

Recent Research in Science and Technology 2011, 3(4): 58-65

ISSN: 2076-5061

www.recent-science.com



ENVIRONMENTAL SCIENCES

DEVELOPMENT OF ENZYMATIC METHOD FOR ENVIRONMENTAL MONITORING OF MONOCROTOPHOS

Uma Maheswara Rao Ganguru¹ and Prameela Devi Yalavarthy^{2*}

¹Center for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad 500085, India

²Environmental Biology Laboratory, Department of Zoology, Kakatiya University, Warangal 506009, India

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 succinate) 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 its 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 (37°C). 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

* Corresponding Author, Email: prameeladevi@rediffmail.com

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 (37°C) and stored in a plastic bag at refrigerated conditions (4°C) to optimize the enzymatic method for detection. The method is same for preparation of both chick liver and *E. coli* enzymatic strips.

Substrate strips

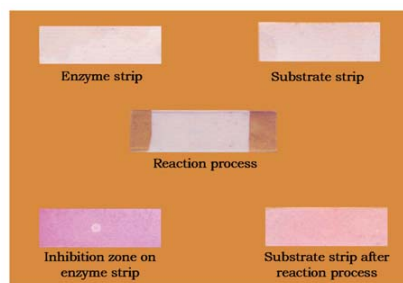
Filter paper strips (wattman no. 3) (5x2 cm) were dipped in freshly prepared chromogenic reagent and dried at room temperature (37°C). If necessary, the strips were dried with hair drier (Prameela Devi and Nanda Kumar, 1983) and stored in a plastic bag at refrigerator (4°C).

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 40°C 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 (37°C) and 1% egg albumin enzyme emulsion in distilled water (w/v) was sprayed. The

plates were incubated at room temperature (37°C) 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 (μ g) |
|------|---------------|-----------------------------|
| 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 recombinant 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)

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 : Ethyl acetate : 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)

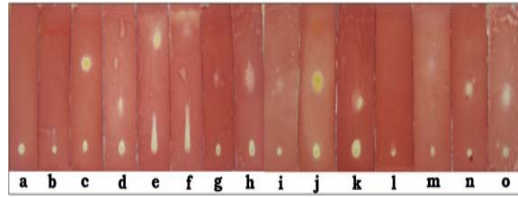


Table 3: Analysis of environmental samples for presence of monocrotophos

| 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.

Acknowledgements

The authors acknowledge UGC, INDIA for the financial support offered to this project and selecting one of the authors, Prof. Yalavarthy Prameela Devi for UGC Research Award 2006. The authors thank JNTUH authorities for providing laboratory facilities in undertaking this research work.

References

- Amr M. (1999). Pesticide monitoring and its health problems in Egypt, a Third World country. *Toxicology Letters*. 107(1-3):1-13.
- Alexander M. Klibanov (2001). Improving enzymes by using them in organic solvents. *Nature*. 409: 241-246.
- Amine A., Mohammadi H., Arduini F., Ricci F., Moscone D. Palleschi G. (2004). Extraction of enzyme inhibitors using a mixture of organic solvent and aqueous solution and their detection with electrochemical biosensors. In: Proceedings of the Eighth World Congress on Biosensors, Granada, Spain, p. 3.7.64.
- Andreescu S., Noguer T., Magearu V. and Marty J.-L., (2002). Screenprinted electrode based on AChE for the detection of pesticides in presence of organic solvents. *Talanta* 57, 169–176.
- Arun Prakash Periasamy, Yogeswaran Umasankar and Shen-Ming Chen (2009). Nanomaterials - Acetylcholinesterase Enzyme Matrices for Organophosphorus Pesticides Electrochemical Sensors: A Review. *Sensors*. 9:4034-4055.
- Bachmann T.T., Leca B., Villat F., Marty. J.L., Fournier D. and Schmid R.D. (2000) Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of *Drosophila* acetylcholinesterase and artificial neural networks. *Biosens. Bioelectron.* 15, 193-201.
- Boni A., Cremisini C., Magano E., Tosi M., Vastarella W. and Pilloton (2004). Optimized biosensors based on purified enzymes and engineered yeasts: detection of inhibitors of cholinesterases on grapes. *Anal. Lett.* 37:1683-1699.
- Choi J.W., Kim Y.K., Lee I.H., Min J. and Lee W.H. (2001). Optical organophosphorus biosensor consisting acetyl cholinesterase/ viologen hetero Langmuir-Blodgett film. *Biosens. Bioelectron.* 16:937-943.
- Ciucu A., Negulescu, C. and Baldwin, R.P. (2003). Detection of pesticides using an amperometric biosensor based on ferrophthalocyanine chemically modified carbon paste electrode and immobilized bienzymatic system. *Biosens. Bioelectron.* 18, 303–310.
- Ciucu. A. and Ciucu. C., (2002). Organic phase amperometric biosensor for detection of pesticides. *Roum. Biotechnol. Lett.* 7: 667-676.
- Crew A., Hart J.P., Wedge R., Marty J.L. and Fournier D. (2004). A screen-printed, amperometric, biosensor array for the detection of organophosphate pesticides based on inhibition of wild type, and mutant acetylcholinesterases, from *Drosophila melanogaster*. *Anal. Lett.* 37:1601-1610.
- Damayanthi P. Balashouri and Prameela Devi Y. (2006). Simple paper and thin layer chromatographic enzymatic methods for separation and detection of zinc sulphate in fresh water. *J. Bios.* 1(4):41-45.
- Defendi V. and Pearson B. (1955). Quantitative estimation of succinic dehydrogenase activity in a single microscopic tissue section. *J Histochem Cytochem.* 3:61.
- Del Carlo M., Mascini M., Pepe A., Compagnone D. and Mascini M. (2002). Electrochemical bioassay for the investigation of chlorpyrifosmethyl in vine samples. *J. Agric. Food Chem.* 50:7206-7210.
- Donnelly Betowski L and Tammy L Jones (1900). Analysis of Organophosphorus Pesticide Samples by High-Performance Liquid Chromatography/Mass Spectrometry and High-Performance Liquid Chromatography/Mass Spectrometry/Mass Spectrometry. *Environ. Sci. Technol.* 22:1430-1434.
- Dzyadevych S.V., Soldatkin A.P., Korpan Y.I., Arkhypova V.N., El'skaya A.V., Chovelon J.M., Martelet C. and Jaffrezic Renault N. (2003). Biosensors based on enzyme field-effect transistors for determination of some substrates and inhibitors. *Anal. Bioanal. Chem.* 377:496-506.
- El Kaoutit M., Bouchta B., Zejli H., Izaoumen N. and Tamsamani K.R. (2004). A simple conducting polymer-based biosensor for the determination of atrazine. *Anal. Lett.* 37:1671-1681.
- Glick D. and Nayyar SN. (1956). Studies in histochemistry, XLII. Further studies on the determination of succinic dehydrogenase in microgram amounts of tissue and distribution of the activity in the bovine adrenal. *J Histochem Cytochem.* 4:389.
- Gulla K.C., Gouda M.D., Thakur M.S. and Karanth N.G. (2002). Reactivation of immobilized acetylcholinesterase in an amperometric biosensor for organophosphorus pesticide. *Biochim. Biophys. Acta.* 1597:133-139.
- Ivanov A., Evtugyn G., Budnikov H.C., Ricci F., Moscone D. and Palleschi G. (2003). Cholinesterase sensors based on screen-printed electrode for detection of organophosphorus and

- carbamic pesticides. *Anal. Bioanal. Chem.* 377:624-631.
- Jeanty G., Ghommidh Gh. and Marty J.L. (2001). Automated detection of chlorpyrifos and its metabolites by a continuous flow system-based enzyme sensor. *Anal. Chim. Acta.* 436:119-128.
- Jeanty G., Wojciechowska A. and Marty J.L. (2002). Flow-injection amperometric determination of pesticides on the basis of their inhibition of immobilized acetylcholinesterases of different origin. *Anal. Bioanal. Chem.* 373:691-695.
- Joshi K.A., Tang J., Haddon R., Wang J., Chen W. and Mulchandani A. (2005). A disposable biosensor for organophosphorus nerve agents based on carbon nanotubes modified thick film strip electrode. *Electroanalysis.* 17:54-58.
- Kalyan Chakravarthi B, Rambabu Naravaneni, Philip GH. and Srinivasulu Reddy C. (2009). Investigation of monocrotophos toxic effects on human lymphocytes at cytogenetic level. *African Journal of Biotechnology.* 8(10):2042-2046.
- Kok F.N., Bozoglu F. and Hasirci V. (2002). Construction of an acetylcholinesterase-choline oxidase biosensor for aldicarb determination. *Biosens. Bioelectron.* 17, 531-539.
- Kun E. and Ahood LG. (1949). Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. *Science* 109: 144.
- Lee H.S., Kim Y.A., Chung D.H. and Lee T.Y. (2001). Determination of carbamate pesticides by a cholinesterase-based flow injection biosensor. *Int. J. Food Sci. Technol.* 36:263-269.
- Lee P.W., Fukuto J.M., Hernandez H. and Stearns SM. (1990). Fate of monocrotophos in the environment. *Journal of Agricultural Food Chemistry.* 38(2):567-573.
- Lee W.E., Thompson H.G., Hall J.G. and Bader. D.E. (2000). Rapid detection and identification of biological and chemical agents by immunoassay, gene probe assay and enzyme inhibition using siliconbased biosensor. *Biosens. Bioelectron.* 14:784-804.
- Michele Saliola, Paola Chiara Bartoccioni, Ilaria De Maria, Tiziana Lodi, and Claudio Falcone (2004). The Deletion of the Succinate Dehydrogenase Gene *Klsdh1* in *Kluyveromyces lactis* Does Not Lead to Respiratory Deficiency, *Eukaryotic Cell*, 3 (3): 589-597.
- Nanda Kumar N.V. and Prameela Devi. Y. (1981). A new paper and micro TLC method for determination of mercuric chloride and detection of copper sulphate. *J. Assn. Offic. Anal. Chem.* 64:729.
- Nikolelis D.P., Simantiraki M.G., Siontorou C.G. and Toth K. (2005). Flow injection analysis of carbofuran in foods using air stable lipid film based acetylcholinesterase biosensor. *Anal. Chim. Acta.* 537:169-177.
- Nunes G.S., Jeanty G. and Marty J.L. (2004). Enzyme immobilization procedures on screen printed electrodes used for the detection of anticholinesterase pesticides Comparative study. *Anal. Chim. Acta.* 523:107-115.
- Paulo Peiti Jr, Elza T Sakamoto Hojo and lice M de Syllos Colus (1996). Genotoxic activity of the insecticide Nuvacron (Monocrotophos) detected by the micronucleus test in bone marrow erythrocytes of mice and in CHO cells. *Brazilian Journal of Genetics.* 19(4):571-576.
- Prameela Devi Y, and Nandha Kumar N. V. (1981). Simple paper and micro thin layer chromatographic method for separation and detection of mercuric chloride, copper sulphate, cadmium Sulphate, and silver nitrate in fresh water. *J. Assoc. Off. Anal. Chem.* 64(6):1301-1304.
- Prameela Devi Y. and Nanda Kumar N. V. (1981). Simple paper and micro thin layer chromatographic method for separation and detection of mercuric chloride, copper sulphate, cadmium Sulphate, and silver nitrate in fresh water. *J. Assoc. Off. Anal. Chem.* 64(6):1301-1304.
- Prameela Devi Y. and Nandha Kumar N. V. (1983). Enzymatic methods for detection and environmental monitoring of select heavy metal compound, Ph.D thesis, Department of Zoology, Sri Venkateswara University, Tirupati. India.
- Sacks V., Eshkenazi I., Neufeld T., Dosoretz C. and Rishpon J. (2000). Immobilized parathion hydrolase: an amperometric sensor for parathion. *Anal. Chem.* 72:2055-2058.
- Schulze H., Vorlova S., Villatte F., Bachmann T.T. and Schmid R.D. (2003). Design of acetylcholinesterases for biosensor applications. *Biosens. Bioelectron.* 18:201-209.
- Simonian A.L., Good T.A., Wang S.S. and Wild J.R. (2005). Nanoparticlebased optical biosensors for the direct detection of organophosphate chemical warfare agent and pesticides. *Anal. Chim. Acta.* 534:69-77.
- Sofia Sotiropoulou and Nicos A. Chaniotakis (2005). Lowering the detection limit of the acetyl cholinesterase biosensor using ananoporous carbon matrix. *Analytica chimica.* 530:199-204.
- Sofia Sotiropoulou., Dider Fournierb and Nikos. A. Chaniotakis (2005). Genetically engineered acetyl cholinesterase based biosensors for attomolar detection of DDVP. *Biosens. Bioelectron.* 20:2347-2352.
- Soldatkin A.P., Volotovskiy V., El'skaya A.V., Jaffrezic Renault N. and Martelet C. (2000). Improvement of urease based biosensor characteristics using additional layers of charged polymers. *Anal. Chim. Acta.* 403:25-29.

- Suprun E.V., Budnikov H.C., Evtugyn G.A. and Brainina Kh.Z. (2004). Bienzyme sensor based on thick-film carbon electrode modified with electropolymerized tyramine. *Bioelectrochemistry*. 63:281-284.
- Suwansa-ard S., Kanatharana P., Asawatreratanakul P., Limsakul C., Wongkittisuksa B. and Thavarungkul P. (2005). Semi disposable reactor biosensors for detecting carbamate pesticides in water. *Biosens. Bioelectron.* 21:445-454.
- Tomlin C. (1994). *The Pesticide Manual*, Tenth Edition, Crop Protection Publications. British Crop Protection Council and Royal Society of Chemistry.
- Tomlin C. (1995). *The Pesticide Manual*. 10th edn. British Crop Protection Council (BCPC).
- Udaya Bhaskar S. and Nanda Kumar N.V. (1980). Colorimetric determination of dimethoate or omethoate. *Talanta*. 27(9): 757- 758.
- Udaya Bhaskar S. and Nanda Kumar N.V. (1981a). Colorimetric determination of malathion as malaaxon in wheat. *Comp. Physiol. Ecol.* 6:502.
- Udaya Bhaskar S. and Nanda Kumar N.V. (1981b). Thin layer chromatographic methods for detection of methyl parathion by liver acetone powder ChE inhibition. *J. Assoc. Offic. Anal. Chem.* 64:1312.
- Uma Maheswra Rao G. and Prameela Devi Y. (2008). Detection, separation and identification of Dichlorvos (DDVP) by enzymatic method. *The Bioscan*. 3(4):773-376.
- Vedrine C., Fabiano S. and Can T.M. (2003). Amperometric tyrosinase based biosensor using an electrogenerated polythiophene film as an entrapment support. *Talanta*. 59:535-544.
- Wan K., Chovelon J.M. and Jaffrezic Renault N. (2000). Enzymeoctadecylamine Langmir Blodgett membranes for ENFET biosensors. *Talanta* 52:663-670.
- White B.J. and Harmon H.J. (2005). Optical solid-state detection of organophosphates using organophosphorus hydrolase. *Biosens. Bioelectron.* 20:1977-1983.
- Xavier M.P., Vallejo B., Marazuela M.D., Moreno-Bondi M.C., Baldini F. and Falia A. (2000). Fiber optic monitoring of carbamate pesticides using porous glass with covalently bound chlorophenol red. *Biosens. Bioelectron.* 14:895-905.
- Zhang S., Zhao H. and John R. (2001). A theoretical model for immobilized enzyme inhibition biosensors. *Electroanalysis*. 13:1528-1534.