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DEVELOPMENT OF ENZYMATIC METHOD FOR ENVIRONMENTAL MONITORING OF MONOCROTOPHOS

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Abstract

In the present study, we have developed a simple, low cost, viable and sensitive enzymatic method for detection, separation and identification of an organophosphorus pesticide, monocrotophos from environmental samples. This technique is based on the inhibition of enzyme, Succinate Dehydrogenase (SDH) (EC.No. 1.3.5.1). The enzyme (SDH) specifically binds to the substrate (sodium succine) and develops colour in the presence of chromogenic reagent. This chromogenic reagent contains INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl tetrazolium chloride) and PMS (N-methyl phenazonium methosulphate). Chicken liver, egg albumin and *Escherichia coli* were used as the sources of SDH enzyme. Simple adsorption technique has applied for the preparation of enzyme and substrate strips using wattman no. 3 filter paper as a solid support system. Aqueous standards of monocrotophos was prepared and tested for the inhibition. The concentration as low as six micrograms of monocrotophos was successfully detected. Separation and identification of monocrotophos was done by micro thin layer chromatography (TLC) combined with the enzymatic method using the same enzyme inhibition principle. The developed enzymatic method was successfully applied for detection, separation and identification of monocrotophos from environmental samples.

Keywords: Monocrotophos, SDH, Detection, Separation, Identification, Environmental samples

Introduction

Monocrotophos, is an organophosphorus pesticide (OPP), widely used against pests to protect economically important crops (Lee et al. 1990; Tomlin, 1994). It is 100% water soluble, hence, may appear in wastewater released from manufacturing units. It may remain as soil residue when sprayed on crops and also enter into surface and ground water through leaching from soil (Tomlin, 1995). Monocrotophos is classified under extremely hazardous category. Like other OPPs, monocrotophos inhibits acetylcholinesterase (AChE) which is an essential enzyme for normal nerve impulse transmission. It is a systemic pesticide and it's action is mainly on organs including skin, eyes and central nervous system (CNS). It has also been shown to cause delayed neuropathy (Lee et al. 1990). Monocrotophos has the ability to interact with genetic material and cause chromosomal damage to mammalian cells (Kalyan et al. 2009; Paulo et al. 1996). Amr (1999) study on Egypt farmers has shown 50% of them being effected neurologically and the signs being superficial or deep sensory loss and decrease or lost reflexes in their ankle or ankle and knee (Amr, 1999).

Residues of monocrotophos from environmental samples can be detected and determined by various instrumental methods like HPLC-MS, GC and GC-MS (Donnelly et al. 1900) or AChE based enzymatic methods (Arun et al. 2009). Use of instrumental methods are cumbersome, time consuming and costly. AChE enzyme based methods are expensive, due to the use of purified enzymes and costly immobilized matrices. The enzymes used in these methods are also not stable for a long time at room temperature (37ºC). The aim of the present study is to develop a simple, sensitive, inexpensive enzymatic method which is rapidly performable with viable result for detection, separation and identification of monocrotophos from environmental samples.

Materials and Methods

Monocrotophos and other materials

Monocrotophos (IUPAC Name: Dimethyl (E) -1-methyl-2-methyl-2- (methylcarbamoyl) vinylphosphate) (98% pure) was procured from Hyderabad chemicals, Hyderabad, India. Fresh broiler chick liver and eggs were purchased from Model Rythu Chicken Bazaar (MRCB), Hyderabad, India. *Escherichia coli* wild strain was previously isolated from soil and identified by the 16s rRNA gene sequencing at biotechnology laboratory, JNTUH, Hyderabad. Wattman no. 3 filter paper sheets (size 60x80 cm) and silica gel coated aluminum TLC (Thin Layer Chromatography) plates (15x12 cm) were supplied by HIMEDIA. Sodium succinate (anhydrous), INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl

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tetrazolium chloride), PMS (N-methyl phenazonium methosulphate) and also media components including nutrient broth and nutrient agar were procured from HIMEDIA. The organic solvents such as absolute alcohol, acetone, benzene, chloroform, ethyl acetate, hexane and methanol were purchased from MERCK.

Crude enzyme

The crude enzyme for Succinate Dehydrogenase (SDH) (EC.No. 1.3.5.1) was prepared from different sources such as: chick liver, egg albumin and microorganisms (*Escherichia coli*). Fresh chick liver was washed twice with distilled water, and the homogenate (10% (w/v)) was prepared in ice cold distilled water. Homogenate was filtered with four layered cheesecloth and filtrate was lyophilized. This lyophilized powder was used as SDH enzyme source. Egg albumin was extracted from fresh egg and an emulsion was prepared at 20% (v/v) concentration in distilled water. This emulsion was lyophilized and the powder was directly used as the source of SDH enzyme. *E. coli* was inoculated in nutrient broth and incubated at 37˚C on aerobic condition. The culture was harvested (at Optical Density - 0.3) by centrifugation at 10,000 rpm, at 4˚C for 5 minutes. Pellet was collected and 5% homogenate was prepared in ice cold distilled water. The homogenate was lyophilized and used as enzyme source.

Chromogenic reagent

Chromogenic reagent was prepared freshly in 10:10:2 ratio by 2.5% of sodium succinate (w/v), 0.4% of INT (w/v), and 0.1% of PMS (w/v) in distilled water.

Enzyme strips

Wattman no. 3 filter paper strips (5x2 cm) were dipped in the emulsion which was prepared by lyophilized egg albumin powder in acetone (w/v). Strips were dried at room temperature (37ºC) and stored in a plastic bag at refrigerated conditions (4ºC) to optimize the enzymatic method for detection. The method is same for preparation of both chick liver and *E. coli* enzymatic strips.

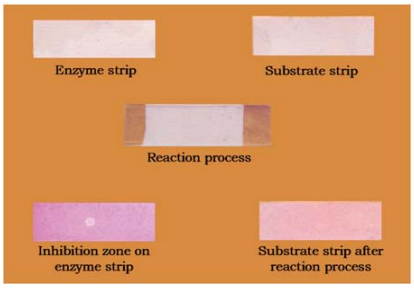
Substrate strips

Filter paper strips (wattman no. 3) (5x2 cm) were dipped in freshly prepared chromogenic reagent and dried at room temperature (37ºC). If necessary, the strips were dried with hair drier (Prameela Devi and Nanda Kumar, 1983) and stored in a plastic bag at refrigerator (4ºC).

Optimization of enzymatic method for detection

The method was optimized by visual technique based on the pink color formazan formed during the enzyme reaction on enzyme strip. As shown in fig 1, the enzyme and substrate strips were placed on separate glass slides. Distilled water or buffer was sprayed on both enzyme and substrate strips and sandwiched between two slides. These slides were incubated to facilitate the enzyme reaction. Optimum pH for SDH enzyme was standardized by spraying 0.2 M potassium phosphate buffer at various pH i.e., 4-9. Optimum temperature and incubation time were standardized at 10 to 80˚C and 1 to 10 minutes respectively. The optimum enzyme concentration was tested in the range of 0.1 to 0.5 % for *E. coli* enzymatic strips and 0.5 to 2% (w/v) for both chick liver and egg albumin enzymatic strips for detection.

Fig 1: Detection process of monocrotophos by enzyme detector strip method



Detection of monocrotophos by enzyme detector strip method

Standards preparation & application

Monocrotophos standards were freshly prepared (10 mg/mL (w/v)) in tightly capped brown glass containers and inhibition studies were conducted within 2-3 hours of standard preparation. Standards were applied (in 2 mm diameter) on enzyme strip with the help of 10 µl graduated micro capillary (fig 1). Precaution was taken to minimize the spread of applied spot by frequent drying.

Detection process

As shown in fig 1, both the enzyme and substrate strips were placed on separate glass slides and distilled water was sprayed. Care must be taken restrict leaching of water through strips. The substrate strip was kept over the enzyme strip and sandwiched between two slides. These slides were incubated at 40ºC in an incubator for 3 minutes. After incubation, the strips were separated from the slides and inhibition zone was observed on the enzyme strip.

Separation and identification of monocrotophos

Standards preparation and application

As revealed in the detection process, standard was prepared and 2 µl of standard was applied on TLC plate.

Separation and identification

As shown in table 2, various solvent systems were attempted for separation of monocrotophos. After separation on TLC plates, the plates were dried at room temperature (37ºC) and 1% egg albumin enzyme emulsion in distilled water (w/v) was sprayed. The

plates were incubated at room temperature (37ºC) for 3 minutes to allow the enzyme-inhibitor reaction. Chromogenic reagent was sprayed on TLC plates and again incubated at 60˚C for 2 minutes. After successful incubation period, the plates were observed for a white chromatogram against pink back ground. Based on the chromatogram, the R_f value was noted (Uma Maheswara Rao and prameela Devi, 2008).

Table 1: Lower detection limits of monocrotophos with various enzyme source by developed enzymatic method

S.No	Enzyme source	Detection limits (µg)
1.	Egg albumin	6
2.	Chick liver	8
3.	E. coli	8

Environmental sample preparation and analysis by the developed enzymatic method

The present work was undertaken to exhibit the applicability of developed enzymatic method for detection, separation and identification of monocrotophos from environmental samples. Water and soil samples were collected from industrial and agricultural areas of Balanagar and Jeedimetla, Hyderabad. All the samples were collected in fresh plastic bags and analyzed within 4 hours of collection.

The water samples were filtered using whatman no. 40 filter paper. The monocrotophos present in the samples (1 litre) were extracted into 20 ml of hexane and further concentrated using rota vapour at 37°C. After complete removal of hexane, the residue was dissolved in 1 ml of distilled water. Soil samples (50 gm) were ground and extracted with 100 ml of distilled water. This water was separated from soil by filtration and concentrated by the above mentioned procedure. After hexane evaporation, the residue was dissolved in 1 ml of distilled water for evaluating monocrotophos. The samples were analyzed by the developed enzymatic method as well as by GC-MS to know the presence of monocrotophos.

Result and Discussion

Basic principle of field method

The principle involved in detection and identification of monocrotophos is dependent on the biochemical reaction between monocrotophos and SDH enzyme (inhibition reaction). Enzymes are extremely specific in catalyzing unique chemical reactions. The SDH is a member of citric acid cycle, that catalyses oxidation of succinate to fumarate (Michele et al, 2004). Activity of this enzyme is generally determined by colorimetric method, based on the reduction of tetrazolium salts to deeply colored, water insoluble, formazan in the presence of substrate (sodium succinate) (Glick and Nayyar, 1956; Defendi, 1955; Kun and Ahood, 1949). The enzyme reaction is inhibited in the presence of inhibitor and no formazan formation takes place. This kind of inhibitory nature is made use for detection and identification of monocrotophos from environmental samples. In the current experiment INT was used as a tetrazolium salt and PMS, as an exogenous electron carrier to speed up the reaction process. The earlier work on the same principle was done for monitoring of some heavy metals (Damayanthi et al., 2006; Prameela Devi and Nanda Kumar, 1981).

Inhibition based methods have been formulated for analysis of various pesticides. There are different types of enzymes which are used in enzymatic methods including Acetylcholinesterase (Joshi et al., 2005; Suwansa-ard et al., 2005; Sotiropoulou and Chaniotakis, 2005; Nikolelis et al., 2005; Crew et al., 2004; Boni et al., 2004; Nunes et al., 2004; Schulze et al., 2003; Dzyadevych et al., 2003; Gulla et al., 2002; Andreescu et al., 2002; Jeanty et al., 2002; Del Carlo et al., 2002; Choi et al., 2001; Zhang et al., 2001; Lee et al., 2001; Xavier et al., 2000; Lee et al., 2000; Jeanty et al., 2001), Butyrylcholinesterase (Suprun et al., 2004; Ivanov et al, 2003; Wan et al., 2000), Choline oxidase (Ciucu et al., 2003; Ciucu and Ciucu. 2002; Kok et al., 2002; Zhang et al., 2001), Horseradich peroxidase (Ciucu and Ciucu. 2002), Organophosphorus-hydrolase (Simonian et al., 2005; White and Harmon, 2005), Parathion hydrolase (Sacks et al., 2000), Polyphenol oxidase (El Kaoutit et al., 2004), Succinate Dehydrogenase (Uma Maheswara Rao and prameela Devi, 2008), Tyrosinase (Vedrine et al., 2003) and also some recombinent enzymes (Sofia et al., 2005; Bachmann et al., 2000).

The enzymatic methods are generally sensitive and specific when compared to the chemical methods (Nanda Kumar and Prameela Devi, 1981; Udaya Bhaskar and Nanda Kumar 1981a, 1981b, 1980). These enzymatic methods can operate under extremely mild conditions of pH and temperature and can generally induce fast reaction rates. Their specificity enables them to catalyze specific chemical reactions. The application of enzymatic methods based on SDH using paper and micro TLC for detection and determination of heavy metal compounds was reported for the first time by Prameela Devi and Nanda Kumar in 1981. A portable method using bio-detector strip was also reported for detection of some heavy metal compounds (Nanda Kumar and Prameela Devi, 1981; Prameela Devi and Nanda Kumar, 1981). However, this is the first enzymatic method for detection,

separation and identification of monocrotophos from environmental samples by SDH enzyme.

Biodetector strips and substrate strips

Immobilization of enzymes for development of enzymatic methods is a critical step. A large number of expensive matrices are employed for immobilization of enzymes, such as; nylon net (Gulla et al., 2002), nanoporous carbon matrix (Sofia and Nikos, 2005), screen printed electrodes (Ivanov et al., 2003) and negatively charged polymer (Soldatkin et al., 2000). However, in the present experiment the authors employed a low cost wattman no. 3 filter paper strips as a matrix and also the simple immobilization technique (adsorption) was used to immobilize both the enzyme and substrate.

Optimum conditions of enzymatic method for detection

Generally, an enzymatic method requires an optimum condition to work and get a proper result. The incubation time was optimized at 3 minutes time for all SDH enzyme sources. The optimum enzyme concentration for *E. coli* enzymatic strip was found at 0.2% and 1% for both the chick liver and egg albumin SDH enzyme strips. The temperature was optimized at 40˚C for the all enzyme sources. pH was found to be optimum at pH 7, when tested with 0.2 M potassium phosphate buffer in the range of pH 4-9. But, in the present experiment, when distilled water was used for spraying on both enzyme and substrate strips, no effect was observed.

Various enzyme sources and detection limit of monocrotophos

As shown in table 1, the lower detection limit of monocrotophos with egg albumin SDH is 6 µg. It is 8 µg with both the chick liver and *E. coli* SDH enzyme source. Egg albumin has shown lowest detection limit and it was recommended as the best enzyme source for detection of monocrotophos. Because of this reason, egg albumin enzyme source was only used in separation and identification of monocrotophos. Prameela Devi and Nanda Kumar (1981), have used the same detector strip method for identification of some selected organophosphorus pesticides using the enzyme cholinesterase (Prameela Devi and Nanda Kumar, 1981). However, this is the first enzymatic method for SDH enzyme based detection of monocrotophos.

Separation and identification of monocrotophos

Technical grade sample of monocrotophos was successfully separated and identified by TLC base enzymatic method. Different solvent systems were tried and acetone was found to be the best solvent system with an R_f value of 0.68, also the chromatograms were compact and clear (table 2, fig. 2). The mixture of pesticides can be separated and the monocrotophos alone can be identified by this TLC based enzymatic method. The white spot against pink back ground on micro TLC represents the inhibition zone of monocrotophos on enzyme SDH (Uma Maheswara Rao and Prameela Devi, 2008)

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Table 2: Optimization of solvent systems for separation and identification of monocrotophos by micro TLC based enzymatic method

S.No.	Solvent system	Rf value	Remark
1.	Hexane	0.00	*
2.	Chloroform	0.00	*
3.	Acetone	0.68±0.01	***
4.	Ethyl acetate	0.28±0.01	**
5.	Methanol	0.78±0.02	**
6.	Distilled water	0.80±0.02	**
7.	Acetone : Hexane (8:2)	0.65±0.01	**
8.	Acetone : Ethyl acetate (1:1)	0.63±0.02	**
9.	Acetone : Hexane(6:4)	0.43±0.02	**
10.	Acetone : Chloroform (6:4)	0.54±0.01	**
11.	Acetone : Ethyl acetate: Hexane(4:4:3)	0.46±0.02	**
12.	Benzene	0.00	*
13.	Absolute alcohol	0.68±0.01	**
14.	Acetone: Ethyl acetate :Hexane :Chloroform (4:3:2:1)	0.46±0.01	**
15.	Chloroform : Ethyl acetate :Acetone (4:3:3)	0.39±0.02	**

* No movement

** The chromatogram is not compact and clear

*** The chromatogram is compact and clear, hence the solvent system is recommended

Data are mean of 3 observations (±SD)

Solvent system and separation and identification of monocrotophos

A huge number of solvent systems are recommended and reported in literature for separation and identification monocrotophos by TLC. Those solvent systems may also be useful for other enzymatic methods. However, the solvent system recommended in the present study for separation and identification of monocrotophos is highly suitable for the developed enzymatic method (table 2, fig. 2). Some organic

solvents act on enzymes by inactivation or denaturation (Amine et al., 2004 and Alexander, 2001). This posed the problem of choosing a solvent system that is enzyme tolerant. The solvent system which was chosen for enzymatic method should not denature or inactivate or inhibit the enzyme and at the same time it should separate compound on micro TLC plate for better resolution.

Analysis of environmental samples

Monocrotophos was detected in both the Balanagar and Jeedimetla pesticide industrial wastewater by the developed enzymatic method. However, it is not detected in Agricultural wastewater samples. Monocrotophos was detected only in Balanagar pesticide industrial area soil sample, but not found in Agricultural land and Jeedimetla pesticide industrial area soil samples (table 3).

Fig 2: Separation and identification of monocrotophos by micro TLC based enzymatic method. Solvent systems: (a) Hexane; (b)Chloroform; (c)Acetone; (d) Ethyl acetate; (e)Methanol; (f) Distilled water; (g) Acetone : Hexane (8:2); (h) Acetone : Ethyl acetate (1:1); (i) Acetone : Hexane(6:4); (j) Acetone : Chloroform (6:4); (k) Acetone : Ethylacetate : Hexane(4:4:3); (l)Benzene; (m)Absolute alcohol; (n) Acetone: Ethyl acetate : Hexane : Chloroform (4:3:2:1); (o) Chloroform : Ethyl acetate : Acetone (4:3:3)

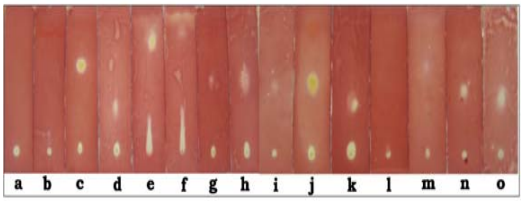

Table 3: Analysis of environmental samples for presence of monocrotophos

S.No.	Sample	By developed enzymatic method		Quantification by GC-MS (ppm)
		Detection	Separation and Identification	
Water Samples				
1.	Agricultural waste water	- ve	- ve	- ve
2.	Balanagar pesticide industrial waste water	+ve	+ve	0.048 ± 0.002
3.	Jeedimetla pesticide industrial waste water	+ve	+ve	0.022 ± 0.006
Soil Samples				
1.	Agricultural land soil	- ve	- ve	- ve
2.	Balanagar pesticide industrial area soil	+ve	+ve	0.021 ± 0.001
3.	Jeedimetla pesticide industrial area soil	- ve	- ve	- ve

Data are mean of 3 observations (±SD)

As shown in table 3, all samples which were detected by enzymatic method have shown positive result in separated and identified. When environmental samples were confirmed by GC-MS, the positive result samples have given positive result by GC-MS. The concentration of monocrotophos in Balanagar and Jeedimetla pesticide industrial waste water is 0.048 and 0.022 ppm respectively. It is 0.021 ppm in Balanagar pesticide industrial area soil sample. Concentration of monocrotophos is very low in environmental samples. Generally, the enzymatic methods are not suitable with these concentrations. But, here the authors developed a procedure for concentration of monocrotophos after extraction from environmental samples.

Conclusion

The enzymatic method developed in the present study, is very useful for quick and timely monitoring of monocrotophos from environmental sample, which otherwise would have been time consuming, expensive

and unsuitable for field use when sophisticated equipment employed.

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