"First Order UV Spectrophotometric and RP-HPLC Method for Determination of Pesticides Residue in Vegetables from North Gujarat Region"

A THESIS SUBMITTED TO

NIRMA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

DAVE HARSHIT JAYESHKUMAR
(12MPH306) B. PHARM.

UNDER THE GUIDANCE OF

Dr. CHARMY S. KOTHARI – GUIDE
Assistant Professor, Department of Pharmaceutical Analysis

Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Ahmedabad-382481
Gujarat, India.

May 2014

CERTIFICATE

This is to certify that the dissertation work entitled "First Order UV Spectrophotometric and RP-HPLC Method for Determination of Pesticides Residue in Vegetables from North Gujarat Region" submitted by Mr. Harshit Jayeshkumar Dave with Regn. No. (12MPH306) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.

Guide

21/5/2014

Dr. Charmy S. Kothari
M. Pharm., Ph.D.
Assistant Professor,
Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Forwarded Through:

Prof. Priti J. Mehta
M. Pharm., Ph.D.
Professor & Head,
Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Prof. Manjunath Ghate
M. Pharm., Ph.D.
Director
Institute of Pharmacy,
Nirma University

Date: 21st May, 2014

DECLARATION

I hereby declare that the dissertation entitled ***"First Order UV Spectrophotometric and RP-HPLC Method for Determination of Pesticides Residue in Vegetables from North Gujarat Region"*** *is based on the original work carried out by me under the guidance of Dr. Charmy s. Kothari, Assistant professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.*

Mr. Harshit Jayeshkumar Dave
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University,
Sarkhej - Gandhinagar Highway,
Ahmedabad-382481,
Gujarat, India

Date: 21st ***May, 2014***

ACKNOWLEDGEMENT

*I thank **Almighty** for making my dream come true. I have, for so many years, dreamt of the day that I would acquire my Master's Degree with some novel research and the day has finally come. I believe GOD is the best manager in the world; he manages everyone's life smoothly with ups to build the confidence in us and downs to prevent the penetration of arrogance in us. Every step, he comes to us in some or the other form, it may be a family or a friend or a teacher or a stranger, to help us out.*

*First and foremost I want to thank my guide **Dr. Charmy S. Kothari**, Dept. of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. It has been an honor to be her M.Pharm student. She has taught me, both consciously and unconsciously, how good experimental pharmacy research is done. I appreciate all her contributions of time and ideas to make my M.Pharm experience productive and stimulating. The joy and enthusiasm she has for her research was contagious and motivational for me, even during tough times in the M.Pharm. Her belief and trust in me was a source of determination for me which led to the conversion of an idea into a realistic method development on a novel technique*

.

*I am also thankful to **Dr. Priti J. Mehta,** Head, Dept. of Pharmaceutical Analysis, for providing me with an excellent example a source of inspiration as a successful woman, an analyst and a professor, moreover a perfectionist. I am highly thankful to **Dr. Manjunath Ghate** (Director, Institute of Pharmacy, Nirma University, Ahmedabad) for providing all necessary help and facility for my work and also for his constant support and encouragement.*

***Mr. Nrupesh R. Patel,** Assistant Professor, Dept. of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University was a constant source of support and guidance with his endless patience, knowledge and depth on the subject matter. The members of the institute have contributed immensely to my personal and professional time at Nirma University. I am extremely grateful to **Tejas sir, Komal mam and Prutha mam,** Dept of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University for their valuable suggestions, continuous encouragement and everlasting support throughout this dissertation work.*

*I would like to thank my colleagues **Maharshi, Gauvrav, Ankit, Dhruv, Pratik, Jwal, Ashish, Palak and Dhara.** I would also like to thank my colleaugues from other department **Harshal, Udit, Yash, Mohit, Kishan, and Abhishek.** I would also like to thank my all batchmates of all the departments as constant source of inspiration.*

*I am also thankful to our Lab Assistant **Satej Bhai, Shreyas Bhai** and also to **Bipin Bhai** for providing all the requirements and material as and when required. We would also like to thank the whole library staff to avail us with library facilities during project work.*

*Lastly, I would like to thank **my dad Jayeshkumar Dave, my mom Sudhaben Dave & my loving sister shivani Dave** for all their love and encouragement. For my parents who raised me with a love of science, supported me in all my pursuits and provided me with a vision and a goal to achieve in future. I would also like to thank **my grandma Chandrikaben and my aunt Minaxiben Odedra** for all their love and encouragement. I would like to thank my friends **Anand Odedra, Jaimin Vaidya, Jaydeep Jalu, Bhavin Patel, Dharak Patel, Ketan Macchi, Manish Patel and Jaydeep Dhobi.***

Thank you.
Last, but not the least, I express my gratitude and apologize to anybody whose contributions, I could not mention in this page.

Date:
Harshit J. Dave
Institute of Pharmacy,
Nirma University,
Ahmedabad.

LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
°C	Degree centigrade
±	Plus or Minus
<	Less than
λ	Lambda
%	Percentage
µg	Microgram
µl	Micro liter
Abs.	Absorbance
AR	Analytical Reagent
CAS No.	Chemical Abstract Service Number
Cm	Centimeter
C_{max}	Maximum Plasma Concentration
T_{max}	Maximum Plasma Concentration Time
$T_{1/2}$	Half Life
Conc.	Concentration
FDA	Food and Drug Administration
FT-IR	Fourier Transform Infrared spectrometry
GC	Gas Chromatography
Hr	Hour
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
i.d.	Internal Diameter
IP	Indian Pharmacopoeia
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry

L	Liter
LC	Liquid Chromatography
LLE	Liquid-liquid Extraction
LOQ	Limit of Quantification
LOD	Limit of Detection
FR	Flow Rate
Max	Maximum
Min	Minimum
mL	Mililiter
min	Minute
mm	Milimeter
MRL	Maximum Residual Limit
MS	Mass Spectrometry
N	Number
No.	Number
Ng	Nanogram
Pg.No.	Page number
ppm	Part Per Million
Ref.No.	Reference number
R_t	Retention time
RP	Reverse Phase
R.S.D.	Relative Standard Deviation
S.D.	Standard Deviation
Sec.	Second
SPE	Solid phase extraction
Sr.No.	Serial number
Std.	Standard

UV/Vis	Ultra violet/Visible
UV	Ultra violet
Vol.	Volume
TLC	Thin Layer Chromatography
v/v/v	Volume/Volume/Volume
w/v	Weight / Volume
Temp.	Temperature

INDEX

LIST OF FIGURES

LIST OF TABLES

Abstract

This project work describes the validated UV spectrophotometric and Reverse phase high-performance chromatography (RP-HPLC) methods for simultaneous estimation of chlorpyrifos and prophenofos in vegetables by solid phase extraction and liquid-liquid extraction. The RP-HPLC separation was achieved on a Hibar C_{18} column (250*4.6mm, 5µg) using mobile phase of acetonitrile:water, (90:10,v/v) as the mobile phase at flow rate 1.0ml/min. The quantification was achieved with UV detection at 219nm. UV spectrophotometric method includes first order derivative spectrophotometry method that involves the measerment of the absorbance at 277nm for chlorpyrifos and 289nm for prophenofos. Both of the methods were validated as per ICH guidelines. The comparison of liquid-liquid extraction and solid phase extraction process was also carried out for vegetable samples. The proposed methods for chlorpyrifos and prophenofos were found to be simple, precise and accurate. These methods are applicable for simultaneous estimation of chlorpyrifos and prophenofos in vegetable samples.

Keywords: Chlorpyrifos, Prophenofos, UV spectrophotometric, RP-HPLC, Solid phase extraction, Liquid-liquid extraction.

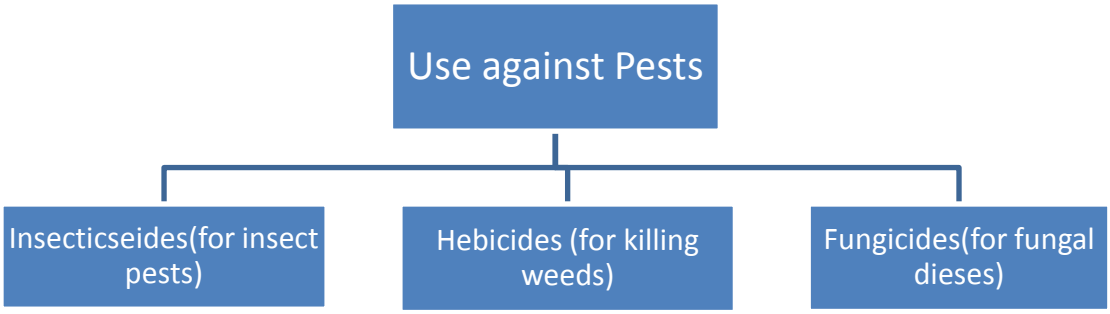
1.1 Introduction of Pesticides:

Pesticides are widely used to improve the quality as well as quantity of crop. Pesticides are chemicals which control and reduce the different types of agricultural pests. In india more than 200 Pesticides are registered for agricultural use to protect agricultural product from different pests. Pesticides are beneficial to improve quality and quantity of crops but overuse of pesticides produce pollution in environment. It is directly affect the human health. Some of the pesticides residue remain in food and agricultural product which consumed by people.[1-3]

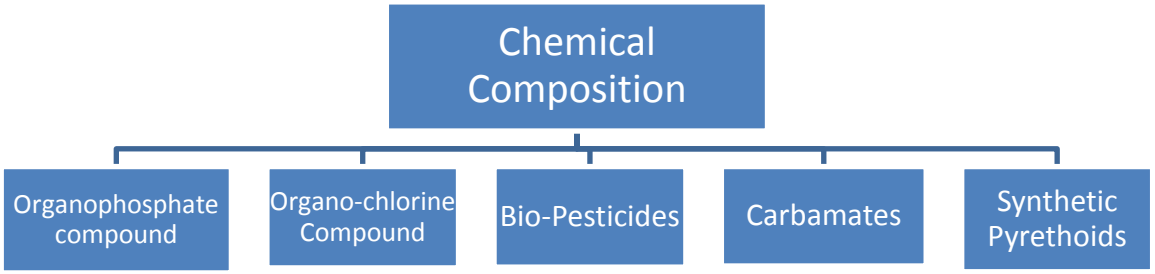
1.1.1 Classification of Pesticides:

Pesticides are classified according to use against pest, Chemical composition and toxicity[6]

A. Use against Pests: This Classification of pesticide is based on mode of action of pesticides on different types of insects, weeds and other organisms. According to the attack of insecticides on food products it's used.

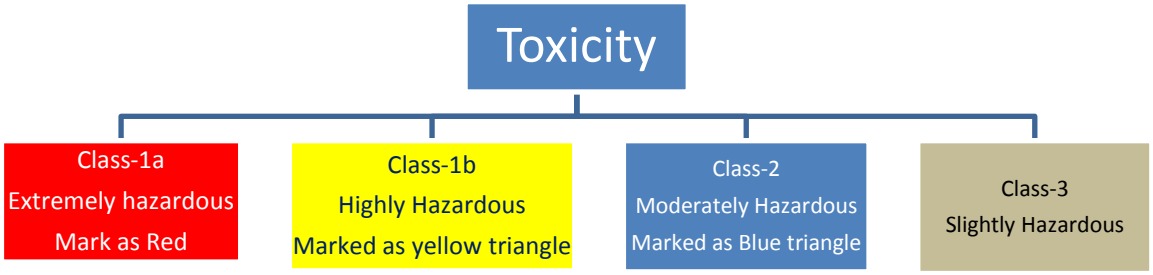


B. Chemical Composition: This classification is based on chemical property of pesticides. Efficacy and mode of action is also different for different classes. For example Organo-chlorine compound- endosulfan, Organophosphate-Chlorpyrifos.



C. Toxicity:

This classification is based on toxicity of pesticides which classified by World Health Organization (WHO). Toxicity classification is beneficial to identify the hazardous effect of pesticides on human. It is easily identify by colour and symbol on container of pesticides.



After application of these kinds of pesticides the active ingredient (compound) of pesticides directly contact with pests and reduce or eliminate the affect of pests but some of the active ingredient remain present in the environment even after use. They are responsible for pollution. The amount of pesticides residue may differ across the sample for different pesticides and region. Due to their hazardous effect on human health it is essential to control the unwanted use of pesticides. So we must check the level of pesticides in environment and different food samples. Specific Sampling techniques and methods are used for determine the level of pesticides in food samples and the environment.

The production and use of the pesticides is regulated by different laws and regulation committees. Central Insecticides Act 1968 which regulate the import, manufacture, storage, transport, distribution and use of pesticides with a view to prevent risk to human beings, animals and the environment. This regulatory body issue licence, quality regulation, registration and determine the residual level in food sample.[4]

Maximum Residual Limit (MRL) indicates the maximum concentration of pesticide residue in food sample. The MRL is different for different pesticides according to the potency of a

pesticide. Pesticides are toxic in nature. To estimate the toxicity of pesticides, different studies are carried out using animal model. From these studies, data are evaluated and tolerance limits are set for use and consumption of pesticides. The MRL is measured in mg/kg. Organophosphorus pesticides are highly or moderately toxic to human.[3][4]

From different pesticides Organophosphorus pesticides Chlorpyrifos and Prophenofos are widely used to protect the different fruits, citrus, maize, tobacco and vegetables in the world.

1.2 Rationale for Selection of Pesticides:

From the literature review and ground level survey Chlorpyrifos and Prophenofos are frequently used to control the pests in cauliflower and cabbage. These vegetables are commonly used in salads and meals in day to day life. Thus, the need of these vegetables are moderately high in Gujarat, to reach the need of vegetables it is necessary to stop the wastage of crop by pests, to improve the quantity of crop sometimes farmer use high amount of pesticides. Regular or frequent use of organophosphorus pesticides increase the chances of these pesticides being found in vegetables which may enter in to body and damage the human health.

Table 1.1. Organophosphorus Pesticides use in vegetables

Vegetables	Organophosphorus Pesticides use
Cabbage	Chlorpyrifos, Prophenofos
Cauliflower	Chlorpyrifos, Prophenofos
Okra	Chlorpyrifos, Monocrotofos

For evaluation of these pesticides it is required to develop a specific extraction and analytical technique. In this project, I have developed and validated methods useful to get a general idea about the pesticides residue in vegetables in north Gujarat region. It is also turns out to be also useful for demographic study of the same pesticides in different region.

1.3 Intorduction to Chemical Profile of Pesticides:

(A)Chlorpyriphos:[1][2][4]

Chlorpyrifos is a broad-spectrum, chlorinated organophosphate (OP) insecticide. Chlorpyrifos is chemically known as 0,0-diethyl 0-(3,5,6-trichloro-2-pyridinyl-phosphorothioate. The Chemical Abstracts Service (CAS) registry number is 2921-88-2.Chlorpyrifos is chemically containing chlorine, Phosphorus and nitrogen containing benzene ring.

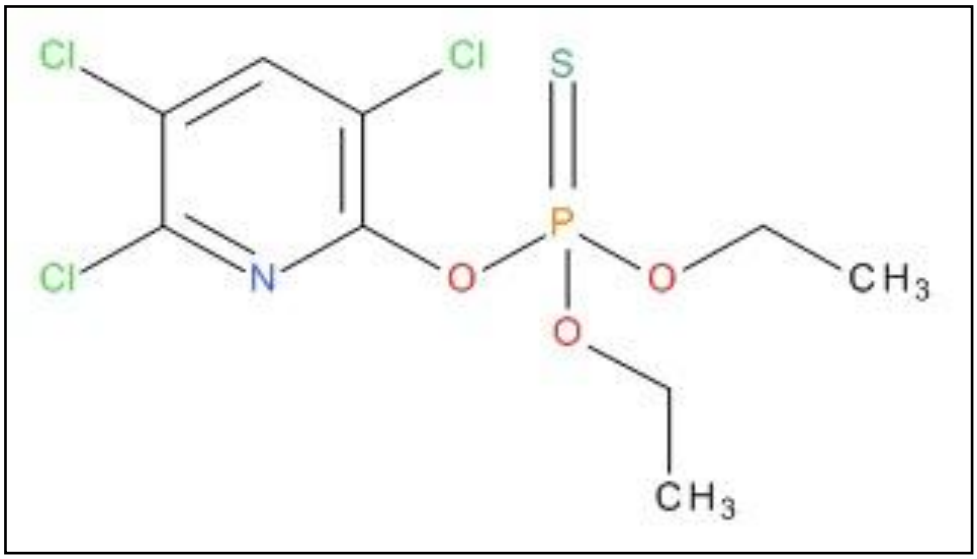


Figure 1.1.: Structure of Chlorpyrifos

- IUPAC Name : O, O-Diethyl-O-3, 5, 6-trichloro-2-pyridyl phosphorothioate
- Chemical Family: Organophosphate
- Molecular Formula: $C_9H_{11}Cl_3NO_3PS$
- Molecular mass: 350.6gm/mol
- CAS registry number: 2921-88-2
- Physicochemical parameter:
    1. Appearance: colorless to white crystalline solid
    2. Odor: Mild Mercaptan like odor

3. Solubility:

   Solubility in water: 1.05 mg/l

   Solubility in organic solvent:

   Hexane: 774 g / l

   Toluene: >4000 g/l

   Dichloromethane: >4000 g/l

   Methanol: 290 g / l

   Acetone: >4000 g/l

   Ethyl acetate: >4000 g/l

4. Melting Point: 41 to 43.5 ^{o}C
5. Octanol-Water Partition co-efficient: LogKow 4.7

- **Mode of Action:**

Chlorpyrifos kills pest by targeting the nervous system. It interferes the normal function of nervous system. It inhibits the breakdown of neurotransmitter Acetylcholine (ACH). When insecticides come directly in contact with the chlorpyrifos the active compound of it affect the functional site of cholinesterase (ChE) enzyme and inhibits the breakdown of ACH in synaptic clefts. Due to this ACH accumulated in synaptic and causes overstimulation of neuronal cells. It leads to neurotoxicity and cell death. Mode of Action of chlorpyrifos is also same in non-targeting organisms.

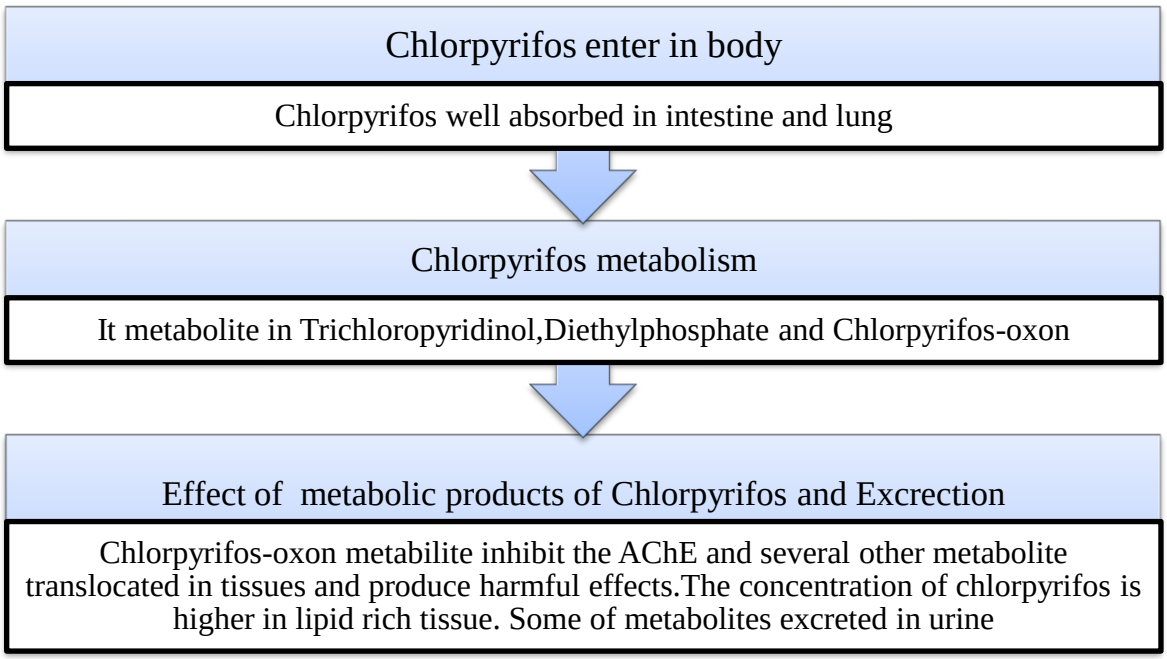
- **Signs and Symptoms of Toxicity :**

Signs and symptoms are typically seen within minutes to hours after exposure of chlorpyrifos. In the initial stage a runny nose, tears in eyes, increased salivation, nausea, and dizziness are seen. After long exposure to it signs of progression include muscle twitching, weakness or tremors, lack of coordination, vomiting, abdominal cramps, diarrhea, and pupil constriction with blurred or darkened vision. Severe toxicity include increased heart rate, unconsciousness, loss of control of the urine or bowels, convulsions, respiratorydepression, and paralysis.

- **Poison Treatment:**

- For Inhalation treatment: Remove person from exposure area to a place with fresh air, If not breathing, give artificial respiration, preferably mouth to mouth, get medical attention.
- Dermal toxicity: Remove the contaminated cloths and isolate the area. Wash the skin with soap and get emergency shower.
- If Swallowed : Immediately contact physician, Poison Control or an emergency centre . Do not induce vomiting or give anything by mouth to an unconscious person.
- Atropine is used as antidote for treatment of Chlorpyrifos poisoning.

- **Biotransformation of Chlorpyrifos in human:**[23][24]



(B) Prophenofos: [2][3][4]

Prophenofos is a broad-spectrum, organophosphorus pesticide. Prophenofos is chemically known as O-(4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate. The Chemical Abstracts Service (CAS) registry number is 41198-07-8.Prophenofos is bromine and chlorine containing pesticides.

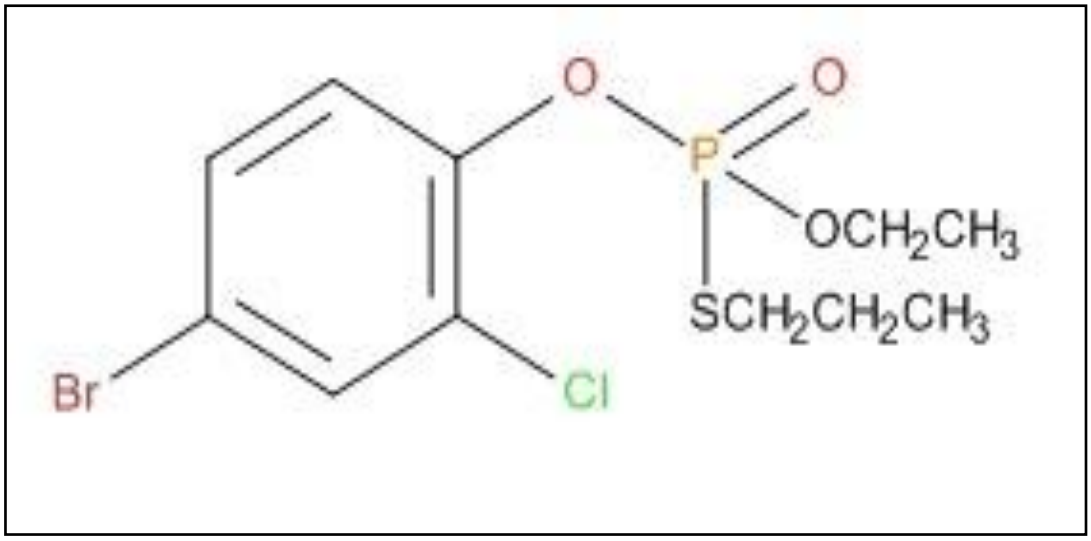


Figure 1.2. Structure of Prophenofos

- IUPAC Name : O-4-bromo-2-chlorophenyl O-ethyl S-propylphosphorothioate
- Chemical Family: Organophosphate
- Molecular Formula: $C_{11}H_{15}BrClO_3PS$
- Molecular mass: 373.6gm/mol
- CAS registry number: 41198-07-8
- Physicochemical parameter:
    1. Appearance: colorless liquid
    2. Odor: Weak odor
    3. Solubility:

Solubility in water: 1.0 mg/l

Solubility in organic solvent:

Hexane: 774 g / l

Toluene: >4000 g/l

Dichloromethane: >4000 g/l

Methanol: 300 g / l

Acetone: >4000 g/l

Ethyl acetate: >4000 g/l

4. Melting Point/ Freezing point: -76^{o}C
5. Octanol-Water Partition co-efficient: LogKow 4.44

- **Mode of Action:**

Prophenofos kills pest by targeting the nervous system. It interferes the normal function of nervous system. When insecticides come directly in contact with the prophenofos the active compound of it inhibit the Acetylcholinesterase enzyme. . It leads to neurotoxicity and cell death. Mode of Action of prophenofos is also same in non-targeting organisms.

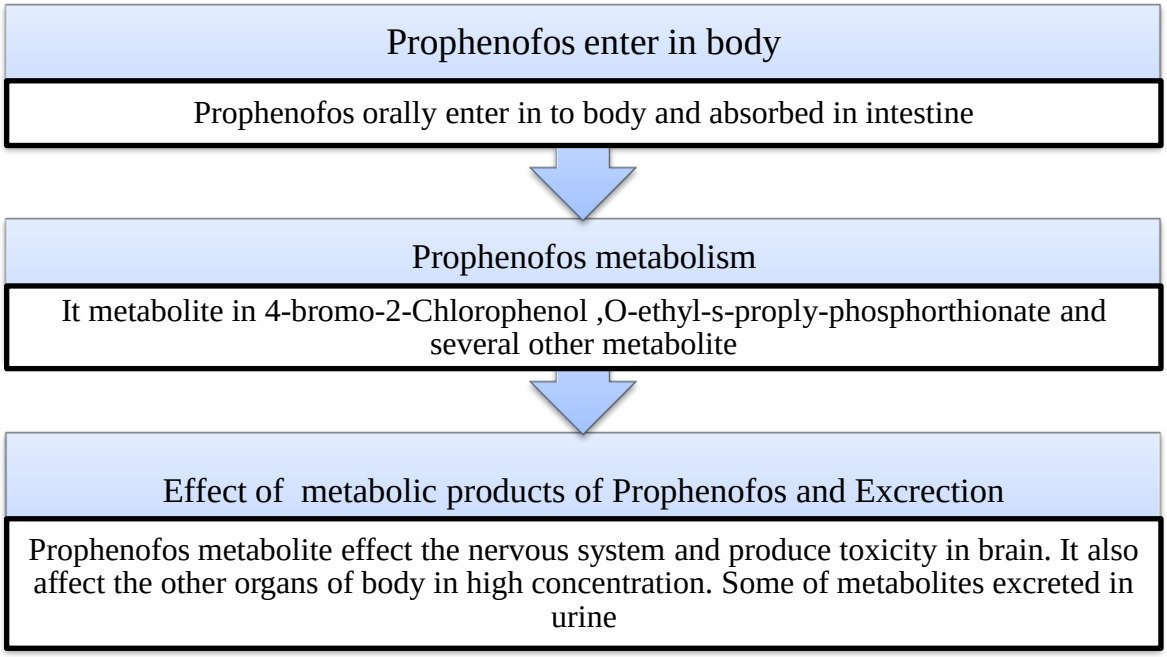
- **Signs and Symptoms of Toxicity :**

Signs and symptoms typically seen within minutes to hours after exposure of Prophenofos. In initial stage runny nose, tearing of eyes, increase saliva, nausea, and dizziness. After long exposure to it Signs of progression include muscle twitching, weakness, lack of coordination, vomiting, abdominal cramps, diarrhoea, and pupil constriction with blurred or darkened vision. Severe toxicity includes increased heart rate, unconsciousness, loss of control of the urine or bowels, convulsions, respiratorydepression, and paralysis.

- **Poison Treatment:**

- For Inhalation treatment: Remove person from exposure area to fresh air, If not breathing, give artificial respiration, preferably mouth to mouth, get medical attention.
- Dermal toxicity: Remove the contaminated cloths and isolate the area. Wash the skin with soap and get emergency shower.
- If Swallowed : Immediately contact physician, Poison Control or an emergency centre . Do not induce vomiting or give anything by mouth to an unconscious person.
- Atropine is used as antidote for treatment of Prophenofos poisoning.

- **Biotransformation of Prophenofos in human:** [25]



1.4 INTRODUCTION TO MULTIRESIDUE ANALYSIS:[8][10]

To protect the agricultural product from pests farmers are using different types of pesticides. Pesticides are frequently used to control the insects, weeds and other agricultural pests. Due to bad effect of these agricultural pests on crops and agricultural production it is necessary to use more than one pesticide. This type of multi pesticide treatment to control the insects gives better quality and quantity of agricultural product. It is beneficial to farmers as well as consumers for better quality and cheaper products. It is also useful for longer storage of agricultural products and reducing the wastage of products. So this technique is very useful for both farmers and consumers.

This frequent or regular use of pesticides increases the chances of pesticides remaining in agricultural products. This remaining Pesticides residue in products is hazardous to human health. So it is necessary to develop the analytical method which can analyze more than one pesticides residue in sample.

Multi residue analysis procedures are now being used more frequently for estimation of pesticides in samples due to their advantages over conventional methods. These multi residue analytical methods are more accurate, precise, quick, sensitive and economical. For the estimation of multi-residue analysis, the instrumental techniques, which are commonly employed, are spectrophotometry, GLC, high performance thin layer chromatography (HPTLC), HPLC LC/MS/MS etc.

⌗ Spectrophotometric multi-component analysis :

Absorption spectroscopy is one of the most useful and widely used technique available for quantitative analysis. Spectrophotometer is one of the economical and cheapest instrument available in most laboratories. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to it being simple, rapid, precise and highly accurate. Spectrophotometric multi-residue analysis can be applied where the spectra of component overlaps. In such cases of overlapping spectra, simultaneous equation derivative spectroscopy, absorption ratio method can be used to obtain the concentration of individual component.

⌗ High performance liquid chromatography (HPLC) :

This technique is based on the separation of the component according to the nature of the analyte. There are different types of separation techniques like adsorption, partition, ion exchange and gel permeation. In HPLC, the mobile phase is pumped through the packed column under high pressure. The technique is most widely used for all the analytical separation techniques due to its sensitivity, its ready adaptability to accumulate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones. In normal HPLC, polar solids such as silica gel; alumina (Al_2O_3) or porous glass beads and nonpolar mobile phase such as heptane, octane or chloroform are used but if the opposite case holds, it is called as reversed phase HPLC (RP-HPLC).

⌗ High performance thin layer chromatography (HPTLC) :

HPTLC also works on principle of separation. Normally, the mobile phase is driven by capillary action. The prominent advantages of this technique include possibilities of separating of more than 50 samples and standard simultaneously on a single plate this leads to high throughout, low cost analogs and the ability to construct calibration curves from standard chromatography under the same condition as the sample. It is widely used for herbal

product analysis which usually contains more components and thereby affords for better separation.

- **Gas chromatography (GC) :**

GC is one of the most extensively used Chromatographic technique in which separation is accomplished by partitioning solute between a mobile phase (gas) and stationary phase (either liquid or solid). It is widely used for detection of pesticides residue in samples. It is sensitive to detect the low level of component in sample.

1.5 INTRODUCTION TO UV VISIBLE SPECTROPHOTOMETRY:[8][10]

Multi-residue analysis is also carried out by UV visible Spectrophotometer. Different methods are used for analysis of the compound according to nature and suitability.

(a) Assay as a single-component sample:

This method is carried out for analysis of one compound. Whole study is carried out on single wavelength. Concentration of a component in a sample is determined on one particular wavelength. This method is comparatively easy, quick and simple for analyst.

(b) Assay using absorbance corrected for interference:

If more than one compound is analyzed and method is not suitable on two different wavelengths or any interference than following methods can be used for analysis:

- Simultaneous Equation Method (Vierodt's method)
- Derivative Spectrophotometric Method
- Absorbance Ratio Method (Q-Absorbance method)
- Dual Wavelength Method
- Ratio spectra Derivative Method

⌗ Derivative Spectrophotometric Method:[10]

Derivative spectrophotometry is a technique which is based on derivative spectra of a basic, zero-order spectrum. Derivative spectroscopy is useful to eliminate the interference and noise from zero-order spectrum. It is mathematical calculation from zero-order spectrum. It can be expressed as:

$$^{n}D_{x,\lambda} = d^{n} A/d\lambda^{n} = f(\lambda)$$

Where, n= Derivative order

$D_{x,\lambda}$ = Value of n order derivative of an analyte at λ

A = absorbance

Now a day Morden software is equipped with mathematical operation like multiplication, subtraction and derivatization. For derivative spectroscopy different orders are applied according to zero order overlapped spectrum.

A first order derivative spectroscopy is rate of change of absorbance with respect to wavelength. A first order spectrum starts and ends at zero. By increasing derivative spectrum order increases elimination of noise and interference. In software First, Second, and Third order spectrum is generated by mathematical calculations. Proper separation of overlapped spectra can be achieved by selecting appropriate order. Lower order derivative is used for wide band spectrums and for narrow bands higher order derivative used.

➢ Application of derivative Spectroscopy:

1. Derivative Spectroscopy is used for multi-component analysis Which is not suitable by other methods like Simultaneous Equation Method, Absorbance Ratio Method etc.
2. Applicable to investigate process kinetics
3. Evaluation of physic-chemical parameter like complexation, bonding of reaction.

1.6 INTRODUCTION TO HPLC:[9]

High performance liquid chromatography (HPLC) is an important analytical technique for the analysis of different types of the components with better separation and quantification. It involves the column which is containing packed silica and small amount of sample (component) passed through column along with mobile phase. In this method complex mixtures (Components) are separated into individual compounds by physical and chemical interaction with packed column, which can be identified and quantified by suitable detectors and data handling systems.

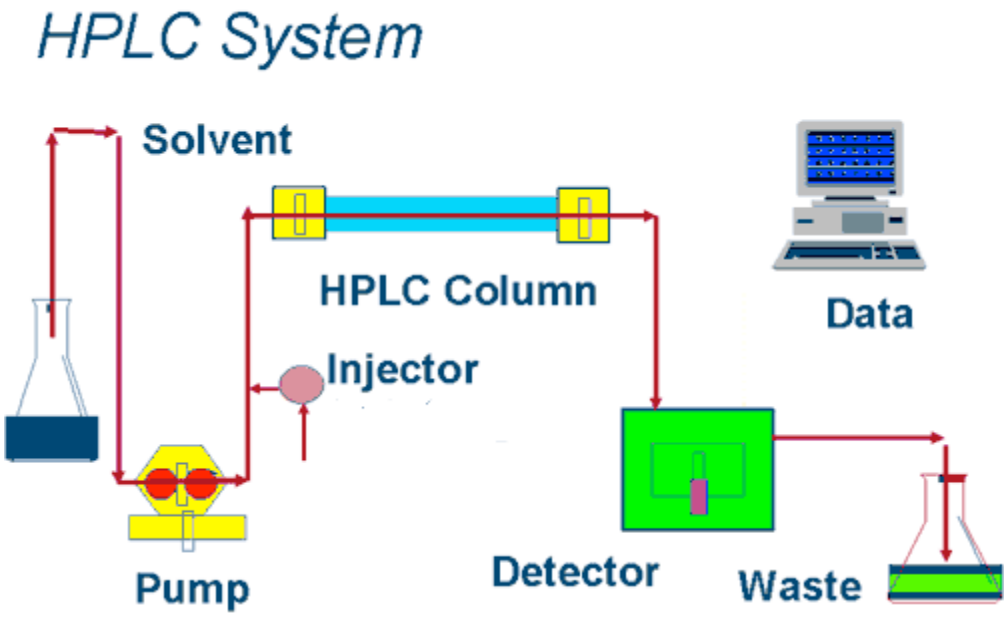


Fig. 1.3. Gradient HPLC System

1.6.1 Working:

HPLC system contains five different components which are pump, injector, column, detector and computer. In this system pump is useful to force mobile phase through column at specific flow rate. Generally flow rate is in the range of 1-2 ml/min. It can deliver a mobile phase at constant rate and maintain the pressure according to it. Second component is injector which is useful to inject analyte in to system. Specific amount of sample can be injected by injector. Third component is column which works as a stationary phase and provide a

separation of injected sample. Fourth component is detector which is useful for detection of separated compound from column; UV and PDA detector are widely used for it. Final component is computer or data management system. It is important component which controls all other components of system and useful for data interpretation like retention time, peak area, peak height etc.

1.6.2 Principle:

HPLC is basically work on general principle of chromatography; separation. The small amount of sample to be analyzed is injected in to a system which is moved through a column by mobile phase. The separation of sample analyte is done by specific chemical or physical interactions with the stationary phase present within the column. The time at which a specific sample elutes from the column (comes out of the end of the column) is called the retention time (Rt). The retention time under specific conditions is considered an identifying characteristic of a injected sample. The smaller particle size column packing (which creates higher backpressure) giving the components less time to diffuse within the column and improving the chromatogram resolution. Common solvents used for mobile phase with combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to improve the separation of the sample components. Different detectors are used for sample analysis, generally UV, PDA, RI etc.

1.6.3 Parameters affecting RP-HPLC:

1.6.3.1 Internal diameter: The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column.

1.6.3.2 Particle size: Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

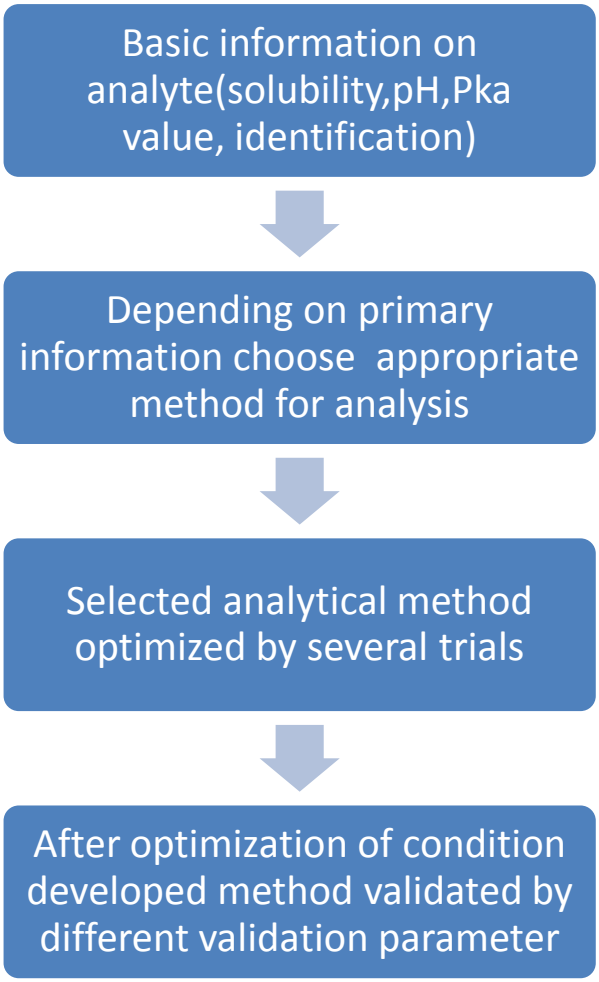
1.6.3.3 Pore size: Many stationary phases are porous to provide greater surface area for separation of analyte. Small pores provide greater surface area and better separation. Larger pore size has better kinetics and useful for larger analytes.

1.6.3.4 Pump pressure: Pumps are one of the major component of system which vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate.

The hyphenated technique is advanced technique for detection of analyte with better quantification and on-line evaluation of sample with less time. These techniques are very accurate and automated which reduces the error. The remarkable improvements in hyphenated analytical methods over the last two decades have significantly broadened their applications in the analysis of chemicals, biomaterials, natural products and toxicological evaluation of hazardous compounds. Few of them are LC-IR, LC-MS and LC-NMR

1.7 INTRODUCTION TO METHOD DEVELOPMENT:

Method Development is initial and crucial stage for developed or validated method. Method of analysis is developed based on prior or existing literature review. The requirement of developing a new method is to improve the sensitivity, quality and efficiency of existing method. Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why. The method development includes following steps:



1.8. Extraction of Pesticides from sample:[13][14]

Extraction is one of the most important step in multi-residue analysis of pesticides. If extraction procedure is efficient than accurate amount of pesticides in samples can be determined. There are different techniques used for extraction of pesticides from food samples.

1.8.1. Liquid-liquid extraction:

Liquid-liquid extraction is widely used technique for extraction of different pesticides from agricultural products. It is easy technique for extraction and not required any instruments. So it can be performed in simple analytical laboratory. Liquid-liquid extraction basically works on the principle of partitioning in one of the two liquids. It provides wide variety of selection of different regents for extraction of different pesticides.

1.8.2. Solid Phase Extraction:

Solid Phase Extraction is most efficient extraction technique for extraction of pesticides residue from the food samples. It works on the principle of adsorbent/partitioning on solid sorbents. It provides better recovery and reduces cross contamination of samples. It is very quick method as compare to liquid-liquid extraction.

1.9 INTRODUCTION TO METHOD VALIDATION:[7][8]

1.9.1 Definition:

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product, meeting its predetermined specifications and quality characteristics].

1.9.2 Objective of Validation:

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Any developed method may be influenced by variables like different elapsed assay times, different days, batches of reagents, instruments, equipments, environmental conditions like temperature, etc. It is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH) , United States Food and Drug Administration (US FDA) , United States Pharmacopoeia (USP) , and World Health Organization (WHO) provide a framework for performing such validations in a more efficient and productive manner.

Green et al. has given a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Wegscheider et al. has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. Seno et al. have described how analytical methods are validated in a Japanese QC laboratory. The AOAC has developed a peer-verified methods validation program with detailed guidelines on exactly which parameters should be validated. Winslow and Meyer recommend the definition and application of master plan for validating analytical methods. Breaux et al. have published a study on analytical methods development and validation.

1.9.3 Validation Parameters:

1.9.3.1 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). The response of the analyte in test mixtures containing the analyte and all potential sample components is compared with the response of a solution containing only the analyte.

1.9.3.2 Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value. NIST reference standards are often used; however, such a well-characterized sample is usually not available for new drug-related analytes. The extraction of the drugs from the formulation product can easily be carried out by accuracy study.

1.9.3.3 Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

1. Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

2. Intermediate Precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

3. Reproducibility: Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

1.9.3.4 Limit of Detection (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit needs to be determined only for impurity methods in which chromatographic peaks near the detection limit will be observed. The detection limit should be estimated early in the method development-validation process.

1.9.3.5 Limit of Quantification (LOQ) :

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

1.9.3.6 Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels, from 50 to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data. A correlation coefficient of > 0.995 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level.

1.9.3.7 Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. In practice, the range is determined using data from the linearity and accuracy studies.

1.9.3.8 Robustness:

Robustness of a method is its ability to remain unaffected by small but deliberate changes in parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature, and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. Obtaining data on the effects of these parameters may allow a range of acceptable values to be included in the final method procedure.

1.9.3.9 Solution Stability:

Solution stability is the stability of sample test solutions in specified period of time at designated storage conditions. Samples and standards should be tested over at least a 48 hr period, and quantitation of components should be determined by comparison with freshly prepared standards. If the solutions are not stable over 48 hr, storage conditions or additives should be identified that can improve stability.

1.9.3.10 System Suitability:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

Table 1.2.ICH validation guideline

Type of analytical procedure characteristic	**Identification**	**Testing for impurities, Quantitative limit.**		**Assay, dissolution (measurement only), Content/potency.**
Accuracy	-	+		+
Precision				
Repeatability	-	+	-	+
Intermediate precision	-	+(1)	-	+(1)
Reproducibility	-	-(2)	-	-(2)
Specificity (3)	+	+	+	+(4)
Detection limit	-	-	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated

+ Signifies that this characteristic is normally evaluated

(1) Intermediate precision is not needed in some case, when reproducibility is checked

(2) May be needed in some cases

(3) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(4) May not be needed in some cases

Validation of analytical methods which are used for various analytical purpose, in general, has been extensively covered in the ICH guidelines Q2 (R1) [(7)]and by USP (Table 1.2).

AIM AND OBJECTIVE

Pesticides are use to protect the crops and for getting better yield of agriculture product. If pesticides are not used according to the guideline than it may produce harmful effect on environment as well as humans. Pesticides enter in our body and metabolite/degraded products produce irreversible damage to body. This kind of damages are long lasting, some of pesticides affect the gene and create a problem for next generation.

Unfortunately not all farmers follow the legal practices. They use huge amount of pesticides for getting more yield. Now days it is necessary to determine pesticides level in different agriculture products. For better determination of the pesticides it required sensitive, specific, accurate and simple technique which can applicable on most of the food samples and agriculture product.

From the literature survey two main pesticides are frequently used for cabbage and cauliflower (Chlorpyrifos, and Prophenofos). For Extraction of pesticides from sample it is necessary to optimize appropriate method. After extraction second important step is applicability of developed method for these pesticides.

So, Main aim of this project work is optimize efficient extraction procedure and develop simple, accurate, precise, and sensitive method for determination of pesticides.

Objective:

- **Development** of the methods which are helpful to monitor the level of pesticides.
- **Optimization** of extraction method for liquid-liquid extraction and solid phase extraction.
- **Validation** of developed methods as per ICH guidelines.
- Confirmation and **quantitative determination** of the frequently used pesticides using developed method.
- Carry out **demographic study**.

Table 3.1. Literature survey for Pesticides

Sr No.	Pesticides detected	Matrix	Method	Mobile Phase and Condition	Reference No.
1	Endosulfan, Carbendazim Chlorpyrifos	Fruits	RP-HPLC	M. phase: Acetonitrile: Water (70:30) Flow rate: 1ml/min Uv detector: 254nm	16
2	Triazophos, Profenofos, Chlorpyrifos Monocrotofos	Honey	RP-HPLC	M. phase: Acetonitrile: Water(75:25) Flow rate: 1.2ml/min Uv detector: 230nm	19
3	Chlorothalonil Folpet, Tetradifon	Fruits	RP-HPLC	M. phase: Acetonitrile: Water(gradient 40to90) Flow rate: 1ml/min DAD detector:200-360nm	14
4	Endosulfan, Carbendazim Monocrotofos	Coconut water	RP- HPLC	M. phase: Methanol: Water(70:30) Flow rate: 254ml/min UV detector:254nm	13
5	Prophenofos Triazofos Chlorpyrifos	Vegetables	RP- HPLC	M. phase: Acetonitrile: Water Flow rate: 1ml/min Uv detector: 254nm	12
6	Imidachloprid Monocrotofos Carbendazim	Vegetables	UPLC	M. phase: Acetonitrile: Water (65:35) Flow rate: 1ml/min Uv detector: 210nm	37
7	Chlorpyrifos	Fruits	UV	UV detection : 289nm	17
8	Chlorpyrifos Imazalil Thiabendazole	Vegetables	LC-MS/MS	M. phase: formic acid : acetonitrile Flow rate: 0.6ml/min Run time: 45min.	36

4. IDENTIFICATION OF PESTICIDES

Identification of chlorpyrifos and prophenofos were carried out by melting point, UV-Visible spectrophotometer and FT-IR spectrophotometer.

Instrumentation

- Melting Point Apparatus: T603160 (EIE Instruments, Pvt. Ltd., Ahmedabad)
- UV-visible spectrophotometer: UV/vis-2450, (Shimadzu Inc., Columbia, MD)
- FT IR Spectrophotometer: JASCO FT/IR-6100 (Jasco, Japan)

4.1. IDENTIFICATION BY MELTING POINT

Melting point of chlorpyrifos and prophenofos had been determined using melting point apparatus. Melting point standard pesticides were taken by capillary method.

Table 4.1. Melting Point determination data of Chlorpyrifos & Prophenofos

Drug	Reported Melting Point(^{o}C)[1][3]	Observed Melting Point(^{o}C)
Chlorpyrifos	41-43.5	41-43
Prophenofos	110	109-110

4.2. IDENTIFICATION OF DRUGS BY UV-VISIBLE SPECTROMETER

For identification of standard chlorpyrifos and prophenofos 10 µg/ml solution was prepare in ethyl-acetate and absorbance maxima was carried out at 290nm for chlorpyrifos and 276nm for prophenofos.

Table 4.2. UV spectra determination data of Pesticides

Drugs	Reported peak(nm) [1][3]	Observed peak(nm)
Chlorpyrifos	290	290.1
Prophenofos	275	275.9

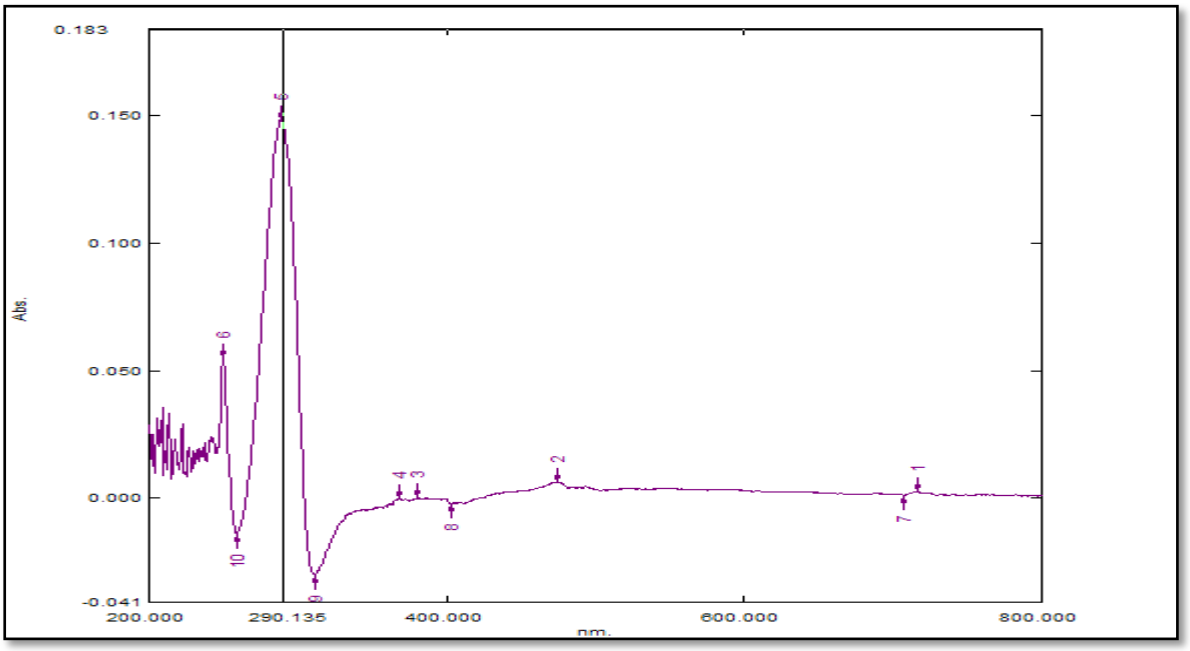


Figure 4.1. UV spectra of Chlorpyrifos (10µg/ ml) in Ethylacetate at 290.1nm

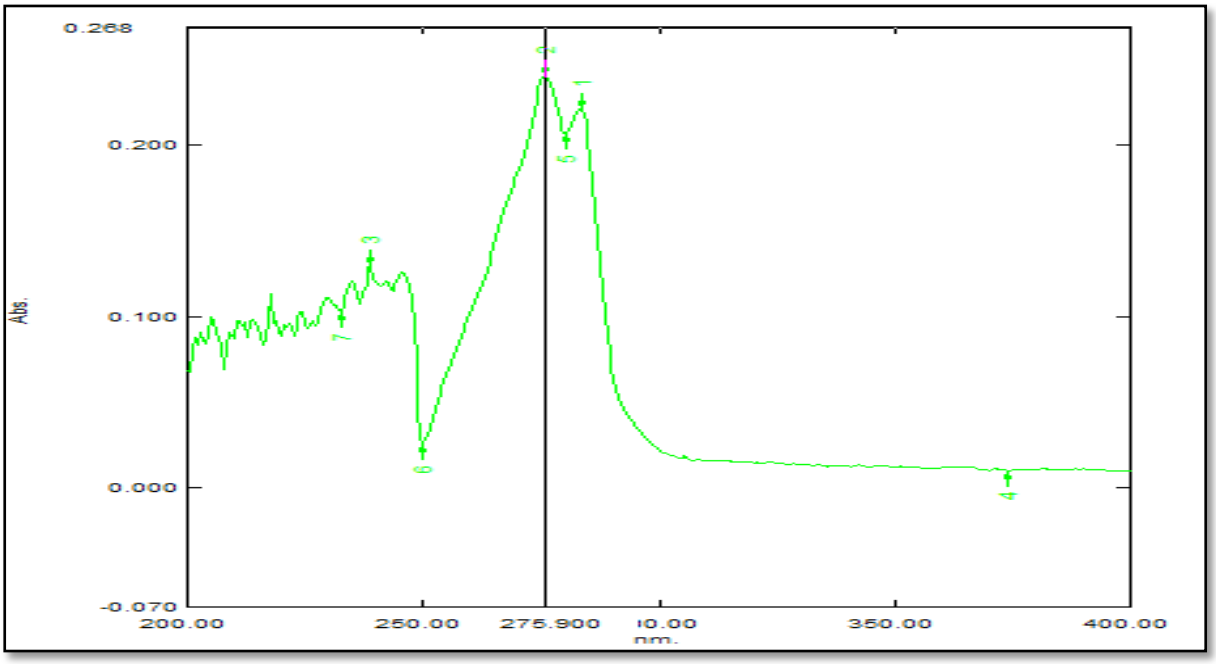


Figure 4.2. UV spectra of Prophenofos (10µg/ ml) in Ethylacetate at 275.9nm

4.3. IDENTIFICATION BY INFRARED SPECTROSCOPY

IR spectra of standard were taken using FT-IR spectrophotometer. IR spectra obtained were verified with reported IR spectra available in literature.

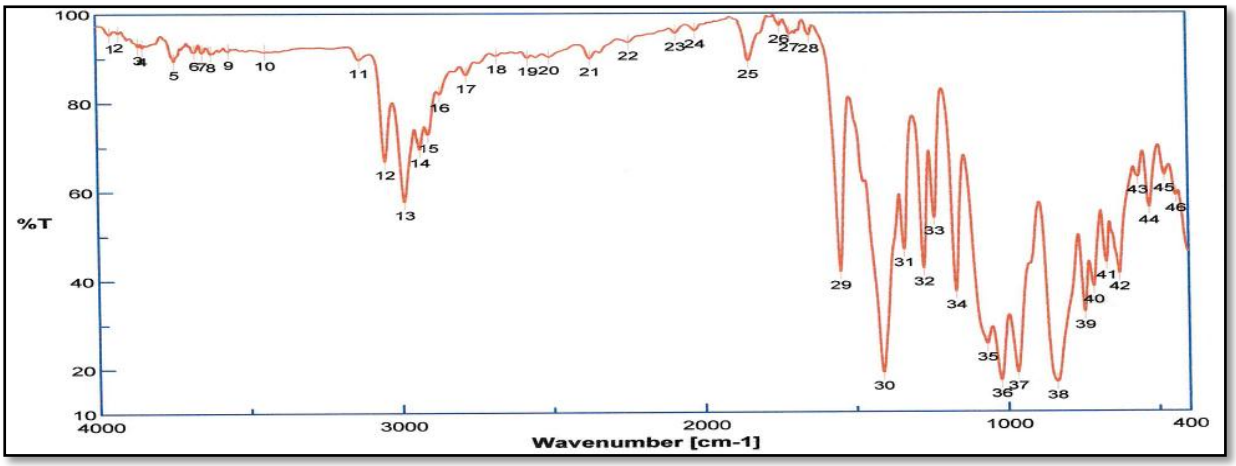


Figure 4.3 Reported IR spectra of Chlorpyrifos

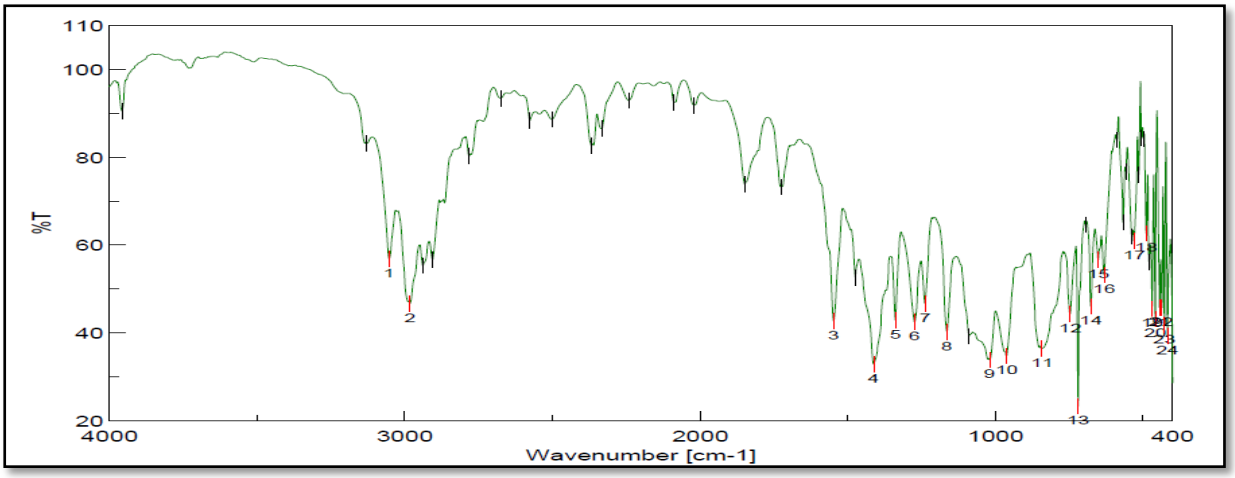


Figure 4.4 Obtained IR spectra of Chlorpyrifos sample

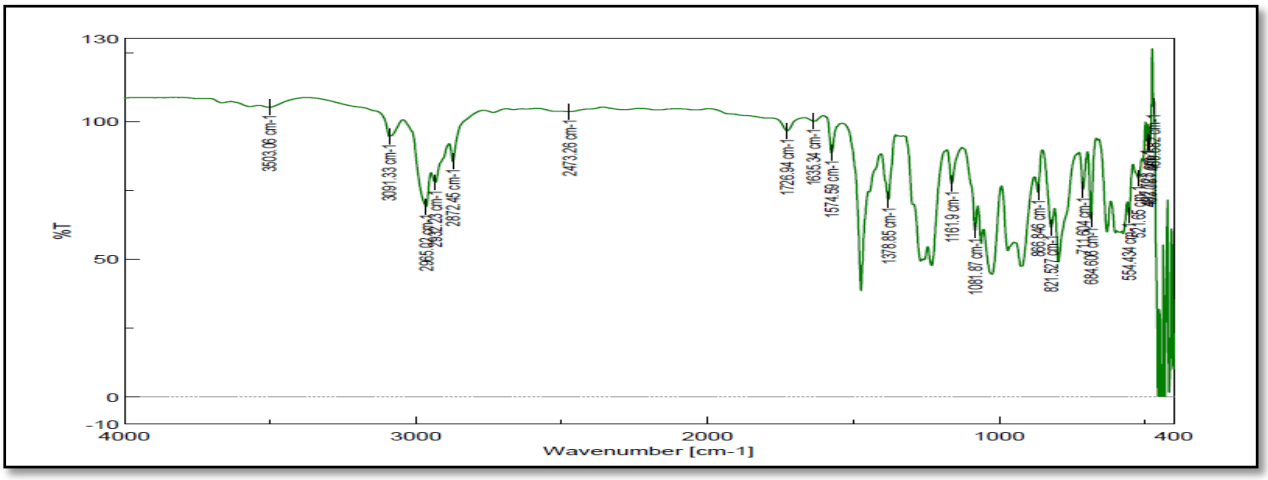


Figure 4.5 Obtained IR spectra of Prophenofos

Table 4.3 Observed FT-IR Peaks Prophenofos

Range of wave number [cm¹]	Functional group
3049	-C-H (aromatic)
2986	-C-H (aliphatic)
1644	-C=N
1549	-C=C (aromatic)
743	-C-Cl
685	-C-Br-

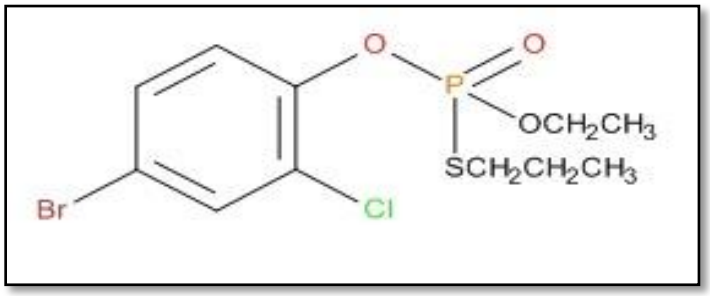


Figure 4.6. Chemical structure of Prophenofos

5.1. INSTRUMENTS

Weigh Balance:

Weighing capacity 220 grams, sensitivity 0.1 mg. (Citizens CX220 ,Mumbai, India)

UV-Visible spectrophotometer:

Shimadzu UV-Visible double beam spectrophotometer, model 2450 PC series, with spectral width of 1 nm, wavelength accuracy of 0.5nm and a pair of 10 mm matched quartz cells (Shimadzu , Japan).

Sonicator: D-compact., Capacity: 2 Lit.(TRANS-O-SONIC, Mumbai, India)

Hot air Oven: EIE 108 (EIE Instruments Pvt. Ltd., Ahmadabad, India)

Solid Phase Extractor: SPE 24 position vaccume manifold set. (Phenomenex., Ahemadabad, India)

Cartridge: Orochem C18, Celerity deluxe 30mg (Ahemdabad, India)

HPLC:

JASCO 200 Series HPLC model (JASCO, Inc JAPAN) with PU-2080 plus pump, MX-2080-31 mixer, Rheodyne model 7125 with 20µl fixed loop injector and Photo Diode Array detector was used. The software used was Borwin version 1.50.

5.2. MATERIALS AND REAGENTS

5.2.1. Standards:

Standard of Chlorpyrifos and Prophenofos were gifted from Meghmani Organics Ltd., Ahemadabad, India.

5.2.2. Reagents

Acetonitrile HPLC grade was purchased from Merk Specialities Pvt. Ltd., Worli, Mumbai. Ethyl acetate, Cyclohexene and toluene AR grade was purchased from Merk specialities Pvt.Ltd., Worli, Mumbai. Sodium bicarbonate, sodium chloride, Magnesium sulphate and Sodium sulphate were purchased from Finar chemicals Ltd., Ahemadabad.

5.3. SAMPLING:

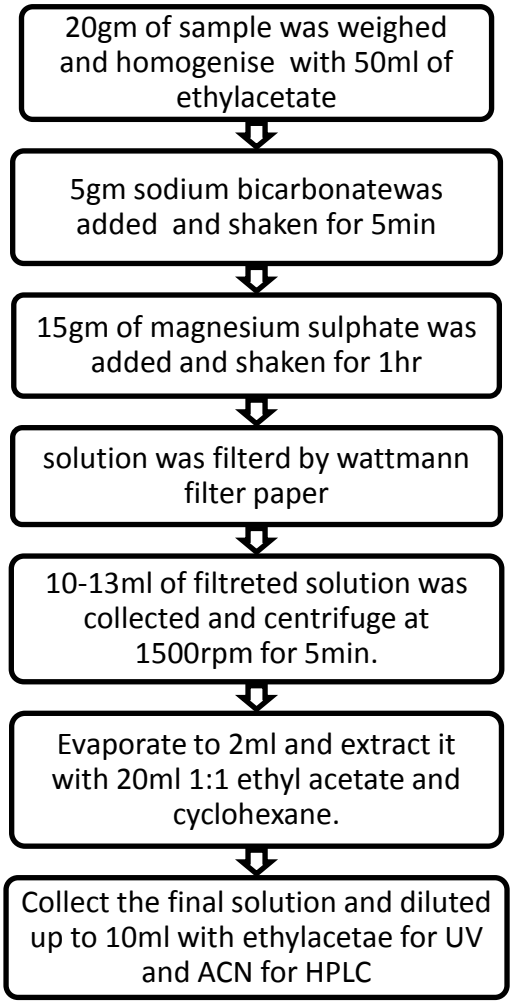
Sample collection is one of the most important part for evaluation of pesticides residue in vegetables and sampling technique affects the final result of analytical method. For estimation of pesticides residues in vegetables random Samples were collected from the Gandhinagar, Ahemadabad and North guajrat region. Different samples of Cabbage and Cauliflower were collected in inert container and store in refrigerator to avoid contamination or damage. Contamination and deterioration of samples were avoided at all stages of sampling because it affects the final result of analysis. Samples were labeled properly to identify the samples. These samples were extracted by Liquid-liquid extraction and solid phase extraction technique and analyzed by UV-spectrophotometry and RP-HPLC method.

5.4. EXTRACTION:

5.4.1. Sample Preparation for liquid-liquid Extraction:

The sample of cabbage and cauliflower (20gm) was weighed accurately and wash with distilled water for 5min. Ethyl acetate (50ml) was added and homogenized using mixture to prepare semisolid or viscous liquid. Then it was transferred into conical flask (250ml) and sodium bicarbonate (5gm) was added and shaken for 5min. Then magnesium sulphate (15gm) was added and shaken for 1hr in mechanical flask shaker. After shaking, solution was filtered by wattmann filter paper and 10-13ml was transferred in centrifuge tube. The soluation in centrifuge tube was centrifuged at 1500rpm for 5min. Then it was evaporate to 2ml and extracts it with 20ml 1:1 ethyl acetate and cyclohexane. Finally diluted up to 10ml with the ethylacetate for UV spectrophotometer and diluted with acetonitrile for HPLC system.

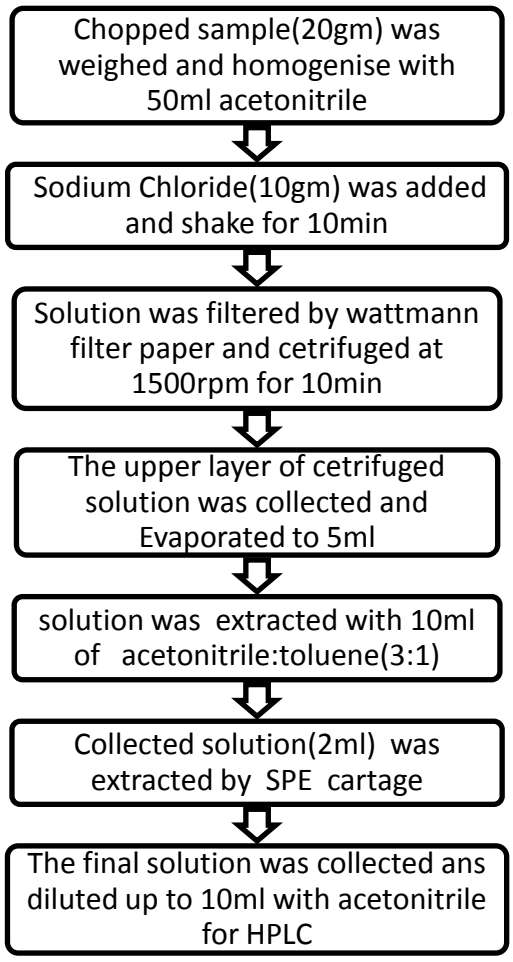
The Flow chart for liquid-liquid extraction is given below:



5.4.2. Sample Preparation for Solid Phase Extraction:

The sample of cabbage and cauliflower (20gm) was weighed accurately and wash with distilled water for 5min. Then acetonitrile (50ml) was added and homogenized using mixture. Then solution was transferred in to conical flask (250ml). Then sodium Chloride(10gm) was added and shaken for 10min. Solution was filtered by wattmann filter paper and transfered in to centrifuge tube to centrifuge at 1500rpm for 5min. The Solution was Evaporated to 5ml and extracted with 20ml of acetonitrile:toluene(3:1). For further extraction SPE cartage (Orochem C_{18}) was treated with 1ml of water and acetonitrile after treating with water and acetonotrile 1ml of sample solution was transferred to SPE cartage for extraction and wash with water and acetonitrile. The final solution was collected and diluted up to 10ml with acetonitrile and injected in HPLC system.

Flow chart of solid phase extraction is given below:



5.5. UV-Visible Spectroscopic method

5.5.1. Preparation of Standard solution

Chlorpyrifos (10mg) standard was accurately weighed and transferred to 100ml volumetric flask and dissolved in ethylacetate. The flask was shaken for 5min. and volume was made up to mark with ethylacetate to get 100 µg/ml standard stock solution of chlorpyrifos.

For prophrnofos (density 1.42gm/cm^3), 0.68ml of standard was taken which is equivalent to 100mg and transferred to 100ml flask and dissolved in ethylacetate. The flask was shaken for 5min. and volume was made up to mark with ethylacetate to get 1000 µg/ml of standard stock solution of Prophenofos. From this 1000 µg/ml 10ml was transferred to 100ml volumetric flask and volume was made up to mark with ethylacetate to get 100 µg/ml working standard solution of Prophenofos.

5.5.2. Preparation of sample solution

For liquid-liquid extraction, final 1ml solution was collected and transferred to 10ml volumetric flask. Then volume was made up to mark with ethylacetate and absorbance was observed at 277nm for chlorpyrifos and 289nm for prophenofos.

For solid phase, extraction final 1ml solution was collected and transferred to 10ml volumetric flask. Then volume was made up to mark with ethylacetate and absorbance was observed at 277nm for chlorpyrifos and 289nm for prophenofos.

5.5.3. UV-Visible method optimized parameter

From standard stock solution of chlorpyrifos (100 µg/ml) and prophenofos (100 µg/ml) 10 µg/ml solution of chlorpyrifos and 10 µg/ml solution of prophenofos was prepared and scanned in 200-400 nm range. Zero order spectra for both the solutions were taken and from that first order spectra were constructed. From spectral observation 277 nm were selected for measuring amplitude of chlorpyrifos and 289 nm was taken for prophenofos.

5.5.4. Method Validation

5.5.4.1. Preparation of Calibration Curve

Appropriate volume of aliquots (0.6, 0.8, 1, 1.2, 1.4 and 1.6ml) from standard chlorpyrifos stock solution were transferred to different 10ml volumetric flask, the volume was made up to mark with ethylacetate to obtain the concentration of 6,8,10,12,14and 16 µg/ml.

Appropriate volume of aliquots (0.6, 0.8, 1, 1.2, 1.4 and 1.6ml) from working standard prophenofos stock solution were transferred to different 10ml volumetric flask, the volume was made up to mark with ethylacetate to obtain the concentration of 6,8,10,12,14and 16 µg/ml.

5.5.4.2. Precision

5.5.4.2.1. Intra-day and Interday Precision

Intra-day and Inter-day precision was determined by measuring the absorbance of both the pesticides three times within a day and on three different days, respectively. For intraday and interday precision of chlorpyrifos 0.6, 1 and 1.4 ml were taken from standard stock solution of chlorpyrifos in 10 ml volumetric flask. The volume was made up to the mark with ethyl-acetate to obtain concentration of 6, 10 and 16 µg/ml. A first order spectrum was generated for chlorpyrifos at 277nm.

For intraday and interday precision of prophrnofos 0.6, 1 and 1.4 ml were taken from working standard solution of prophenofos in 10 ml volumetric flask. The volume was made up to the mark with ethylacetate to obtain concentration of 6, 10 and 16 µg/ml. A first order spectrum was generated for prophenofos at 289nm.

5.5.4.2.2 Repeatability

To study the repeatability, six determinations at 100% test concentrations (i.e. 10µg/ml of chlorpyrifos and prophenofos) were carried out. For chlorpyrifos, standard stock solution(100 µg/ml) 1 ml was taken in 10ml volumetric flask. Then volume was made up to 10 ml by ethylacetate to obtain 10µg/ml and a first order spectrum was generated at 277nm. For

prophenofos, working standard stock solution (100 µg/ml) 1 ml was taken in 10ml volumetric flask. Then volume was made up to 10 ml by ethylacetate to obtain 10µg/ml and a first order spectrum was generated at 289nm.

5.5.4.3. Accuracy

Accuracy of the method was determined by %recovery study using standard addition method at three different levels (80%, 100% and 120%) of assay concentration.

For chlorpyrifos accuracy study, different 9 volumetric flasks were taken in each of them prequantified synthetic mixture which was containing 6 µg/ml chlorpyrifos and 6 µg/ml prophenofos. From them, each 3 flasks were spiked with 0.48, 0.6 and 7.2 ml chlorpyrifos standard stock solution to produce 80%, 100% and 120% standard spiked recovery samples.

For prophenofos accuracy study, different 9 volumetric flasks were taken in each of them prequantified synthetic mixture which was containing 6 µg/ml chlorpyrifos and 6 µg/ml prophenofos. From them, each 3 flasks were spiked with 0.48, 0.6 and 7.2 ml prophenofos standard stock solution to produce 80%, 100% and 120% standard spiked recovery samples.

5.5.4.4. Robustness

Robustness was performed using of 10µg/ml concentration for chlorpyrifos and prophenofos. Robustness of the method was determined by making change in λ_{max} of both the pesticides by ±2 nm. The % Assay values were calculated and compared with that of standard. Results were reported in terms of % RSD.

5.5.4.5. Limit of Detection and Limit of Quantification

For this determination Calibration curve for both the drugs was repeated three times The LOD & LOQ were measured by using mathematical equations given below.

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where, σ = Standard deviation of the Intercept

S = slope of calibration curve

5.6. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.6.1. Preparation of standard solution

Chlorpyrifos (10mg) standard was accurately weighed and transferred to 100ml volumetric flask and dissolved in acetonitrile. The flask was shaken for 5min. and volume was made up to mark with acetonitrile to get 100 µg/ml standard stock solution of chlorpyrifos.

For prophrnofos(density 1.42gm/cm^3), 0.68ml of standard was taken which is equivalent to 100mg and transferred to 100ml flask and dissolved in acetonitrile. The flask was shaken for 5min. and volume was made up to mark with acetonitrile to get 1000 µg/ml of standard stock solution of Prophenofos. From this 1000 µg/ml 10ml was transferred to 100ml volumetric flask and volume was made up to mark with acetonitrile to get 100 µg/ml working standard solution of Prophenofos.

5.6.2. Preparation of sample solution

For liquid-liquid extraction final 1ml solution was collected and transferred to 10ml volumetric flask. Then volume was made up to mark with acetonitrile and 20 µl was injected in RP-HPLC system.

For solid phase extraction final 1ml solution was collected and transferred to 10ml volumetric flask. Then volume was made up to mark with acetonitrile and 20 µl was injected in RP-HPLC system.

5.6.3. Preparation of Cleaning Solution

For Preparation of cleaning solution Warm water was prepared by heating the 250ml water on water bath at 60^0C for 10min. This warm water was used for removing pesticides from vegetables.

For preparation of 5% soda-salt solution, 2.5gm sodium chloride and 2.5gm sodium bicarbonate was weighed and dissolved in 100ml water.

Vegetables were treated by these solutions for 10min.

5.6.4. Optimized condition for HPLC

- Stationary phase: Hibar Column C 18 (250 mm × 4.6mm i.d., 5µm)
- Mobile phase: Acetronitrile : Milli Q Water(90:10)
- Wavelength: 219 nm
- Runtime: 8 minutes
- Flow rate: 1ml/min
- Diluent : ACN
- Retention time for Chlorpyrifos : 3.20 Min.
- Retention time for Prophenofos: 5.52 Min.

5.6.5. HPLC Method Validation:

5.6.5.1. Preparation of calibration Curve

Suitable aliquots of standard stock solution (100µg/ml) of chlorpyrifos and working standard stock solution (100µg/ml) of prophenofos were mixed and diluted with acetonitrile to obtain different mixture solutions containing chlorpyrifos and prophenofos in concentration of solution in range of 0.01to1.5 µg/ml were prepared for calibration curve of these two pesticides. Calibration curve of peak area v/s concentration was plotted for each of the pesticides.

5.6.5.2. Precision

5.6.5.2.1. Intraday and Interday Precision

For intraday precision, the experiment was repeated three times in a day using three different concentrations for chlorpyrofos(0.05,0.5and1.5µg/ml) and prophenofos (0.05,0.5 and 1.5 µg/ml)

For Interday precision, the experiment was repeated on three different days using three different concentrations for chlorpyrifos (0.05, 0.5and1.5µg/ml) and prophenofos (0.05, 0.5and1.5µg/ml). The results were reported in terms of % Relative standard deviation(%RSD).

5.6.5.2.2. Repeatability

Repeatability was analyzed for Chlorpyrifos (0.1µg/ml) and Prophenofos (0.1µg/ml), 6 times and peak area was recorded. Repeatability of sample scan was measured in terms of %RSD.

5.6.5.3. Accuracy

Accuracy of the method was determined by % recovery study by standard addition method at three different levels (80%, 100% and 120%) of assay concentration.

For accuracy studies of chlorpyrifos, known amount of working stock solution (10µg/ml) of chlorpyrifos 0.4, 0.5, and 0.6ml were added to three different 10ml volumetric flask containing synthetic mixture solution (chlorpyrifos 0.5µg/ml & prophenofos 0.5µg/ml) and volume was made up with acetonitrile to produce final concentration of chlorpyrifos (0.9, 1 and 1.2µg/ml).

For accuracy studies of prophenofos, known amount of working stock solution (10µg/ml) of prophenofos 0.4, 0.5, and 0.6ml were added to three different 10ml volumetric flask containing synthetic mixture solution (Chlorpyrifos 0.5µg/ml & Prophenofos 0.5µg/ml) and volume was made up with acetonitrile to produce final concentration of prophenofos (0.9, 1 and 1.2µg/ml).

5.6.5.4. Robustness

Robustness was performed using of 0.1µg/ml concentration for chlorpyrifos and prophenofos. Robustness study was carried out by changing following parameters one by one and observed their effect on system suitability.

- Change in flow rate ±0.2ml/min to 0.8ml/min and 1.2ml/min.
- Change in wave length was done by ±2nm of optimum wavelength to 217nm and 221nm.

5.6.5.5. Specificity

Separated chromatographic peaks of two pesticides were analyzed for peak purity (specificity) by scanning in the range of 200-400 nm with the help of borwin PDA software.

For specificity both Chlorpyrifos and Prophenofos were studied for its standard spectra. The peak purity of chlorpyrifos and prophenofos were determine by peak start(S), peak apex (M) and peak end (E) by UV-PDA detector.

5.6.5.6. Limit of Detection and Limit of Quantification

For determination of LOD/LOQ, all linearity range solution was prepared in triplicate as described in section of linearity. Chromatograms of all these solutions are recorded. 3 linearity curves of peak area v/s concentration were plotted. From these curves, mean slop value and SD of intercept was calculated. From all these values, LOD/LOQ was calculated using following equation.

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

Where, σ = Standard deviation of intercept

S = Slope of the linearity curve

7.1 CONCLUSION:

- We **ascertained** that there are **pesticide residues present** in the vegetables that we consume. The study was conducted on a **sample taken** in the state of **Gujrat**. However keeping in mind the fact that agricultural trends are more or less the same in other agricultural states in India, the results could be extrapolated to states sharing somewhat demographic and agricultural similarities.

- During the course of this study, we developed and validated a **method on RP-HPLC and UV spectrophotometer** which could determine the pesticide residue in vegetables. It was found that the said method was **accurate, precise and easy to apply** so much so that it could be applied en masse to address public health concerns.

- For the extraction study, **solid phase and liquid-liquid extraction we recompared**. The results of the multitude of tests carried out indicated that of these two methods, the **solid phase extraction** technique was **easier and quicker** as compared to the liquid-liquid extraction. Therefore, it is suggested that in future work on this, a solid phase extraction be used as long as the pesticides in concern share physiochemical properties with the ones considered in this particular study.

- Of the samples collected from different regions in the state, **chlorpyrifos** were found to be of the concentration of **0.018mg/kg. Prophenofos** were **not found** in any of the analysed samples. On the other hand, the **MRL for Chlorpyrifos** was **0.01mg/kg**. This value is above the acceptable limits as dictated by the standards issued for food pesticide residue levels. This is one of the reasons that prompted further inquiry into effective and cost efficient methods of getting vegetables rid of pesticide residues.

- Of the different solutions tested for pesticide removal from vegetables meant for consumption, as a washing **solution of soda-salt (5%)** was found to be the most effective. It had the **highest relative cleaning capacity** as compared to the other cleaning agents tested.

7.2 FUTURE SCOPE

- As this happens to be a **vast topic** with little research being done as compared to applied and pure investigative aspects of pharmaceutical sciences, there are many possibilities in the future.

- Sensitivity can be worked upon to help improve the detection of residues in the samples subject to testing. Towards this end, we can perform **Gas Chromatography, with LC/MS**. This will attribute to an **increased sensitivity**. This was not carried out in the study due to technical limitations at that point in time.

- To add validity to the claims, a **Plasma study** can also be carried out**.** This doesn't need extra apparatus. It can easily be **carried out by the RP-HPLC method** developed in this document for the detection of pesticide residues in vegetables.

- There are no constrains as to the number and **variety of fruits and vegetables** that may be subject to this method of testing for pesticide residue. The author strongly urges the researcher to apply this method and evaluate it. Its **applicability is not limited** to the particular vegetable and fruit samples discussed in the report. It is understood that those are meant as indicative specimens; not exhaustive of the method.

- As the focus of this study was Gujarat, it may need to be carried out for other sample states so as to have a nationwide applicability. This however requires a considerable investment in time and resources. A **more extensive Demographic study which** encompasses various regions of the state, and if feasible – the country, could be carried out. This will help build pesticide residue profiles for different states and possibly the regions within states.

1. Centre, N. P. I. Chlorpyrifos. Oregon State University, 310 Weniger Hall, Corvallis 2009.
2. European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance bitertanol. EFSA Journal 2010;8(10):1850, 63.
3. www.efsa.europa.eu/efsajournal.htm
4. Chandra B., Bhardwaj A. and Misra S., 2013, State of Pesticide Regulations in India, Centre for Science and Environment, New Delhi.
5. Chromatographic determination of pesticides in foods and food products, Eur. Chem. Bull.2012, 1(3-4), 58-68.
6. Ningombam L. D. and Raha P., Contamination of Organochlorine pesticides (OCPs) in India, Bulletin of Environmental and Scientific Research, 2013, Vol. 2, Issue(1), 9-14.
7. ICH, Q2(R1), Validation of Analytical Procedures: Text and Methodology, 2005.
8. Beckett A.H., Stenlake J.B., Practical Pharmaceticals Chemistry CBS Publishers and distributors, 1997, 4th Ed., Part-2, 275-333.
9. Snyder L.R., Kirkland J.J., Glajch J., Practical HPLC Method Development 1988, 3rd Ed., 2-21.
10. http://www.intechopen.com/books/macro-to-nano-spectroscopy/derivative spectrophotometry and analytical application
11. Chandran S., Singh R.S.P., Comparison of various international guidelines for analytical method validation, Pharmazie, 2007,6,4-14.
12. Ahmad B., Ahmad A.,Muhammad A. and Muhammad R., "Determination of the Organophosphorus Pesticide in Vegetables by High-Performance Liquid Chromatography"American-Eurasian J. Agric. & Environ. Sci., 6 (5): 513-519, 2009.
13. Britoa N.M., Navickienea S., Polesea L., "Determination of pesticide residues in coconut water by liquid–liquid extraction and gas chromatography with electron-capture plus thermionic specific detection and solid-phase extraction and highq performance liquid chromatography with ultraviolet detection", Journal of Chromatography A, 957 (2002) 201–209

14. Sibel T., Ozhan G., Alpertunga B., "Simultaneous determination of various pesticides in fruit juices by HPLC-DAD", Food Control 16(2009),87–92.
15. Dasika R., Tangirala S., Naishadham P. "Pesticide residue analysis of fruits and vegetables",Journal of Environmental Chemistry and Ecotoxicology Vol. 4(2), (2012) pp. 19-28, 22.
16. Paranthaman, R., A. Sudha and S. "Determination of Pesticide Residues in Banana by Using High Performance Liquid Chromatography and Gas Chromatography-Mass Spectrometry"Kumaravel,American Journal of Biochemistry and Biotechnology,2012, 8 (1): 1-6.
17. Yoshio M., Meilan L., Seiichi O., Yoshinori k.," Nondestructive detection of pesticides on fruits and vegetables using UV camera", The University of Tokyo, Japan
18. Mr Makoto Irie. Prophenofos. Ministry of Agriculture, Forestry and Fisheries, Tokyo,japan 2008, 1, 1375-1456.
19. Rao T.N., Simultaneous Determination of Toxic Organophosphorus Insecticide. Open Access Scientific Reports 2012, 1 (6), 1-6.
20. Shailendra Singh Chauhan, Monitoring of pesticides residues in farmgate. Wudpecker Journal of Agricultural Research 2012, 1 (7), 250-256.
21. Hameed, A. M. Quantification of Pesticide Residues in Vegetables. Journal of Chromatography Separation Technique 2013, 4, 1-4.
22. Rahman, M. A. Supercritical fluid extraction and quantification of chlorpyrifos. Agriculture and Biology Journal of North America 2010, 1 (4), 548-555.
23. Keehner D., M. Shamim, Environmental Risk Assessment for Prophenofos, United State Environmental Protection Agency, Washington, 1999, 1-26
24. Eaton D., Daroff. R.,Autrup. H., Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment, Critical Reviews in Toxicology, Informa healthcare, 2008,S2:1–125.
25. Marable BR, Maurissen JP, Mattsson JL and Billington R , Differential sensitivity of blood, peripheral,and central cholinesterases in beagle dogs following dietary exposure to chlorpyrifos. Regul Toxicol Pharmacol,2007, 47:240−248.

26. Manal M., Yehya M., Yousef M. Pathological and Biochemical Studies of Profenofos Toxicity on Rats, Egypt. J. Comp. Path. & Clinic. Path, 2008, Vol. 21 No. 4 ,75 – 92.
27. Kuruganti K., Dharmender G. R., Pesticides, Residues and Regulation:A case of vegetables in Hyderabad Market, Centre for Sustainable Agriculture 12-13-445, Street no-1,Tarnaka, Secunderabad-500017,2010.
28. Hubert P. T., Recent development in analysis of persistent organic pollutants under the Stockholm Convention, Trends in Analytical Chemistry, 2013,Vol. 45.
29. George Y., Laseter J., and Charles M., Persistent Organic Pollutants in Serumand Several Different Fat Compartments in Humans, Hindawi Publishing Corporation Journal of Environmental and Public Health , 2011,Volume 1, 1-8.
30. Zhang Y., LI Xiao-p., LIU H., Study on universal cleaning solution in removing blended pesticide residues in Chinese cabbage, Journal of Environment Chemistry and Ecotoxicity, , 2013,Volume 5(8).
31. Qingming Z., Lusheng Z., Chang H., Analysis of chlorpyrifos and TCP residues in agricultural soil and apples by HPLC, Journal of Food, Agriculture & Environment , 2011,Vol.9 (1): 659 -662.
32. Venugopal N.,Sumalatha B., Rao B. and Veeribabu G., Spectrophotometric Determination Of Organophosphate Insecticide (Chlorpyrifos) Based On Diazotisation With Anthranilic Acid, The Malaysian Journal of Analytical Sciences, 2012,Vol 16 No 2 180 – 186.
33. T ao S., Xuf L. , Wang X J., Organochlorine Pesticides in Agricultural Soil and Vegetables from Tianjin, China, Environ. Sci. Technol. 2005, 39, 2494-2499.
34. Zuin VG, Yariwake JH, Langas FM, Analysis of pesticide residues in Brazilian plants. Braz Chem Soc, 2003, 14: 304-309.
35. Singh M., Production and utilization of pesticides in Asia and the Pacific, Asian Productivity Organisation Tokyopp, 2002, 190-199.
36. Fernndez-Alba AR, Garca-Reyes JF, Large-scale multi-residue methods for pesticides and their degradation products in food by advanced LC-MS. Trac-Trend. Anal. Chem., 2008, 27 (11): 973-990.

37. Bilehal D.C., Chetti M. B., Goroji P.T. and Khethagoudar M.C., A Simple, reversed-phase Ultra performance liquid chromatographic method for the simultaneous determination of Monocrotophos, Thiram, carbendazim, Carbaryl and imidacloprid pesticides by Quechers method in chilli samples, International Multidisciplinary Research Journal 2012, 2(9):01-04.
38. Porta M, Puigdomenech E, Ballester F, Selva J, Ribas-Fito N, Llop S, Monitoring concentrations of persistent organic pollutants in the general population: the international experience, Environ Int, 2008, 34:546–61.
39. Trejo-Acevedo A., Rivero-Perez N., Flores-Ramirez R., Assessment of persistent organic pollutants levels in blood samples from Quintana Roo, Mexico, International Journal of Hygiene and Environmental Health 216 (2013) 284– 289.
40. Zwir-Ferenc A., Biziuk M., Solid Phase Extraction Technique – Trends, Opportunities and Applications, Polish J. of Environ. Stud. Vol. 15, No. 5 (2006), 677-690.