction. t_2 will depend on the type of spectroscopic measurement and the instrument and its configuration. Typically, t_2 is around five seconds. t_1 is typically one minute and so t_c is around 260s where $n = 4$.

Generally, a minimum of two readings must be taken to establish the slope, preferably three or more to obtain an accurate linear slope. Where two readings are taken, for an average reaction, one would expect t_c to fall in the range 0.5 to 5 minutes. For fast reactions this range could be five to thirty seconds whereas for slow reactions the range could be two minutes to several hours.

The incubation time of the modulator for irreversible inhibitors such as organophosphates falls generally in the range ten to twenty minutes although its sensitivity can be increased by lengthening the incubation time to one hour or more. Where the modulator is trypsin, the incubation time can be two minutes to overnight, depending on the sensitivity required.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described in further detail, by way of example only, with reference to accompanying drawings in which:

FIGURE 1 shows the activity of acetylcholinesterase under standard conditions over 567 seconds;

FIGURE 2 shows replicate assays of acetylcholinesterase activity.



performed in the same microtitre well;

FIGURE 3 shows the effect of sweet potato homogenate on acetylcholinesterase activity;

FIGURE 4 shows the effects of sweet potato, chilli and chinese lettuce homogenate on acetylcholinesterase activity;

FIGURE 5 shows inhibition curves of meth in vegetable samples on acetylcholinesterase activity;

FIGURE 6 shows the basic steps of a standard irreversible modulator assay;

FIGURE 7 shows the basic steps of a reversible modulator assay;

FIGURE 8 shows a plan of the mode of analysis for a semi-quantitative assay using a microtitre plate; and

FIGURE 9 shows the effect of trypsin concentration on acetylcholinesterase activity.

DESCRIPTION OF THE EMBODIMENT OF THE INVENTION

Mode of Analysis

The assay can basically be operated in two modes:

1. Quantitative or semi-quantitative analysis of a modulator

The steps are shown in Figure 6, e.g. quantitation of the amount of pesticide present using immobilized acetylcholinesterase. In this instance, a standard curve must first be obtained using known amounts of the modulator. For a quantitative assay, the modulator standards are generally analysed together with the unknowns as in any other quantitative assay. The advantages of the assay system in this case are:

- (a) The assay can be performed in a 96-well microtiter plate. Therefore, even if one 8-well row is used as the standard, one can potentially analyze 88 other unknown samples.



(b) Each well serves as its own uninhibited control. An uninhibited control is performed in all the wells before the modulator is added. There is always going to be variability in the amount of enzyme present in each well. The present assay gives results of improved reproducibility because modulation detection in one well is not compared to an unmodulated control in another well, as would be the case in a conventional free enzyme assay. Rather, each well has its own V_0 value, representing the true amount of enzyme present in that test well. After incubation with the modulator, the V_1 detected is compared with the V_0 of the same well.

When only semi-quantitative analysis is required, then the positive control consists of a specific concentration of modulator to which the test samples would be compared. The scheme for a general semi-quantitative assay is shown in Figure 8.

2. **Screening for presence of modulating activity** This assay, when performed in a microtiter well, is extremely useful for screening simultaneously many compounds for their effects on enzymes. The screening for beta-lactamase inhibitors can be used as an example to illustrate this utility.

Beta-lactamase is a very important enzyme in clinical biochemistry because it is responsible for the hydrolysis of the beta-lactam ring in the penicillin family of antibiotics. Its activity can be monitored by a well-



characterised colorimetric assay using cephalosporin as substrate. In the present assay, beta-lactamase is immobilized onto the reaction vessel, such as a 96-well microtitre plate, and various compounds added to check for inhibition effects. These compounds may be newly purified or extracted natural or synthetic compounds. They may also be part of an existing repertoire of a pharmaceutical company which modulating activity on beta-lactamase is unknown. If irreversible inhibitors are being screened, then the assay steps would be as shown in Figure 6.

In the screening mode, the modulation of different compounds is being tested, so quantitation is not relevant. Obviously, a standard curve is not necessary in this case, and the only control needed would be the incubation with the extraction buffer in the absence of the compound to be screened.

In both modes of analysis, a reversible inhibitor can instead be screened if the assay is modified as shown in Figure 7. The second wash step is omitted, so that the modulated enzyme activity assay is performed in the presence of the modulator. This is possible even if the sample is coloured e.g. a herbal extract.

Detection principles

Many enzymes give a classic Michaelis type kinetic reaction. This means that in a relatively large volume, and at a defined substrate concentration where mixed order kinetics occur, the initial reaction rate V_0 would be constant over time. As the substrate is consumed, the concentration drops, and the enzyme



reaction rate begins to slow down.

The present assay exploits the linear portion of the kinetic reaction by taking two to several readings at fixed time intervals as the reaction proceeds in each well. In this way, the initial reaction rate V_0 is determined. The substrate is then washed away and the modulator added. After incubation, the modulator is washed away and the enzyme kinetic assay repeated. The reaction rate after modulation is denoted V_1 .

The effect of the modulator on the immobilized enzyme is determined using the simple general relationship :

$$\% \text{ modulated activity} = \frac{V_1}{V_0} \times 100 \% \quad \text{eqn. (1)}$$

If the modulator is an enhancer, then the amount of enhancement can be expressed as :

$$\% \text{ enhancement} = \frac{V_1 - V_0}{V_0} \times 100 \quad \text{eqn. (2)}$$

if the modulator is an inhibitor, then the percent residual activity can be expressed as in eqn. (1).

Example

Detection of methamidophos in vegetables

Fresh vegetables are consumed in large quantities in Asia. Intensive farming leads to the frequent use of insecticides. As such, it is extremely important that pesticide residues be monitored closely to ensure that every vegetable consignment is



fit for consumption. Methamidophos (meth) is one of the most frequently used pesticides in S.E. Asia. Therefore it has been chosen as the modulator (inhibitor) as an example of the applications of the present technique.

5

Acetylcholinesterase (AChE) is an enzyme found in nervous tissue that is inhibited by organophosphate and carbamate pesticides. Its activity can be monitored in the Ellman's assay using acetylthiocholine iodide (ATCI) and 5,5"-dithio-bis-2-nitrobenzoic acid (DTNB) in the following reactions where 5-thio-2-nitrobenzoate (TNB) can be monitored photometrically at 405nm:

10

AChE

ATCI + H₂O ----- > acetic acid + thiocholine

15

thiocholine +DTNB --- > 2-nitrobenzoate-5-mercaptothiocholine
+
TNB

20 In the present system, AChE is covalently immobilized onto microtiter plates activated with maleic anhydride. V₀ of its reaction was monitored by doing 3 absorbance readings with 30- or 60-second shaking intervals. V₀ is expressed as the change in absorbance at 405nm (A₄₀₅) with time or dA/dt. The Ellman's reagent was then rinsed off and the assay repeated with fresh
25 Ellman's reagent. The assay was performed for a total of three times. This serves as the unmodulated control. After rinsing off the Ellman's reagent the third time, methamidophos at various concentrations are added into their respective wells.

30

Materials and Methods



Acetylthiocholine Iodide (ACTI), 5'5-dithio-bis-2-nitrobenzoic acid, bovine serum albumin (BSA) and acetylcholine esterase (AChE) from electric eel purchased from Sigma Chemical Company. Reacti-Bind Maleic Anhydride Activated Polystyrene Plates from
5 Pierce (Reacti-Bind is a trade mark). Trizma base from Sigma. Methamidophos (analytical grade) was from Hoechst. Glycine from Merck.

Ellman's assay reaction mixture

10 This assay consisted of 1.5mM DTNB, 6mM ATCI in 0.01M Tris-HCl, pH 8.5 ATCI was prepared as a 10X stock in distilled water and added to the reaction mixture immediately before each set of assays.

15 Immobilization of AChE in Reacti-Bind Maleic Anhydride Activated Polystyrene Plates

All reagents, plate and samples were brought to room temperature before use. 100ul of 3U/ml AChE was introduced into each well of a Reacti-Bind plate. The plate was then incubated at 37°C
20 overnight for optimal binding. AChE was removed the next day and plate washed with 0.01% BSA + 0.05% Tween 20, pH 7.2 PBS ten times. The blocking buffer, containing 1M glycine + 0.05% Tween 20 in PBS, was delivered to the wells and incubated at 4°C for 3 hours. The plate was then washed with PBS Tween, pH 7.2 ten
25 times.

AChE activity assay

AChE activity was tested by introducing 200ul of Ellman's reaction mixture into each well using an 8-channel micropipetter.
30 The microtitre plate reader was programmed to shake for 10 seconds before an absorbance reading at 405 nm was taken. The

cycle was repeated 3 times so that a total of 4 readings were taken. The 4 readings can be plotted as one kinetic curve. An example is assay 1 in Figure 2. The rate of that reaction (V_0) was calculated from the slope of that kinetic curve.

5

To ensure the reproducibility of the assay, the activity test in each well was repeated three times i.e. four separate kinetic measurements were obtained. As a result, a total of 4 kinetic curves were obtained as assays 1 to 4 in Figure 2. The slopes
10 (V_0) of the 4 independent kinetic curves were calculated and shown in Table 1. The mean of the four V_0 is calculated as V_0 .

Homogenization of vegetable material

Vegetables were obtained from the Primary production Department
15 of Singapore. The plant materials were weighed and homogenized in equal volume of TB (1g of plant material was assumed to have a volume of 1 ml) in a Waring blender and filtered with filter paper. The pH of the filtrate was raised to 8.5 with sodium hydroxide and used for AChE incubation without further treatment.
20 For methamidophos inhibition of AChE, the appropriate amount of meth was spiked into the vegetable homogenate from a concentrated stock solution.

25 Inhibition of AChE by methamidophos

Three AChE activity assays were performed by adding 200ul of methamidophos of various concentrations into the different wells. The plate was then incubated on an orbital shaker at room temperature for 10 mins. After the incubation, the plate was
30 washed gently with Tris-HCl two times and AChE activity assay performed three times again. Residual activity of the enzyme





after incubation was then calculated using the equation below:

$$\frac{V_0}{V_1} \times 100 \%$$

5

Results and Discussion

Linearity range The assay system works under a substrate concentration in which the initial reaction rate (V_0) has a linear range of minutes. In this way, the absorbance readings of the many wells of a microtitre plate can be taken simultaneously, even though the substrate solution may be added sequentially using a multichannel or multiple dispensing pipetter.

10

15

A simple experiment was performed to determine the linear range of the immobilized AChE activity as shown in Figure 1. Results show that at the substrate concentration used in these experiments, the initial AChE reaction rate is constant over 10 minutes. Therefore, in all subsequent experiments, 2 or 3 absorbance readings obtained within this time frame was used to calculate V_0 .

20

Reproducibility Multiple Ellman's assays were performed in the same well to determine the reproducibility of the system. For each assay, three absorbance readings were taken to determine V_0 . Figure 2 shows 4 Ellman's assays performed on the same well. Although the absolute absorbance fluctuated, V_0 was very reproducible. The mean of four V_0 is calculated as V_0 and is shown under well 1 of Table 1. Results of assays performed on 8 other wells are also shown in Table 1. The standard deviations in all cases were below 3% of V_0 .

25

30



Table 1 Results of 4 Ellman's assays performed in each of 8 wells. Assay conditions were as described in Figure 2.

Well no	1	2	3	4	5	6	7	8
V_o (dA/min)	0.038	0.037	0.042	0.042	0.040	0.042	0.044	0.035
	0.038	0.037	0.042	0.040	0.040	0.041	0.045	0.036
	0.039	0.037	0.042	0.040	0.039	0.043	0.045	0.036
	0.039	0.038	0.043	0.041	0.041	0.043	0.045	0.037
Mean (V_o)	0.039	0.037	0.042	0.041	0.040	0.042	0.045	0.036
SD (% of V_o)	1.498	1.340	1.182	2.346	2.040	1.943	1.116	2.267

Effect of vegetable extract on AChE activity An important advantage of the assay system is that even complex extracts could be analyzed without extensive sample cleanup. Figure 3 shows the effect of an aqueous homogenate of sweet potato on immobilized AChE. Each data point is a mean of 3 Ellman's assays. V_o was $0.0414 \pm 1.30\%$. while V_i after 5 minutes incubation with the sweet potato extract was $0.0406 \pm 1.3\%$. V_i/V_o is 98%, showing that there is a minimal effect of 2%. Two other types of vegetables (red chili and Chinese lettuce) were also tested and their effect on AChE shown in Figure 4. Each bar represents the mean of the V_o of 8 separate wells. These results show that even when AChE was incubated with complex matrices such as vegetables that have simply been homogenized and filtered, good activity is recovered.

Effect of meth in vegetable extract on AChE activity When known amounts of meth were spiked into vegetable samples, inhibition was observed. Figure 5 shows the increasing inhibitory effects of increasing amounts of meth. Each data point represents the mean of 3 replicate wells. If 15% inhibition is taken as significant, the limit of sensitivity of this assay would be 8 and 13 mg/kg plant material for Chinese lettuce and Chilli



respectively.

Example 2

5 Detection of trypsin

All reagents and method for AChE activity assay are the same as in Example 1. The general procedure is shown in Figure 6. Ellman's reagent was used to obtain V_0 of AChE immobilized onto the walls of the microwells. The assay was repeated twice to get
10 three V_0 readings and V_0 obtained. After washing off the substrate, 0.2ml of PBS pH 7.4 containing 0 to 2 mg/ml trypsin were introduced into the wells in triplicates. AChE was incubated with the trypsin solution for 20 minutes at room temperature with shaking. After incubation, the trypsin was
15 washed off and the AChE activity assay repeated. The result is shown in Figure 9. PBS pH 7.4 shows no inhibitory effect on AChE after 20 minutes incubation. In the presence of 62 u/ml trypsin, AChE activity was reduced to 81.3%. Increasing amounts of trypsin causes increased AChE inactivation.

20

Conclusion

There are many advantages to the assay system

Superior reproducibility This assay relies on the slope of the enzyme kinetic reaction rather than an end point. As a result,
25 the starting time of the reaction, and the exact volume of substrate added in each well is not crucial, and multiple samples can be manipulated comfortably, and yet superior, reproducible results can be obtained.

30 **Little or no sample pretreatment required** The modulator can be in an extract that interferes with detection by the microtiter



plate reader. e.g. it has a deep colour. For an irreversible inhibitor the modulator extract is washed away before the enzyme reaction is monitored, so no interference would occur. As a result, sample cleanup can be eliminated. In the application
5 used here, the vegetable was simply homogenized in buffer and filtered through paper. No further clean up was required. For reversible inhibitors in which the sample has to be present together with the substrate in the microtitre well, there is still good tolerance for baseline interference from the sample.
10 As long as the test sample does not affect V_0 in the absence of the modulator, a shift in the baseline absorbance reading will result in a parallel line as in Figure 3. V_0 would not be affected unless the modulator is present.

15 **High sample throughput** Up to 95 samples can be tested in a very short time. In the case of meth detection, 95 samples can potentially be analyzed in less than 1 hour, since each of the 6 Ellman's assay takes approximately 5 minutes, and incubation with the vegetable extract takes 10 minutes.



CLAIMS:

1 1. A method for assaying an enzyme modulator, which method
2 comprises:

3 (a) immobilizing an enzyme on an interior wall of a reaction
4 vessel to form an immobilized enzyme;

5 (b) conducting in the reaction vessel in the presence of the
6 immobilized enzyme a reaction catalysed by the enzyme and
7 determining spectroscopically the initial rate of the reaction
8 (V_0) in the reaction vessel;

9 (c) incubating with the immobilized enzyme a sample potentially
10 containing an amount of an enzyme modulator so as to form an
11 immobilized modulated enzyme;

12 (d) repeating step (b) in the reaction vessel in the presence
13 of the immobilized modulated enzyme and determining
14 spectroscopically the initial rate of the modulated reaction (V_1)
15 in the reaction vessel; and

16 (e) comparing V_1 with V_0 to determine the effect of the enzyme
17 modulator.

1 2. A method according to claim 1, wherein the reaction
2 vessel comprises a microtitre well or a cuvette.

1 3. A method according to claim 2, wherein the reaction
2 vessel comprises a microtitre well in a microtitre plate and the
3 initial rates of reaction are determined using a microtitre plate
4 reader.

1 4. A method according to any one of claims 1 to 3, wherein



1 the modulator is a pesticide or a protease.

1 5. A method according to any one of the preceding claims,
2 wherein the enzyme comprises acetylcholinesterase.

3
1 6. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined by measuring
3 the progress of each reaction at a plurality of time points or
4 continuously with time.

1 7. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined
3 spectroscopically by photometry, fluorometry or luminescence.

1 8. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined
3 spectroscopically using detection means remote of the reaction
4 vessel.

1 9. A method according to any one of the preceding claims,
2 wherein each of steps (b), (c) and (d) are conducted by pipetting
3 appropriate solutions into the reaction vessel.

1 10. A method according to any one of the preceding claims,
2 which further comprises a step of washing the immobilized enzyme
3 between steps (b) and (c).

1 11. A method according to any one of the preceding claims,
2 which further comprises a step of washing the immobilized enzyme-
3 modulator complex between steps (c) and (d).



1 12. A method of screening samples for their enzyme
2 modulator activities, which comprises assaying a plurality of
3 samples, each in accordance with the method of any one of the
4 preceding claims, each in a separate reaction vessel, and
5 comparing the effect of each sample with one another or with a
6 blank containing no enzyme modulator.



SUMMARY OF THE INVENTION

The present invention seeks to overcome the disadvantages of the prior art by providing a simpler method for assaying an enzyme modulator. The method comprises:

- (a) immobilizing an enzyme on an interior wall of a reaction vessel to form an immobilized enzyme;
- (b) conducting in the reaction vessel in the presence of the immobilized enzyme a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel;
- (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modular so as to form an immobilized modulated enzyme;
- (d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining spectroscopically the initial rate of the modulated reaction (V_1) in the reaction vessel; and
- (e) comparing V_1 and V_0 to determine the effect of the enzyme modulator.



ABSTRACT

The present invention is a simple method for assaying an enzyme modulator comprising the steps: (a) immobilizing an enzyme on an interior wall of a reaction vessel to form an immobilized enzyme; (b) conducting in the reaction vessel in the presence of the immobilized enzyme a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel; (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modulator so as to form an immobilized modulated enzyme; (d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining spectroscopically the initial rate of the modulated reaction (V_1) in the reaction vessel; and (e) comparing V_1 and V_0 to determine the effect of the enzyme modulator. By conducting each reaction in the same reaction vessel, each vessel therefore serves as its own control, effectively eliminating variability from one reaction vessel to the next. Because the initial rate of reaction is determined, the enzyme reaction is not required to be stopped at a certain end point and no stopping solution is needed. As a result, the enzyme is not destroyed and the same well can be used for the modulator assay. Moreover, if the modulator is found to be absent, the well can actually be recycled for another round of detection with another sample.

This Sheet Has Been Replaced



1/9

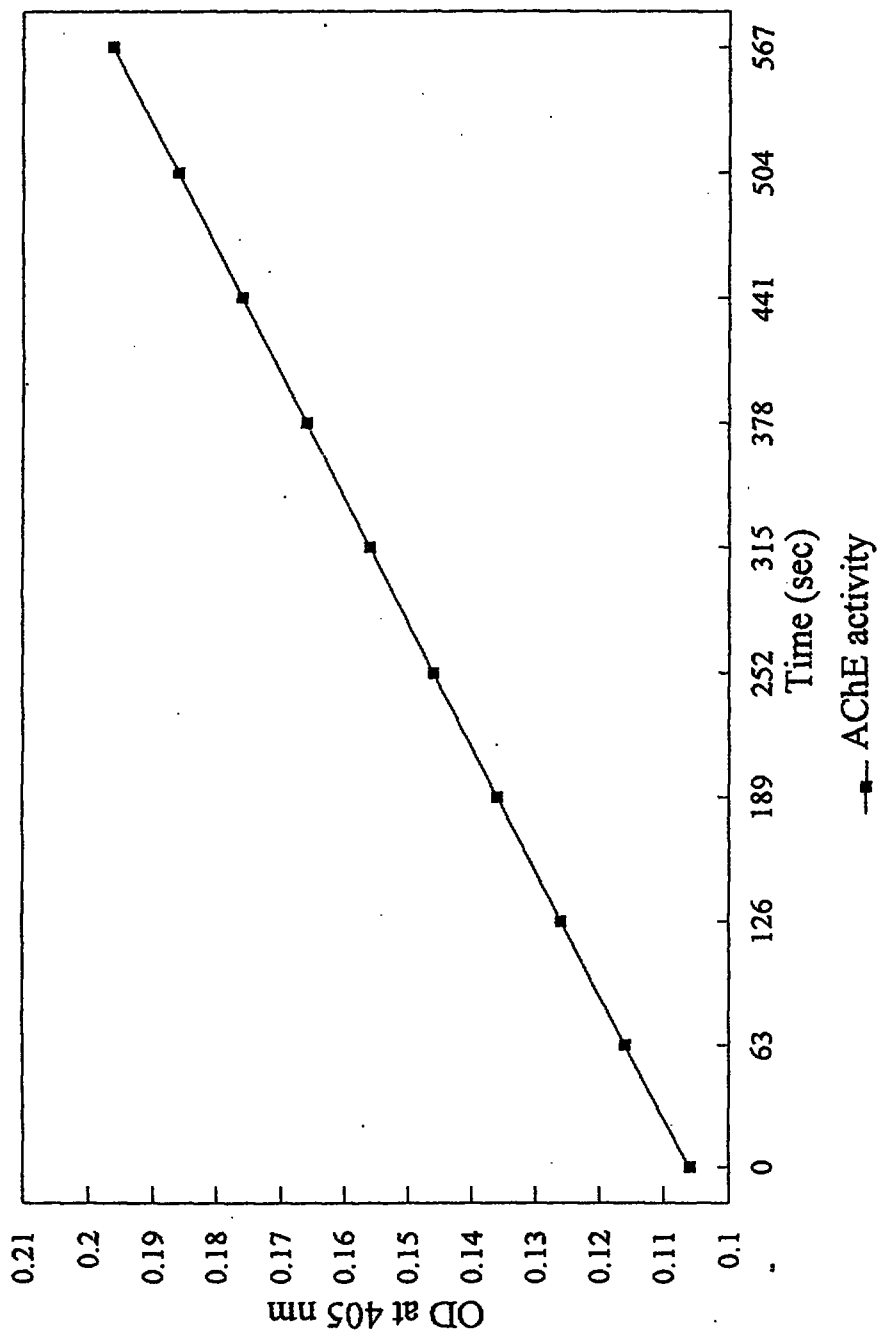


FIGURE 1

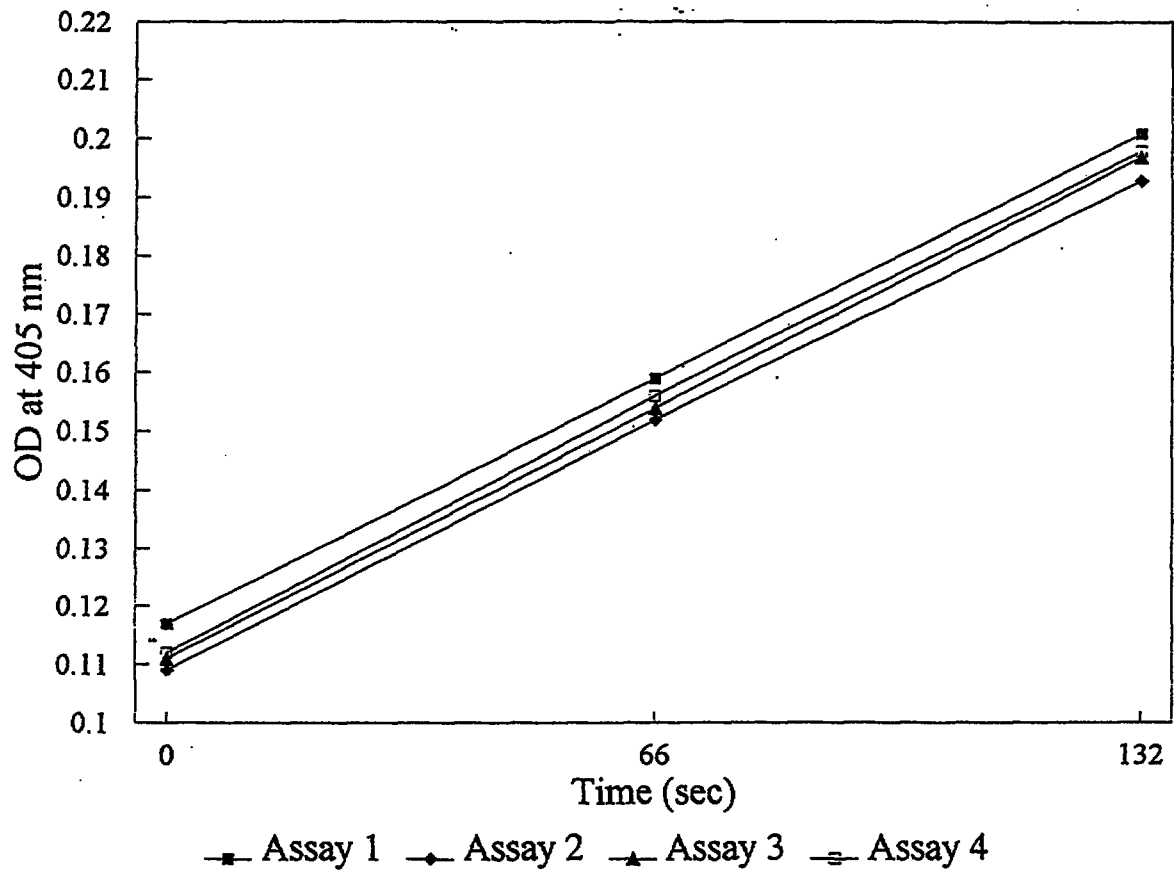


FIGURE 2

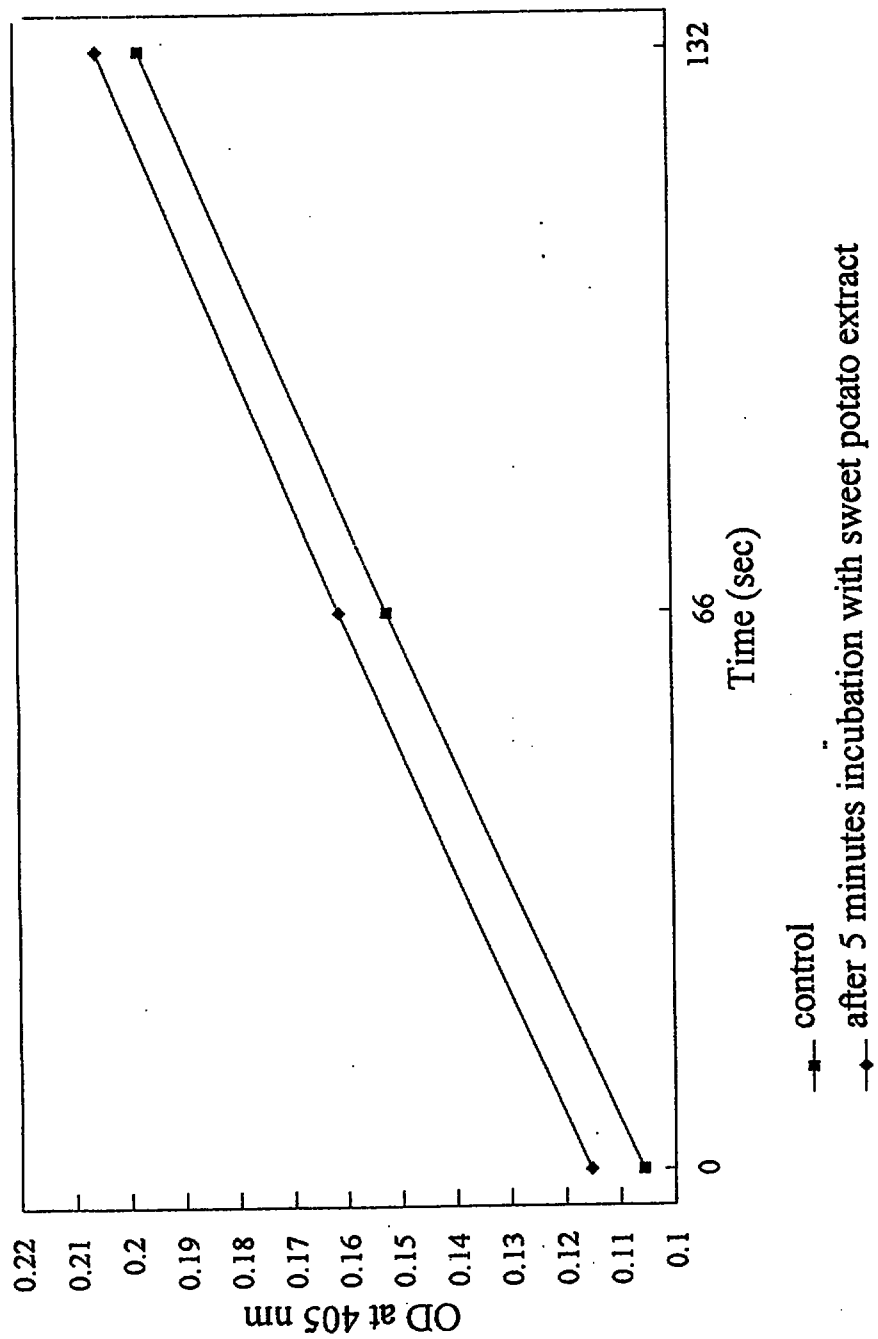


FIGURE 3

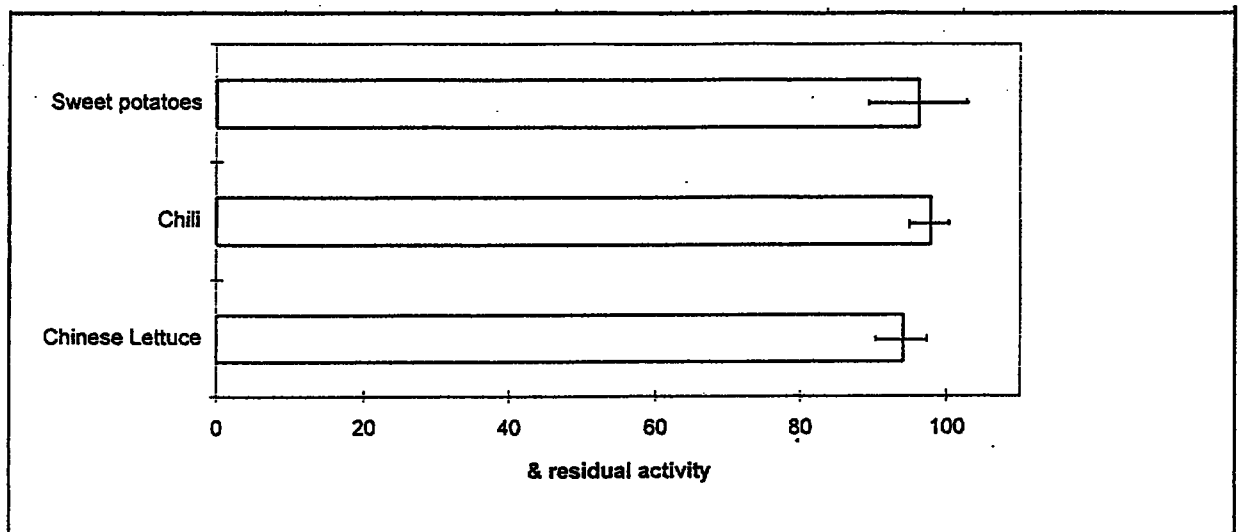


FIGURE 4

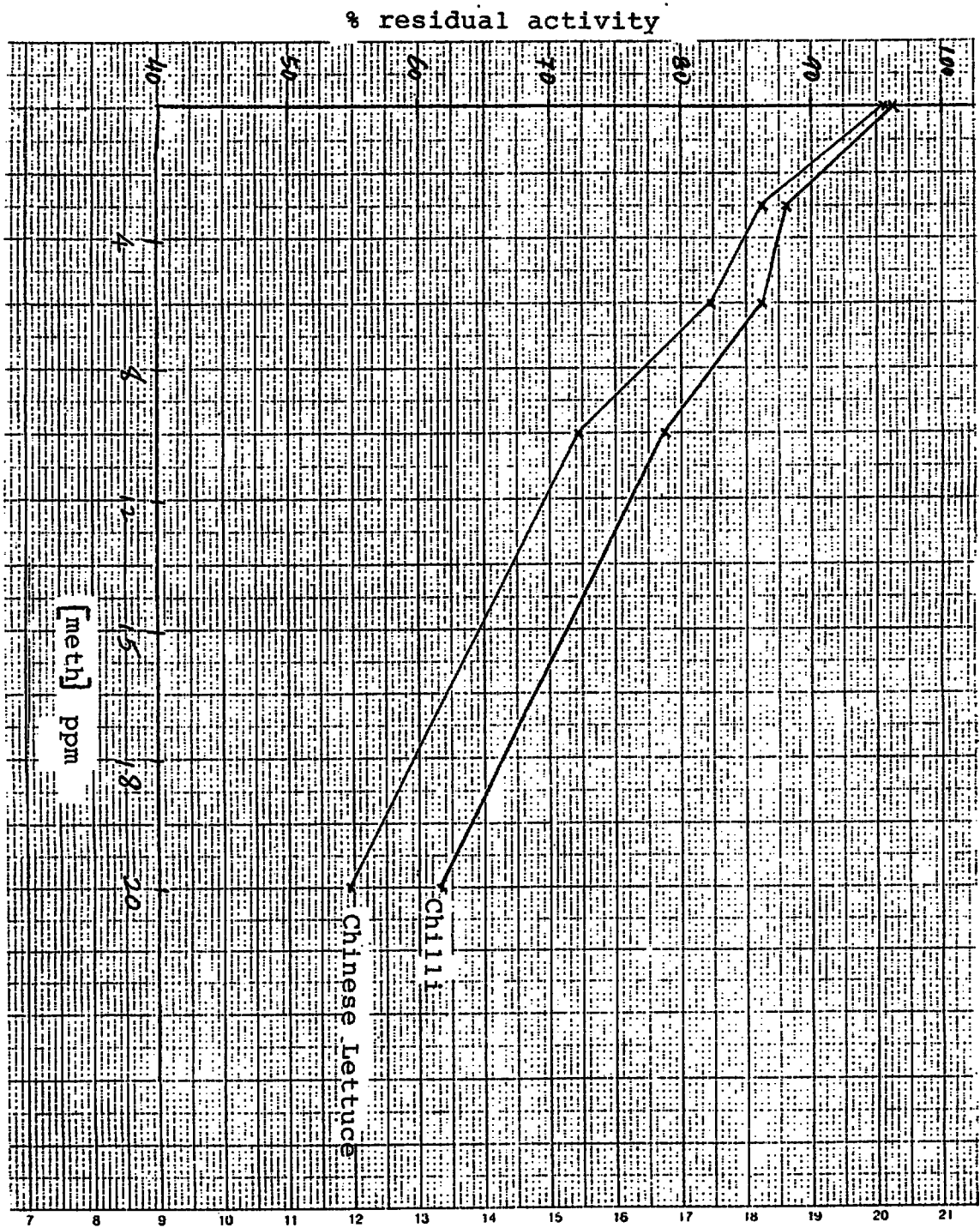
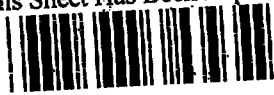
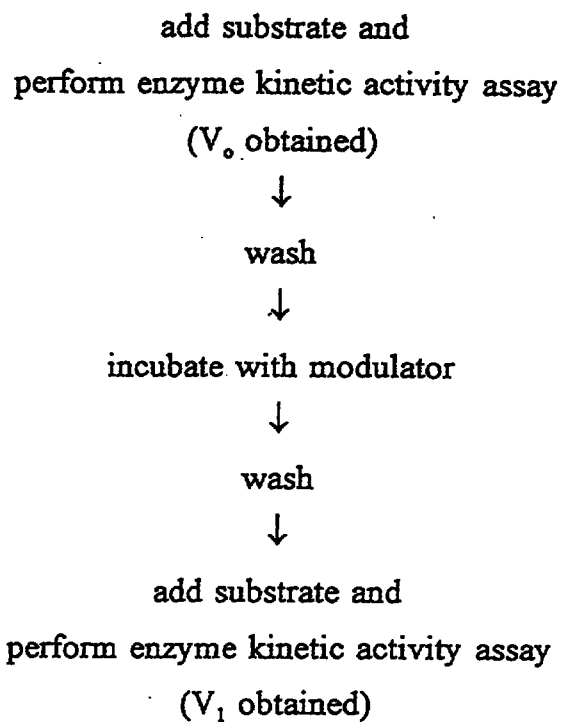
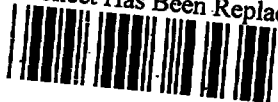
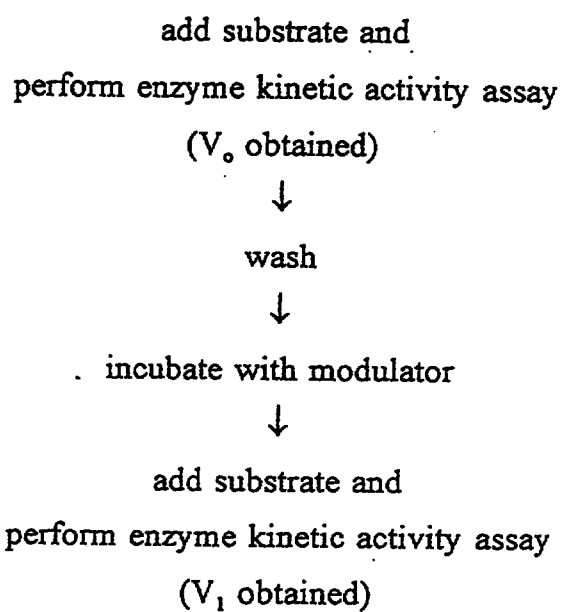


FIGURE 5



STEPS OF A STANDARD IRREVERSIBLE MODULATOR
ASSAY

FIGURE 6



STEPS OF REVERSIBLE MODULATOR ASSAY

FIGURE 7



	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Negative control			Positive control							
B				Duplicate of test samples								
C												
D												
E												
F												
G												
H												

Figure 8



9/9

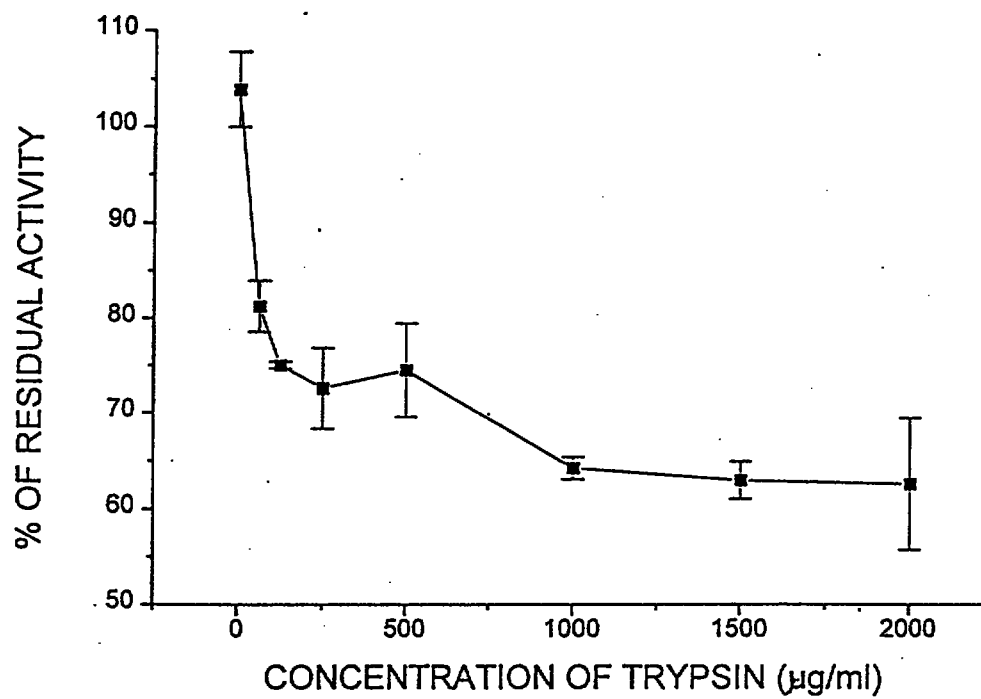


Figure 9

REPLACED SHEETS SG

☐ Description : paged to paged
☐ Claim(s) : paged to paged
☒ Drawing(s) : paged ..119.. to paged ..919..
☒ Abstract : paged ..25.. to paged

1

4500505-9 Pg 15 missing

IPOS

REPLACEMENT SHEETS SG

☒ Description : paged ...1. to paged ...21.
☒ Claim(s) : paged ...22. to paged ...24.
☒ Drawing(s) : paged ...119. to paged ...919.
☒ Abstract : paged ...25. to paged

DATE OF RECEIPT 24-5-96

ASSAY METHOD

FIELD OF THE INVENTION

The invention relates to a method for assaying enzyme modulators, particularly inhibitors, using immobilized enzymes.

5

BACKGROUND OF THE INVENTION

Intensive farming leads to the frequent use of pesticides, such as insecticides. It is therefore extremely important that pesticide residues be monitored closely to ensure that every vegetable consignment is fit for consumption. This problem is prevalent particularly in South East Asia where methamidophos (meth) is one of the most frequently used pesticides.

10

Gas chromatography is a sensitive and reliable method for pesticide residue detection. However, the instrument is expensive, and requires highly skilled personnel and sophisticated laboratories for its effective operation. It uses expensive reagents, and the organic solvents used are often toxic, resulting in additional expenses in waste disposal. There is therefore the need to design cheaper methods that can handle large numbers of samples in a short period of time. Ideally, the method should be fast, simple and robust, and can be used in whole sale markets with simple laboratory equipment for mass screening.

15

20

25

Enzyme immobilization onto various matrices using various cross-linkers is well-known. In general, enzymes may be immobilized onto gels, membranes, electrode surfaces, magnetic particles, nylon net and glass beads.

30

For example, Marty et al describe in Electroanalysis, 4(1992)

4-2033080

This is the replacement sheet
 Date: 24 MAY 1996

249-252, a biosensor for the detection of organophosphate and carbamate insecticides. This article primarily relates to the immobilization of choline oxidase and acetylcholinesterase on cellulose nitrate membranes so as to determine by amperometry inhibition by paraoxon. This approach is described in further detail in Biosensors and Bioelectronics 8(1993) 273-280 in relation to other organophosphorous and carbamate pesticides.

Shekhovtsova and Chernetskaya report in Analytical Letters 27(15) (1994), 2883 to 2898 a method for determining mercury using "immobilized horse radish peroxidase". These authors describe a complexation or "immobilization" of horse radish peroxidase with chitozan. Detection of enzyme activity appears to be performed after the chitozan is dissolved into solution in a microtiter plate or on paper.

Rogers et al report in Fundamental and Applied Toxicology, 16:810 (1991) a flow cell method for determining the effect of insecticides on acetylcholinesterase. A biosensor is reported in which acetylcholinesterase is immobilized on a quartz fibre within the flow cell, to which is tagged a fluorescein isothiocyanate (FITC). Inhibition studies were performed in which the quartz fibre operates as a fibre-optic evanescent fluorosensor integral with the flow cell. The fluorophore emission from the FITC is detected by the fluorosensor. Methodology of this type involves expensive materials and specialized equipment.

A sophisticated approach is also taken by Kindervarten et al in Analytica Chimica Acta 234(1990) 113-117 and by Gunther and Biliteswki in Analytica Chimica Acta 300(1995) 117-125. In these

This is the replacement sheet

Date: 12 4 MAY 1996

papers the authors describe a³ method for assaying various pesticides using a magnetic reactor in a stopped flow apparatus. Acetylcholinesterase was bound to magnetic particles positioned in a magnetic reactor in which the enzyme reaction is performed. After a fixed incubation period, the reaction mixture is passed into a detector for photometric or electrochemical detection. A difficulty with this methodology is that small fluctuations in the assay conditions give rise to fluctuations in the results obtained. In order to overcome these difficulties, it is necessary to automate the flow injection system using computer-controlled pumps and injection valves. Equipment of the type necessary to make these prior art methods sufficiently reliable can therefore be costly or not easily portable.

15 SUMMARY OF THE INVENTION

The present invention seeks to overcome the disadvantages of the prior art by providing a simpler method for assaying an enzyme modulator. The method comprises:

- 20 (a) immobilizing an enzyme on an interior wall of a reaction vessel to form an immobilized enzyme;
- (b) conducting in the reaction vessel in the presence of the immobilized enzyme a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel;
- 25 (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modulator so as to form an immobilized modulated enzyme;
- (d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining spectroscopically the initial rate of the modulated reaction (V_1)
- 30 in the reaction vessel; and

This is the replacement sheet

Date: 24 MAY 1996

4

(e) comparing V_1 with V_0 to determine the effect of the enzyme modulator.

By conducting each reaction in the same reaction vessel, each vessel therefore serves as its own control, effectively eliminating variability from one reaction vessel to the next. Because the initial rate of reaction is determined, the enzyme reaction is not required to be stopped at a certain end point and no stopping solution is needed. As a result, the enzyme is not destroyed and the same well can be used for the modulator assay. Moreover, if the modulator is found to be absent, the well can actually be recycled for another round of detection with another sample.

On a general level, the interior of the reaction vessel may be made from any material which does not interfere with the reaction and which is capable of acting as a substrate for immobilizing enzymes. Suitable materials include plastics such as polystyrene, or glass. Microtitre wells, particularly of plastics such as polystyrene, are especially useful as reaction vessels in the present invention. Microtitre wells are conveniently supplied in microtitre plates such as those commonly used for ELISA assays having an 8x12 format. As described in further detail below, a microtitre plate reader can be used in measurements on the microtitre plates so as to measure the initial rate of reaction in a large number of samples.

Cuvettes made of glass or plastic are also useful reaction vessels. These are convenient because they are portable and because a simple device can be constructed from them using a filter and light-emitting diode in combination with a photodiode

This is the replacement sheet

Date:

24 MAY 1996

for the purpose of monitoring⁵ the desired wavelength.

Various products are commercially available for the purpose of immobilizing enzymes, such as Hybond-N+ membrane from Amersham, 5 Cellulose acetate membrane from Sartorius, or Nylon mesh from Cole-Palmer. Typically, cross-linkers are employed to couple the enzymes to the interior wall. For example, nylon membranes can be used in conjunction with glutaraldehyde as the cross-linker. Alternatively, photo-cross-linkable polymers such as polymers of 10 polyvinyl alcohol bearing styrylpyridinium groups can be cross-linked to entrap the enzyme. The latter photo-cross-linkable polymers are available from Toyo Gosei Co., Tokyo, Japan. It is also possible to immobilize enzymes onto quartz, although this can be expensive.

15 The enzyme modulator may be any compound or molecule which alters the activity of the enzyme. Thus, the modulator may be an inhibitor or, alternatively, a potentiator of enzyme activity. The modulator may act on the enzyme either reversibly or 20 irreversibly and the method according to the present invention can be tailored to assay the action of the modulator as described in further detail below. An important class of modulators are the pesticides, such as organophosphorous or carbamate pesticides, including methamidophos, triazophos, parathion, 25 chlorpyrifos, profenofos, prothiophos and carbofuran. Proteins can also act as modulators. In the case of protease enzymes such as trypsin, irreversible inhibition of the enzyme can take place by proteolytic degradation. Another modulator is phosphate, which is a competitive inhibitor of alkaline phosphatase. 30 Further examples of modulators are heavy metal ions and other ions and salts.

This is the replacement sheet

Date: 24 MAY 1996

A broad range of enzymes is useable in the present invention and includes any biomolecules which catalyse biological or chemical reactions, particularly proteins. The reactions in question can be any reactions which are capable of being assayed directly or indirectly, including redox reactions. It is therefore possible to use as the enzyme in the present invention all types of enzymes including electron transport proteins. The enzyme activities may be assayed directly by measuring the disappearance of reactant or the appearance of product or they may be measured indirectly by the use of a secondary reaction linked to the disappearance of reactant or appearance of product from the first reaction. A good example of the enzyme and its assay is acetylcholinesterase, which catalyses the production of thiocholine from acetylthiocholine iodide. This reaction is described in further detail below. The enzyme is assayed by making use of the secondary reaction of thiocholine with 5,5"-dithio-bis-2-nitrobenzoic acid (DTNB) to form a product which can be monitored photometrically at 405nm. Acetylcholinesterase activity is found to be inhibited in the presence of various important pesticides. As a further example, horse radish peroxidase can be inhibited by mercury or other heavy metal ions.

The initial rates of reaction in the absence and presence of the modulator are preferably determined by measuring the progress of each reaction at a plurality of time points or continuously with time. Provided that the measurements are made in the linear portion of the kinetic reaction as is observed, for example, in the presence of excess substrate, the initial rate of reaction is simply the gradient of a plot of the appearance of product (disappearance of substrate) against time. It is advantageous to determine the initial rate in this manner because the accuracy

This is the replacement sheet

Date: 24 MAY 1996

of the determination of the rate is unaffected by the starting point of the reaction.

The initial rates of reaction may be determined by any convenient method of spectroscopy. Photometry, fluorometry and luminescence are preferred on account of their ready application to commonly-used reaction vessels such as microtitre wells, plates and cuvettes. Enzyme assay systems can be chosen, as discussed above, so that the appearance of product or disappearance of substrate is measured by one of these techniques. For example, the appearance of TNB may be monitored photometrically at 405nm. Advantageously, the initial rates of reaction are determined spectroscopically using detection means remote of the reaction vessel. In this way, there is no need for detection means to be used which is dedicated to the reaction vessel. This enables the assay to be performed in commonly-available reaction vessels such as cuvette or microtitre wells in conjunction with pre-existing standard detection means such as a spectrophotometer or spectrofluorimeter, for example in conjunction with a standard microtitre plate reader.

The assay method may be conducted discontinuously involving standard apparatus of the type described. For example, each of steps (b), (c) and (d) may be conducted by pipetting appropriate solutions into the reaction vessel. This has the advantage of simplicity in that no special apparatus needs to be used for performance of the assay.

One or more washing steps may be incorporated into the method, depending on the type of modulator being assayed. Advantageously, between steps (b) and (c) a further step of

This is the replacement sheet

Date: 24 MAY 1996

washing the immobilized enzyme may be performed so as to remove any reaction mixture from the reaction vessel prior to incubation of the enzyme modulator with the immobilized enzyme. Where the enzyme modulator acts irreversibly to form the immobilized enzyme-modulator complex, after incubation with the enzyme modulator, a further washing step may be performed to remove from the reaction vessel all of the test sample. This has the advantage that substances in the test sample which might interfere with the assay are washed away. Because the immobilized enzyme-modulator complex is formed irreversibly, little or no bound modulator would be expected to be washed away with the sample.

Where the action of the modulator is reversible, the second wash step is omitted so that the modulated enzyme activity assay is performed in the presence of the modulator. Although the presence of the test sample may interfere with the enzyme assay, this generally results only in an increase in the base line of the assay (absolute absorbance in photometry) but does not affect the slope of the reaction with respect to time. Thus, the initial rate of reaction is still readily determinable.

The temperature at which the reaction is conducted will depend, to some extent, upon the organism from which the enzyme is purified. For many enzymes it is possible to conduct the reaction at ambient outdoor temperatures from near freezing to as high as 45°C, typically in the temperature range 10 to 40°C. However, for enzymes purified from thermophilic organisms, it is possible to conduct the reaction at more extreme temperatures of up to, say, 95°C.

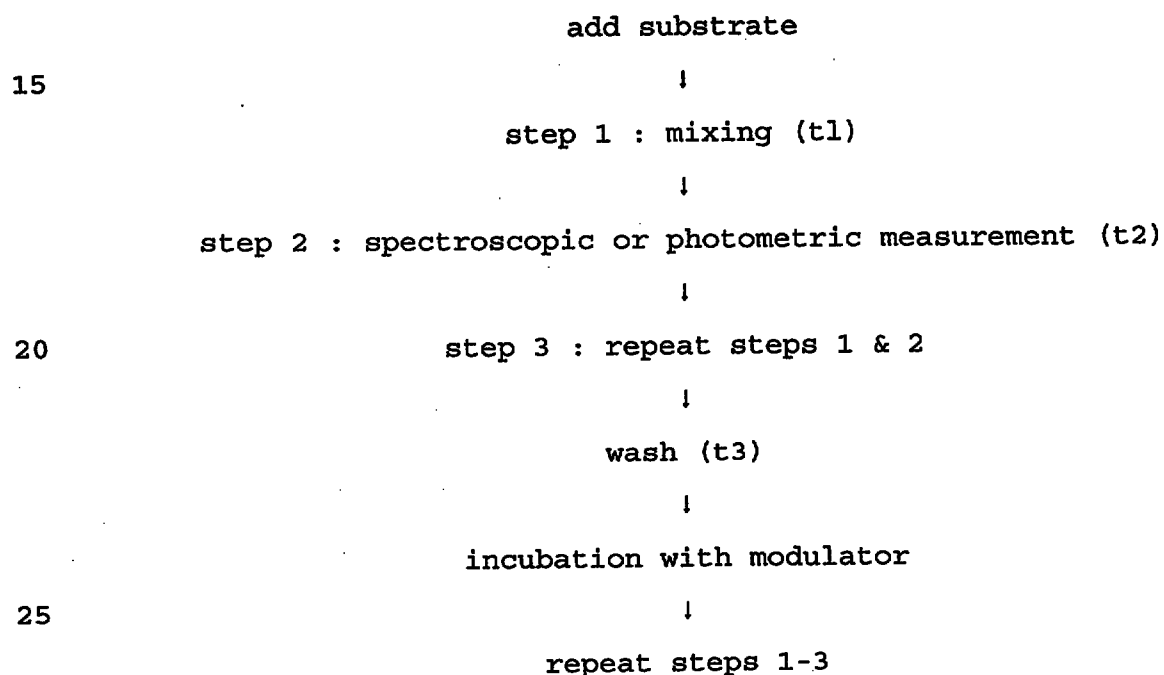
This is the replacement sheet

Date: 24 MAY 1996

For a typical assay there are three different sets of timings to consider; measurement time, mixing time and washing time. Additionally, there is the length of incubation time of the enzyme with the modulator. Referring to the flow diagram below,

5 in the case of a microtitre assay, step (1) has a mixing step to allow efficient mass transfer of the substrate to the immobilized enzyme and this is followed by an almost instantaneous spectroscopic or photometric measurement in step (2). This would

10 yield the first point of a curve from which the rate of reaction will subsequently be generated. This cycle would be repeated at least one more time to generate at least two points for the slope determination:



The total time necessary for one assay with n number of points for determining the slope of the reaction rate would be $t_t = n(t_1 + t_2)$. Typically, n can be as little as 2 or as much as 10 or

30 more, preferably 3 or 4. If the average rate is calculated by

a microtitre plate reader or spectrophotometer automatically, it would be necessary to ensure that t_c is within the linear range of the enzyme reaction. t_2 will depend on the type of spectroscopic measurement and the instrument and its configuration. Typically, t_2 is around five seconds. t_1 is typically one minute and so t_c is around 260s where $n = 4$.

Generally, a minimum of two readings must be taken to establish the slope, preferably three or more to obtain an accurate linear slope. Where two readings are taken, for an average reaction, one would expect t_c to fall in the range 0.5 to 5 minutes. For fast reactions this range could be five to thirty seconds whereas for slow reactions the range could be two minutes to several hours.

15

The incubation time of the modulator for irreversible inhibitors such as organophosphates falls generally in the range ten to twenty minutes although its sensitivity can be increased by lengthening the incubation time to one hour or more. Where the modulator is trypsin, the incubation time can be two minutes to overnight, depending on the sensitivity required.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described in further detail, by way of example only, with reference to accompanying drawings in which:

FIGURE 1 shows the activity of acetylcholinesterase under standard conditions over 567 seconds;

FIGURE 2 shows replicate assays of acetylcholinesterase activity

This is the replacement sheet

Date: 24 MAY 1996

performed in the same microtitre well;

FIGURE 3 shows the effect of sweet potato homogenate on acetylcholinesterase activity;

FIGURE 4 shows the effects of sweet potato, chilli and chinese lettuce homogenate on acetylcholinesterase activity;

FIGURE 5 shows inhibition curves of meth in vegetable samples on acetylcholinesterase activity;

FIGURE 6 shows the basic steps of a standard irreversible modulator assay;

FIGURE 7 shows the basic steps of a reversible modulator assay;

FIGURE 8 shows a plan of the mode of analysis for a semi-quantitative assay using a microtitre plate; and

FIGURE 9 shows the effect of trypsin concentration on acetylcholinesterase activity.

15

DESCRIPTION OF THE EMBODIMENT OF THE INVENTION

Mode of Analysis

The assay can basically be operated in two modes:

1. Quantitative or semi-quantitative analysis of a modulator

The steps are shown in Figure 6, e.g. quantitation of the amount of pesticide present using immobilized acetylcholinesterase. In this instance, a standard curve must first be obtained using known amounts of the modulator. For a quantitative assay, the modulator standards are generally analysed together with the unknowns as in any other quantitative assay. The advantages of the assay system in this case are:

- (a) The assay can be performed in a 96-well microtiter plate. Therefore, even if one 8-well row is used as the standard, one can potentially analyze 88 other unknown samples.

This is the replacement sheet

Date: 24 MAY 1996

12
(b) Each well serves as its own uninhibited control. An uninhibited control is performed in all the wells before the modulator is added. There is always going to be variability in the amount of enzyme present in each well. The present assay gives results of improved reproducibility because modulation detection in one well is not compared to an unmodulated control in another well, as would be the case in a conventional free enzyme assay. Rather, each well has its own V_0 value, representing the true amount of enzyme present in that test well. After incubation with the modulator, the V_1 detected is compared with the V_0 of the same well.

When only semi-quantitative analysis is required, then the positive control consists of a specific concentration of modulator to which the test samples would be compared. The scheme for a general semi-quantitative assay is shown in Figure 8.

2. **Screening for presence of modulating activity** This assay, when performed in a microtiter well, is extremely useful for screening simultaneously many compounds for their effects on enzymes. The screening for beta-lactamase inhibitors can be used as an example to illustrate this utility.

Beta-lactamase is a very important enzyme in clinical biochemistry because it is responsible for the hydrolysis of the beta-lactam ring in the penicillin family of antibiotics. Its activity can be monitored by a well-

characterised colorimetric assay using cephalosporin as substrate. In the present assay, beta-lactamase is immobilized onto the reaction vessel, such as a 96-well microtitre plate, and various compounds added to check for inhibition effects. These compounds may be newly purified or extracted natural or synthetic compounds. They may also be part of an existing repertoire of a pharmaceutical company which modulating activity on beta-lactamase is unknown. If irreversible inhibitors are being screened, then the assay steps would be as shown in Figure 6.

In the screening mode, the modulation of different compounds is being tested, so quantitation is not relevant. Obviously, a standard curve is not necessary in this case, and the only control needed would be the incubation with the extraction buffer in the absence of the compound to be screened.

In both modes of analysis, a reversible inhibitor can instead be screened if the assay is modified as shown in Figure 7. The second wash step is omitted, so that the modulated enzyme activity assay is performed in the presence of the modulator. This is possible even if the sample is coloured e.g. a herbal extract.

25

Detection principles

Many enzymes give a classic Michaelis type kinetic reaction. This means that in a relatively large volume, and at a defined substrate concentration where mixed order kinetics occur, the initial reaction rate V_0 would be constant over time. As the substrate is consumed, the concentration drops, and the enzyme

This is the replacement sheet

Date: 24 MAY 1996

14
reaction rate begins to slow down.

5 The present assay exploits the linear portion of the kinetic reaction by taking two to several readings at fixed time intervals as the reaction proceeds in each well. In this way, the initial reaction rate V_0 is determined. The substrate is then washed away and the modulator added. After incubation, the modulator is washed away and the enzyme kinetic assay repeated. The reaction rate after modulation is denoted V_1 .

10

The effect of the modulator on the immobilized enzyme is determined using the simple general relationship :

$$15 \quad \% \text{ modulated activity} = \frac{V_1}{V_0} \times 100 \% \quad \text{eqn. (1)}$$

If the modulator is an enhancer, then the amount of enhancement can be expressed as :

$$20 \quad \% \text{ enhancement} = \frac{V_1 - V_0}{V_0} \times 100 \quad \text{eqn. (2)}$$

if the modulator is an inhibitor, then the percent residual activity can be expressed as in eqn. (1).

25

Example

Detection of methamidophos in vegetables

30 Fresh vegetables are consumed in large quantities in Asia. Intensive farming leads to the frequent use of insecticides. As such, it is extremely important that pesticide residues be monitored closely to ensure that every vegetable consignment is

This is the replacement sheet

Date: 24 MAY 1996

Acetylthiocholine Iodide (ACTI), 5'5-dithio-bis-2-nitrobenzoic acid, bovine serum albumin (BSA) and acetylcholine esterase (AChE) from electric eel purchased from Sigma Chemical Company. Reacti-Bind Maleic Anhydride Activated Polystyrene Plates from
5 Pierce (Reacti-Bind is a trade mark). Trizma base from Sigma. Methamidophos (analytical grade) was from Hoechst. Glycine from Merck.

Ellman's assay reaction mixture

10 This assay consisted of 1.5mM DTNB, 6mM ATCI in 0.01M Tris-HCl, pH 8.5 ATCI was prepared as a 10X stock in distilled water and added to the reaction mixture immediately before each set of assays.

15 Immobilization of AChE in Reacti-Bind Maleic Anhydride Activated Polystyrene Plates

All reagents, plate and samples were brought to room temperature before use. 100ul of 3U/ml AChE was introduced into each well of a Reacti-Bind plate. The plate was then incubated at 37°C
20 overnight for optimal binding. AChE was removed the next day and plate washed with 0.01% BSA + 0.05% Tween 20, pH 7.2 PBS ten times. The blocking buffer, containing 1M glycine + 0.05% Tween 20 in PBS, was delivered to the wells and incubated at 4°C for 3 hours. The plate was then washed with PBS Tween, pH 7.2 ten
25 times.

AChE activity assay

AChE activity was tested by introducing 200ul of Ellman's reaction mixture into each well using an 8-channel micropipetter.
30 The microtitre plate reader was programmed to shake for 10 seconds before an absorbance reading at 405 nm was taken. The

cycle was repeated 3 times so that a total of 4 readings were taken. The 4 readings can be plotted as one kinetic curve. An example is assay 1 in Figure 2. The rate of that reaction (V_0) was calculated from the slope of that kinetic curve.

5

To ensure the reproducibility of the assay, the activity test in each well was repeated three times i.e. four separate kinetic measurements were obtained. As a result, a total of 4 kinetic curves were obtained as assays 1 to 4 in Figure 2. The slopes
10 (V_0) of the 4 independent kinetic curves were calculated and shown in Table 1. The mean of the four V_0 is calculated as V_0 .

Homogenization of vegetable material

Vegetables were obtained from the Primary production Department
15 of Singapore. The plant materials were weighed and homogenized in equal volume of TB (1g of plant material was assumed to have a volume of 1 ml) in a Waring blender and filtered with filter paper. The pH of the filtrate was raised to 8.5 with sodium hydroxide and used for AChE incubation without further treatment.
20 For methamidophos inhibition of AChE, the appropriate amount of meth was spiked into the vegetable homogenate from a concentrated stock solution.

25 Inhibition of AChE by methamidophos

Three AChE activity assays were performed by adding 200ul of methamidophos of various concentrations into the different wells. The plate was then incubated on an orbital shaker at room temperature for 10 mins. After the incubation, the plate was
30 washed gently with Tris-HCl two times and AChE activity assay performed three times again. Residual activity of the enzyme

This is the replacement sheet

Date: 24 MAY 1996

18

after incubation was then calculated using the equation below:

$$\frac{V_0}{V_1} \times 100 \%$$

5

Results and Discussion

Linearity range The assay system works under a substrate concentration in which the initial reaction rate (V_0) has a linear range of minutes. In this way, the absorbance readings of the many wells of a microtitre plate can be taken simultaneously, even though the substrate solution may be added sequentially using a multichannel or multiple dispensing pipetter.

15 A simple experiment was performed to determine the linear range of the immobilized AChE activity as shown in Figure 1. Results show that at the substrate concentration used in these experiments, the initial AChE reaction rate is constant over 10 minutes. Therefore, in all subsequent experiments, 2 or 3 absorbance readings obtained within this time frame was used to calculate V_0 .

Reproducibility Multiple Ellman's assays were performed in the same well to determine the reproducibility of the system. For each assay, three absorbance readings were taken to determine V_0 . Figure 2 shows 4 Ellman's assays performed on the same well. Although the absolute absorbance fluctuated, V_0 was very reproducible. The mean of four V_0 is calculated as V_0 and is shown under well 1 of Table 1. Results of assays performed on 8 other wells are also shown in Table 1. The standard deviations in all cases were below 3% of V_0 .

30

This is the replacement sheet

Date: 24 MAY 1996

Table 1 Results of 4 Ellman's assays performed in each of 8 wells. Assay conditions were as described in Figure 2.

Well no	1	2	3	4	5	6	7	8
V ₀ (dA/min)	0.038	0.037	0.042	0.042	0.040	0.042	0.044	0.035
	0.038	0.037	0.042	0.040	0.040	0.041	0.045	0.036
	0.039	0.037	0.042	0.040	0.039	0.043	0.045	0.036
	0.039	0.038	0.043	0.041	0.041	0.043	0.045	0.037
Mean (V ₀)	0.039	0.037	0.042	0.041	0.040	0.042	0.045	0.036
SD (% of V ₀)	1.498	1.340	1.182	2.346	2.040	1.943	1.116	2.267

Effect of vegetable extract on AChE activity An important advantage of the assay system is that even complex extracts could be analyzed without extensive sample cleanup. Figure 3 shows the effect of an aqueous homogenate of sweet potato on immobilized AChE. Each data point is a mean of 3 Ellman's assays. V₀ was 0.0414±1.30%. while V₁ after 5 minutes incubation with the sweet potato extract was 0.0406±1.3%. V₁/V₀ is 98%, showing that there is a minimal effect of 2%. Two other types of vegetables (red chili and Chinese lettuce) were also tested and their effect on AChE shown in Figure 4. Each bar represents the mean of the V₀ of 8 separate wells. These results show that even when AChE was incubated with complex matrices such as vegetables that have simply been homogenized and filtered, good activity is recovered.

Effect of meth in vegetable extract on AChE activity When known amounts of meth were spiked into vegetable samples, inhibition was observed. Figure 5 shows the increasing inhibitory effects of increasing amounts of meth. Each data point represents the mean of 3 replicate wells. If 15% inhibition is taken as significant, the limit of sensitivity of this assay would be 8 and 13 mg/kg plant material for Chinese lettuce and Chilli

This is the replacement sheet

Date: 24 MAY 1996

respectively.

Example 2

5 Detection of trypsin

All reagents and method for AChE activity assay are the same as in Example 1. The general procedure is shown in Figure 6. Ellman's reagent was used to obtain V_0 of AChE immobilized onto the walls of the microwells. The assay was repeated twice to get
10 three V_0 readings and V_0 obtained. After washing off the substrate, 0.2ml of PBS pH 7.4 containing 0 to 2 mg/ml trypsin were introduced into the wells in triplicates. AChE was incubated with the trypsin solution for 20 minutes at room temperature with shaking. After incubation, the trypsin was
15 washed off and the AChE activity assay repeated. The result is shown in Figure 9. PBS pH 7.4 shows no inhibitory effect on AChE after 20 minutes incubation. In the presence of 62 u/ml trypsin, AChE activity was reduced to 81.3%. Increasing amounts of trypsin causes increased AChE inactivation.

20

Conclusion

There are many advantages to the assay system

Superior reproducibility This assay relies on the slope of the enzyme kinetic reaction rather than an end point. As a result,
25 the starting time of the reaction, and the exact volume of substrate added in each well is not crucial, and multiple samples can be manipulated comfortably, and yet superior, reproducible results can be obtained.

30 **Little or no sample pretreatment required** The modulator can be in an extract that interferes with detection by the microtiter

This is the replacement sheet

Date: 24 MAY 1996

- plate reader. e.g. it has a deep colour. For an irreversible inhibitor the modulator extract is washed away before the enzyme reaction is monitored, so no interference would occur. As a result, sample cleanup can be eliminated. In the application
- 5 used here, the vegetable was simply homogenized in buffer and filtered through paper. No further clean up was required. For reversible inhibitors in which the sample has to be present together with the substrate in the microtitre well, there is still good tolerance for baseline interference from the sample.
- 10 As long as the test sample does not affect V_0 in the absence of the modulator, a shift in the baseline absorbance reading will result in a parallel line as in Figure 3. V_0 would not be affected unless the modulator is present.
- 15 **High sample throughput** Up to 95 samples can be tested in a very short time. In the case of meth detection, 95 samples can potentially be analyzed in less than 1 hour, since each of the 6 Ellman's assay takes approximately 5 minutes, and incubation with the vegetable extract takes 10 minutes.

**CLAIMS:**

- 1 1. A method for assaying an enzyme modulator, which method
2 comprises:
3 (a) immobilizing an enzyme on an interior wall of a reaction
4 vessel to form an immobilized enzyme;
5 (b) conducting in the reaction vessel in the presence of the
6 immobilized enzyme a reaction catalysed by the enzyme and
7 determining spectroscopically the initial rate of the reaction
8 (V_0) in the reaction vessel;
9 (c) incubating with the immobilized enzyme a sample potentially
10 containing an amount of an enzyme modulator so as to form an
11 immobilized modulated enzyme;
12 (d) repeating step (b) in the reaction vessel in the presence
13 of the immobilized modulated enzyme and determining
14 spectroscopically the initial rate of the modulated reaction (V_1)
15 in the reaction vessel; and
16 (e) comparing V_1 with V_0 to determine the effect of the enzyme
17 modulator.
- 1 2. A method according to claim 1, wherein the reaction
2 vessel comprises a microtitre well or a cuvette.
- 1 3. A method according to claim 2, wherein the reaction
2 vessel comprises a microtitre well in a microtitre plate and the
3 initial rates of reaction are determined using a microtitre plate
4 reader.
- 1 4. A method according to any one of claims 1 to 3, wherein

This is the replacement sheet

Date: 24 MAY 1996



1 the modulator is a pesticide or a protease.

1 5. A method according to any one of the preceding claims,
2 wherein the enzyme comprises acetylcholinesterase.
3

1 6. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined by measuring
3 the progress of each reaction at a plurality of time points or
4 continuously with time.

1 7. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined
3 spectroscopically by photometry, fluorometry or luminescence.

1 8. A method according to any one of the preceding claims,
2 wherein the initial rates of reaction are determined
3 spectroscopically using detection means remote of the reaction
4 vessel.

1 9. A method according to any one of the preceding claims,
2 wherein each of steps (b), (c) and (d) are conducted by pipetting
3 appropriate solutions into the reaction vessel.

1 10. A method according to any one of the preceding claims,
2 which further comprises a step of washing the immobilized enzyme
3 between steps (b) and (c).

1 11. A method according to any one of the preceding claims,
2 which further comprises a step of washing the immobilized enzyme-
3 modulator complex between steps (c) and (d).

This is the replacement sheet

Date: 24 MAY 1996



24

1 12. A method of screening samples for their enzyme
2 modulator activities, which comprises assaying a plurality of
3 samples, each in accordance with the method of any one of the
4 preceding claims, each in a separate reaction vessel, and
5 comparing the effect of each sample with one another or with a
6 blank containing no enzyme modulator.

This is the replacement sheet

Date: 24 MAY 1996



ABSTRACT

ASSAY METHOD

The present invention is a simpler method for assaying an enzyme modulator. The method comprises: (a) immobilizing an enzyme on an interior wall of a reaction vessel to form an immobilized enzyme; (b) conducting in the vessel a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel; (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modular so as to form an immobilized modulated enzyme; (d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining spectroscopically the initial rate of the modulated reaction (V_1) in the reaction vessel; and (e) comparing V_1 and V_0 to determine the effect of the enzyme modulator.

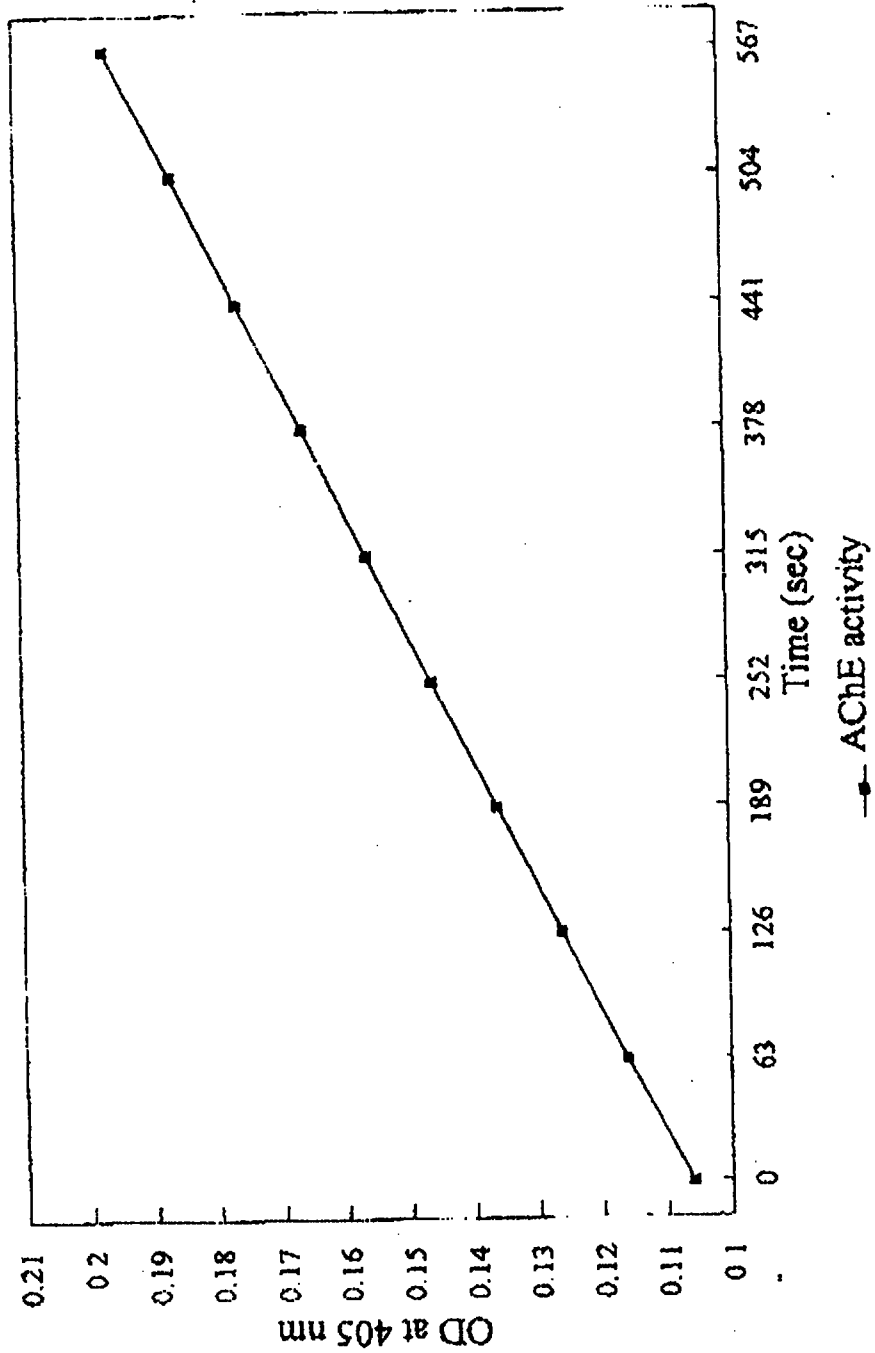


FIGURE 1

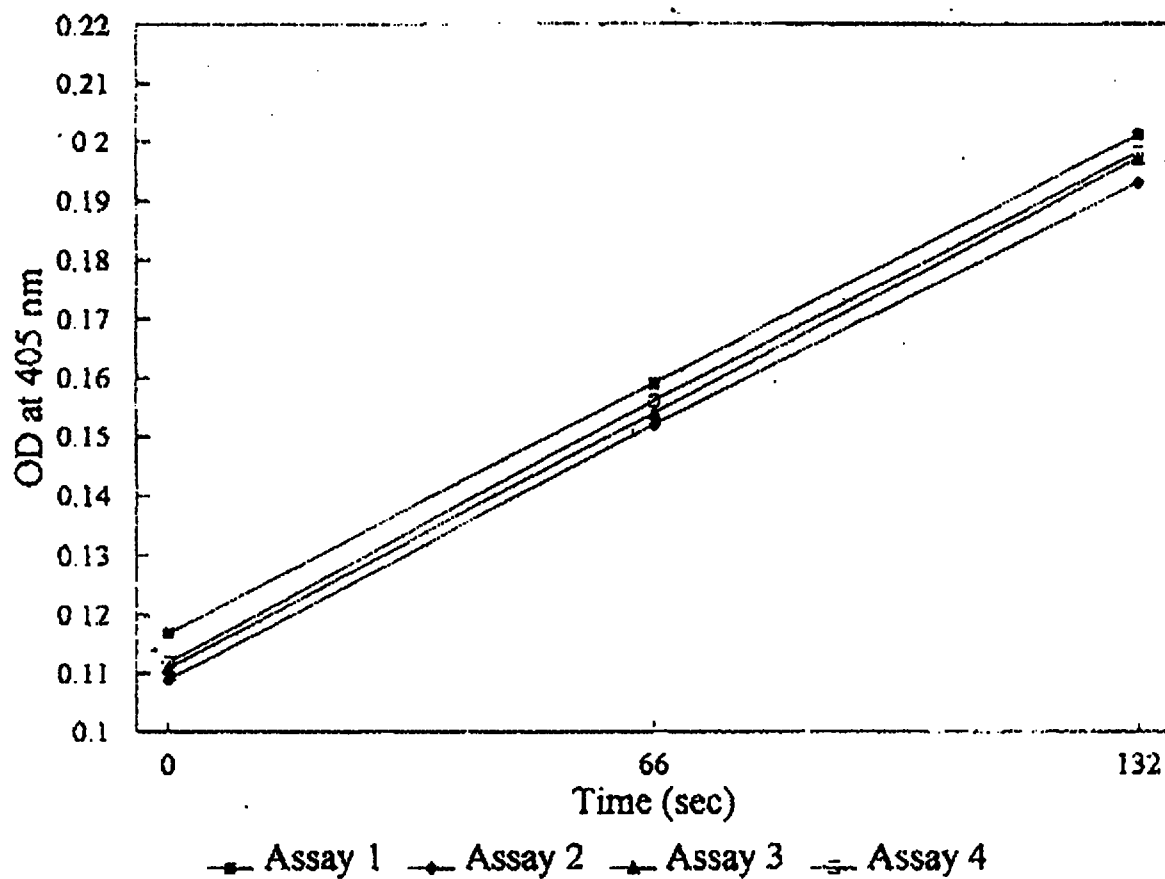


FIGURE 2

This is the replacement sheet

Date: 24 MAY 1996

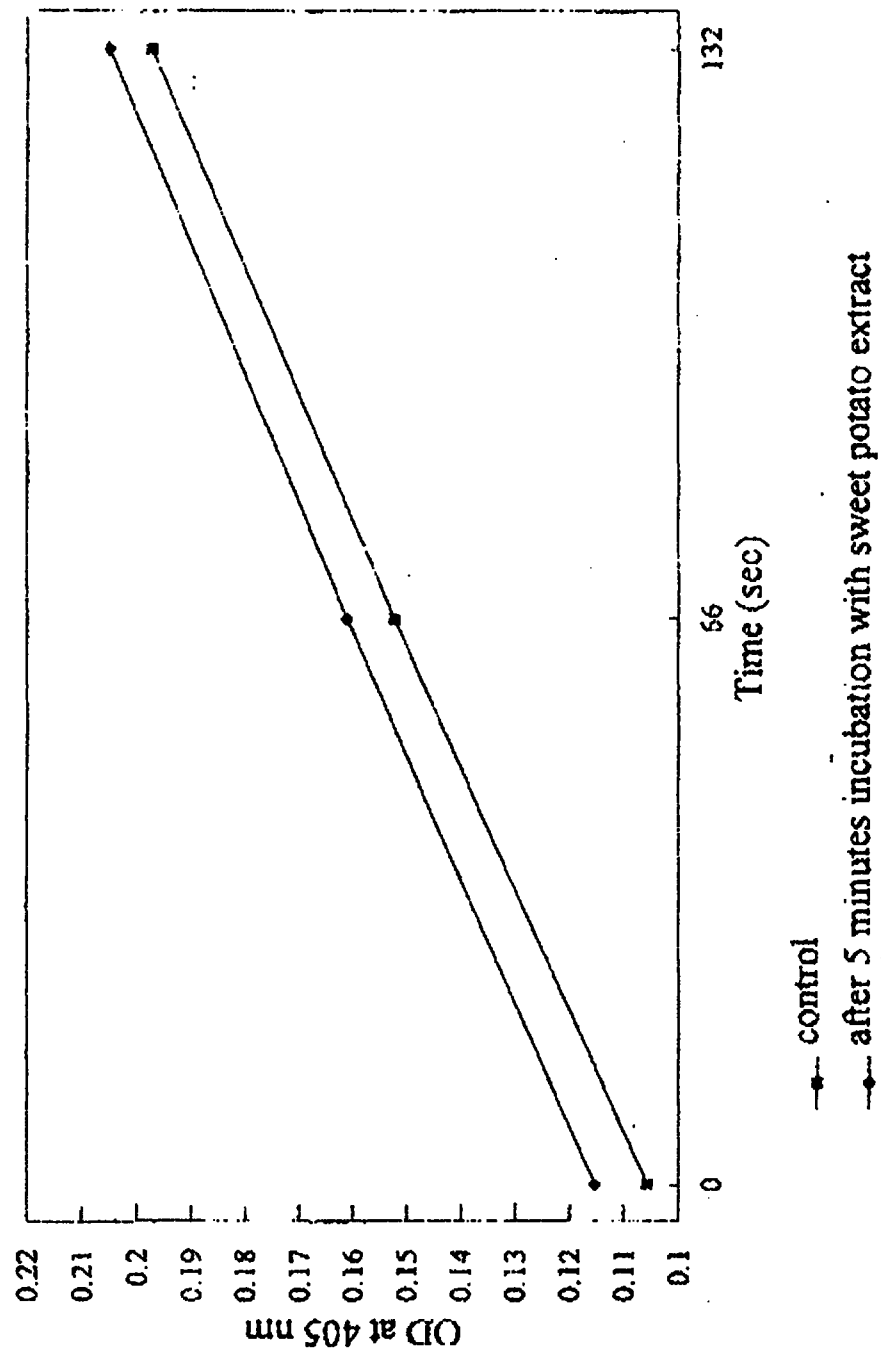


FIGURE 3

This is the replacement sheet
Date: 24 MAY 1996

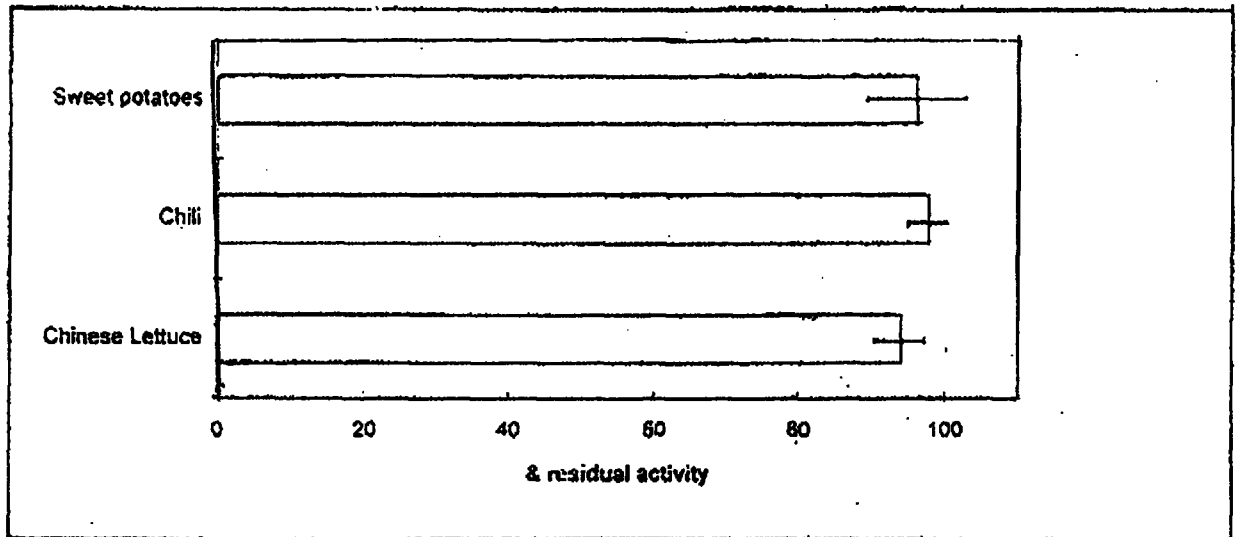


FIGURE 4

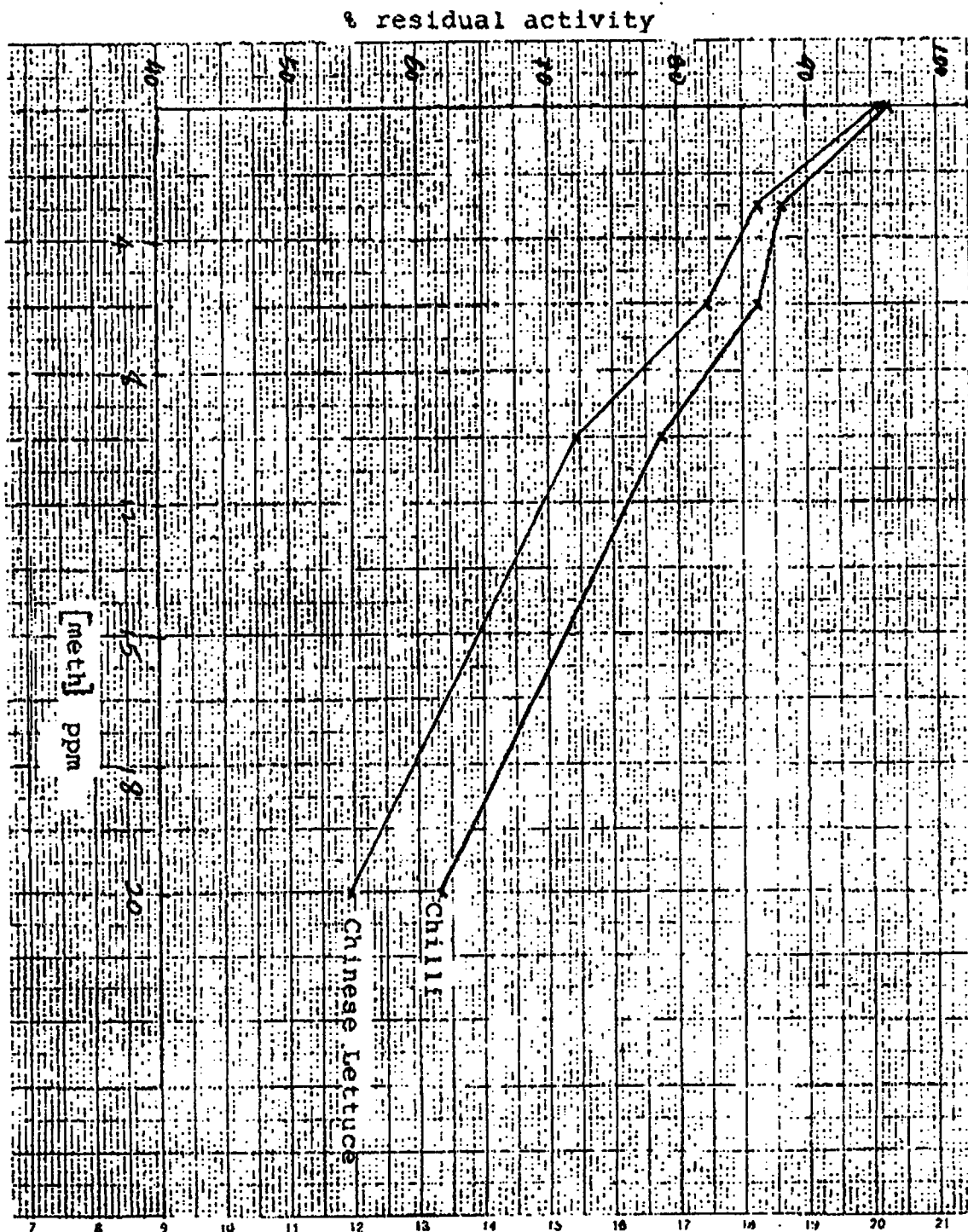
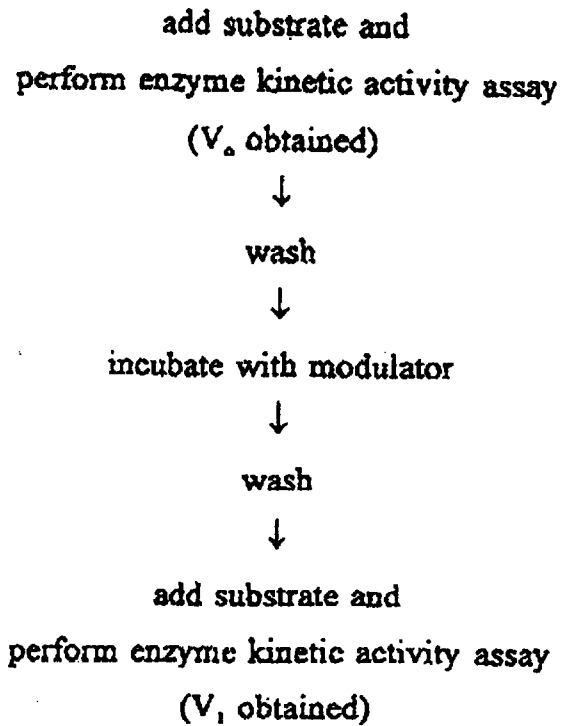


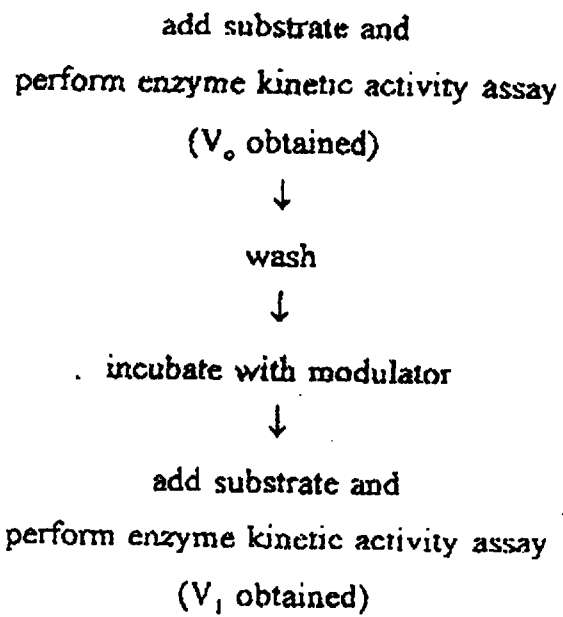
FIGURE 5



STEPS OF A STANDARD IRREVERSIBLE MODULATOR
ASSAY

FIGURE 6

This is the replacement sheet
Date: 24 MAY 1996



STEPS OF REVERSIBLE MODULATOR ASSAY

FIGURE 7

This is the replacement sheet

Date: 24 MAY 1996



	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Negative control		Positive control								
B			Duplicate of test samples									
C												
D												
E												
F												
G												
H												

Figure 8

This is the replacement sheet

Date: 24 MAY 1996

This Sheet Has Been Replaced



9/9

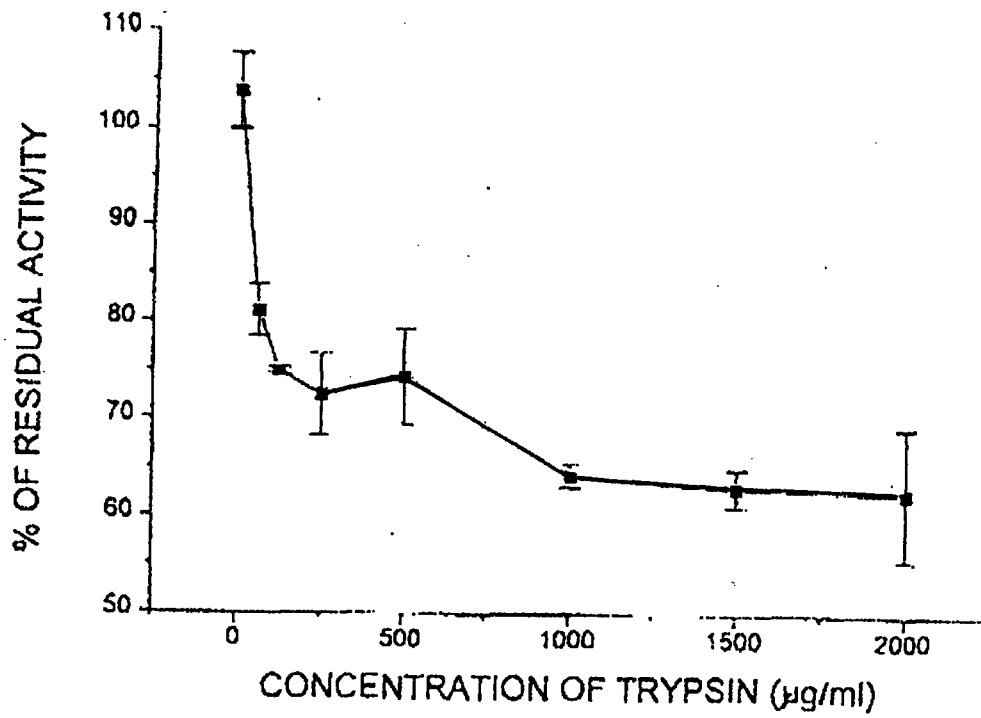


Figure 9

This is the replacement sheet

Date: 24 MAY 1996

ABSTRACT**ASSAY METHOD**

The present invention is a simpler method for assaying an enzyme modulator. The method comprises: (a) immobilizing an enzyme on an interior wall of a reaction vessel to form an immobilized enzyme; (b) conducting in the vessel a reaction catalysed by the enzyme and determining spectroscopically the initial rate of the reaction (V_0) in the reaction vessel; (c) incubating with the immobilized enzyme a sample potentially containing an amount of an enzyme modular so as to form an immobilized modulated enzyme; (d) repeating step (b) in the reaction vessel in the presence of the immobilized modulated enzyme and determining spectroscopically the initial rate of the modulated reaction (V_1) in the reaction vessel; and (e) comparing V_1 and V_0 to determine the effect of the enzyme modulator.

Fig. 6

REPLACEMENT SHEETS		SG
☐	Description : paged to paged	
☐	Claim(s) : paged to paged	
☒	Drawing(s) : paged 119 to paged 919	
☒	Abstract : paged 25 to paged	
DATE OF RECEIPT 3-8-96...		

This is the replacement sheet
 Date: 03 AUG 1996

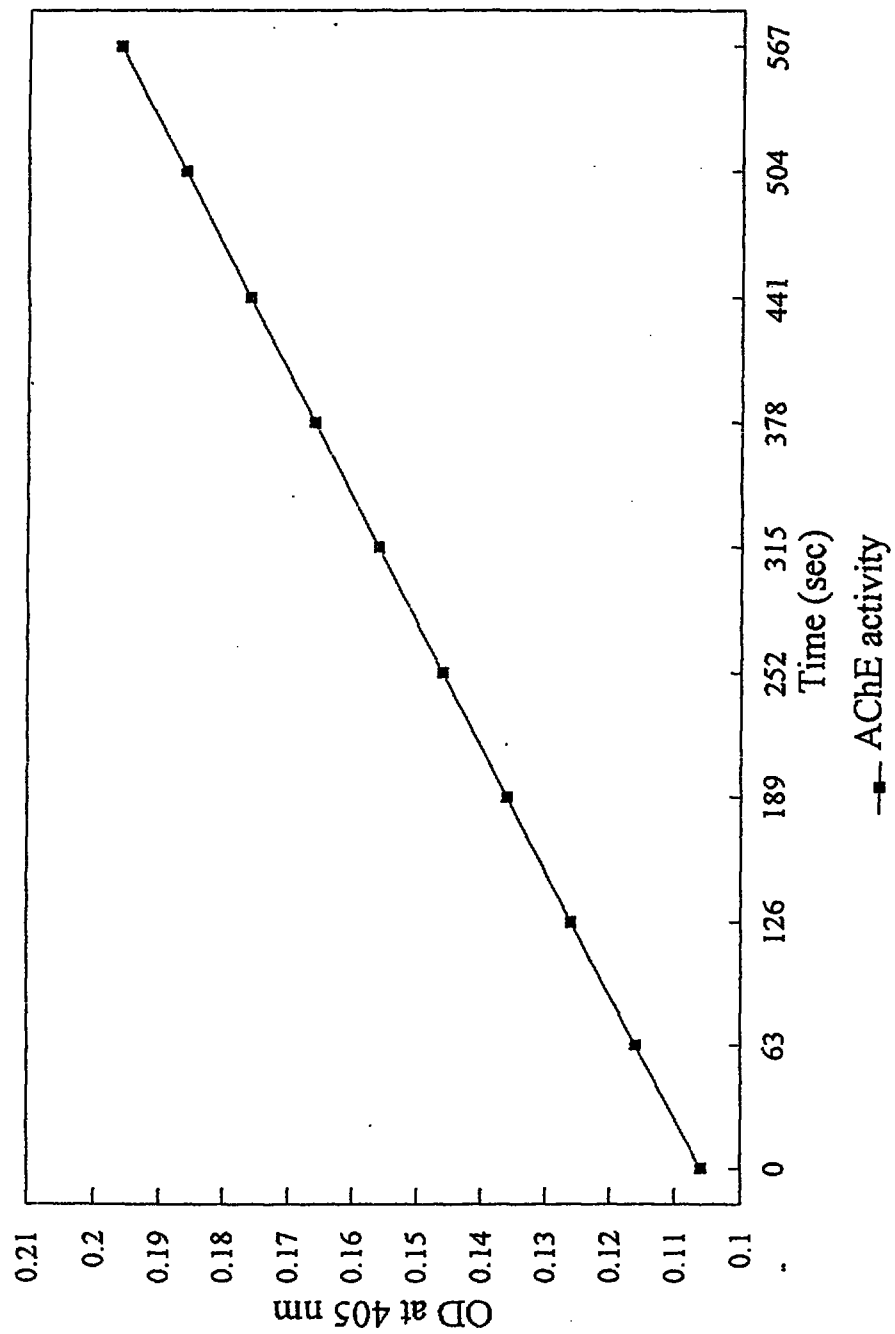


FIGURE 1

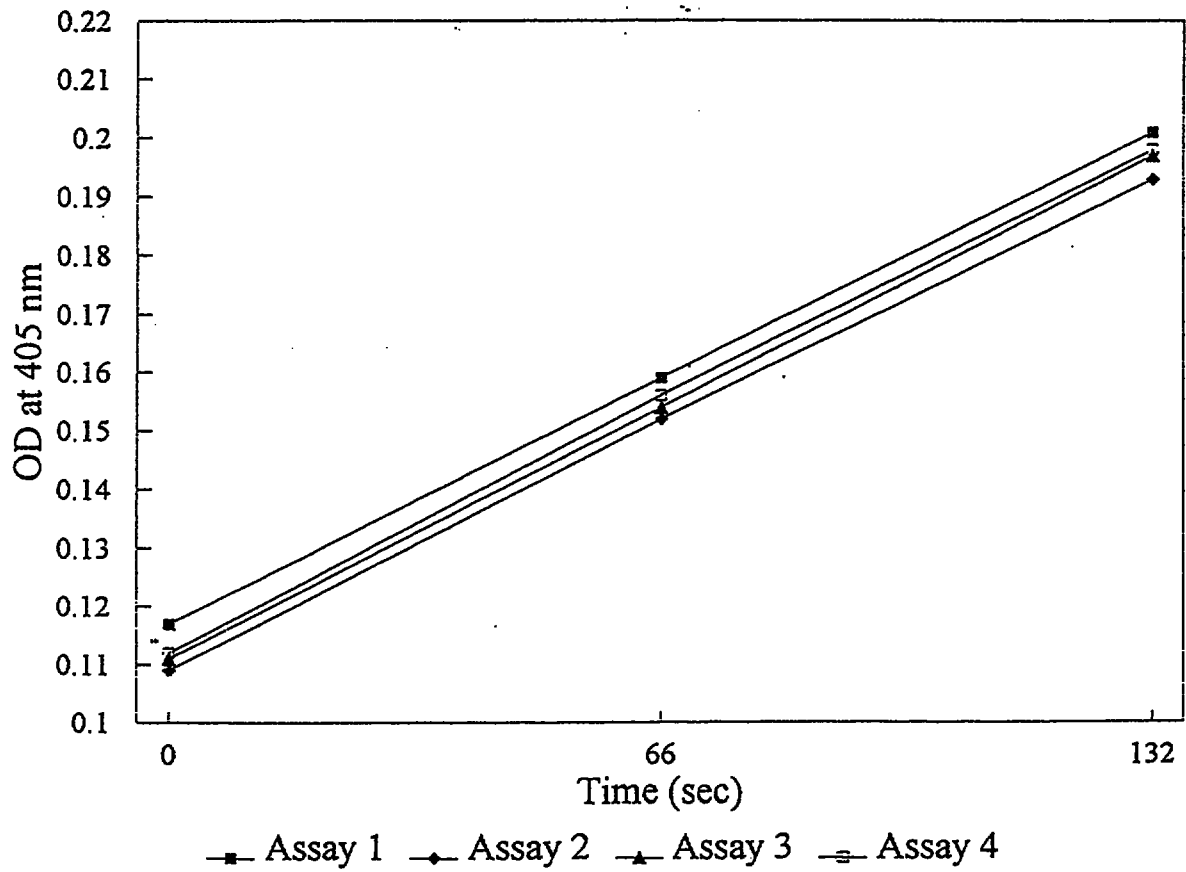


FIGURE 2

This is the replacement sheet

Date: 03 AUG 1996

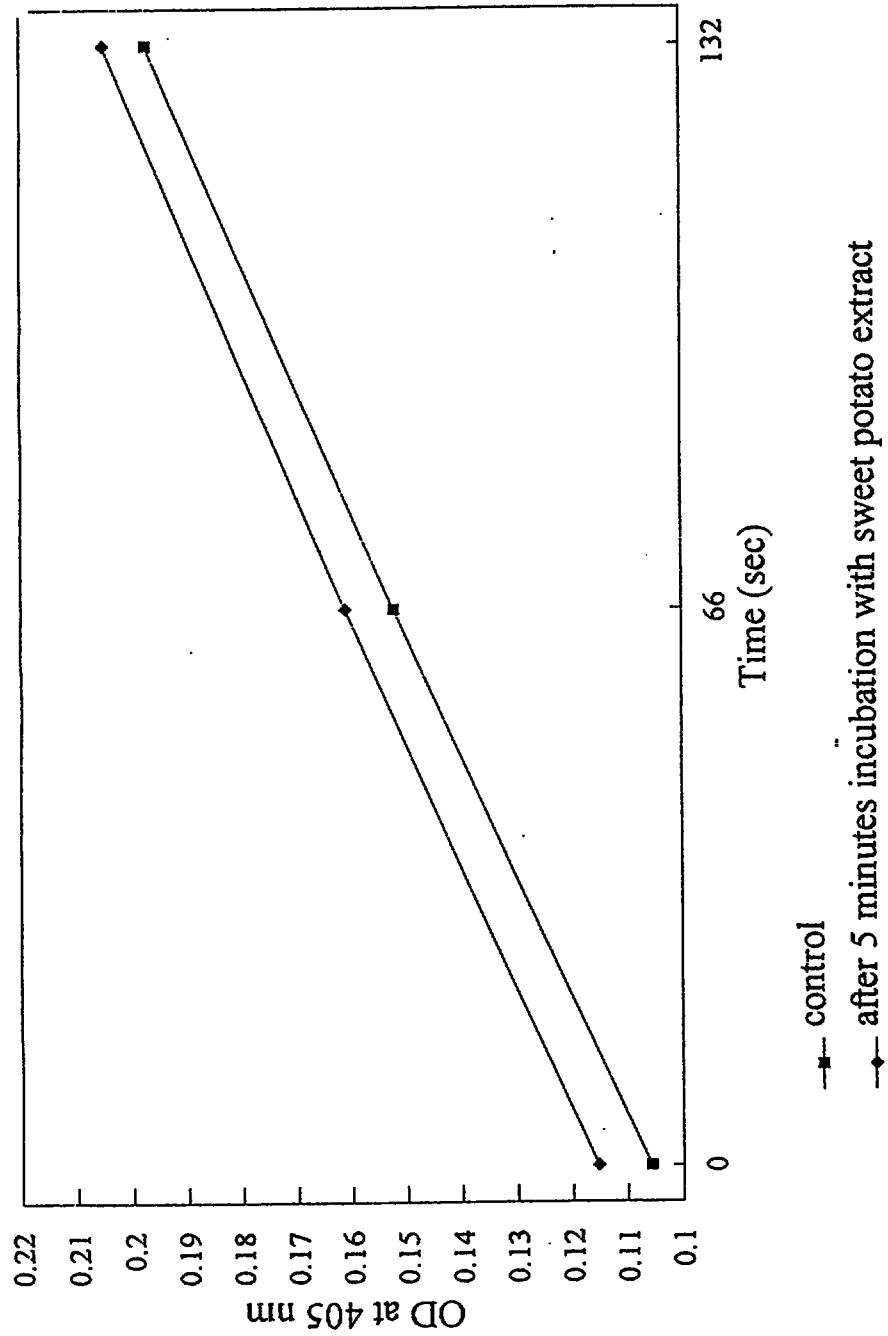


FIGURE 3

This is the replacement sheet

Date: 03 AUG 1996

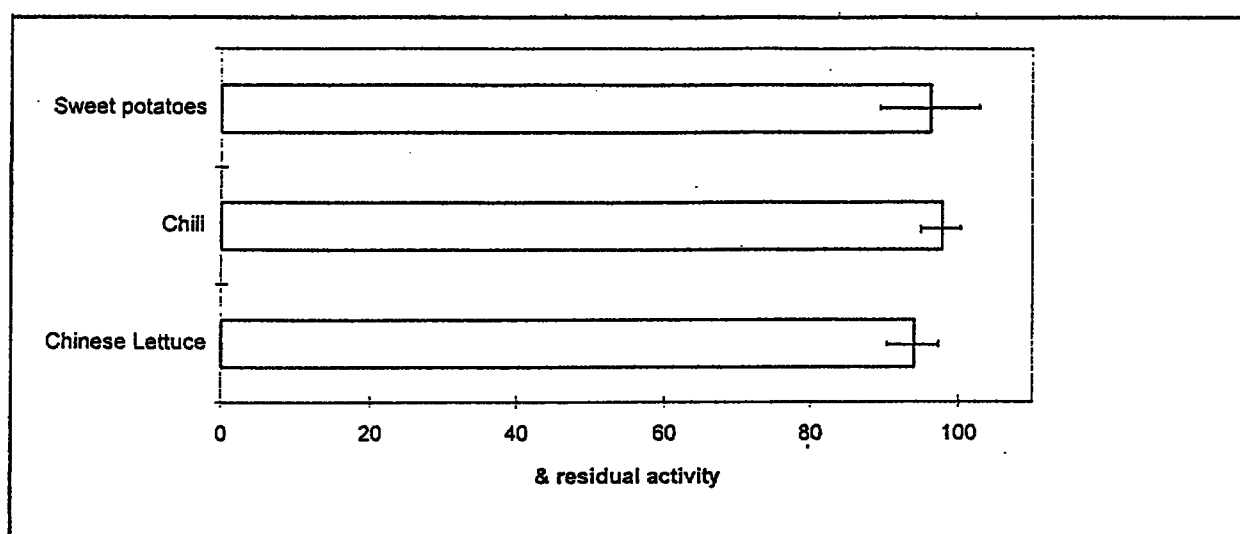


FIGURE 4

This is the replacement sheet

Date: 03 AUG 1996

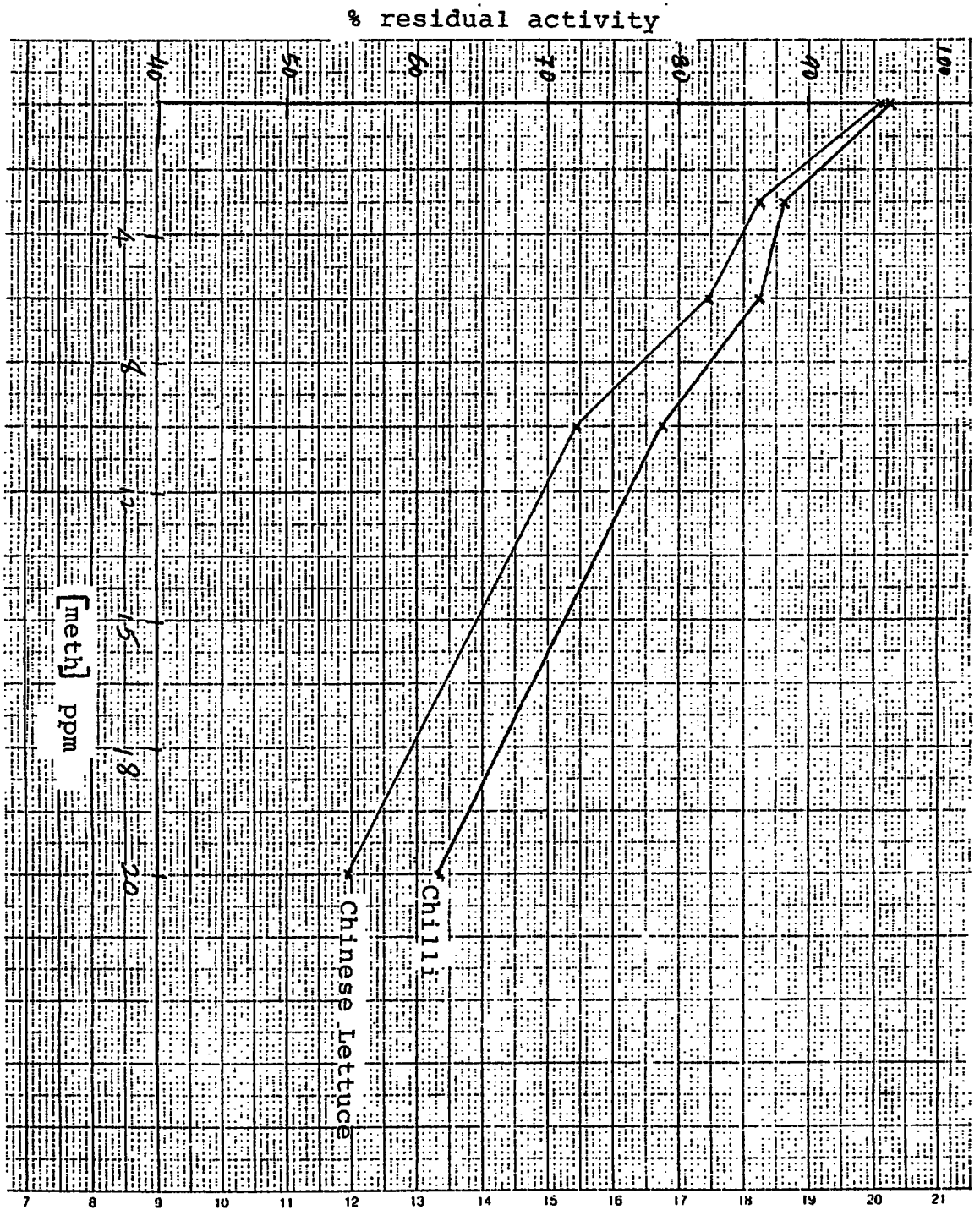
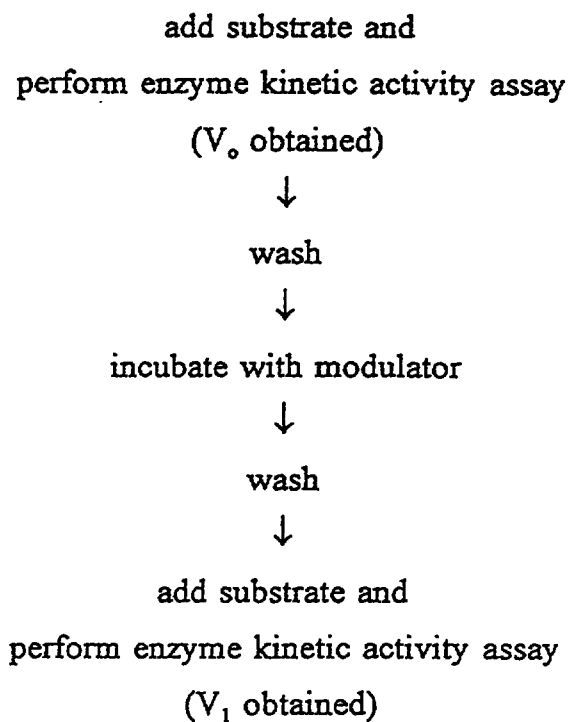


FIGURE 5

this is the replacement sheet

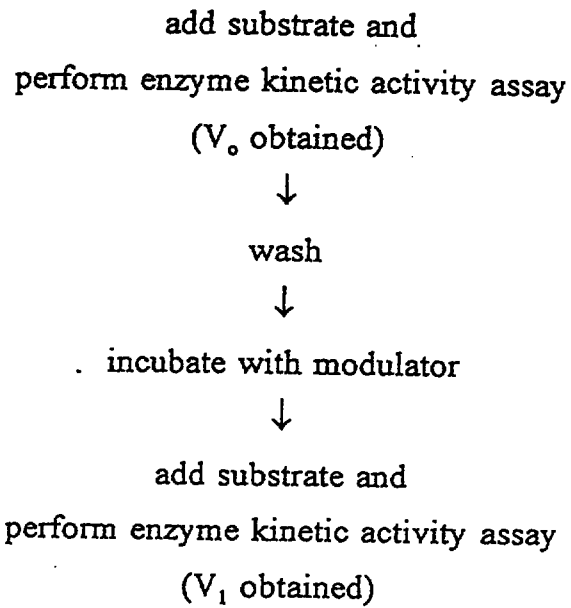
Date: 03 AUG 1996



STEPS OF A STANDARD IRREVERSIBLE MODULATOR
ASSAY

FIGURE 6

This is the replacement sheet
Date: 03 AUG 1996



STEPS OF REVERSIBLE MODULATOR ASSAY

FIGURE 7

This is the replacement sheet

Date: 03 AUG 1996

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank		Negative control			Positive control						
B				Duplicate of test samples								
C												
D												
E												
F												
G												
H												

Figure 8

This is the replacement sheet

Date: 03 AUG 1996

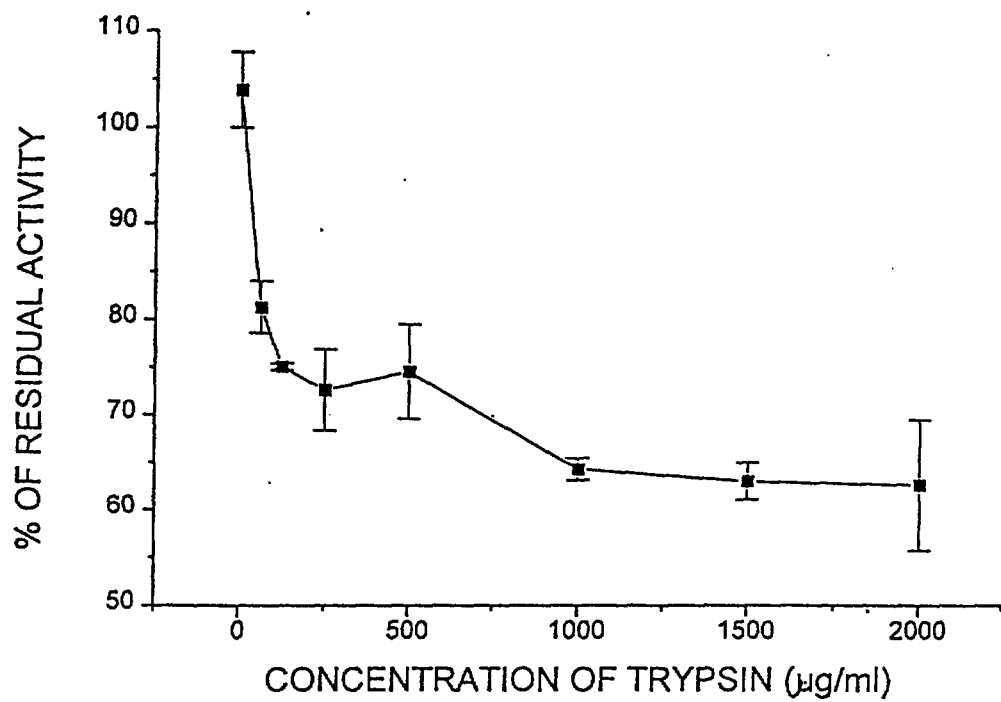


Figure 9

This is the replacement sheet

Date: 03 AUG 1996

fit for consumption. Methamidophos (meth) is one of the most frequently used pesticides in S.E. Asia. Therefore it has been chosen as the modulator (inhibitor) as an example of the applications of the present technique.

5

Acetylcholinesterase (AChE) is an enzyme found in nervous tissue that is inhibited by organophosphate and carbamate pesticides. Its activity can be monitored in the Ellman's assay using acetylthiocholine iodide (ATCI) and 5,5"-dithio-bis-2-nitrobenzoic acid (DTNB) in the following reactions where 5-thio-2-nitrobenzoate (TNB) can be monitored photometrically at 405nm:

10

AChE

ATCI + H₂O ----- > acetic acid + thiocholine

15

thiocholine +DTNB --- > 2-nitrobenzoate-5-mercaptothiocholine
+
TNB

20 In the present system, AChE is covalently immobilized onto microtiter plates activated with maleic anhydride. V_o of its reaction was monitored by doing 3 absorbance readings with 30- or 60-second shaking intervals. V_o is expressed as the change in absorbance at 405nm (A₄₀₅) with time or dA/dt. The Ellman's reagent was then rinsed off and the assay repeated with fresh
25 Ellman's reagent. The assay was performed for a total of three times. This serves as the unmodulated control. After rinsing off the Ellman's reagent the third time, methamidophos at various concentrations are added into their respective wells.

30

Materials and Methods

This is the replacement sheet

Date: 28 FEB 2000