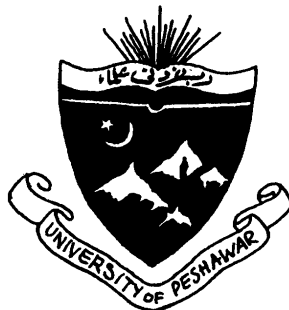


**INVESTIGATION OF NEW METHODS FOR THE
DETERMINATION OF TRIAZINE GROUP OF
HERBICIDES AND ITS APPLICATION TO
SAMPLES OF ENVIRONMENTAL IMPORTANCE**



Ph. D Thesis

By

BEHISHT ARA

**INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR
PAKISTAN**

September 2011

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DISSERTATION

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PAKISTAN**

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INSTITUTE OF CHEMICAL SCIENCES
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Certified that **Ms Behisht Ara** D/O Zia ul Haq has carried out her research and experimental work on the topic entitled as “**Investigation of New Methods for the Determination of Triazine Group of Herbicides and its Application to the Sample of Environmental Importance**” under our guidance and supervision. Her research work is original and her dissertation is worthy of presentation to the university of Peshawar for the award of degree of **Doctor of Philosophy in Chemistry**.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

Dedicated

To

To my Loving Parents

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*Behisht Ara
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LIST OF ABBREVIATIONS

%	Percent
°C	Degree celsius
µg	Microgram
µgmL ⁻¹	Microgram per milliliter
µm	Micrometer
ANOVA	Analysis of variance
ATR	Atrazine
BCG	Bromocresol green
BET	Brunauer emmet teller
C _e	Equilibrium concentration
C _f	Final concentration
C _i	Initial concentration
cm	Centimeter
cm ⁻¹	Per centimeter
CMIA	Carbonyl metallo immunoassay
CZE	Capillary zone electrophoresis
DA	Deaminometribuzin
DADK	Deaminodiketometribuzin
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
df	Degree of freedom
DK	Diketometribuzin
DLLME	Dispersive liquid–liquid microextraction technique
DMAB	<i>P</i> -dimethylaminobenzaldehyde

ε	Molar absorptivity
EDX	Energy dispersive X-ray
EFIA	Enzyme flow immunoassay
ELISA	Enzyme-linked immunosorbent assay
g	Gram
gmol^{-1}	Gram per mole
HEM	Hydroxyethyl methacrylate
HPLC	High performance liquid chromatographic
K_1	Pseudo-first-order rate constant
K_2	Pseudo-second-order rate constant
K_D	Distribution constant
K_F	Freundlich adsorption isotherm constant
K_L	Langmuir sorption isotherm constants
L	Liter
LC–ESI-MS/MS	Liquid chromatography- electron spray ionization mass spectrometry
ln	Natural logarithm
LOD	Limit of detection
log	Common logarithm
LOQ	Limit of quantification
LVSS	Large volume sample stacking
M	Molarity
MAE	Microwave assisted extraction
MAILME	Microwave assisted ionic liquid microextraction
MCPD	Methylchlorophenoxypropionic acid

MEC	Micellar electrokinetic chromatographic
mg	Milligram
mg g^{-1}	Milligram per gram
min	Minute
mL	Milliliter
mol	Mole
$\text{mol L}^{-1} \text{cm}^{-1}$	Mole per liter
MWCNT	Multiwall carbon nanotubes
nm	Nanometer
PDMS–DVB	Polydimethylsiloxane-divinylbenzene
PLE	Pressurized liquid extraction
PU	Polyurethane foams
q_e	Sorption capacity at equilibrium
Q_o	Monolayer sorption capacity
q_t	Sorption capacity at time t
r^2	Correlation coefficient
RSD	Relative standard deviation
S	Standard deviation
SBSE	Stir bar sorptive extraction
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SLM	Supported liquid membrane
SPME–GC–MS	Solid-phase microextraction–gas chromatography–mass spectrometry
SPMTE	Solid phase membrane tip extraction

SPR	Surface plasmon resonance
SWV	Square wave voltammetry
T	Time
TFMAA	2-(trifluoromethyl) acrylic acid
TTAB	Tetradecylammonium bromide
TTAB	Tetradecylammonium bromide
UAE	Ultrasonic-assisted extraction
UV	Ultra violet
Wt	Weight
ΔG°	Change in standard Gibbs free energy
ΔH°	Change in standard enthalpy
ΔS°	Change in standard entropy

Summary

Weeds are a serious challenge for crop production where increasing world population demand increasing supply of food. Chemical weed control is an efficient means of weed control, increase in crop production, decrease in tillage requirements and facilitating an increase in farm size. The discovery and use of triazine herbicides was a major milestone in providing the tools required for our modern crop production systems and it had resulted in the control of many weed species with one application. However increase in use of these chemicals has resulted in the increased accumulation of these pollutants in the environment (atmosphere, agricultural products, ground and waste waters). Triazine herbicides are toxic and are not only environmental risk but are also a health hazard.

Various chromatographic and electrochromatographic methods have been developed for the separation and quantitative determination of herbicide residues in different matrices such as food, biological fluids and soil. Most of the method mentioned above requires expensive instrumentation on one hand and specialized personnel on the other. Some of these even require round the clock power supply and as such are not suited for our environment. Therefore, there is a need for alternate methods based on simple instrumentation and at the same time need to be sensitive and reliable enough which could be used for multiresidue determination in soil, food and water samples. The work presented in this dissertation is an effort for the same.

The first chapter of this dissertation includes general introduction to weeds, weed control, herbicides, their history, classification and mode of action. Specific characteristic (physical and chemical properties) of the selected triazine herbicides (atrazine, metribuzin, ametryn and terbutryn), their mode of action and toxicology are also briefly discussed.

The second chapter deals with relevant literature of the reported methods for determination of selected triazine herbicides. Most of the reported methods for determination of triazine herbicides involve non aqueous capillary electrophoresis, molecularly imprinted polymer, micellar-electrokinetic capillary chromatography, high performance liquid chromatography, and gas chromatography after liquid-liquid

extraction, solid-phase extraction or solid phase micro extraction. These reported methods have been reviewed in the light of their scope, sample preparation, extraction and quantitative determination in samples, linearity range, limits of detection, limits of quantification and percent recovery.

The third and the last chapter deals with experimental work which includes brief discussion on preliminary investigations of each method developed, optimization of various parameters, application of these methods to environmental samples and results and discussion of the proposed methods. This chapter is divided into eight parts; each part is dedicated to a new method or procedure. The first four parts (from part I to part IV) presents spectrophotometric methods developed for the determination of the selected herbicides, which involves derivatization and complexation reaction, where as the last four parts (from part V to part VIII) includes various extraction methods proposed for the HPLC analysis of selected triazine herbicides.

The first method presented in Part I of chapter third describes a spectrophotometric method for determination of atrazine. This method is based on nucleophilic substitution reaction of atrazine with pyridine to form glutaconic dialdehyde. The glutaconic dialdehyde group was coupled with sulfanilic acid to form a yellow colored product having $\lambda_{\max}$ 450 nm or alternatively coupled with aniline to form orange red colored product having $\lambda_{\max}$ 480 nm. The Beer's law was obeyed over the concentration range from 0.1 to 25 $\mu\text{g mL}^{-1}$ with molar absorptivity of $1.5 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ for sulfanilic acid, and from 0.08 to 12 $\mu\text{g mL}^{-1}$ with molar absorptivity of $1.3 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ for aniline. Atrazine was satisfactorily determined by the proposed method with limit of detection (LOD) 0.029 $\mu\text{g mL}^{-1}$ and 0.021 $\mu\text{g mL}^{-1}$ and limit of quantification (LOQ) 0.098 $\mu\text{g mL}^{-1}$ and 0.041 $\mu\text{g mL}^{-1}$ with sulfanilic acid and aniline respectively. The proposed method has been successfully applied for the analysis of commercial formulations and real samples with average recoveries in the range of 92.2 % $\pm$ 0.20 – 97.0 % $\pm$ 0.70 from corn as well as sugarcane samples. A recovery test was also applied to the commercial formulations and average recoveries found with sulfanilic acid was in the range of 93.33 % $\pm$ 0.47 – 98.33 % $\pm$ 0.05 while with aniline the recovery was in the range of 90.0 % $\pm$ 0.10 – 96.67 % $\pm$ 0.30.

The second method deals with development of a spectrophotometric method for determination of ametryn and its application to real samples. The method is based on reaction with pyridine and further coupling with sulfanilic acid to form a colored product. The absorbance was measured at 400 nm with a molar absorptivity of $2.1 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$. The method shows a linear range from 0.2–20.0 $\mu\text{g mL}^{-1}$. The method has been successfully applied for determination of ametryn in sugarcane juice and commercial formulations after separation of ametryn from triazine herbicides using solvent extraction. Recovery values were found to be in the range of $96.0 \% \pm 0.2$ to $98.4 \% \pm 0.1$.

The third method is a spectrophotometric method for the determination of metribuzin herbicide and its application to real samples. Metribuzin was reacted with copper and to form a stable complex in the presence of ammonia (0.2 M) at pH 10.5. The resulting yellow colored complex was extracted in chloroform which showed absorption maxima at 340 nm. Beer's law was obeyed in the concentration range of 0.8–25.0 $\mu\text{g mL}^{-1}$ with molar absorptivity of $5.67 \times 10^3 \text{ Lmol}^{-1}\text{cm}^{-1}$. The composition of the complex was studied by Job's method of continuous variation and the results indicated that the mole ratio of metribuzin: Cu (II) is 2:1. A two-level factorial design was also used to investigate the effect of different parameters and their interaction on metribuzin: Cu (II) complex formed. The method was successfully applied for the determination of metribuzin in commercial formulations and real samples with recovery values found in the range of $86.0 \% \pm 0.9$ to $91.7 \% \pm 0.2$.

The fourth method is for determination of metribuzin herbicide and is based on the reaction of *p*-dimethylaminobenzaldehyde (DMAB) with sodium hydroxide via Cannizzaro's reaction at 100°C to form a *p*-dimethylaminobenzoic acid. The resultant *p*-dimethylaminobenzoic acid is reacted with metribuzin herbicide in acidic media at 100 °C and the yellow colored product obtained was measured at 455 nm. A linear plot between absorbance and concentration over the range from 0.2 to 20.0 $\mu\text{g mL}^{-1}$ was found with molar absorptivity of $2.1 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.05 $\mu\text{g mL}^{-1}$ and 0.2 $\mu\text{g mL}^{-1}$ respectively. The proposed method has been successfully applied for the analysis of commercial

formulation and potato sample. The recoveries of the method were found to be in the range of $92.16 \% \pm 0.06$ to $96.66 \% \pm 0.18$. Two level factorial designs of 2^3 and 2^2 were used to optimize all parameters, determine the influence of different parameters and their interactions on the final product formation.

Part V of third chapter deal with a microwave assisted extraction (MAE) procedure and separation followed by HPLC determination is presented for selected triazine herbicides. The procedure is based on MAE of soil samples for 4 min at 80 % of 850-W magnetron outputs in the presence of mixture of solvents (methanol/acetonitrile/ethylacetate). Related important factors influencing the MAE efficiency, such as the solvent type and volume, irradiation energy and time were optimized in detail. Calibration curve ranges established using HPLC for metribuzin, atrazine, ametryn, and terbutryn are 1.0–19.0, 0.9–18.0, 0.6–11.0, and 0.7–11.0 $\mu\text{g mL}^{-1}$, respectively. The limits of detection of metribuzin, atrazine, ametryn, and terbutryn are 0.30, 0.24, 0.16 and 0.20 $\mu\text{g mL}^{-1}$ while limits of quantification are 1.0, 0.80, 0.50 and 0.60 $\mu\text{g mL}^{-1}$, respectively. A Plackett–Burman factorial design was used as a screening method in order to select the variables that influence MAE extraction. The recoveries at three different spiked levels were $83.33 \% \pm 0.12$ – $96.33 \% \pm 0.23$ by analyzing real soil samples.

Part VI of third chapter deals with comparison of extraction performance of microwave-assisted extraction (MAE), ultrasonic assisted extraction (UAE) and shake-flask traditional extraction for analysis of triazine herbicides in corn sample. The extracted triazines were analyzed by HPLC using C_{18} column with UV detector. MAE provided better extraction efficiency than UAE and shake-flask extraction. Maximum recoveries were achieved with 2 min at 850 W magnetron out puts of MAE in comparison to 15 min of UAE and 60 min of shaking flask at three different spiked levels for control samples. The proposed MAE method allows extracting the triazine in a small volume of solvent and faster than the other extraction methods. The proposed method was validated using standard addition and average recoveries obtained with MAE were in the range of $79.0 \% \pm 0.17$ to $98.8 \% \pm 0.12$.

Part VII of third chapter deals a method proposed for the preconcentration and determination of triazine herbicides in irrigation water and sugarcane juice samples using sawdust followed by HPLC determination. The removal efficiency of selected triazine herbicides shows that the proposed method may could be used for decontamination as well as preconcentration of the target herbicides. The kinetic studies shows the equilibration time for deriving the adsorption isotherm is 6 min. According to the temperature studies, the adsorption of the target herbicides decreases with increase in temperature. The experimental results show that the ametryn and terbutryn can be completely separated using different aqueous solution of methanol. The Langmuir isotherm was the best approach for adsorption equilibrium data correlation. The developed method was successfully applied for the removal of selected herbicides from various aqueous and sugarcane sample.

Part VIII of third chapter describes a procedure for preparation of uniformly-sized, molecularly imprinted polymers (MIPs) for ametryn herbicide by precipitation polymerization method using divinyl benzene (DVB) as a cross linker and methacrylic acid (MAA), maleimide and 4-vinyl pyridine as a functional monomer. The MIP's synthesized using MAA showed better binding results for ametryn herbicide than polymers prepared using other functional monomer. Uniform monodispersed particles with narrow size distribution were obtained using maleimide as functional monomer. The MAA polymer also showed good binding selectivity for chloro triazine, atrazine, simazine, propazine and methylthio triazine prometryn, terbutryn. Different compositions of the mixture of solvent methyl ethyl ketone (MEK) and heptane was investigated as a reaction solvent. A solvent ratio of 30:70 MEK and heptane provided the best results for the MAA-based ametryn imprinted polymer.

INTRODUCTION

1. INTRODUCTION

1.1 Pesticides¹

A pesticide is constituent or combination of substances, synthetic or natural, formulated for the control of some pests that contest with humans for food or spreads diseases. The word pest comprises weeds, insects, microbes and others.

Pesticides exist usually as chemical substances. The active part of a pesticide, recognized as the active ingredient, is usually provided by the manufacturer as emulsifiable concentrate or in solid particles (wetable powder or soluble powder, granules or dust particles). Most of the market formulations are to be diluted with water for use and may have adjuvant to increase pesticide holding and absorption by shoots or leaves. Pesticides are divided into different classes based on the nature of their use. One of the important pesticide groups is of herbicides which are used against weeds and other plants growing in places where they are not wanted.

The use of pesticide has been increased 50-times since 1950 and about 2.5 million tons of commercial pesticides are used nowadays every year. According to the European Crop Protection Association Annual Report 2001 –2002, the frequent use of pesticide are in the agricultural areas of Asia, Europe, and North America with 22.6 %, 23.8 % and 31.9 % respectively in 2001. Amount of pesticides used in a particular environmental area rest on the conditions of climate and on the outburst of pests and infections in specific year. However, herbicides stand as main group of pesticides in use worldwide, followed by fungicides and insecticides.

1.2 Weed

Weed is a plant species growing where it is not desired (an out of place plant) or a plant that is more harmful in a place, it is not needed. Weeds limit the yield of crops, by competing for nutrients, light, moisture and space in the soil. Crops have the ability to tolerate annual weeds to some extent depending on its growth stage. Seedling crops do not suffer much from seedling weeds but can be damaged later when the weeds grow vigorously. Ripening plants are not affected much from weeds at the later stage, but the

weeds if left are likely to shed seeds, which will harm the upcoming crops. The middle period is critical and the weeds have greater influence on the growth of the crops. Removing weeds in this period makes a significant difference to the final results.

1.3 Weed Control ²⁻⁴

Weed Control is the process of holding and limiting weed invasions. Weed control of an individual species is determined by the growth habits and methods of reproduction. The important factors which influence control practices include the stage of growth (life cycle), time of year it germinates and grows, soil type, climatic conditions, crop and species of weeds. It is more difficult to selectively control weeds which have growth characteristics similar to the associated crop. The traditional and modern ways of weed control are discussed briefly as under:

1. Traditional ways of weed control

- a. Mechanical removal of weeds from the soil. The methods such as hand pulling, hoeing and spading, tillage (disturbing root systems with mechanical implements), mowing, flooding (physically removing oxygen from the soil) and smothering (a physical barrier, such as a black plastic sheet, at the soil surface which the weeds cannot penetrate) are adopted.
- b. Crop competition is effective where the growth habits of the crop favors the crop in relation to the weeds. In this way, the crop competes more efficiently for sunlight, water, nutrients, and space than the weeds. This is the most economical and informal method of weed control.
- c. Biological control uses natural predators to control weeds with little or no harm to the crop. Insects, parasitic plants, selective grazing and competitive replacement plants are some of the biological control agents.
- d. Crop rotation is another traditional means of controlling weeds by providing a strong competitive crop on disturbed soil during all periods of a growing season. Weeds flourish prior to crop establishment and after harvest.
- e. Fire is also an effective tool for weeds removal. Intense heat can burn green vegetation which usually dries in 10 to 14 days. Burning can destroy seeds and emerging seedlings

of the weeds. Burning is more effective in controlling small annual weeds in comparison to perennial species.

2. Modern method of control

Chemicals are the most efficient means of controlling weeds. Herbicides are products of modern technology and a tool for controlling weeds. Selective herbicides are being used since early twentieth century, and the greatest advances have occurred in the last four decades. Herbicides provide better weed control than cultivation, hoeing, and hand pulling. Herbicides along with other agriculture chemicals, such as fertilizers, and pesticides provide a solution for combating the rising costs and labor shortage. However, these chemicals are not a replacement for good management practices and conscientious farming.

1.4 Herbicides²

Herbicides are chemicals used to kill plants especially weeds or interrupt their normal growth. The agriculture system of today is heavily depended on herbicides as they form integral part of weed controlling practices. Presently there seems to have nearly no reports of plant destruction by weeds that cannot be resolved by the use of herbicides. Various herbicides that were in use in sixties and seventies of the last century are being replaced by some fresh and extra effective herbicides synthesized relatively recently. Usage of certain back date herbicides has been significantly reduced, restricted and even abolished after the understanding of its toxicological and environmental consequences and also because of the accessibility of additionally effective and nontoxic chemicals.

1.5 Classification of Herbicides²

There are more than 400 herbicides discovered and synthesized in the past five decades. These can be classified on the bases of following categories:

- 1) Structural similarity and chemical affinity
- 2) Method of application
- 3) Mode of action
- 4) Time of application

1) Classification based on chemical structure

This system of classification is based on similarity in chemical structure. The best collective means of determining the new herbicides is by making structural modifications in the established herbicide chemical family or nucleus. Therefore, herbicides which come from one chemical family or nucleus show structural resemblance and similarities and this provides the foundations of chemical classification.

2) Method of Application⁵

Grouping based on this category, classify herbicides into two main groups i.e. foliage-applied and soil-applied. Herbicides used as preplanting or preemergence come under the umbrella of soil-applied group and the one applied to the plant parts at postemergence are called as foliage-applied herbicides. These chemicals kill weeds either by contact or by systemic action. **Contact herbicides** kill only the parts of plant which it touches. **Systemic herbicides** get riveted by the roots or the foliage and are passed to all parts through the plant. Systemic herbicides are principally active against perennial weeds as the chemical spreads to all plant parts even to woody stems and deep roots, which seem to be rather unreachable. Contact herbicides are commonly used for the control of annuals and biennials and are considered by the fast die-back they do. Systemic can proceed through a little longer time to offer the wanted results up to few weeks, or even extended time for woody perennials. Herbicide action is either selective or nonselective. Selective herbicides kill weeds without substantial or no damage to adjacent plants. These are used for decreasing weed competition in crops, ornamental plants and lawns. Nonselective herbicides kill all existing plants if used at suitable rate. These are particularly for no plant growth in places such as ditch banks, drive banks, fence rows, parking lots and roadsides. The selectivity of herbicides can differ depending on the rate of application. Large amount of selective herbicides generally harm all plants at the site of application. Certain nonselective herbicides can be used selectively if applied at a lower rate. The other reasons that affect selectivity comprise the method and its time of application, stage of plant growth and the environmental conditions.

There are several causes for increasing a plant's vulnerability to herbicides and are mentioned below.

- i) The location of growing plants:** Plants that are covered or sited underneath the soil surface are not touched by the spray of contact herbicide.
- ii) The shape of leaf:** Herbicides incline to spring or overspill on the narrow and upright leaves. Flat and broad leaves hold the herbicide for longer time.
- iii) The cuticle and wax:** The foliar sprays can be stopped from going to the leaf by a thick cuticle and wax layer. Waxy surface also form droplets of spray solution and flow off the leaves surface.
- iv) The leaf hairs:** A thick cover of leaf hairs clasps the herbicide droplets off the leaf surface, letting a lesser amount of chemical to be taken into the plant. On the other hand, a thin sheet of leaf hairs let the chemical to stay on the surface of leaves for longer permitting more chemicals to harm the plant.
- v) The age and size:** Young and rapidly rising plants are more liable to herbicides than are larger and more developed plants.
- vi) Herbicide resistance ability of plants:** Certain plants can build up herbicides resistivity and become less prone to damage from these chemicals. These plants can become dominant over a certain period of time if the same herbicides are used frequently.
- vii) The Stage in the life Cycle:** Seedlings seem more sensitive to herbicides and to utmost other weed control methods. The stages of vegetation and early bud formation are more subject to translocated herbicides. Plants with seeds or in the maturity stage are the least susceptible to weed control practices.
- viii) Time and stages in the Life Cycle:** The plants that can sprout and grow at dissimilar times than the crops possibly will be liable to cautiously timed herbicide uses.

3) Mode of Action⁶⁻⁷

Understanding about the mode of action of herbicide is valuable in detecting herbicide damage complaints. Indeed a very large number of herbicides are marketed

commercially, a number of these have similar properties and activity. Herbicides having common properties are placed into "families." These herbicide families represent an easy way of gathering information about them. Moreover, among these herbicide families some have the similar mode of action in the plant and shows same injury symptoms and herbicide activity. The following information describes the features of broadly used families of herbicides grouped according to their mode of action. The chief modes of action are: amino acid synthesis inhibition, seedling growth inhibition, lipid synthesis inhibition, photosynthesis inhibition, pigment inhibition, cell membrane disruption and growth regulation.

I. Amino Acid Synthesis Inhibitors

These inhibitors activate a particular enzyme which prevents the production of target amino acids that are important constituents for normal development and growth of plants. Such herbicides stop the release of three main branched amino acids by destroying one basic enzyme of plant. On the whole, symptoms of injury develop slowly within 1 or 2 week time and have restricting effect on plant growth and eventually lead to slow plant death. Herbicides of this families show action on both annual and perennial grass weeds and broadleaf and are foliar or soil applied. Amino acid synthesis inhibitors are nonselective and their place of uptake in the plant is the foliage. Herbicides of this family travel through the phloem to different plant parts; they have tremendous perennial weed control activity and are also active against annual weeds.

II. Seedling Growth Inhibitors

These types of herbicides restrict fresh plant growing, thus decreasing the capability of seedlings to progress in the normal way in the soil. Herbicides of this family must be applied to the soil. The plants can uptake these growth inhibitors after sprouting till the seedling appears on the soil. As a result, the herbicides are simply effective on seedling perennial and annual weeds. Plants which can succeed in emerging from the soil unharmed are expected to stay unaffected. Such inhibitors remain active by two central locations, the emerging root and shoot. These herbicides stop the cells of plant from dividing, which then inhibits the shoot propagation and also the lateral root formation.

The uptake of the herbicide is performed by the developing shoots and roots. As the herbicide transfer in the plant is restricted, herbicide damage is limited mainly to the plant shoots and roots. These herbicides are transferred to developing shoots and roots and moves through xylem to the areas of fresh growth. There is confirmation which proposes that these seedling growth chemicals can upset numerous sites of a plant and mostly interfere with protein and lipid synthesis.

III. Lipid Synthesis Inhibitors

Lipid synthetic inhibitors prevent the development of fatty acids constituents vital for the manufacture of plant lipids. Lipids components are essential for the long life of the cell membranes and also for the new plant growth. These herbicides prevent a distinct important enzyme used by plant body in fatty acid biosynthesis. The broadleaf plants show tolerance to this herbicide family, but nearly all annual and perennial grasses are vulnerable. The Injury signs are developed slowly within one to two weeks and appear first on fresh leaves developing from the spiral of the grass plant. Such herbicides enter plant through the foliage and travel in the phloem to areas of plant with new growth.

IV. Photosynthesis Inhibitors

Triazines, benzothiadiazoles, phenylureas, nitriles and uracils are included in the photosynthetic inhibitors family. These inhibitors actually shut down the photosynthesis in the target plant by attaching to particular sites of the chloroplasts in plants. Blocking of photosynthetic process could result in a slow death of the plant; conversely, the plant shows a rapid loss which is supposed to be as a result of production of some other toxic substances from these herbicides. The injury indicators consist of yellowing of tissue of leaf followed eventually by the tissue death. Three of photosynthetic inhibitor families (phenylureas, triazines and uracils) enter the plant by the foliage or roots and travel in xylem of the plant to the leaves. Consequently, damage symptoms appear first on the older leaves, beside the leaf edge. After the application, these herbicides become less mobile and stay inside the tissues of the leaf. Two of herbicide families (benzothiadiazole and nitrile) are not that mobile in the plants and are called as postemergence contact herbicides so they have no activity in soil. The contact herbicides must completely cover

a disposed plant's foliage if thorough elimination is to be attained. The herbicide of this group may control perennial or annual broadleaf weeds or grass. Information about the use, common characteristic, injury symptoms and site of action are discussed in detail in as following:

- Use: Ametryn in combination with atrazine for corn, sugarcane Atrazine for sugarcane, corn and sorghum. Metribuzin for potatoes and soybeans
- Common Characteristic of Photochemical System II Inhibitors: The CO₂ fixation rate decreases within few hours of application. The growth of the plant root is not directly affected by this group of herbicides when used according to the specified rates. All most all of the members of this group travel through xylem, therefore the perennial weeds are killed by application of these herbicides to soil. The foliage should be thoroughly covered when these herbicides are applied as postemergence spray as there is a slight besipetal movement of herbicide and their action on weeds become contact instead of systemic form. Surfactants are regularly added to raise foliar action. Plants become at risk to post emergence spray when they are under low light in the days before spraying and at abundant light after the spraying. Generally, translocation of these herbicides in soil is small to modest, but it depends upon the compound, rainfall and soil in the particular area. Their presence in the soil varies from one month to few years depending on the soil, climate, nature and amount of herbicide applied.
- Injury Symptoms: These herbicides do not stop plantlets from growing or emerging. Symptoms of damage only occur after the appearance of first cotyledons or first leave. Early injury signs include staining of the leaf tips and margins. Bruising in the middle of the leaf veins may take place in broad leaf plants. Larger and older leaves are affected ahead of other parts as they take in more of the herbicide solution and they serve as the main photosynthetic tissue of a plant. Harmed tissues of leaf will ultimately become brown and will die. Based on the chemical nature of the herbicide/soil relationship, injury warning signs are expected to increase with increase in the soil pH i.e. > 7.2.
- Site of Action: The specific D-1 quinine-binding protein of electron transport chain of photosynthesis.

V. Cell Membrane Disrupters

This family includes the bipyridylum and diphenylether. Cell membrane disrupters are postemergence contact herbicides which are triggered by sunlight to form oxygen containing compounds such as hydrogen peroxide. These herbicides can damage plant tissue by rupturing cell membranes of plants. Damage of cell membranes gives a rapid browning of the plant tissues. Herbicide damage indications can be visible in 1 to 2 hours on a sunny and bright day. As cell membrane disrupters are one of the contact herbicides, that is why they show excellent property of damaging foliage and the postemergence control of annual weeds. Weeds (perennial) frequently regrow because there is no herbicide movement to subversive shoot and root systems. This kind of herbicides has very little activity in soil.

VI. Pigment Inhibitors

These inhibitors stop the plants from developing photosynthetic tinctures. Consequently, the pretentious parts of the plant become white and then transparent. This herbicide enters by plant shoots and roots and moves in the plant xylem to their leaves. The freshly developed vegetation of numerous plant species is so delicate to these herbicides that even very slight amounts can bleach the new plant growth. Norflurazon, a herbicide applied to soil, is the only herbicide of this family in use nowadays. It enters the plant roots and passes to the developing parts of subject plants. At risk weeds emerges as gray plants before vanishing.

VII. Growth Regulators

Growth regulators family includes the subsequent herbicide: the pyridines, phenoxy acetic acids and the benzoic acids. The growth regulator herbicides act on many sites in a plant to upset hormone equilibrium and also the protein production and therefore are a cause of a range of plant growth irregularities. These growth controller herbicides carefully destroy broadleaf weeds; never the less, these are capable of damaging grass crops. Herbicides of this group have the ability to move inside the plant body through the xylem and also through the phloem to the areas of new plant growth. Thus, several

herbicides of this group are active on annual and perennial broadleaf weeds. The herbicides are taken up mostly through the leaves but root uptake is also possible. Damage symptoms are most apparent on freshly rising leaves.

4) Time of Application

Several ways are used for application of herbicides. These include preplant, preplant incorporated, postemergence, preemergence and lay-by. In Preplant application herbicides is applied before planting a crop. Preplant incorporated means when the herbicide has to be added to the soil prior to a crop is implanted. In case of postemergent herbicide is applied after weed or crop emergence. Preemergence application of herbicide is after implanting the crop but prior to weed or crop emergence. Herbicide is applied next to the last cultivation in case of lay-by application. This terminology generally speaks of application of herbicides relative to crops; but, it can also refer to use in relation to weeds.

1.6 Triazine Herbicides⁸

The very first triazine herbicide was discovered by J.R. Geigy, Ltd. in Switzerland in 1952. Today a variety of the triazine herbicides are listed in more than 100 countries of the world with broad-range weed control in crops and noncrop areas.

Triazine herbicides are divided into four different classes based on structural differences. These are chlorotriazines, methylthiotriazines, methoxytriazines, and typical or asymmetrical triazines. The chlorotriazine group includes atrazine, cyanazine, propazine, simazine and terbuthylazine. The methylthiotriazine group contains ametryn, prometryn, and terbutryn. The methoxytriazine group consists of prometon and sebumeton. Hexazinone and metribuzin represents triazinone group of triazine.

Chlorotriazines are used for preemergence and postemergence control of broadleaf and grassy weeds in corn, sorghum, sugarcane, and other crops. Methylthiotriazines controls preemergence and post-directed broadleaf and grass weeds in banana, cotton, corn, celery, pineapple, sugarcane, and also in the noncrop areas. Methoxytriazines are used mostly for weeds control in the noncrop areas. Metribuzin is used for preemergence and

postemergence control of many grasses and broad leaf weeds in corn, potato, sugarcane, soybean and many cereals.

1.6 Specific Characteristic of Triazine Herbicide^{2,6,9}

Ametryn

Ametryn has the chemical formula name as *N*-ethyl-*N'*-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine. This herbicide has tremendous contact activity. It is available in the white crystalline solid form. At 20.0 °C it has a vapour pressure of 8.4×10^{-7} mm of Hg. This triazine has a solubility of 200.0 $\mu\text{g mL}^{-1}$ in water and a half-life of 60.0 days in soil. In the tropical areas, it shows better weed control and its residence life in the soil exceed to several months. Although it is stable but is also prone to UV decomposition.

Metribuzin

The chemical formula name of this triazine herbicide is 4-amino-6-*tert*-butyl-3-methylthio-1,2,4-triazin-5(4*H*)-one. It belongs to the triazinone herbicide family, which was discovered in 1964. It is also crystalline white solid with 1.2×10^{-7} mm of Hg pressure with solubility of 1100.0 $\mu\text{g mL}^{-1}$ in water at 20 °C. Metribuzin is a relatively stable triazine herbicide and is not effected by UV light. It has a half life of 30.0 to 60.0 days. Metribuzin residue do not damage the vulnerable crops grown one year after application of this herbicide but few of the crops with roots such as potato, onion and sugar beets can show susceptibility.

Atrazine

Atrazine is one of the member compounds of chloro-triazine herbicide. The properties of this herbicide make it liable to runoff and leaching, particularly in rains. The test procedure provided by Goss and Wauchope in 1990 gives atrazine a “huge” potential for leaching or moving in the surface solution and an “average” potential for adsorption to residue particles. Atrazine has a half life of 60.0 to 150.0 days in loamy soils. Nevertheless, after change of environment in soils from aerobic to anaerobic the speed of degradation slows down considerably. Besides this, degradation of atrazine is very slow when it enters the water channel. The half-life of this herbicide in water reservoirs can be up to 2.0 years. This type of slowing down in rate of degradation of atrazine has

significant environmental consequences. Water polluted with atrazine leach down in the soil and moves away from the root zone of the plant. Similarly, the presence of atrazine herbicide in ground water and in the badly aerated areas of the surface waters is also predictable to have higher concentration as compared to the well aerated waters. Hence, literature explains that atrazine herbicide and its degradation products keep on staying in various soils and in reservoirs. Based on data about atrazine monitoring in ground and surface water, it is predictable that if the amount of the degraded compounds are also considered, the atrazine total residues in groundwater would be approximately double the amount of atrazine alone.

1.7 Mechanism of action and toxicology: triazines¹⁰

Mechanism of action

Triazine herbicides kill the weeds by meddling with their photosynthetic course. Many of the triazine compounds interfere with Photosystem II of the photosynthetic process, which uses chlorophyll to transform short-wavelength light in the form of chemical energy for plants survival. Without this conversion of energy, the plants cannot produce sufficient energy for their growth. Vulnerable plants, particularly growing seedlings, go yellow and die. These compounds when applied to the soil are taken up by the plant's roots. The efficiency of triazines is dependent upon their comparative rates of uptake and breakdown in the plants. Some proof has related the mode of toxicity of these triazines in mammals to an interference of the vitamins metabolism.

Acute toxicity

Triazines herbicides have a little acute toxicity for mammals. Atrazine have oral rat LD₅₀ values of 3000.0 mgkg⁻¹day⁻¹. Most of the other members of this group also have high oral LD₅₀ values. Classic symptoms of acute toxic effects from these compounds include eye and skin irritation, vomiting, nausea, muscle weakness, diarrhea and salivation. Acute skin rashes and sub-acute skin rashes have also been reported in the former U.S.S.R. from contact with chlorotriazines. The vital symptoms include burning and reddening which could last up to 5 days.

Reproductive effects

In the study of three-generation of rat in which $5.0 \text{ mgkg}^{-1}\text{day}^{-1}$ was controlled, toxic effect varied from a no adverse effect to a decrease in the mean of parental body weights for different triazine herbicides. Triazines caused a reduction in second-generation rats' body weights and another study showed some extent of toxic effects in fetal rabbits. Thus, particular triazine compounds can cause hostile reproductive effects.

Teratogenic effects

Feeding of simazine or atrazine to rats showed no teratogenic effects ($5.0 \text{ mgkg}^{-1}\text{day}^{-1}$). Amounts of $100.0 \text{ mg kg}^{-1}\text{day}^{-1}$ of propazine in rats had no maternal harmfulness or teratogenic properties. A quantity of $300.0 \text{ mgkg}^{-1}\text{day}^{-1}$ or higher, resulted in declines in maternal and fetal body weight and also in feed ingestion.

Carcinogenic effects

In 2-year study of triazine with high oral doses of atrazine and propazine, an increase of mammary tumors in female rats was noticed.

Organ toxicity

Various triazines have showed liver damages with high doses in test animals.

Fate in humans and animals

The triazine herbicides that have been ingested are freely absorbed into the gastrointestinal system. In different animal's studies, more than half of different triazines were expelled in the urine or in the feces in about four days. Trace amounts of these herbicides can bio accumulate in the muscles and fats of the animals.

LD₅₀ values of some triazine herbicide¹¹

Ametryn	110.0 mg/kg
Metribuzin	322.0 mg/kg
Atrazine	3000.0 mg/kg

Soil Influences⁶

Triazines herbicides get reversibly attached by clay and organic matter. In the study of 25.0 soil types having five triazine herbicides added to them, the adsorption decreased in the following order: prometryn > prometon > simazine > atrazine > propazine. Methoxy and methylthiotriazine adsorption on soil was reported to be highly linked to clay composition, and adsorption of chlorotriazine (propazine, simazine, atrazine) was somewhat more affected by the organic matter. A reduction in phototoxicity of triazine herbicides is associated with increasing amount of clay and organic matter in soil.

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LITERATURE REVIEW

2. LITERATURE REVIEW

Melo, *et al.*, (1) investigated high-performance liquid chromatographic (HPLC) determination of six pesticides in tomato samples. NH_2 (aminopropyl) and silica-based C_{18} (octadecyl) solid-phase extraction (SPE) sorbents, made in the laboratory, were used for sample extraction. By thermal immobilization of proper polysiloxanes onto $40.0\ \mu\text{m}$ silica surfaces, SPE materials were obtained and were used in sample preparation for analysis of the pesticides: tebuthiuron and diuron (urea herbicides), atrazine, simazine and ametryn (triazines herbicides) and benomyl (benzimidazol fungicide). The results obtained were compared with similar commercial materials. Reversed-phase high-performance liquid chromatography was used for analysis of these pesticides using Purospher RP-18 $5\ \mu\text{m}$ column and acetonitrile: 0.01 % aqueous ammonium hydroxide, pH 8.4 (35:65, v/v) as mobile phase, at $0.7\ \text{ml min}^{-1}$, with 235.0 nm ultraviolet detection. The Method was validated using three fortified levels (100.0 , 200.0 , $1000.0\ \mu\text{g L}^{-1}$). Limits of detection and quantification showed that the methods developed can be used to determine the pesticides in concentrations below the maximum residue levels set by the Codex Alimentarius, European Union, United States of America and Brazilian legislations. The results showed that aminopropyl were a better sorbent than octadecyl. The results obtained with laboratory-made materials were comparable to commercial sorbents for residue analysis, with recoveries and precisions in agreement with directives for method validation.

Nascimento, *et al.*, (2) determined seven low-dosed pesticides in saline concentrates for hemodialysis by solid-phase extraction (SPE) procedure for cleanup and preconcentration followed by high performance liquid chromatography with ultraviolet (HPLC-UV) detection. The target compounds were ametryn, desmetryn, molinate, prometryn, terbutryn, triallate and butylate. Three types of polyethylene, teflon, polyurethane and polystyrene, in powder form, were investigated as adsorbents for solid-phase extraction of the analytes from the saline samples. Quantification done using reverse phase chromatography on a LiChrosorb RP-18 ($5\ \mu\text{m}$, $125\ \text{mm} \times 4\ \text{mm i.d.}$) column] on gradient elution mode with water/acetonitrile as mobile phase. A single chromatographic

run was 18 min including the time for column reconditioning. The retaining efficiency of the different SPE substrates for the analytes from the highly concentrated saline (HSC) samples was discussed. Best results were achieved with polystyrene as SPE material considering preconcentration factor, precolumn clogging, reusability and similarity between the mobile phases for SPE and HPLC procedures. Determination of analyte concentrations as low as $1.0 \mu\text{gL}^{-1}$ were achieved in spiked HCS samples after preconcentration on polystyrene SPE precolumns. Recoveries obtained were 98.7 and 102.2 % from commercial spiked samples. Detection limits obtained, ranged from 4.8 (for prometryn) to $46.0 \mu\text{gL}^{-1}$ (for butylate) without preconcentration. The relative standard deviations ($n = 9$) calculated within a day ranged from 2.3 to 4.8 %.

Bagheri and Khalilian., (3) developed an immersed solvent microextraction (SME) method for the enrichment of s-triazine herbicides from aquatic samples. Extraction solvent used was a microdrop of butyl acetate. The microdrop was introduced into a gas chromatography–mass spectrometry (GC–MS) injection port after extraction. Extraction parameters such as type of solvent, stirring rate, extraction time and temperature were optimized. High enrichment factor was obtained by a microdrop volume of $3.0 \mu\text{L}$, sampling temperature of 60.0°C and by the use of butyl acetate. The linearity and enrichment factor were studied by preconcentration of 1.0 ml of high performance liquid chromatography grade rice farm water and river water samples spiked with a known solutions of triazine herbicide at a concentration range of $0.1\text{--}100.0 \mu\text{gL}^{-1}$ (relative standard deviation $< 11.0\%$). The correlation coefficient obtained was satisfactory ($r^2 > 0.98$) for all the studied triazine herbicides. The limits of detection achieved using HPLC-grade and river water sample was in the range of $0.015\text{--}0.4 \mu\text{gL}^{-1}$. The matrix effect on extraction was also studied using real water samples.

Ribeiro, *et al.*, (4) evaluated the applicability of the electrokinetic process for atrazine determination soil when submitted to an electric field. Two polluted soils were used on with and the other without atrazine residues and the latter was spiked. Four electrokinetic experiments were studied at a laboratory scale. Atrazine determination was done by enzyme-linked immunosorbent assay (ELISA). The results showed that the electrokinetic process was able to efficiently carry atrazine herbicide in soil solution towards the anode

compartment. The results showed that 30.0–50.0 % of the initial amount of atrazine is removed from the soil within the first 24.0 hr. One-dimensional model was developed for the electrokinetic treatment of a saturated soil samples having atrazine. The movement of atrazine was studied by the diffusion transport mechanism resulting from atrazine concentration gradients and also by reversed electro-osmotic flow at low pH of soil.

Piletska, *et al.*, (5) developed molecularly imprinted polymers (MIPs) for simazine determination and its controlled release in water. Three polymers were studied using computational modelling. Hydroxyethyl methacrylate (HEM) and methacrylic acid (MA) were selected as monomers on the basis of the results obtained using the Leapfrog™ algorithm. A cross-linked polymer with no functional monomers was tested as a control. The release of simazine from the three polymers was studied. The results showed that the presence of monomers is important for polymer affinity and also for controlled release of simazine herbicide. The correlation values of the speed for release of the herbicide with binding characteristics were calculated. The high-affinity MA-based polymer released 2.0 % while the low-affinity HEM polymer released 27.0 % of the template over a period of 25.0 days. The kinetics of simazine released from HEM polymer showed that the saturation of an aqueous environment could be achieved over a period of 3.0 weeks which will correspond to the maximum simazine solubility in water.

Zhoua, *et al.*, (6) described a sensitive method for the determination of atrazine and simazine herbicides on solid phase extraction using carbon nanotubes and high performance liquid chromatography with diode array detector. Important factors affecting the preconcentration factor of multiwalled carbon nanotubes such as the volume of eluent and the water samples, sample flow rate and pH were optimized. At optimum conditions, multiwalled carbon nanotubes was achieved with tremendous enrichment efficiency for simazine and atrazine. The detection limits of the simazine and atrazine were 9.0 and 33.0 ngL⁻¹, respectively. The recoveries of the atrazine and simazine from spiked samples were in the range of 82.6–103.7 %. Good analytical performance was achieved with the proposed method for real water samples (river, reservoir, tap and wastewater after primary pretreatment). The results indicated the potential use of this method as alternate for the analysis of simazine and atrazine in environmental water samples.

Bruzzoniti, *et al.*, (7) optimized a solid phase extraction (SPE) procedure for the gas chromatography–mass spectrometry (GC–MS) determination of herbicides of different families like carbamate (molinate), atrazines (ametryne, atrazine, propazine, cyanazine, terbutylazine, simazine, deethylterbutylazine, deethylatrazine), dinitroaniline (trifluralin, pendimethalin) and chloroacetamide (metolachlor, alachlor). Different solid substrates such as C₁₈, cyano, phenyl, styrene–divinylbenzene, graphitic carbon have been compared. Variables such as the elution solvent and its volume, type of conditioning and the sample flow rate affected the recovery of these herbicides. Experimental conditions optimized were C₁₈ column conditioned with 3.0 mL acetone, loaded with 1.0 L water sample at 5.0 mLmin⁻¹, and eluted with 3mL acetone. Recoveries in the range of 79.0 % - 99.0 % and relative standard deviation between (2.0 % and 12.0 %) have been obtained for all the analytes, except for deethylatrazine (with recovery of 46.0 ± 7.0 %). A non-porous graphite carbon (when coupled to the C₁₈ phase, keeping the other optimized parameters constant) increased the recovery of deethylatrazine to 94.0 ± 17.0 %. The optimized method was successfully applied for the analysis of the selected herbicides in raw and drinking water samples, with quantification limits as low as 0.01 µgL⁻¹. The developed method was applied for the analysis of herbicides in water and more than one thousand samples were analysed.

Huertas-Perez, *et al.*, (8) developed a micellar electrokinetic chromatographic (MEC) method for the analysis of metribuzin and its major conversion products (diketometribuzin, deaminometribuzin, and deaminodiketometribuzin) in soils. MCE was considered effective method for the separation of these compounds, providing good efficiency and short analysis times. Parameters such as the buffer concentration, pH, sodium dodecyl sulphate concentration, injection conditions and applied voltage were studied. The target compounds showed excellent separation in about 7.0 min. These analytes were extracted from soil samples using methanol in an ultrasonic bath and then pre-concentrated by using a LiChrolut EN sorbent column. The limit of detection obtained was at the low µgkg⁻¹ level. The proposed method was applied for the determination of the target herbicides in soil samples and showed recoveries ranging from 86.7 % to 104.2 % and thus represented an alternative to HPLC method.

Bichon, *et al.*, (9) investigated a liquid chromatography- electron spray ionization mass spectrometry (LC–ESI-MS/MS) method for the determination of several triazine and phenylurea herbicides and their transformation products in oysters at the low $\mu\text{g/kg}$ level. Pressurised liquid extraction of lyophilised samples required liquid/liquid extraction combined with successive SPE for providing relatively clean extracts for onward determination by liquid chromatography mass spectrometry. This procedure was validated in according with 2002/657/EC analytical decision. This analytical method led to confirmatory CC α values ranging from 0.1 to $14.0 \mu\text{gkg}^{-1}$ with relative standard deviation value in the range of 14.0 % to 66.0 %. Recovery ranging from 32.0 % to 46.0 % for phenylureas and from 29.0 % to 75.0 % for triazines herbicides were obtained respectively.

Agostino, *et al.*, (10) synthesized a MIP for atrazine determination using a potentiometric sensor. The MIP worked like a solid ion selective electrode where the specific carriers were the imprinted sites. The protonated atrazine was positively charged. Determination was carried out in solution at pH lower than 1.8, in which atrazine was prevalently monoprotionate to form the positively charged ion. Under these conditions, the membrane potential increased with increase in atrazine concentration over a range of 3.0×10^{-5} to 1.0×10^{-3} M. The slope for potential versus log atrazine concentration of about 25.0 mV/decade showed that a biprotonated atrazine form was sorbed on MIP. The detection limits were determined by the high concentration of atrazine released by the membrane in the sample solution but was independent of the atrazine concentration in the internal solution of the sensor, and depended on the acidity of the solution. The response time was less than 10.0 s and the sensor could be used for more than 2.0 months.

Carabias-Martínez, *et al.*, (11) prepared a MIP via precipitation polymerization method using methacrylic acid as functional monomer and propazine as template for the extraction of methylthio and chloro-triazines and some of their hydroxylated and dealkylated metabolites. Interaction between the functional monomer and the template was confirmed by proton NMR analyses. The spectra showed that the nitrogen and hydrogen of the propazine amino group were involved in the formation of hydrogen bonds with the functional monomer. The propazine-MIP for the extraction of triazine

herbicides and their derivatives were studied in aqueous and organic media and different behaviours were observed. In organic medium, selective extractions of methylthio and chloro-triazines by using the optimization extraction process. Recovery values higher than 75.0 % were obtained in all cases. Though, the hydroxylated metabolites were not retained on the propazine-MIP in organic medium, the chloro and the dealkylated chloro-triazines were retained on the propazine-MIP by specific interactions in aqueous medium. It was observed that the recoveries obtained were related to their polarity. The hydroxylated metabolites were retained on the propazine-MIP through non-specific hydrogen-bridge interactions. The methylthio-triazines were poorly recognized in aqueous medium.

Katsumata, *et al.*, (12) proposed a sensitive and selective column adsorption procedure for the preconcentration and determination of simazine and atrazine. These herbicides were preconcentrated on heat-treated diatomaceous earth as an adsorbent and determined by high-performance liquid chromatographic system. Several parameters were investigated for the recoveries of the analytes. The experimental results showed the possibility of obtaining quantitative analysis (when the solution pH was 2.0 using 100.0 mL of validation solution containing 1.5 μg of triazines and 5.0 mL of ethanol as an eluent). Recoveries of atrazine and simazine obtained were $95.7 \pm 4.2 \%$ and $75.0 \pm 1.9 \%$ with a relative standard deviation for seven determinations as 4.7 % and 2.7 % under optimum conditions. The maximum preconcentration factor obtained was 100.0 for triazines when 500.0 mL of sample solution volume was used. The linear calibration curves range for atrazine and simazine were $1.0\text{--}150 \text{ ngmL}^{-1}$ and $1.0\text{--}300 \text{ ngmL}^{-1}$, respectively. Correlation coefficients of 0.999 and the detection limits (3 Signal-to-Noise) of 0.24 ngmL^{-1} and 0.21 ngmL^{-1} were achieved for atrazine and simazine respectively. The adsorbent capacity was found to be 0.8 mgg^{-1} and 1.3 mgg^{-1} for atrazine and simazine. The proposed method was effectively applied for the determination of triazines in tap and river water samples with high precision and accuracy.

Ross and Filipov., (13) developed a broad and sensitive LC-MS method for the analysis of atrazine and its metabolites in mouse urine and plasma. Simultaneous measurement of atrazine and its major mammalian metabolites, which include didealkyl atrazine,

desisopropyl atrazine, desethyl atrazine, atrazine -glutathione conjugate, and atrazine -mercapturate, were achieved using preparation procedures that used small sample volumes of plasma and urine (0.25 and 0.5 mL, respectively). In addition, derivatization of analytes prior to analysis was not required. To demonstrate the utility of this method, plasma and urine samples were analysed following single in vivo oral exposures of a limited number of mice to atrazine (doses, 5.0–250.0 mgkg⁻¹ body weight). The results obtained suggested that atrazine was rapidly metabolized in mice. Didealkyl atrazine was the most abundant metabolite detected in the urine and plasma samples (1000.0 µM in 24.0-hr urine and 100.0 µM in plasma following the highest dose of atrazine), with lesser quantities of mono N-dealkylated metabolites and thio conjugates of atrazine. This methodology was also used in a preliminary study of cytochrome P450-catalyzed metabolism of atrazine in vitro. The study suggested that this method will be a useful tool for the atrazine and its metabolites determination in future pharmacokinetic studies and for the subsequent development and refinement of biologically based models of atrazine disposition.

Tran, *et al.*, (14) described the application of different extraction techniques for the preconcentration of ten acidic and non-acidic polar herbicides (2,4-D, atrazine, bensulfuron-methyl, clomazone, dicamba, diuron, MCPA, metolachlor, simazine and triclopyr) in the aqueous environment. Extraction efficiency of these herbicides in different matrices were compared by Liquid–liquid extraction using dichloromethane and solid-phase extraction (SPE) (using Oasis HLB cartridges or SDB-XC EmporeTM disks) for water samples from contaminated agricultural drainage water containing high concentrations of particulate matter. These herbicides were separated and quantified by high performance liquid chromatography with ultraviolet detector. Target herbicides in the Murrumbidgee Irrigation Area (NSW, Australia) were determined by applying SPE using SDB-XC EmporeTM disks during a two-week survey from October 2005 to November 2005. The daily aqueous concentrations of herbicides from 24.0-hr composite samples detected at two sites increased after run-off from a storm event and were in the range of: < 0.1–0.9, 0.1–17.8 and 0.2–17.8 µgL⁻¹ at site 1; < 0.1–0.2, < 0.1–3.5 and < 0.2–3.2 µgL⁻¹ at site 2 for atrazine, simazine and diuron herbicides respectively.

Sambe, *et al.*, (15) prepared uniformly-sized, molecularly imprinted polymers (MIPs) for atrazine, ametryn and irgarol by a multi-step swelling and polymerization method using ethylene glycol dimethacrylate as a cross-linker and methacrylic acid (MAA), 2-(trifluoromethyl) acrylic acid (TFMAA) or 4-vinylpyridine either as a functional monomer or not. Good molecular recognition abilities for chlorotriazine herbicides (atrazine) were obtained with MAA, while the MIPs for ametryn and irgarol using TFMAA showed excellent molecular recognition abilities. A restricted access media-molecularly imprinted polymer (RAM-MIP) for irgarol was prepared followed by hydrophilic surface modification (in situ) using glycerol dimethacrylate and glycerol monomethacrylate as hydrophilic monomers. The selective pretreatment and enrichment of methylthiotriazine herbicides, simetryn, ametryn and prometryn, in river water was done by using RAM-MIP, followed by their determination via column-switching HPLC with UV detection. The calibration graphs of these compounds showed good linearity in the range of 50.0–500.0 pgmL^{-1} ($r^2 > 0.999$ with a loading of a 100.0 mL river water sample). The quantitation limits of simetryn, ametryn and prometryn were 50.0 pgmL^{-1} , and the detection limits were 25.0 pgmL^{-1} . The recoveries of ametryn, prometryn and simetryn at 50.0 pgmL^{-1} were 95.6 %, 95.1 % and 101.0 % respectively. This procedure was effectively applied for the determination of ametryn, prometryn and simetryn in river water.

Nagaraju and Huang., (16) reported a simple and rapid dispersive liquid–liquid microextraction technique (DLLME) combined with gas chromatography–ion trap mass spectrometric detection (GC–MS) for the analysis of triazine herbicides in water samples. In this method, a mixture of acetone (1.00 mL) and chlorobenzene (12.0 μL) as an extraction and disperser solvent respectively, was injected by syringe into the water sample (5.00 mL) containing 4.0 % (w/v) sodium chloride. Triazines in the water sample were extracted into droplets of chlorobenzene followed by centrifugation (at 6000 rpm for 5.0 min), the fine droplets of chlorobenzene were settled in the bottom of the conical test tube ($8.0 \pm 0.3 \mu\text{L}$). The 2.0 μL of settled phase was injected into the GC–MS for the separation and determination of triazine herbicides. Some important parameters such as salt effect on DLLME, extraction time, type of extraction solvent, identity and volume of

disperser solvent were studied. The preconcentration factors and extraction recoveries under optimum conditions were high and ranged between 151.0–722.0 and 24.2–115.6 % respectively. The linear range was from (0.2–200 μgL^{-1}) with detection limits obtained between 0.021 and 0.12 μgL^{-1} for most of the analytes. The relative standard deviations for 5.00 μgL^{-1} of triazines in water were in the range of 1.36–8.67 %. The relative recoveries of triazine herbicides from river and tap water spiked at level of 5.0 μgL^{-1} were 85.2–114.5 % and 87.8–119.4 %, respectively. The method was compared against hollow fiber protected liquid-phase microextraction and solid-phase microextraction methods. The DLLME analysis required 3.0 min and showed high enrichment factors and recoveries for the extraction of triazine herbicides from water samples.

Hu, *et al.*, (17) prepared a novel molecularly imprinted polymer (MIP) solid-phase microextraction fiber connected to high-performance liquid chromatography for the determination of prometryn herbicide in different soil and vegetables samples with prometryn as the template molecule. Characteristics of SPME fiber were investigated and it was showed by electron microscope photographs that the MIP coating with average thickness of 25.0 μm was porous and homogeneous. The extraction efficiency of prometryn with the MIP-coated fibers was 10.0 times as much as that with the non-imprinted polymer coated fibers. Specific selectivity of the modified fiber for other triazines having similar structure to prometryn was discovered with the MIP-coated fibers. Extraction conditions were optimized, and the limits of detection for the triazines were within the range of 0.012–0.090 μgL^{-1} at optimum conditions. This method was applied for the determination of five target triazine herbicides in the spiked corn, lettuce, soybean and soil samples with the recoveries of 78.0–103.5, 82.4–113.4, 75.5–83.4, and 81.0–106.1 % respectively. MIP-coated fibers were suitable for the selective extraction of trace triazines in complex samples.

Quesada-Molina, *et al.*, (18) reported a capillary zone electrophoresis method with ultraviolet detection for monitoring of the major degradation products of metribuzin that is deaminodiketometribuzin (DADK) deaminometribuzin (DA) and diketometribuzin (DK). The dissociation acid constants were estimated by capillary zone electrophoresis and no important differences were observed with the results obtained by applying other

techniques. Separation of the target compounds was achieved in less than 9.0 min using *p*-aminobenzoic acid as internal standard and applying a voltage of 15.0 kV at 25.0 °C in 40.0 mM with sodium tetraborate buffer of pH 9.5. The sensitivity was increased by large volume sample stacking (LVSS) with polarity switching applied as on-line pre-concentration methodology. Detection limits obtained for DADK, DA and DK were 10.0, 10.0 and 20.0 ngmL⁻¹ respectively. The method was applied to soil samples after pressurized liquid extraction (PLE). Samples were extracted with methanol as extraction solvent and sodium sulphate as drying agent at high temperature (103.0 °C and 1500.0 psi). PLE method was followed by an off-line pre-concentration and sample clean-up (using a LiChrolut EN sorbent column). The last two extraction methods were suitable for the direct treatment of groundwater samples before capillary zone electrophoresis analysis. Significant improvement in sensitivity was achieved by combination of the off-line and on-line pre-concentration procedures. LVSS provided pre-concentration factors of 36.0, 4.0 and 28.0 for DA, DK and DADK, respectively. SPE showed a pre-concentration of 500.0-fold for water samples and of 2.5-fold in case of soil samples. The method was suitable for the determination of these residues in environmental samples with high sensitivity, precision and recoveries.

Baranowska, et al., (19) determined optimal conditions for atrazine determination by means of an antigen-coated tube enzyme-linked immunosorbent assay (ELISA) in trophic chain samples. ELISA was used for analysis of selected samples and the results and method requirements were compared with that of HPLC analysis. A minimum detection limit of 0.05 ngmL⁻¹ and a dynamic range 0.1–2.0 ngmL⁻¹ was achieved using 2.0 hr competitive ELISA. Atrazine concentration in trophic chain samples indicated atrazine residue (µgkg⁻¹) in samples in the range of 3.2–85.4 for soil, 32.9–148.9 µgkg⁻¹ vegetable roots, 67.7–136.4 for green parts of plants, 42.4–91.5 for cereals and 1.3–8.4 for samples of animal origin. The correlation obtained between the results of HPLC and ELISA methods was 0.97. Moreover, the detection limits of simazine and atrazine content determined by the HPLC method was 0.3 µgg⁻¹ and 0.2 µgg⁻¹ respectively. The residue level (µgkg⁻¹) of simazine in soil samples was 13.5–15.5, in vegetables roots 29.5–93.7, in green parts of plants 34.6–72.6 and in cereals 158–189. This study

demonstrated the successful utility of ELISA method in a non-immunoassay laboratory for the analysis of food and environmental samples. The method was found ideal for the rapid screening of large numbers of samples in laboratories where access to HPLC facilities is limited or lacking. In addition the results showed the presence of significant levels of atrazine and simazine in samples of trophic chain collected from different areas of the region. Highest concentration of both herbicides was found in plants.

Valera, *et al.*, (20) described a novel impedimetric immunosensor for atrazine detection. The sensor was made up of on coplanar non-passivated interdigitated metallic μ -electrodes and the differential measurement of the impedance frequency spectrum. There was no redox electrode incorporated in the sensor. Chemical recognition layer was deposited on top area of the interdigitated μ -electrodes (inter-digits space and fingers). Immunochemical detection of the atrazine herbicide was obtained by a non-label competitive reaction. Moreover specifically developed immunoreagents were used to detect atrazine. The immunosensor and its functionalization steps were characterized by impedance spectroscopy and chemical affinity methods. Development of immuno sensor and the study of its physico-chemical and analytical characteristic were investigated in this method. The immunosensor showed a detection limit of $8.34 \pm 1.37 \mu\text{gL}^{-1}$, lower than the maximal residue level ($100.0 \mu\text{gL}^{-1}$) established by European Union for residues of atrazine as herbicide in the wine grapes and other foodstuff products.

Zhao, *et al.*, (21) developed a method for the determination of simazine and atrazine herbicides in environmental aqueous samples. It was based on solid-phase extraction of these herbicides on bamboo charcoal followed by determination by HPLC with UV detection. Factors influencing extraction efficiency, such as the kind of eluent and its volume, flow rate, pH and volume of the sample were optimized. Experimental results showed that linearity obtained ranged from of $0.5\text{--}30.0 \text{ gL}^{-1}$ with correlation coefficients 0.9982 and 0.9991 for simazine and atrazine respectively. The relative standard deviations of target compounds were 8.3 and 8.7 %, respectively. The method was applied successfully to the analysis of well and tap water samples. Reasonable recoveries were obtained in the range of 75.2–107.1 % for the two spiked herbicides. The results

indicated the applicability of this method as an alternative for the analysis of these herbicides in the environmental samples.

Koohpaei, *et al.*, (22) investigated solid-phase extraction techniques, for ametryn herbicide based on a molecular imprinted polymer (MIP) phase. In this method, the synthesis of the corresponding ametryn MIP was accomplished and the factors influencing its efficiency were optimized. The template amount, type and the amounts of solvents and functional monomer, cross-linker, initiator and temperature were optimized. The optimum conditions for the polymerized sorbent considering the recovery efficiency were monomer: 5.41 mmol of methacrylic acid; 6.41 mL of solvent, acetonitrile; template: 1.204 mmol; initiator: 2.03 mmol; cross-linker: 27.07 mmol and temperature: 40.86 °C. The optimum molar ratio achieved among the template, monomer and cross-linker for ametryn was 1:4.49:22.48. Reversed-phase HPLC with UV detection was used for the ametryn determination, using an isocratic mode (acetonitrile: water, 60:40) with a flow-rate of 0.8 mL min⁻¹) and ultraviolet detection at 220.0 nm. The obtained results could be increased in precision and accuracy by using central composite design for the synthesis and optimization of MIP for ametryn.

Rocha, *et al.*, (23) determined simazine and atrazine and three chloroacetamide herbicides (acetochlor, metolachlor and alachlor) by solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME–GC–MS) in aqueous samples from a midwest United States agricultural drainage drain for two growing seasons. Different factors such as, effects of salt concentration, sample volume, extraction and injection time on the extraction efficiency (using a 100.0 µm polydimethylsiloxane-coated fiber) were investigated. Detection limit of 0.5 µg L⁻¹ for simazine and 0.25 µg L⁻¹ for atrazine, acetochlor, alachlor, and metolachlor were achieved in ditch water samples after optimization of the different parameters. The optimum NaCl concentration found was 83.0 %, while peak area was negligibly affected by sample volume (10.0 or 20.0 mL). The optimum extraction time and injection time was 40.0 min and 15.0 min, respectively. Atrazine levels in the drain water surpassed the United States maximum contaminant level for drinking water by 12.0 %, and was the most frequently detected among the

analytes studied. Trace concentration of herbicides in tile-fed drain water were successfully detected by SPME–GC–MS.

Djoza and Ebrahimi., (24) reported a polymerization strategy to prepare a monolithic solid phase micro extraction (SPME) fiber based on molecularly imprinted polymer coupled with gas chromatography (GC) and gas chromatography mass spectrometry GC–MS for selective extraction and determination of triazine herbicides. A fiber was made by copolymerization of methacrylic acid–ethylene glycol dimethacrylate imprint using atrazine template. Factors that influenced polymerization were investigated and optimized. The fiber synthesized at the optimized conditions was firm and thermally stable up to 280.0 °C. Also parameters such as the influence of pH, extraction temperature and time on the extraction efficiency of target compounds were optimized. The fibers selectivity in relation to triazine herbicides and some of the other pesticide was investigated. High extraction efficiency was achieved for atrazine, ametryn, cyanazine, simazine, terbutryn, propazine and prometryn yielding detection limits of 81.0, 80.0, 20.0, 70.0, 80.0, 88.0, 69.0 and 68.0 ngmL⁻¹, respectively with high recoveries. SPME developed was applied to spiked samples such as onion, rice and tap water samples.

Salmain, *et al.*, (25) described a new approach to transduce antigen–antibody association optically, without a label, using IR spectroscopy for the analysis of organic thin films at the surface of reflective materials with high sensitivity. This new technique was applied to the immunodetection of the herbicide atrazine as a proof the principle. Capture layer consisting of a protein derivative of the herbicide atrazine which was covalently bonded to a self-assembled monolayer containing a carboxy-terminated thiolate was used to cover Gold-coated chips. A change of the IR absorption properties of the organic film at the sensor surface was observed as a result of successive binding of anti-atrazine antibody and secondary anti-rabbit JgG antibody. The IR spectra with prominent amide I and II bands were taken for semi quantitative analysis of the adsorbed protein amount. Intensity of IR signal yielded a small increase by the presence of increased amounts of atrazine, which resulted in the progressive inhibition of antibodies binding to the sensors. The standard curves obtained, displayed a sigmoidal shape typical of competitive inhibition assays. The test midpoint (IC₅₀) and the limits of detection (IC₈₀) were in the

nano molar range and very close to those measured by an in-house enzyme-linked immunosorbent assay using the same antibody and the same antigen competitor.

Portugal, *et al.*, (26) developed a polyurethane foams (PU) and applied it as new generation polymeric phases for stir bar sorptive extraction (SBSE) of seven triazinic herbicides (simazine, atrazine, prometon, ametryn, propazine, prometryn and terbutryn) in water matrices. It was demonstrated by the assays performed for PU synthesis and characterization that seven formulations presented remarkable stability and excellent mechanical and chemical resistance, for which the P6 formulation showed the best results. Assays were performed on 25.0 mL of water samples spiked at the 10.0 $\mu\text{g L}^{-1}$ level and it was established that the best experimental conditions using stir bars coated with P₆ were: an equilibrium time of 6.0 hr (1250 rpm), 5.0 % of methanol as organic modifier followed by liquid desorption with methanol as back extraction solvent under ultrasonic treatment (20.0 min) and high performance liquid chromatography with diode array detection (SBSE (PU)-LD-HPLC-DAD). This method provided good recoveries (20.4–62.0 %) and remarkable reproducibility and relative standard deviation of < 7.0 %. Moreover, excellent linear dynamic ranges (between 0.9 and 16.7 $\mu\text{g L}^{-1}$, correlation coefficient > 0.9949) and detection limits (0.1–0.5 $\mu\text{g L}^{-1}$) at trace level were achieved. Application of the proposed method to the triazinic herbicides analysis in ground and superficial water matrices showed remarkable results while the matrix effects were minimized by using the standard addition methodology. The recoveries attained by comparison of the best PU formulation (P₆, 71.0 μL) to the commercial stir bars coated with polydimethylsiloxane (PDMS of 126.0 μL), were normalized to the polymeric volume up to five times higher than atrazine. The ability of PU foams to extract the more polar compounds rather than PDMS made this polymer a very valuable contribution for SBSE.

Mohammadi, *et al.*, (27) presented a rapid and simple headspace solid-phase microextraction (HS-SPME) method for the simultaneous determination of ametryn and atrazine in water and soil samples by ion mobility spectrometry (IMS). A dodecylsulfate-doped polypyrrole (PPy-DS) was prepared using electrochemical method as a laboratory-made fiber for SPME. The sample temperature in the HS-SPME system was adjusted

with a circulating water bath and a cooling system on the upper part of the sample vial. The properties of the fiber (used for extraction of atrazine and ametryn from spiked water and soil samples) were examined by using a HS-SPME device and thermal desorption in injection port of IMS. Parameters that influenced the extraction efficiency such as the volume of the added water to the soil, pH, salt effect, extraction and desorption time, extraction and desorption temperatures were investigated. This procedure was simple, sensitive and provided good repeatability (relative standard deviation < 10.0 %) and shorter times of analysis for spiked soil (200.0 ngg^{-1}) and water samples (100.0 and 200.0 ngmL^{-1}). The calibration curves were linear in the range of $200.0\text{--}4000.0 \text{ ngg}^{-1}$ and $50.0\text{--}2800.0 \text{ ngmL}^{-1}$ for water and soil samples respectively with correlation coefficient value of 0.99. Detection limits for ametryn and atrazine were 37.0 and 23.0 ngg^{-1} in soil sample and 15.0 and 10.0 ngmL^{-1} in water sample respectively. The accuracy of the proposed method was evaluated by determination of ametryn and atrazine herbicides in the two well water samples and three kinds of soil samples. The HS-SPME using PPy-DS fiber was compared with other reported methods for extraction and determination of selected triazines. The results showed, this method was comparable in detection limits and relative standard deviations and had good linear ranges.

Kueseng, *et al.*, (28) reported the analysis of atrazine on a newly synthesized molecularly imprinted polymer (MIP) using non-covalent method. Binding capacity of the MIP was 1.00 mgg^{-1} of polymers. Selectivity and recovery were investigated with various pesticides for both similar and different chemical structure to atrazine. The competitive recognition between atrazine and structurally similar compounds was studied and it was found that the MIP provided highest recovery and selectivity for atrazine than the other compounds. The results showed that highest recovery was obtained from MIP compared with non-imprinted polymer, a commercial C_{18} and a granular activated carbon sorbent. This method provided high recoveries ranged from 94.0 to 99.0 % at two spiked levels with relative standard deviations less than 2.0 %. The lower detection limit of the method was 80.0 ngL^{-1} . The method was well applied successfully for analysis of environmental water samples.

Grcic, *et al.*, (29) studied degradation of atrazine in model waste water by UV/FeZSM-5/H₂O₂ system chosen as optimal for application of advanced oxidation process (AOP) in batch photo reactor. Statistical studies of the process were performed using two-level full factorial experimental design for three process parameters. Individual parameters and their interaction effects on atrazine degradation were investigated and optimal operating conditions were established. This approach was given a broader insight of the processes that were occurring in the reaction system, which finally led to simplification in terms of kinetics. Degradation of atrazine was described by pseudo-first-order kinetics with observed rate constant $k' = 2.0 \times 10^{-3} \text{ s}^{-1}$.

Moreno, *et al.*, (30) tested the disposal of composted, vermicomposted and natural olive cake for extraction of triazine herbicides (terbuthylazine, cyanazine, simazine and prometryn). Experimental design (desirability function plus surface response methodology) were applied to multicriteria optimization for evaluating type and dose of amendment to retain the analytes and to develop simple and low cost analytical methods for extracting triazine herbicides from soil. Classical and D-optimal designs were employed from methodological point of view. The central composite designs were used for finding the best combination of solvent ratio and soil amount. Under the optimized conditions, triazines were recovered in the range of 87.0–107.0 % for ultrasonic extraction and 75.0–85.0 % for shaking extraction. The reliability of the estimations was confirmed by D-optimal design. Addition of natural olive cake to the soil at a high dose (8.0 %) reduced the herbicide recovery to 50.0–60.0 % for prometryn, simazine and terbuthylazine while cyanazine was negligibly recovered. Design of experiment provided an efficient means for exploring the conditions for optimizing several responses simultaneously.

Vazqueza, *et al.*, (31) encapsulated three microalgal species (*Dictyosphaerium chlorelloides* (D.c.), *Scenedesmus intermedius* (S.i.) and *Scenedesmus* sp. (S.s.) in silicate sol–gel matrices and the increase in the amount of chlorophyll fluorescence signal was used to quantify simazine. Several parameters that influenced the preparation of the sensing layers were evaluated like effect of pH on sol–gel gelation time, effect of algae density on sensor response and influence of glycerol (%) on the membrane stability. Long

term stability of the sensing layer was tested as the fluorescence signal remained stable for at least 3 weeks. The lowest detection limits of $3.6 \mu\text{g L}^{-1}$ with D.c. biosensor and the broadest dynamic calibration range of $19.0\text{--}860.0 \mu\text{g L}^{-1}$ with IC_{50} of $125.0 \pm 14.0 \mu\text{g L}^{-1}$ for simazine were achieved. Biosensor was validated by high performance liquid chromatography with ultra violet and diode array detection. The biosensor showed response to those herbicides that inhibit the photosynthesis at photosystem II (triazines: atrazine, simazine, propazine, terbuthylazine; urea based herbicides: linuron). However, no significant increases of fluorescence response was observed for similar concentrations of 2,4-D (hormonal herbicide) or Cu(II). The combined use of biosensors with two different genotypes, showed its sensitivity and resistivity to simazine and allowed improving microalgae biosensor specificity.

Liang, *et al.*, (32) described a polymeric membrane ion-selective electrode for determination of melamine. A molecularly imprinted polymer was synthesized by using methacrylic acid as a functional monomer, melamine as a template molecule and ethylene glycol dimethacrylate as a cross-linking agent. The membrane electrode showed near-Nernstian response (54.0 mV/decade) to the protonated melamine in the concentration range between 5.0×10^{-6} to $1.0 \times 10^{-2} \text{ mol L}^{-1}$. The electrode exhibited a short response time of $\sim 16.0 \text{ s}$ and was stable for more than 2.0 months. The potentiometric sensor was successfully applied to the determination of melamine in milk samples when combined with flow analysis system. Interference from high concentrations of ions co-existing in milk samples such as K^+ and Na^+ were effectively eliminated by on-line anion and cation-exchanger tandem columns placed upstream, while melamine as neutral molecules in milk of pH 6.7 flow through the ion-exchanger columns and were measured downstream by the proposed electrode in an acetate buffer solution of pH 3.7.

Djozana, *et al.*, (33) fabricated a monolithic molecular-imprinted polymer as new solid-phase microextraction (SPME) fiber, coupled with gas chromatography (GC) and gas chromatography mass spectrometry for the selective extraction and determination of triazine herbicides. Methacrylic acid, ametryn and ethylene glycol dimethacrylate bear role of functional monomer, cross-linker and template respectively. Under optimized conditions, the fiber showed better molecular recognition abilities for methylthiotriazine

than chloro-triazine herbicides. Bi-Langmuir isotherm model was used to evaluate equilibrium constants for ametryn. The equilibrium constants and binding sites values obtained were 0.01, 890.69 μM^{-1} and 129.98, 5.82 nmol g^{-1} , respectively. High extraction efficiencies were obtained for ametryn, prometryn, terbutryn, atrazine, simazine, propazine, and cyanazine, yielding the detection limits of 14.0, 28.0, 45.0, 56.0, 85.0, 95.0 and 74.0 ng mL^{-1} , respectively using GC with flame ionization detection. The usability of the prepared fiber for extraction of ametryn and other analogues in real samples was confirmed by using spiked tap water, maize, rice and onion samples.

Beale, *et al.*, (34) described the application of tris(2,2'-bipyridyl)ruthenium(III) for the detection of atrazine and related triazine herbicides in water by flow injection chemiluminescence analysis. The optimized experimental conditions determined were: [sample and carrier flow rates of 4.6 mL min^{-1} , sample (pH 9.0) buffered with 50.0 mM borax, and reagent concentration of 1.0 mM tris(2,2'-bipyridyl)ruthenium(III) in 20.0 mM H_2SO_4 (pH 1.0)]. Under the optimized conditions, the logarithm of the chemiluminescence intensity versus concentration was linear in the range of 2.15–2150 $\mu\text{g L}^{-1}$ for samples in MilliQ water and detection limit of atrazine determined in water was $1.3 \pm 0.1 \mu\text{g L}^{-1}$. The method was validated using direct injection high performance liquid chromatography. The presence of natural organic matter, masked the signal generated by atrazine which significantly increased the chemiluminescence. The target analyte was isolated via solid phase extraction (SPE) prior to analysis, which removed the interference and concentrated the samples and resulted in a greatly improved sensitivity with a detection limit of $14.0 \pm 2.0 \text{ ng L}^{-1}$.

Mhaka, *et al.*, (35) reported a selective extraction technique based on the combination of molecularly imprinted polymers (MIP) and liquid membrane (microporous membrane liquid–liquid extraction) for triazine herbicides residue (simazine, atrazine and propazine) determination in food samples. The hydrophobic porous membrane formed part of the acceptor phase and was saturated with toluene. The selected herbicides were re-extracted onto MIP particles in the acceptor phase. The extraction parameters such as, the amount of molecularly imprinted polymers, extraction time, type of organic acceptor and desorption solvent were optimized. Extraction time of 90.0 min and 50.0 mg amount of

MIP were found to be optimum. The result showed higher triazines bonded onto MIP particles with toluene as an acceptor phase as compared to hexane and the combination of diethyl ether and hexane. The best desorption solvent was found to be 90.0 % methanol in water as compared to acetonitrile and methanol:water ratio. Selectivity of this technique was proved by spiked lettuce and apple extracts where clean chromatograms were obtained compared to extracts obtained by liquid membrane extraction or by using the microporous membrane liquid-liquid extraction – non-imprinted polymer combination. MIP showed some degree of group specificity among the target herbicides. The extraction efficiency in lettuce extract was 79.0 % (0.72) for simazine, 98.0 % (1.55) for atrazine and 86.0 % (3.08) for propazine.

Sanchez-Ortega, *et al.*, (36) reported an automated thermal desorption-gas chromatography–mass spectrometry method for the determination of triazine herbicides in aqueous solution with excellent sensitivity. This method was based on the use of stir bar sorptive extraction. Important parameters such as sample volume, extraction time, addition of salt and organic modifiers, desorption temperature, flow and time which affect the efficiency of the proposed methodology were studied. The proposed method was sensitive and showed a good linearity within a range of $10.0\text{--}10^4.0\text{ ngL}^{-1}$ and correlation coefficients higher than 0.998. Quantitation limits in all cases were below the limits accepted by European legislation for human waters consumption and ranged between 11.3 ngL^{-1} and 0.7 ngL^{-1} . Relative standard deviation values were lower than 8.0 % for all analytes. Determination of 10 triazines in underground water samples was successfully performed using this method. The average concentrations obtained in the analysis of the spiked samples (at two different concentration levels) corresponded to mean recovery value ranging from $94.4 \pm 5.1\%$ to $106.0 \pm 6.3\%$ for a significance level of 0.05.

See, *et al.*, (37) developed a novel microextraction technique termed solid phase membrane tip extraction (SPMTE). To evaluate the extraction performance, selected triazine herbicides were employed as model compounds and multiwall carbon nanotubes (MWCNTs) were used as an adsorbent enclosed in SPMTE device. Several important extraction parameters were comprehensively optimized for the SPMTE procedure

(performed in semi-automated dynamic mode). The method showed good linearity in the range of 1.0–100.0 $\mu\text{g L}^{-1}$ under optimum extraction conditions. Acceptable reproducibility (relative standard deviation of 6.0–8.0 %, $n = 5.0$), low detection limits (0.2–0.5 $\mu\text{g L}^{-1}$), and satisfactory relative recoveries (95.0–101.0 %) were achieved. The SPMTE device could be regenerated and used for 15 analyses with no analyte carry-over effects. Comparison of SPMTE was made with commercially available solid phase extraction-molecular imprinted polymer cartridge for triazine herbicides. The developed method was simple, feasible and showed comparable and even better results against reference method.

Kleiera, *et al.*, (38) demonstrated that at contact times consistent with drinking water distribution system residence times, a transformation of atrazine could be observed, although the herbicide atrazine has been reported not to react measurably with free chlorine during drinking water treatment. Some of the transformation products detected through the use of high performance liquid chromatography–electrospray mass spectrometry were consistent with the formation of N-chloro atrazine. Effects of applied chlorine, pH and reaction time on the transformation reaction were studied to help understand the practical implications of the transformation on the accurate determination of atrazine in drinking waters. The errors in the determination of atrazine were a function of the type of dechlorinating agent used during sample preparation and the analytical instrumentation utilized. The quantification of the atrazine was inaccurate, ranging from 2.0-fold at pH 7.5 to 30.0-fold at pH 6.0 with a reductive dechlorinating agent, such as sodium sulfite or ascorbic acid. The results suggested that the use of high performance liquid chromatography-ultraviolet detection and ammonium chloride quenching was best for accurate quantification. Hence, the results appeared to have implications for both compliance monitoring and health affects studies that utilized gas chromatographic analysis with sodium sulfite or ascorbic acid as the quenching agents.

Paul, *et al.*, (39) reported adsorption of herbicide simazine on moderately acid-activated beidellite. The non-silicon atoms after acid activation, such as Al, Fe and Mg located in the octahedral sheet, whereas sodium ions between the clay mineral layers were leached from the parent clay mineral. The acid activation increased the basal spacing. Removal of

simazine was achieved by acid activated beidellites. Simazine molecules entered and adsorbed onto the destroyed alumino silicate interlayer from contaminated water spaces.

Tadeo, *et al.*, (40) published a review on the application of ultrasound-assisted extraction (UAE) for environmental and food samples. The technique had been used in the method development for the determination of numerous contaminants, including organic compounds (pesticides, pharmaceuticals, polyhalogenated flame retardants, polycyclic aromatic hydrocarbons etc.) and heavy metals. The advantages and disadvantages of this technique with the possibility of coupling UAE with other analytical techniques were also discussed.

Li, *et al.*, (41) presented a novel application of second-order calibration method based on an alternating penalty trilinear decomposition algorithm for data treatment obtained from high performance liquid chromatography-diode array detection. Atrazine, ametryn and prometryne were accurately and reliably analyzed in soil, river sediment and waste water samples by this method. Satisfactory results were obtained although the elution and spectral profiles of the analytes were heavily overlapped with the background from environmental samples. The average recoveries obtained for atrazine, ametryn and prometryn are 99.7 ± 1.5 , 98.4 ± 4.7 and 97.0 ± 4.4 % in soil samples, 100.1 ± 3.2 , 100.7 ± 3.4 and 96.4 ± 3.8 % in river sediment samples and 100.1 ± 3.5 , 101.8 ± 4.2 and 101.4 ± 3.6 % in waste water samples, respectively. The accuracy and precision of the proposed method were evaluated with the elliptical joint confidence region test. This work showed a new avenue for quantitative determination of herbicides in environmental samples with a simple pretreatment procedure and also provided the scientific basis for an improved environmental management through a better understanding of the waste water-soil-river sediment system as a whole.

Wang, *et al.*, (42) developed a dispersive liquid-liquid microextraction high-performance liquid chromatography (HPLC) method for triazine herbicides determination in honey samples. An ionic liquid, 1-hexyl-3-methylimidazolium hexafluorophosphate [C6MIM][PF₆], was used as an extraction solvent and a Triton X 114 was used as dispersant. The 20.0 mL honey sample was injected by syringe containing a mixture of

175 μL [C6MIM][PF6] and 50.0 μL of 10.0 % Triton X 114 . After extraction, phases were separated using centrifugation and the sediment phase was analyzed by HPLC. Experimental parameters, such as volume and type of extraction solvent, concentration of dispersant and salt, extraction time and pH value of sample solution were optimized. The detection limits for chlortoluron, prometon, linuron, propazine and prebane are 6.92, 5.84, 8.59, 8.55 and 5.31 μgkg^{-1} , respectively. The proposed method was simple in operation, low cost with high enrichment factor and required small extraction volume at microliter level. The results obtained from this method indicated that the proposed method provided acceptable recoveries and precisions.

Bealea, *et al.*, (43) presented a simple, relatively inexpensive method for the detection of a group of herbicides (atrazine, simazine and hexazinone) in natural waters using large-volume direct injection high performance liquid chromatography with a monolithic column and a single wavelength ultraviolet–visible detector. This system gave good results with a mobile phase of acetonitrile and water in a 30:70 ratio, a flow rate of 2.0 mLmin^{-1} , and a detector wavelength of 230.0 nm. Retention times of less than three minutes and detection limits of 5.7 μgL^{-1} for atrazine, 4.7 μgL^{-1} for simazine and 4.0 μgL^{-1} for hexazinone were achieved. The performance of the method developed was validated with an inter-laboratory trial against a National Association of Testing Authorities accredited liquid chromatography–mass spectrometry/mass spectrometry method commonly used in commercial laboratories.

Gao, *et al.*, (44) determined phenylurea and triazine herbicides in milk based on microwave assisted ionic liquid microextraction (MAILME) coupled with high performance liquid chromatographic separation. The experimental parameters of the MAILME, including type and amount of ionic liquid, salt concentration in sample, microwave extraction power and time were evaluated by a univariate method and orthogonal screening. The target compound scan was isolated from milk (4.0 mL) with 60.0 μL of 1-hexyl-3-methylimidazolium hexafluorophosphate as extraction solvent. MAILME was fast (7.0 min) and simple. The limit of detection of isoproturon, monolinuron, linuron, propazine, prometryne, terbutryn and trietazine were 0.46, 0.78,

1.00, 1.21, 1.96, 0.84 and 1.28 μgL^{-1} , respectively. Recoveries of the analytes obtained ranged from 88.4 to 117.9 % relative standard deviations lower than 7.43 %.

Hu, *et al.*, (45) developed a stir bar coated with molecularly imprinted polymer (MIP) as a sorptive extractant for triazine herbicides. The MIP was chemically bonded to the glass bar to improve stability. The stir bar surface was modified with a homogeneous and porous structure. The result showed that the MIP-coated stir bar had stronger affinity for the template molecule terbuthylazine as compared to the reference stir bar without a template. The stir bar obtained also demonstrated specific selectivity to the structural related-compounds of nine triazine herbicides, and could be applied for simultaneous determination of these compounds from complex samples. Four samples (rice, lettuce, apple and soil) were used to evaluate the proposed method. The limits of detection obtained was in the range of 0.04–0.12 μgL^{-1} , and the recoveries for the spiked rice, lettuce, apple and soil samples were 80.8–107.7, 72.0–109.8, 80.6–107.8 and 89.0–114.8 % with relative standard deviation from 1.2 to 7.9 % respectively. In addition, MIP-coated stir bar was firm, durable and could be prepared easily with reproducibly. The coating method developed would be useful for preparation of a range of selective stir bars for extraction in complex sample analysis.

Katsumata, *et al.*, (46) proposed a sensitive and selective multiwalled carbon nanotubes (MWCNTs) disk, for the determination of atrazine and simazine in water samples. These herbicides were extracted on MWCNTs disk and determined by gas chromatography–mass spectrometry. Several parameters based on the enrichment factor of the analytes were investigated. Experimental results showed the possibility of obtaining quantitative analysis with the solution of pH 5.0 (using 200.0 mL of validation solution containing 0.1 μg of triazines and 5.0 mL of acetone as an eluent). Maximum enrichment factors for atrazine and simazine with 200.0 mL of sample solution were 3900.0 ± 250.0 and 4000.0 ± 110.0 , respectively. Relative standard deviations were 6.9 % (atrazine) and 3.0 % (simazine) for seven determinations under optimum conditions. The linear range of calibration curves were 0.1 to 1.0 ngmL^{-1} for both atrazine and simazine with good correlation coefficients. The limits of detection (3S/N) were 2.5 and 5.0 pgmL^{-1} for atrazine and simazine, respectively. The method developed was successfully applied to

the determination of atrazine and simazine in samples of environmental water with high precision and accuracy.

Passeport, *et al.*, (47) reported a solid-phase microextraction gas chromatography–mass spectrometry multiresidue analytical method for 16 pesticides (diphenyl ether, triazine, ureas, acetamides, benzofuran, thiocarbamate, pyridine carboxamides, chloronitrile, piperidine, and azoles). Methodology design was applied for optimization of different experimental parameters. Extraction time, desorption temperature and NaCl addition were the factors which showed significant effects on pesticide extraction. Validation of the method was carried out through: (model adequacy and specificity tests, limits of detection and quantification determination, and full uncertainty assessment). Calibrations of all pesticides were obtained with good first- and second-order model adequacy. Limits of detection were in the range of 0.05–0.5 $\mu\text{g L}^{-1}$ and specificity recoveries varied from 75.0 to 140.0 %. The results obtained were acceptable for research purposes on highly concentrated agricultural flows. Uncertainty values were calculated for several steps like standard preparation, calibration model selection and use. The average real sample concentration uncertainties were lower than 10.0 %, indicating that the method was performed very well. The method was applied to 61.0 real water samples which confirmed the presence of some pesticide concentrations in relation to farmer use, whereas other molecules were either not detected or not quantified.

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EXPERIMENTAL

3.1 SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF ATRAZINE HERBICIDE

3.1.1 Method development strategy for spectrophotometric determination of atrazine

The chemical structure of atrazine is given below in figure 3.1.1

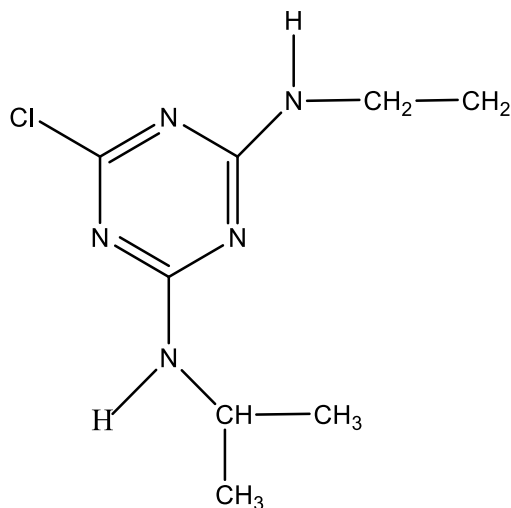
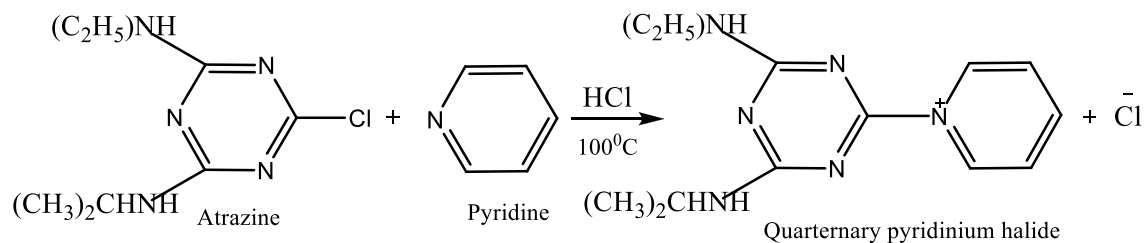


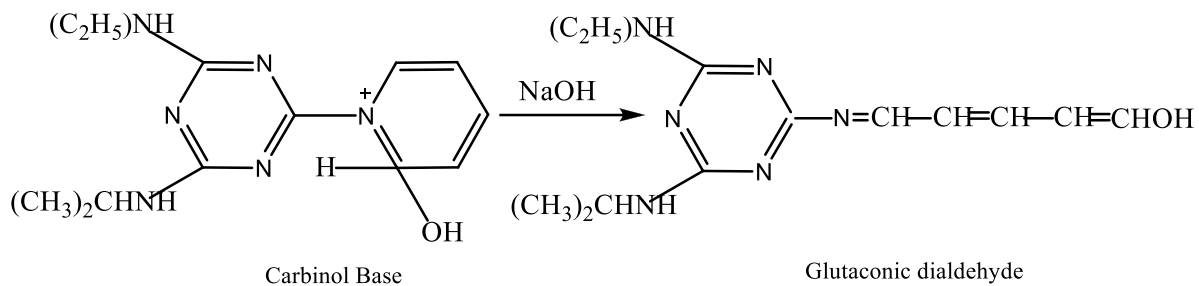
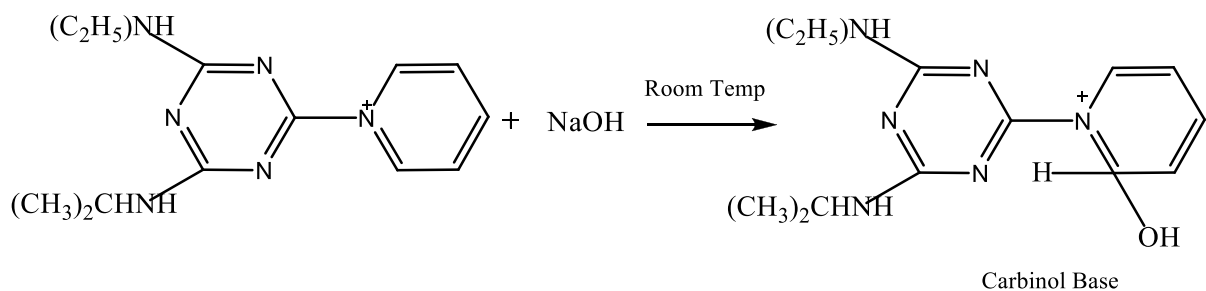
Figure 3.1.1 Chemical structure of atrazine herbicide

The chemical name of atrazine is 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine. From the structure it is expected that atrazine may undergo nucleophilic substitution reaction at the chloride position by reacting with a strong nucleophile like pyridine in acidic media. The quaternary halide thus formed could undergo addition of a hydroxyl group resulting in carbinol base. The resulting glutaconic dialdehyde formed could be stabilized by reacting with a suitable coupling reagent like sulfanilic acid or aniline to give a colored product. The product could be determined spectrophotometrically, from which the amount of atrazine could be determined. The proposed reaction mechanism is given in figure 3.1.2.

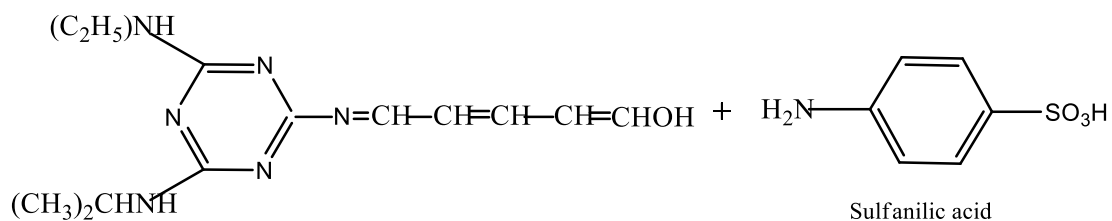
Step 1



Step 2



Step 3



Or with aniline

Step 4

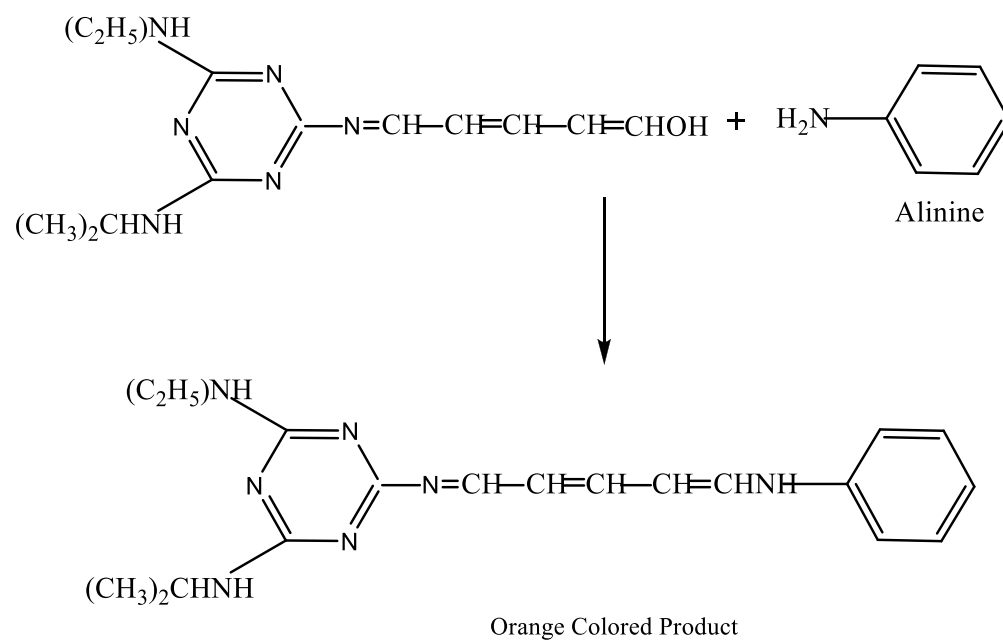


Figure 3.1.2 Proposed reaction mechanism for spectrophotometric determination of atrazine herbicide

3.1.2 Preliminary investigation of the possibility of nucleophilic substitution reaction and coupling of the glutaconic dialdehyde for spectrophotometric determination of atrazine.

Preliminary studies were conducted to investigate the possible nucleophilic substitution reaction and the subsequent coupling of the resulting glutaconic dialdehyde for the formation of a colored product. Initially high concentration of atrazine ($1000.0 \mu\text{g mL}^{-1}$), relatively large volume of hydrochloric acid and pyridine were used to ensure the substitution reaction. The resultant glutaconic dialdehyde formed was coupled with reagents like aniline and sulfanilic acid to form the expected colored product. The formation of the colored product indicated the possibility of reaction and the subsequent spectrophotometric determination of atrazine by this method. Therefore further studies were focused on optimization of various parameters for establishing of the method and are given below.

3.1.3 Optimization studies for spectrophotometric determination of atrazine herbicide using nucleophilic substitution reaction

After the preliminary reaction, it was evident that atrazine undergo nucleophilic substitution reaction with pyridine and the resulting glutaconic dialdehyde formed could be determined by spectrophotometric method after coupling with suitable coupling reagent and formation of a colored product. For quantitative determination it was important to optimize various parameters involved either in the reaction or determination step.

3.1.3.1 Investigation of various parameters for the determination of atrazine herbicide

Instruments

A UV–Visible spectrophotometer (Unico 2100 United Products Instrument Inc., Dayton, NJ, USA) with 1.0 cm cells was used for all spectral measurements. A pH meter model pH-422 (wissenschaftlich-Technische Werkstätten, W. Germany) was used for pH measurements.

Reagents and chemicals

All chemicals used were of analytical reagent grade or similar purity. Pyridine (79.1 g mol^{-1}), Hydrochloric acid (37.0 %), methanol, sodium hydroxide and sulfanilic acid (Merck, Darmstadt, Germany) were used throughout the work without further purification. Reference standard of atrazine was purchased from Dr Ehrenstofer GmbH (Germany). Commercial formulations were purchased from the local market.

Solution Preparation

- i) **Atrazine solution** ($1000.0 \text{ } \mu\text{g mL}^{-1}$): Atrazine stock solution ($1000.0 \text{ } \mu\text{g mL}^{-1}$) was prepared by dissolving 0.1 g of standard atrazine in 40.0 mL methanol and diluted with distilled water up to 100.0 mL. Working standard of $100.0 \text{ } \mu\text{g mL}^{-1}$ and $10.0 \text{ } \mu\text{g mL}^{-1}$ solutions were prepared from a stock solution by dilution.
- ii) **Sulfanilic acid solution** (1.0 %): Prepared by dissolving 1.0 g in 36.0 mL of concentrated hydrochloric acid and diluted up to 100.0 mL with distilled water.
- iii) **Aniline solution** (6.0 %): Aniline was prepared by mixing 6.0 mL of aniline in methanol and diluted up to 100.0 mL with methanol.

Procedure

Atrazine solutions of $0.1\text{--}25.0 \text{ } \mu\text{g mL}^{-1}$ were taken in reaction flasks followed by the addition of 0.35 mL of pyridine (79.1 g mol^{-1}) solution and 0.1 mL of concentrated hydrochloric acid solution (37.0 %) to get 0.86 M pyridine and 0.15 M hydrochloric acid solutions after dilution. These solutions were heated on boiling water bath for 25.0 min. After cooling, 4.0 mL of 1.75 M sodium hydroxide were added followed by the addition of 6.0 mL of 1.0 % sulfanilic acid. These solutions were diluted to 100.0 mL with distilled water. The same procedure was applied for aniline as a coupling reagent. The absorbance was measured with increasing wavelength and each wavelength was calibrated with blank solution. The results are shown in figure 3.1.3.

For optimization of pyridine concentration, atrazine solutions ($25.0 \text{ } \mu\text{g mL}^{-1}$) were taken in six separate 100.0 mL reaction flasks. To each flask pyridine (79.1 g mol^{-1}) solution was added varied from 0.1-0.5 mL which gives a molar concentration in the range of

(0.2-1.4 M) after dilution. Color was developed in the same way as mentioned before. The results are shown in figure 3.1.4.

For optimization of hydrochloric acid concentration, to each of the separate reaction flasks different volume of hydrochloric acid (37.0 %) solution were added in the range of 0.04-0.6 mL giving a final molar concentration in the range of 0.05-0.5 M after dilution. Color was developed in the same way as mentioned before. The results are shown in figure 3.1.5.

For optimization of the heating time of the nucleophilic substitution reaction, the solutions were heated on boiling water bath and heating time was varied from 5.0-30.0 min while rest of the procedure was kept the same. Absorbance of the colored solution was measured at optimum wavelength and the results are shown in figure 3.1.6.

For the formation of glutaconic dialdehyde, the concentration of sodium hydroxide was optimized. For this purpose concentration of sodium hydroxide was varied in the range of 0.8-2.1 M keeping the rest of the procedure same. The results are shown in figure 3.1.7.

Similarly for finding optimum volume of sodium hydroxide, varied volume of 1.75 M sodium hydroxide (2.0-10.0 mL) was used and absorbance was measured, the results are shown in figure 3.1.8.

Similarly for optimization of the amount of the coupling reagent, 5.0 mL of sulfanilic acid was used in the range of 0.5–5.0 % and 2.0 mL of aniline was studied in the range of 1.0–9.0 %. For finding the optimum volume of the coupling reagent, different volume (2.0-10.0 mL) of 1.0 % sulfanilic acid and 6.0 % aniline was used and the absorbance was measured and the results are shown in figure 3.1.9.

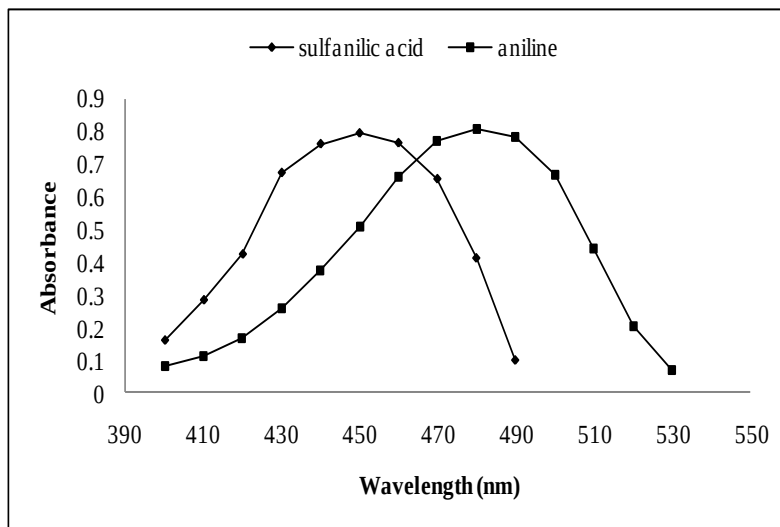


Figure 3.1.3 Investigation of suitable wavelength for the spectrophotometric determination of atrazine

Conditions

$\lambda_{\max}$	Varied
Atrazine solution	25.0 $\mu\text{g mL}^{-1}$
Pyridine solution (79.1 g mol^{-1})	0.35 mL
HCl solution	0.15 M
NaOH solution (1.75 M)	4.0 mL
Sulfanilic acid solution (1.0%)	6.0 mL
Aniline solution (6.0 %)	1.0 mL

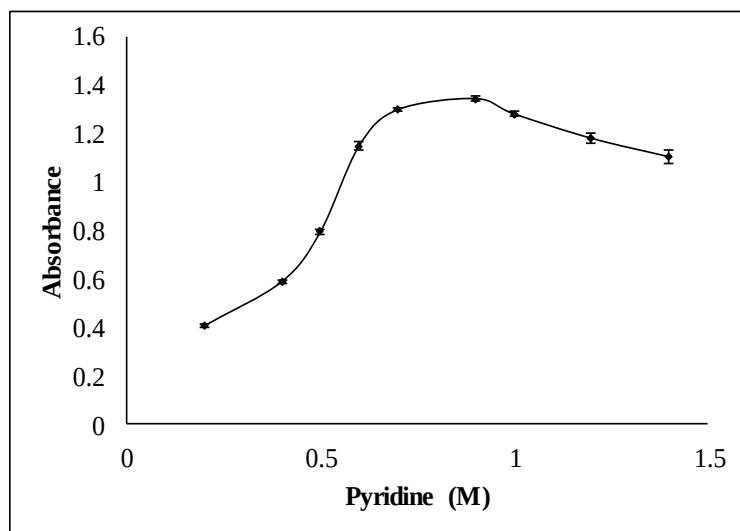


Figure 3.1.4 The effect of pyridine concentration on reaction with atrazine

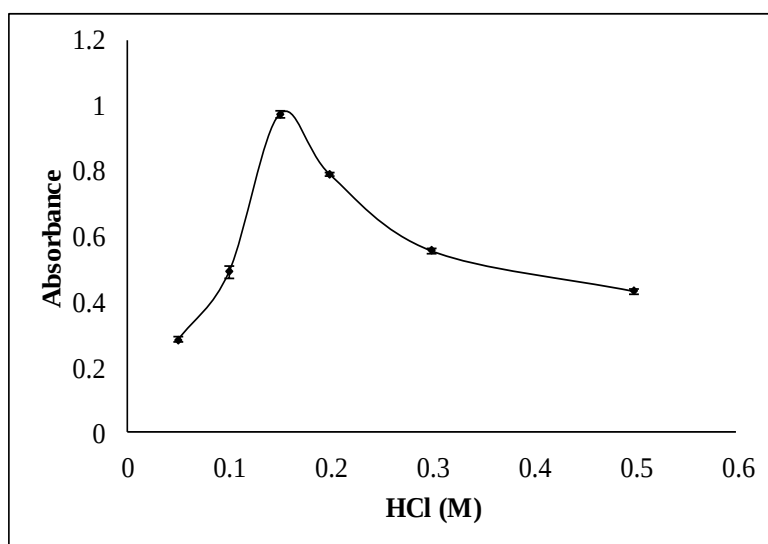


Figure 3.1.5 The effect of hydrochloric acid concentration on reaction of pyridine with atrazine

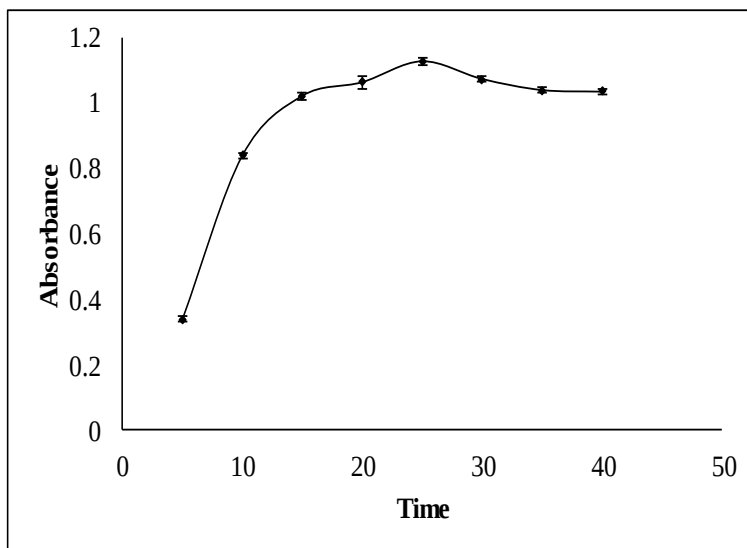


Figure 3.1.6 The effect of heating time on reaction of pyridine with atrazine

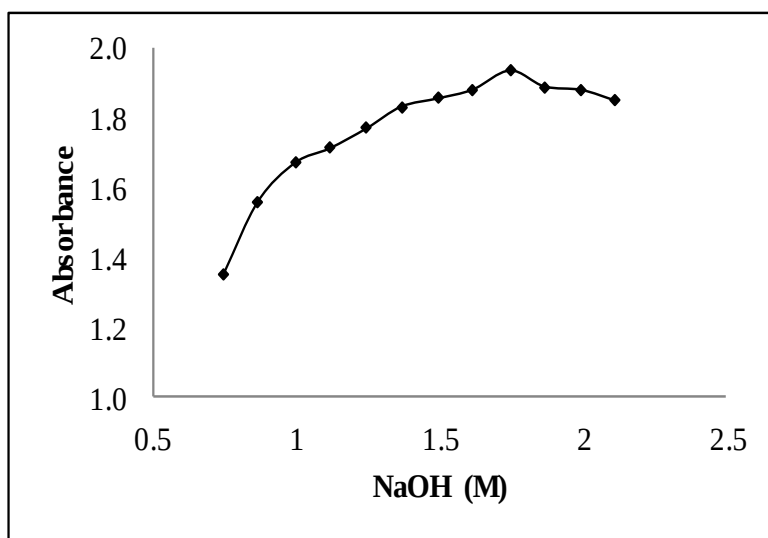


Figure 3.1.7 The effect of sodium hydroxide concentration on reaction of atrazine

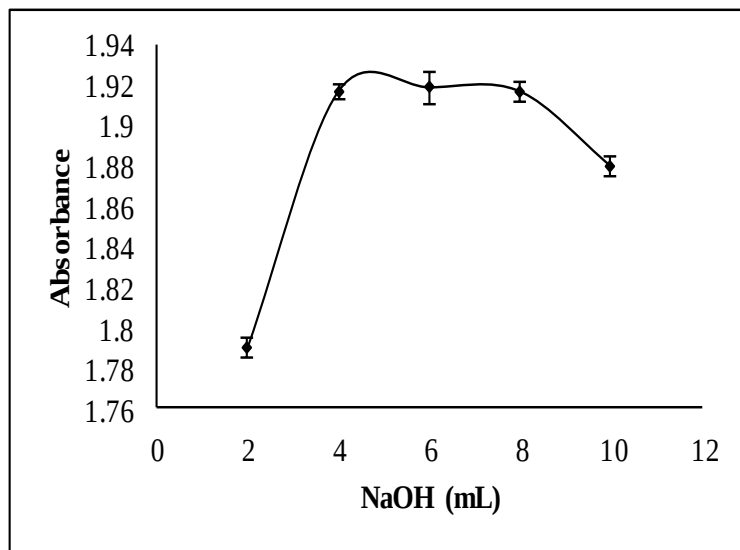


Figure 3.1.8 The effect of sodium hydroxide volume on reaction of atrazine

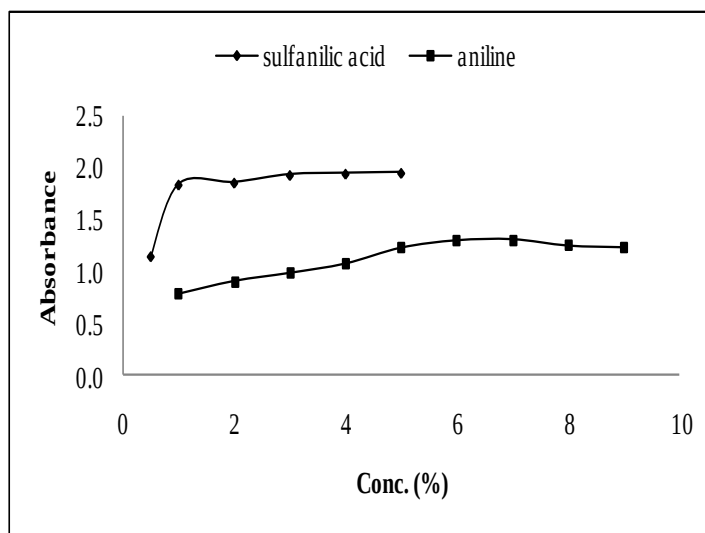


Figure 3.1.9 The effect of concentration of coupling reagents on the formation of colored product formation

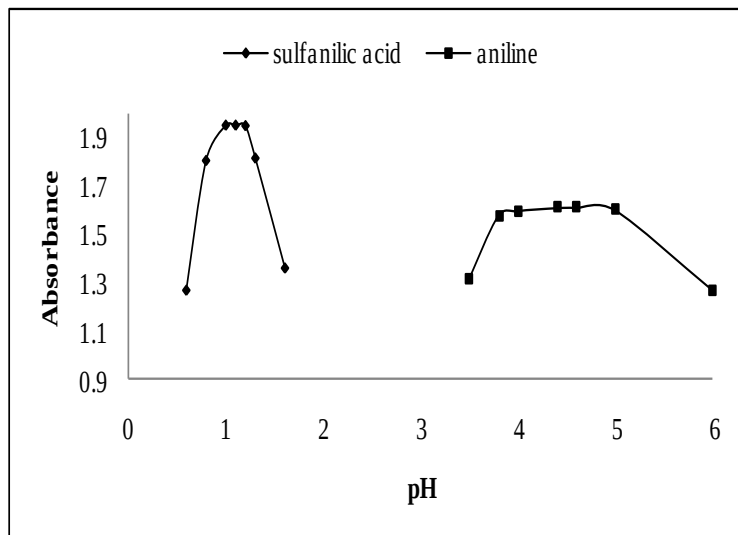


Figure 3.1.10 The effect of pH on the colored product formation

3.1.3.2 Verification of Beer's law and determination of detection and quantification limit for atrazine determination

Instrument: The same as mentioned before

Reagents: The same as above

Solution: The same as mentioned before

Procedure:

From standard stock solution of atrazine ($100.0 \mu\text{g mL}^{-1}$) different volume for $0.1\text{--}25.0 \mu\text{g mL}^{-1}$ were taken in separate 100.0 mL reaction flasks followed by the addition of 0.35 mL of pyridine (79.1 g mol^{-1}) and 0.1 mL of concentrated hydrochloric acid solution (37.0%) to have 0.86 M pyridine and 0.15 M hydrochloric acid solutions after dilution. These solutions were heated on boiling water bath for 25.0 min . After cooling, 4.0 mL of 1.75 M sodium hydroxide were added followed by the addition of 6.0 mL of 1.0% sulfanilic acid. The resulting solutions were diluted to 100.0 mL with distilled water. The same procedure was applied when using aniline as a coupling reagent instead of sulfanilic acid. The absorbance of the colored products was measured at 450.0 nm for sulfanilic acid and 480.0 nm for aniline against reagent blank. The results are given in table 3.1.1. A

calibration plot of absorbance vs. concentration was constructed and is shown in figure 3.1.11. The molar absorptivity was calculated by using the following formula.

$$\text{Molar absorptivity } (\epsilon) = A/C \times L$$

Where A= absorbance

C= concentration (molL^{-1})

L= path length (cm)

LOD and LOQ were calculated based on six replicates solution having lowest quantifiable concentration ($\mu\text{g mL}^{-1}$) by the following formula.

$$\text{Limit of Detection (LOD)} \quad (\text{for concentration}) = 3 \times S$$

$$\text{Limit of Quantification (LOQ)} \quad (\text{for concentration}) = 10 \times S$$

Where S = Standard deviation

Relative standard deviation (RSD) was calculated by using the following formula.

$$\text{RSD} = \text{Standard deviation} / \text{Mean} \times 100$$

All the statistical values are given in table 3.1.2.

Table 3.1.1: Effect of concentration on absorbance behavior of atrazine

Conc. of atrazine ($\mu\text{g mL}^{-1}$)	Absorbance	
	Sulfanilic acid	Aniline
0.08	-	0.017
0.1	0.013	-
0.2	0.018	0.034
0.4	0.026	-
0.6	0.062	0.06
0.8	0.067	0.012
1.0	0.077	-
2.0	0.148	0.157
4.0	-	0.287
6.0	0.419	0.352
8.0	0.633	0.525
10	0.678	0.701
12	0.759	0.716
15	0.857	-
20	1.753	-
25	1.811	-

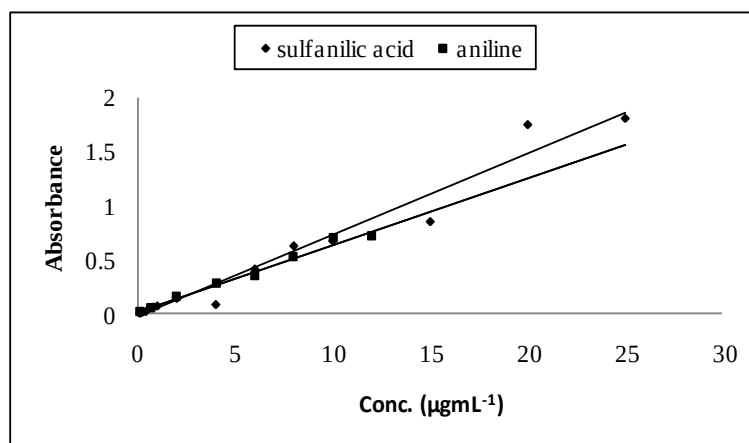


Figure 3.1.11: The effect of concentration on absorbance behavior of atrazine herbicide

Table 3.1.2: Analytical characteristic of the proposed method

Characteristics	Value	
	Sulfanilic acid	Aniline
λ max (nm)	450	480
Color	Yellow	Orange red
Linearity range ($\mu\text{g mL}^{-1}$)	0.1-25.0	0.08-12.0
Slope	0.0806	0.064
Correlation coefficient (r^2)	0.999	0.998
SD	9.8×10^{-3}	4.1×10^{-3}
RSD	6.66	5.10
Limit of detection ($\mu\text{g mL}^{-1}$)	0.029	0.012
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.098	0.041
Molar absorptivity ($\text{L mol}^{-1} \text{cm}^{-1}$)	1.5×10^4	1.3×10^4

3.1.4 Percent recovery of atrazine herbicide from real samples and commercial formulations

To check the applicability of the method in different matrices, recovery test was performed on sugarcane, corn samples and commercial formulations, fortified with known concentration of atrazine solution.

Sample extraction procedure

The sugarcane and corn samples under study were first washed properly with cold water and then with hot water in order to remove adhering dust and dirt. Known amounts (60.0 mL) of sugar cane juice and corn (15.0 g in 60.0 mL of distilled water) were taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then divided into three equal parts. The samples were spiked with different

fortified level of atrazine herbicide. All these spiked samples were kept in dark for 24.0 hr at room temperature. Atrazine was extracted from the sample twice by using 10.0 mL of chloroform each time. The combined chloroform extract was evaporated to dryness and the residue was dissolved in 5.0 mL methanol. The methanolic solution was then analyzed by the proposed method.

For checking the applicability to commercial formulations, a known amount (0.25 g in 20.0 mL of distilled water) of herbicide A (bestrazine) and herbicide B (mixture of atrazine and ametryn) were taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then diluted with water up to 100.0 mL. Atrazine was extracted from the sample by step wise addition of 2 x 10.0 mL of chloroform. The combined chloroform extract was evaporated to dryness and the residue was dissolved in 50.0 mL methanol and diluted with distilled water in a 100.0 mL volumetric flask. Known volume of this sample solution was then spiked with different standard concentration and was analyzed by the proposed method in triplicate.

Results of percent recovery of atrazine in real samples and formulation are given in tables 3.1.3- 3.1.5. Percent recovery was calculated using the following formula.

% Recovery = $\frac{\text{Total } \mu\text{g found in fortified samples} - \mu\text{g found in sample}}{\mu\text{g of standard added}} \times 100$

Table 3.1.3: Determination of atrazine herbicide in fortified samples using sulfanilic acid (n=6)

Samples	Residue found ($\mu\text{g mL}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Corn	0.360	6.0	6.0	95.1 $\pm$ 0.15
		7.4	7.5	96.0 $\pm$ 0.10
		9.8	9.9	97.0 $\pm$ 0.20
Sugar cane	0.53	6.0	6.0	92.2 $\pm$ 0.20
		7.8	8.1	97.0 $\pm$ 0.70
		9.7	9.8	93.0 $\pm$ 0.25

Table 3.1.4: Determination of atrazine herbicide from fortified commercial formulations using sulfanilic acid as coupling reagent (n=6)

Samples	Sample conc. taken ($\mu\text{g mL}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Bestrazine	8.1	6.0	14.0	98.3 $\pm$ 0.05
		9.0	16.9	97.8 $\pm$ 0.20
		10.0	17.8	97.0 $\pm$ 0.24
Atrazine + Ametryn	5.8	6.0	11.4	93.3 $\pm$ 0.47
		8.2	17.6	94.0 $\pm$ 0.56
		9.8	15.2	95.9 $\pm$ 0.26

Table 3.1.5: Determination of atrazine herbicide from commercial formulations using aniline as a coupling reagent (n=6)

Samples	Sample conc. taken ($\mu\text{g mL}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Bestrazine	2.0	6.0	7.8	96.7 $\pm$ 0.3
		9.0	10.5	94.5 $\pm$ 0.1
		10.0	11.5	95.0 $\pm$ 0.1
Atrazine + Ametryn	2.0	6.0	7.4	90.0 $\pm$ 0.1
		8.0	9.3	91.3 $\pm$ 0.1

3.1.5 Application of the investigated method for the analysis of atrazine herbicide in real samples and commercial formulations

Instrument: The same as mentioned before

Samples: Two samples each of sugarcane and corn were collected from different agriculture fields, where atrazine was sprayed and were first analyzed for the presence of residue. Known amounts (60.0 mL) of sugarcane juice and corn (15.0 g in 60.0 mL of distilled water) were taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then divided into three equal parts. Atrazine was extracted from the sample twice by using 10.0 mL of chloroform each time. The combined chloroform extract was evaporated to dryness and the residue was dissolved in 5.0 mL methanol. The methanolic solution was then analyzed by the proposed method. The results are given in table 3.1.6.

For checking the amount of active ingredient in commercial formulations, a known amount (0.25 g in 20.0 mL of distilled water) of herbicide A (bestrazine) and herbicide B (mixture of atrazine and ametryn) were taken in three separate beakers and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then diluted with water up to 100.0 mL in volumetric flask. Atrazine was extracted from the sample

by step wise addition of 2 x 10.0 mL of chloroform. The combined chloroform extract was evaporated to dryness and the residue was dissolved in 50.0 mL methanol and diluted with distilled water in a 100.0 mL volumetric flask. Known volume of this sample solution was analyzed by the proposed method in triplicate. The results are shown in table 3.1.7.

For checking the amount of active ingredient in the Bestrazine formulation, known volume (0.27 mL) of the formulation was taken and dissolved in 50.0 mL methanol in a beaker and was filtered to remove the suspended particles by using Whatman filter paper (NO: 40). This solution was further diluted to 100.0 mL in a volumetric flask with distilled water. Known volume (0.2 mL) was taken from this solution and the color was developed in the same way as mentioned before. The results are shown in table 3.1.7.

Table 3.1.6: Residue level of atrazine herbicide in real samples

S.NO	Samples	Residue (μgg^{-1})
1	Corn-1*	0.15 $\pm$ 0.20
	Corn-2*	0.36 $\pm$ 0.16
2	Sugar cane-1*	1.20 $\pm$ 0.20
	Sugarcane-2*	0.53 $\pm$ 0.23

1 Collected from district Swabi

2 Collected from district Charsadda

Table 3.1.7: Concentration of atrazine herbicide in commercial formulations

Commercial formulations	Label Amount	Batch Analysis
Bestrazine	0.4 μgmL^{-1}	0.39 $\pm$ 0.17 μgmL^{-1}
Atrazine + Ametryn	0.37 μgg^{-1}	0.35 $\pm$ 0.19 μgg^{-1}

3.1.6 Results and discussion

Atrazine was reacted with pyridine in alkaline media leading to a nucleophilic attack between atrazine through its strongly electron attracting chlorine and pyridine at the unshared electron pair of nitrogen forming the quaternary pyridinium salt. This undergoes addition of a hydroxyl group resulting in carbinol base. Glutaconic dialdehyde is formed from carbinol base due to the breaking of heterocyclic ring of the carbinol base in the presence of alkali. Glutaconic dialdehyde is an unstable yellow colored product and can be stabilized by reacting with sulfanilic acid leading to the formation of a new yellow colored product with maximum absorbance at 450.0 nm or with aniline giving an orange red colored product formed with maximum absorbance at 480.0 nm. The proposed reaction mechanism is given in figure 3.1. 2.

The effect of various reagent concentrations for the reactions transforming on the reaction of atrazine into yellow colored product formation was studied. Pyridine solution concentration was investigated in the range of 0.22–2.0 M. The absorbance increased from 0.22 to 0.86 M (Figure 3.1.4). Beyond this decrease in absorbance was observed with increase in concentration of pyridine solution. Pyridine reacted with atrazine in acidic solution to form quaternary pyridinium halide. Because the reaction is acid dependent, the effect of hydrochloric acid concentration was studied. The maximum reaction product was formed when the hydrochloric acid solution concentration reached up to 0.15 M in the final solution (Figure 3.1.5).

Formation of carbinol base from quaternary pyridinium halide takes place in the presence of alkali. The concentration of sodium hydroxide was studied in the range of 0.75–2.0 M. Maximum absorbance was observed at 1.75 M (Figure 3.1.6). More than 1.75 M sodium hydroxide results in hydrolyzing the carbinol base and destroying the aromaticity of the pyridine ring yielding glutaconic dialdehyde.

Sulfanilic acid and aniline were used as coupling reagents to stabilize the unstable glutaconic dialdehyde. The effect of sulfanilic acid concentration was investigated in the range of 0.5–5.0 % and aniline was studied in the range of 1.0–9.0 % (Figure 3.1.7). The absorbance increased from 0.5 to 1.0 % sulfanilic acid and no effect was observed with

further increase in sulfanilic acid concentration, while with aniline maximum absorbance was observed at 6.0 % aniline solution.

Maximum absorbance of yellow colored product was observed when the solution containing glutaconic dialdehyde with sulfanilic acid was heated on a boiling water bath for 25.0 min. The effect of pH on the yellow colored product formation was studied in the range of 1.0–2.5 pH and it was found that the final product formed only in strongly acidic medium with sulfanilic acid at pH 1.0–1.2 and aniline formed orange red colored stable product with glutaconic dialdehyde at pH 4.0–5.0 (Figure 3.1.8).

The effect of time on the stability of yellow dye was investigated up to 24.0 hr. The coupling product with sulfanilic acid and aniline was found to be stable for 24.0 hr at room temperature.

The concentration vs. absorbance curve was found linear over the concentration range of 0.1–25.0 $\mu\text{g mL}^{-1}$ and 0.08–12.0 $\mu\text{g mL}^{-1}$ with sulfanilic acid and aniline as a coupling reagent, respectively. The absorbance with aniline used as a coupling reagent is high but found linear over a smaller concentration range, whereas in the case of sulfanilic acid as a coupling reagent the absorbance value is linear over a wider concentration range. The linearity of the calibration graph was proved by the high value of the correlation coefficient (r^2). The limit of detection was determined by establishing the minimum level at which atrazine can be detected reliably using six replicate determinations and the limit of quantification was calculated by establishing the lowest concentration of atrazine that can be measured with acceptable precision and accuracy also with six replicate determinations for each coupling reagent. The validity of the method was evaluated by statistical evaluation of the regression lines. The precision of the method was tested by the standard deviation (SD) and relative standard deviation (RSD). The small values of the SD and RSD show low scattering of the point on the calibration curve. The results are summarized in table 3.1.2.

A recovery test was performed on samples fortified with known concentration of atrazine solution. Six replicates of the fortified samples were analyzed using the proposed method. The average recoveries were found in the range of 92–97 % from corn as well as sugar

cane samples. A recovery test was also applied to the commercial formulations (Tables 3.1.3 and 3.1.5). The average recoveries found with sulfanilic acid was in the range of $93.33 \% \pm 0.47 - 98.33 \% \pm 0.05$ while with aniline the recovery was in the range of $90.0 \% \pm 0.10 - 96.67 \% \pm 0.30$. In order to evaluate the analytical applications of the proposed method, it was applied for atrazine determination in commercial formulations. The values obtained were comparable with the label values. The method was also applied for residue determination in corn and sugar cane samples. The values for atrazine were found to be $0.15 \pm 0.2 \mu\text{g mL}^{-1}$ and 0.360 ± 0.16 in corn samples and $1.2 \pm 0.2 \mu\text{g mL}^{-1}$ and 0.53 ± 0.23 in sugarcane samples respectively.

3.2 SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF AMETRYN HERBICIDE

3.2.1 Method development strategy for spectrophotometric determination of ametryn herbicide

The chemical structure of ametryn is given below in figure 3.2.1

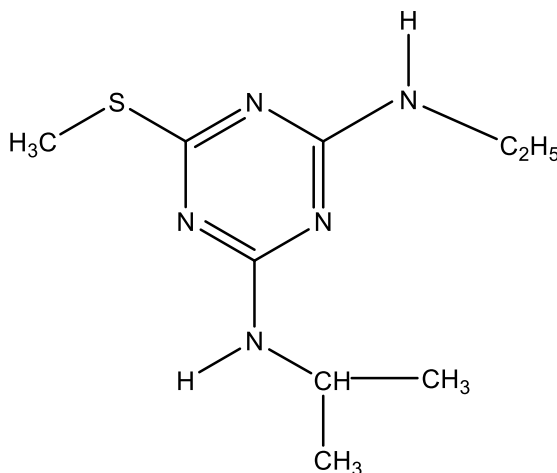


Figure 3.2.1 Chemical structure of ametryn herbicide

The chemical name of ametryn is *N*-ethyl-*N'*-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine. From the structure, it was expected that ametryn may undergo base hydrolysis followed by nucleophilic substitution reaction with pyridine to form a quaternary ammonium hydroxide. The product thus formed could undergo addition of a hydroxyl group resulting in carbinol base. The resulting glutaconic dialdehyde formed could be reacted with a suitable coupling reagent like sulfanilic acid to give a colored product. The product could be determined spectrophotometrically, from which the amount of ametryn could be determined. The proposed reaction mechanism is given in figure 3.2.2.

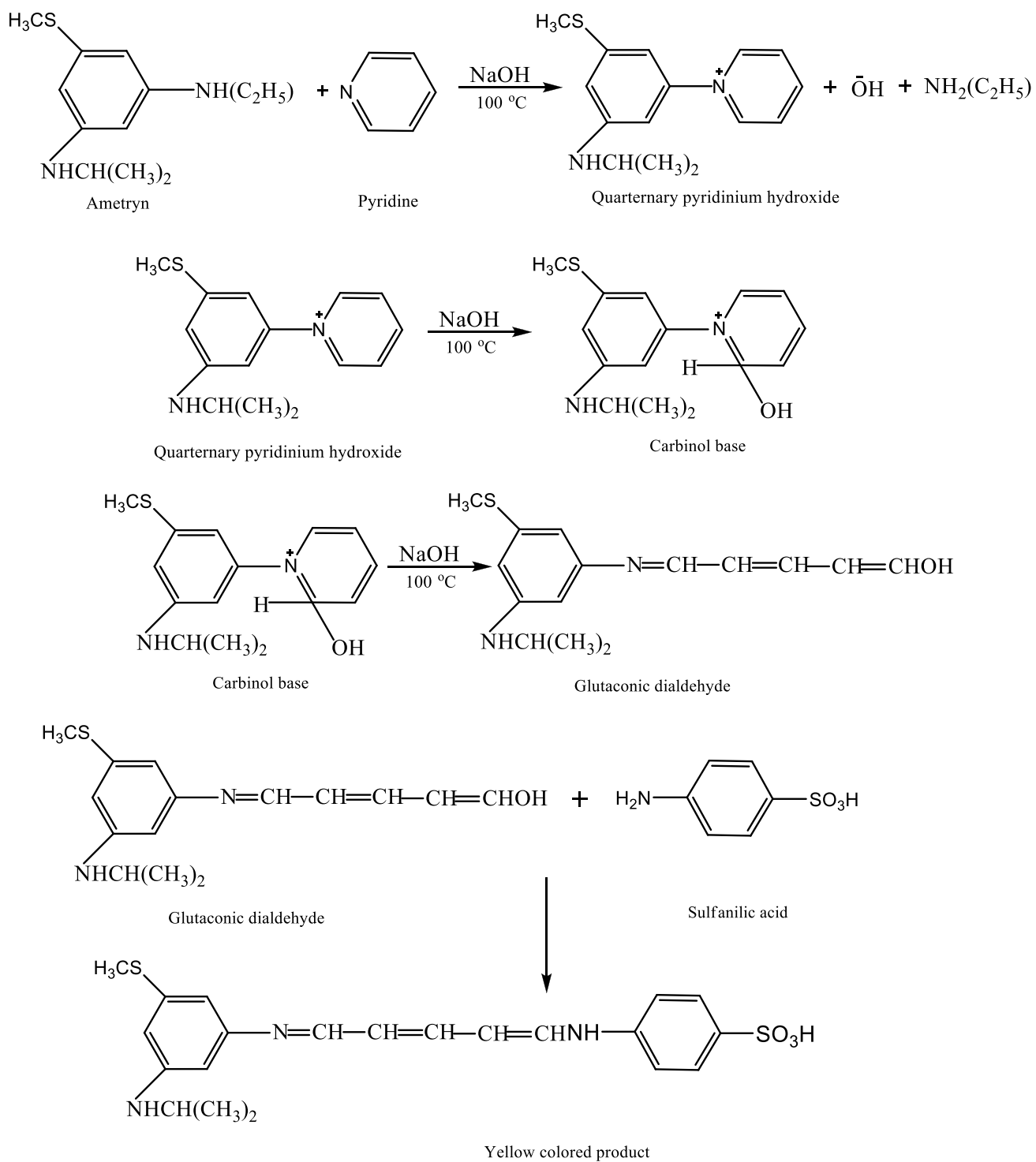


Figure 3.2.2: Proposed reaction mechanism for spectrophotometric determination of ametryn herbicide

3.2.2 Preliminary investigation of the possibility of nucleophilic substitution reaction and coupling of the glutaconic dialdehyde for spectrophotometric determination of ametryn herbicide.

Preliminary studies were conducted to investigate the possible nucleophilic substitution reaction and the subsequent coupling of the resulting glutaconic dialdehyde for the formation of a colored product. Initially high concentration of ametryn ($1000.0 \mu\text{g mL}^{-1}$), sodium hydroxide and pyridine were used to ensure the substitution reaction. The resultant glutaconic dialdehyde formed was coupled with reagents like sulfanilic acid to form the expected colored product. The formation of the colored product indicated the possibility of reaction and the subsequent spectrophotometric determination of ametryn by this method. Therefore further studies were focused on optimization of various parameters for establishing of the method and are given below.

3.2.3 Optimization studies for spectrophotometric determination of ametryn herbicide using nucleophilic substitution reaction

After the preliminary reaction, it was evident that ametryn could undergo base hydrolysis and subsequent nucleophilic substitution reaction with pyridine to form glutaconic dialdehyde which could be coupled with suitable coupling reagent to form a colored product. The colored product could be determined by spectrophotometric methods. For quantitative determination, it was important to optimize various parameters involved either in the reaction or determination step.

3.2.3.1 Investigation of various parameters for the determination of ametryn herbicide

Instruments

A UV–Visible spectrophotometer (Unico 2100 United Products Instrument Inc., Dayton, NJ, USA) with 1.0 cm cells was used for all spectral measurements. A pH meter model pH-422 (wissenschaftlich-Technische Werkstätten, W. Germany) was used for pH measurements.

Reagents and chemicals

All chemicals used were of analytical reagent grade or similar purity. Pyridine (79.1 g mol^{-1}), methanol, sodium hydroxide and sulfanilic acid (Merck, Darmstadt, Germany) were used throughout the work without further purification. Reference standard of ametryn was purchased from Dr Ehrenstofer GmbH (Germany). Commercial formulations were purchased from the local market.

Solution Preparation

- i) **Ametryn solution** ($1000.0 \text{ } \mu\text{g mL}^{-1}$): Ametryn stock solution ($1000.0 \text{ } \mu\text{g mL}^{-1}$) was prepared by dissolving 0.1 g of standard ametryn in 40.0 mL methanol and diluted with distilled water up to 100.0 mL. Working standard of $100.0 \text{ } \mu\text{g mL}^{-1}$ and $10.0 \text{ } \mu\text{g mL}^{-1}$ solutions were prepared from a stock solution by dilution.
- ii) **Sulfanilic acid solution** (1.0 %): Prepared by dissolving 1.0 g of the reagent in 36.0 mL of concentrated hydrochloric acid and diluted up to 100.0 mL with distilled water.
- iii) **Sodium hydroxide solution** (0.75 M): Sodium hydroxide solution was prepared by dissolving 3.0 g of sodium hydroxide in 100.0 mL distilled water.

Procedure

Ametryn solution of $20.0 \text{ } \mu\text{g mL}^{-1}$ was taken in three separate reaction flasks followed by the addition of 0.9 mL of pyridine (79.1 g mol^{-1}) solution (to get 1.0 M pyridine after dilution) and 2.0 mL of 0.75 M sodium hydroxide. These solutions were heated on boiling water bath for 8.0 min. After cooling, 2.0 mL of 1.0 % sulfanilic acid was added to each of the solutions and diluted up to 100.0 mL with distilled water. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are shown in figure 3.2.3.

For optimization of pyridine concentration, ametryn solutions of $20.0 \text{ } \mu\text{g mL}^{-1}$ were taken in three separate 100.0 mL reaction flasks. Pyridine (79.1 g mol^{-1}) solution was added in the range of 0.2-1.2 mL which gave a molar concentration in the range of 0.1-1.3 after

dilution followed by the same procedure as mentioned before. The results are shown in figure 3.2.4.

For optimization of sodium hydroxide concentration, molarity of sodium hydroxide was varied in the range of 0.25-1.25 M, while the rest of the procedure was the same. The results are shown in figure 3.2.5.

Similarly for finding optimum volume of sodium hydroxide, varied volume of 0.75 M sodium hydroxide (2.0-10.0 mL) was used and absorbance was measured, the results are shown in figure 3.2.6.

For optimization of heating time of the nucleophilic substitution reaction, the solutions were heated on boiling water bath and heating time was varied from 1.0-11.0 min. The absorbance of the colored solution was measured at the maximum wavelength using the same procedure. The results are given are shown in figure 3.2.7.

Similarly for optimization of the sulfanilic acid concentration, % concentration of sulfanilic acid was varied in the range of 0.5–2.0 %. For finding the optimum volume of the coupling reagent, different volume (0.5-4.0 mL) of 1.0 % sulfanilic acid was used and the absorbance was measured and the results are shown in figure 3.2.8.

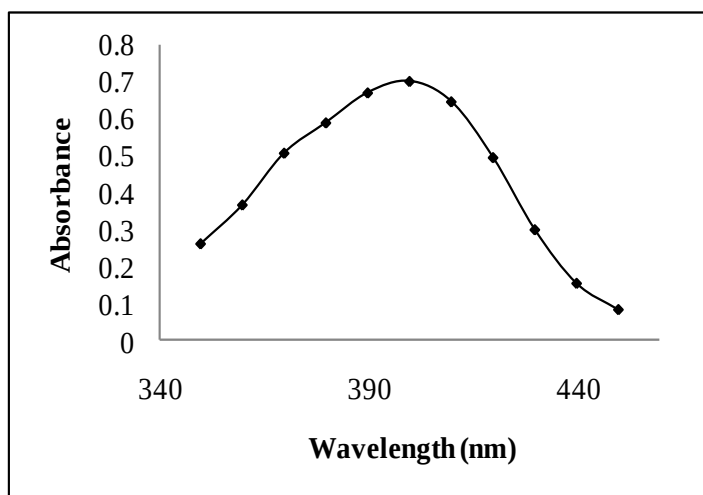


Figure 3.2.3: Investigation of suitable wavelength for the spectrophotometric determination of ametryn herbicide

Conditions

$\lambda_{\max}$	Varied
Ametryn solution	20.0 $\mu\text{g mL}^{-1}$
Pyridine solution (79.1 g mol^{-1})	0.9 mL
NaOH solution (0.75 M)	2.0 mL
Sulfanilic acid solution (1.0%)	2.0 mL

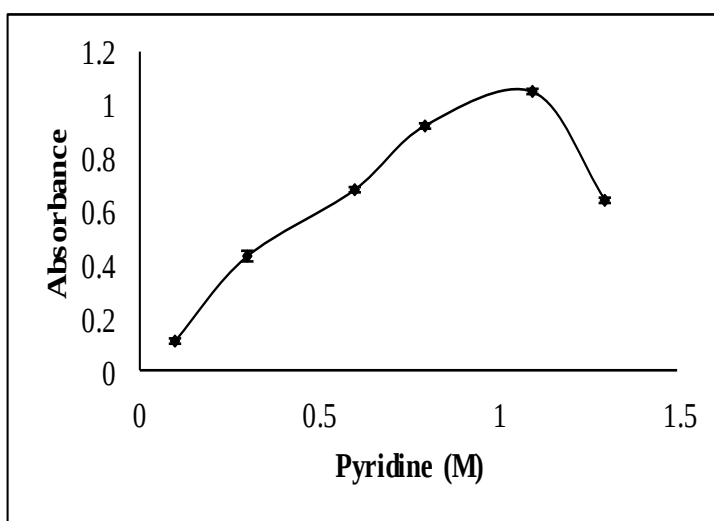


Figure 3.2.4: The effect of pyridine concentration on reaction with ametryn herbicide

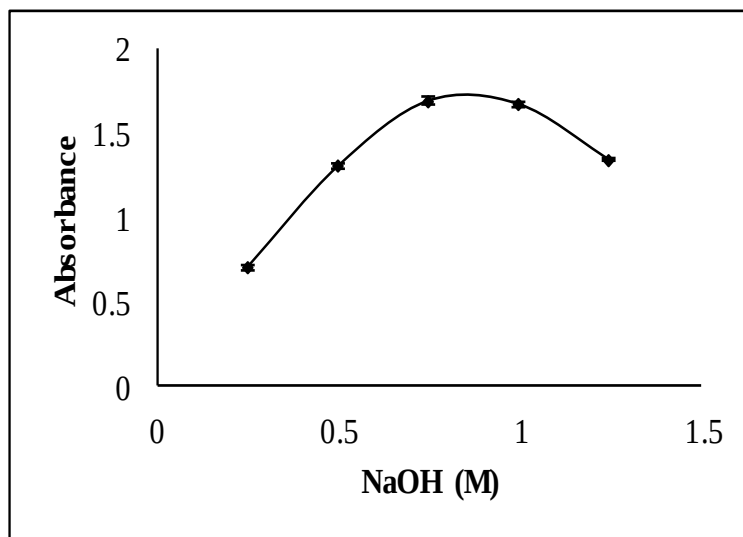


Figure 3.2.5: The effect of sodium hydroxide concentration on reaction of ametryn herbicide

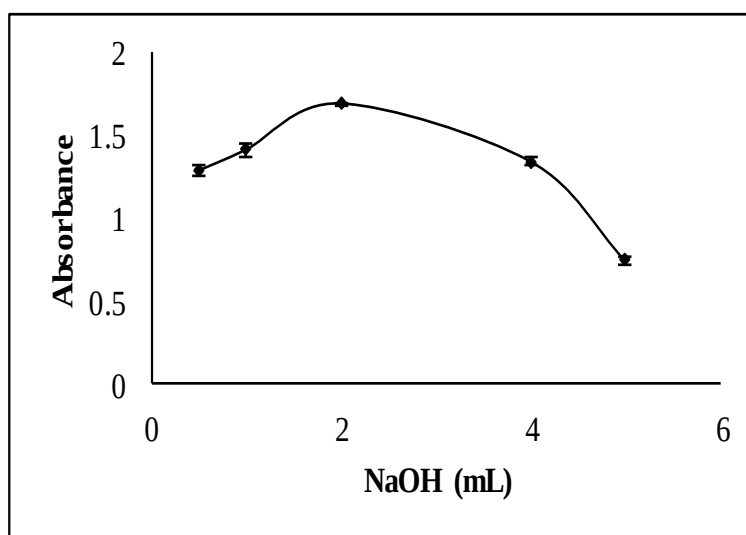


Figure 3.2.6: The effect of volume of sodium hydroxide on reaction of ametryn herbicide

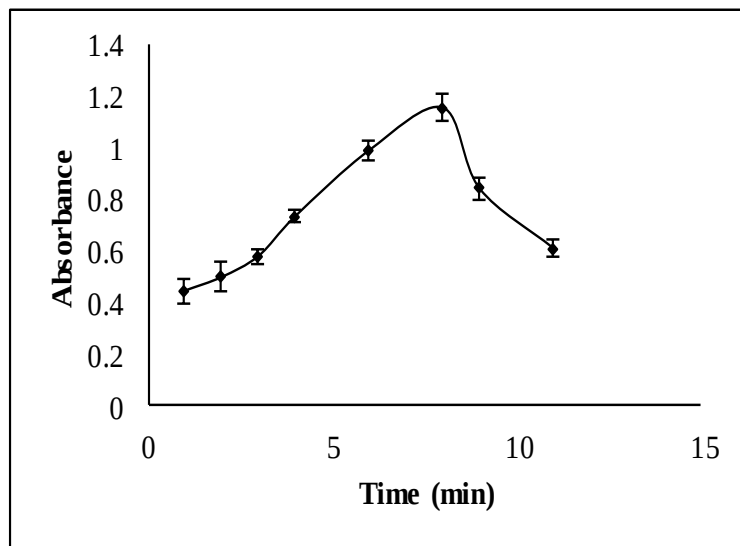


Figure 3.2.7: The effect of heating on reaction of pyridine with ametryn herbicide

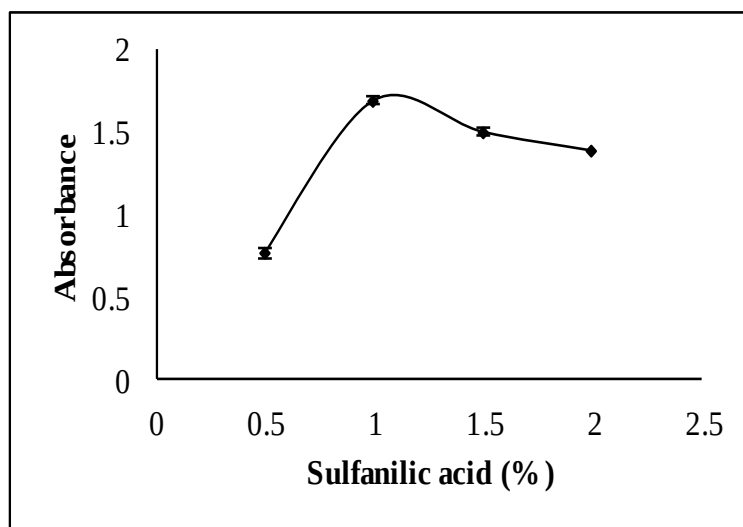


Figure 3.2.8: The effect of concentration of coupling reagent on the formation of colored product

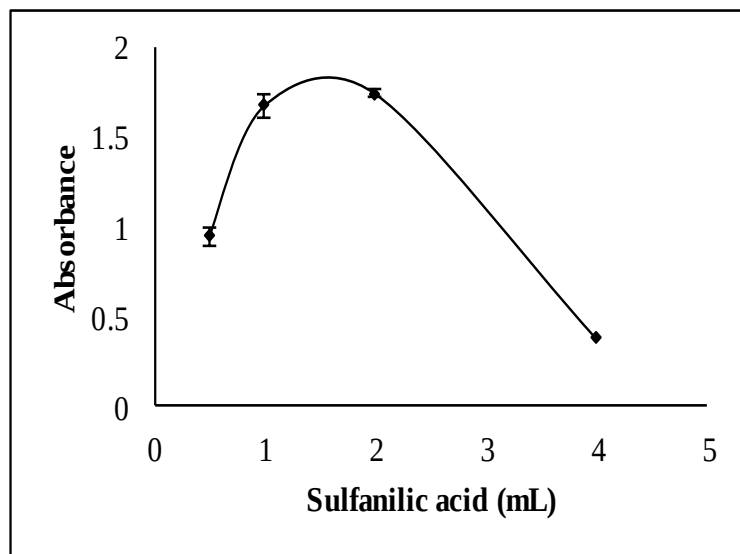


Figure 3.2.9: The effect of volume of coupling reagent on the formation of colored product

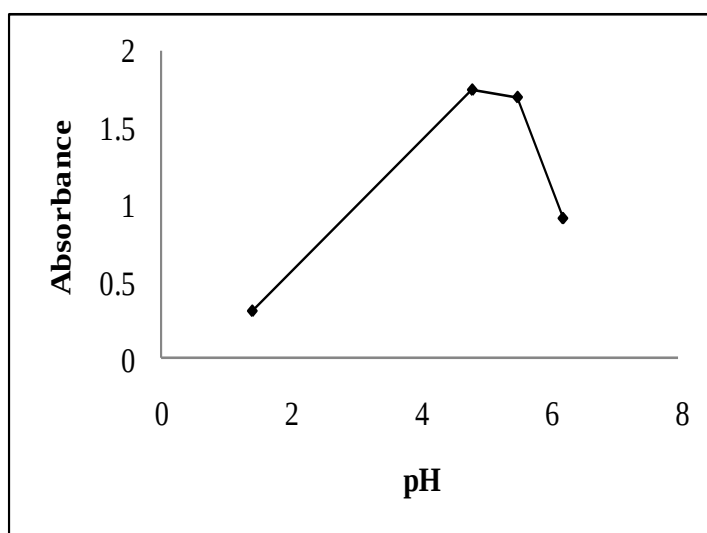


Figure 3.2.10: The effect of pH on the colored product formation

3.2.3.2 Application of factorial design for optimization of different variables for ametryn determination

A 2^4 factorial design with a complete repetition performed in one run resulting in a total of 16 experiments was chosen for optimization of heating temperature and concentration

of pyridine, sodium hydroxide and sulfanilic acid. The designs with four-fold repetition of the optimization and their levels, lowest (-) and highest (+) are given in table 3.2.1. The four factors mentioned above were varied but the total volume was kept constant for all the experiments. The effect of changing a factor from a low to a high level value was studied on absorbance measurement. The highest response was found in the sixteenth run of the table 3.2.1 giving optimum heating temperature to be 100.0 °C, pyridine concentration of 1.0 M, sodium hydroxide and sulfanilic acid concentration of 0.75 M and 1.0 % respectively. For further optimization of the method, a 2³ factorial designs was applied involving a total of 8.0 experiments. The highest response was found in the eighth run of the table 3.2.3 giving optimum heating time to be 8.0 minutes, volume of sodium hydroxide (0.75 M) and sulfanilic acid (1.0 %) to be 2.0 mL each.

Using the data found in table 3.2.1, the effects of the main factors and their interactions with each other were investigated for 2⁴ factorial design. The results are given in table 3.2.2 and are shown in figure 3.2.11. Among the major four factors, the most pronounced effect on the absorption of the yellow dye is heating temperature (A=0.61), concentration of pyridine (B=0.41) and sodium hydroxide (C=0.23). Positive effects from interactions of two factors were AB (0.40), AC (0.21), AD (0.008), BC (0.20), BD (0.018), and three factors were ABC (0.21), ABD (0.01) while CD (-0.0005), ACD (-0.01), BCD (-0.009), ABCD (-0.01) have negative effect on the dye formation. The effects of the main factors and their interactions with each other were also evaluated for 2³ factorial design, given in table 3.2.4 and are shown in figure 3.2.12. All the three factor A (0.98), B (0.31) and C (0.085) show prime impact on dye formation. The AB (0.19) and AC (0.055) have positive effect while BC (-0.016) and ABC (-0.023) have negative effect on the formation of final product.

To study the magnitude of effects for 2⁴ factorial design, the analysis of variance (ANOVA) was carried out and the results are given in table 3.2.5. These results confirm that A, B and C are statistically the main factors for ametryn determination. Note that factor A (3.60) is more significant than factors B (1.48), C (0.72) and D (0.23) and the interaction of AB (0.63), AC (0.17), AD (8.7 x 10⁻⁴), BC (0.19), BD (5.5 x 10⁻⁴), ABC (0.18) and ABD (1.2 x 10⁻³) have significant effect on dye formation. The analysis of

variance (ANOVA) was also carried out for 2^3 factorial design and the results are given in table 3.2.6. Factor A (1.93) is more significant than factors B (0.20), C (0.014) and AB (0.74) and AC (6.1×10^{-3}) are significant among all possible combinations of interaction.

Figure 3.2.13a is a three dimensional response of absorption intensity verses the main influential factors A and B based on the empirical model for 2^4 factorial design. It is clear that absorption increased as heating temperature and pyridine concentration increased. Figure 3.2.13b represents a contour plot for the response derivations. The line curvature is due to the impact of interactions of the two most significant factors and it confirms the experimental results. The three dimensional response of absorption intensity verses the main influential factors A and B and contour plot is shown in figure 3.2.14a and 3.2.14b.

Table 3.2.1: The 2⁴ experimental design

Run	A	B	C	D	Heating temp (°C)	Pyridine Conc. (M)	Sodium hydroxide Conc. (M)	Sulfanilic acid (%)	R Absorbance
1	-1	-1	-1	-1	70	0.30	0.25	0.5	0.227
2	+1	-1	-1	-1	100	0.30	0.25	0.5	0.443
3	-1	+1	-1	-1	70	1	0.25	0.5	0.242
4	+1	+1	-1	-1	100	1	0.25	0.5	0.789
5	-1	-1	+1	-1	70	0.30	0.75	0.5	0.239
6	+1	-1	+1	-1	100	0.30	0.75	0.5	0.455
7	-1	+1	+1	-1	70	1	0.75	0.5	0.292
8	+1	+1	+1	-1	100	1	0.75	0.5	1.691
9	-1	-1	-1	+1	70	0.30	0.25	1	0.236
10	+1	-1	-1	+1	100	0.30	0.25	1	0.454
11	-1	+1	-1	+1	70	1	0.25	1	0.263
12	+1	+1	-1	+1	100	1	0.25	1	0.899
13	-1	-1	+1	+1	70	0.30	0.75	1	0.275
14	+1	+1	+1	+1	100	0.30	0.75	1	0.476
15	-1	-1	+1	+1	70	1	0.75	1	0.291
16	+1	+1	+1	+1	100	1	0.75	1	1.732

A=heating temperature (°C)

B=pyridine concentration (M)

C=Sodium hydroxide concentration (M)

D=Sulfanilic acid concentration (%)

Table 3.2.2: The effect list of main factors and their interactions for 2⁴ factorial design

Error or included in model	Term	Effect	Sum of Square	% Contribution
Model	A	0.61	1.51	41.79
Model	B	0.41	0.69	20.01
Model	C	0.23	0.21	0.260
Model	D	0.03	0.03	0.003
Model	AB	0.40	0.005	0.10
Model	AC	0.21	0.64	17.89
Model	AD	0.008	0.17	4.67
Model	BC	0.20	2×10^{-4}	0.02
Model	BD	0.02	0.17	0.01
Error	CD	5×10^{-4}	0.001	0.005
Model	ABC	0.21	$1 \times 10^{-0.006}$	4.86
Model	ABD	0.01	0.18	0.03
Error	ACD	-0.01	5×10^{-4}	0.007
Error	BCD	-0.010	2×10^{-4}	0.01
Error	ABCD	-0.01	2×10^{-4}	1.5×10^{-3}
Lenth's ME 0.041			Lenth's SME 0.085	

A=heating temperature (°C)

B=pyridine concentration (M)

C=Sodium hydroxide concentration (M)

D=Sulfanilic acid concentration (%)

Design-Expert® Software
absorbance

A: heating temp
B: pyridine conc
C: NaOH conc
D: sulfanilic acid
■ Positive Effects
■ Negative Effects

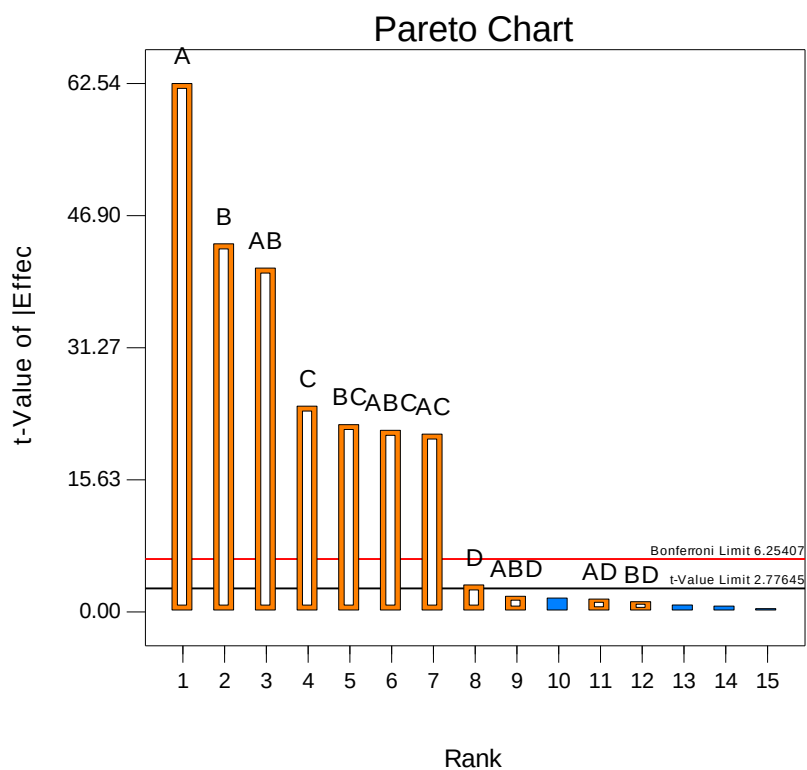


Figure 3.2.11: Standardized Pareto charts for heating temperature and concentration of pyridine, sodium hydroxide and sulfanilic acid

Table 3.2.3: The 2³ experimental design

Run	A*	B*	C*	Heating time (min)	Vol. of 0.75 M sodium hydroxide (mL)	Vol. of 1 % Sulfanilic acid (mL)	R Absorbance
1	-1	-1	-1	1	0.5	0.5	0.443
2	+1	-1	-1	8	0.5	0.5	1.155
3	-1	+1	-1	1	2	0.5	0.557
4	+1	+1	-1	8	2	0.5	1.699
5	-1	-1	+1	1	0.5	2	0.466
6	+1	-1	+1	8	0.5	2	1.334
7	-1	+1	+1	1	2	2	0.593
8	+1	+1	+1	8	2	2	1.800

A=Heating time (min)

B=Volume of sodium hydroxide (0.75 M)

C=Volume of Sulfanilic acid (1.0 %)

Table 3.2.4: List of main factors and their interactions for 2³ factorial design

Error or included in model	Term	Effect	Sum of Square	% Contribution
Model	A	0.98	1.93	86.87
Model	B	0.31	0.20	8.81
Model	C	0.09	0.014	0.65
Model	AB	0.19	0.074	3.33
Model	AC	0.06	6.1 x 10 ⁻³	0.27
Error	BC	-0.016	5.2 x 10 ⁻⁴	0.02
Error	ABC	-0.023	1.0 x 10 ⁻³	0.03
Lenth's ME 0.0419		Lenth's SME 0.085		

A=Heating time (min)

B=Volume of sodium hydroxide (0.75 M)

C=Volume of Sulfanilic acid (1.0 %)

Design-Expert® Software
absorbance

A: heating time

B: vol of 0.75 M (NaOH)

C: vol of % sulfanilic acid

■ Positive Effects

■ Negative Effects

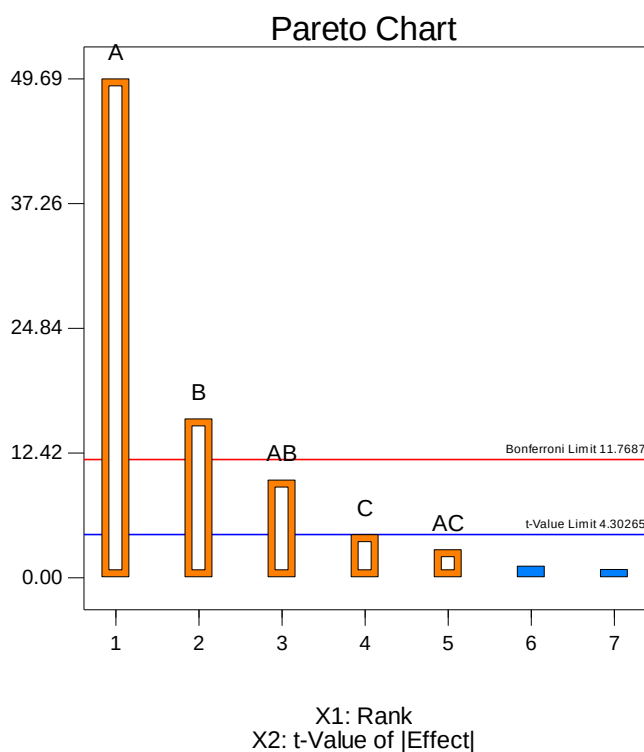


Figure 3.2.12: Standardized Pareto charts for heating time and volume of sodium hydroxide and coupling reagent

Table 3.2.5: Analysis of variance (ANOVA) for main factors for 2⁴ design

source	Sum of squares	df	Mean square	F value	P value Prob > F	The model is significant
Model	3.60	11	0.33	860.9	< 1 x 10 ⁻⁴	
A	1.48	1	1.48	3911.0	< 1 x 10 ⁻⁴	
B	0.72	1	0.72	1896.4	< 1 x 10 ⁻⁴	
C	0.23	1	0.23	593.09	< 1 x 10 ⁻⁴	
D	3.8 x 10 ⁻³	1	3.8 x 10 ⁻³	10.13	0.03	
AB	0.63	1	0.63	1656.5	< 1 x 10 ⁻⁴	
AC	0.17	1	0.17	442.8	< 1 x 10 ⁻⁴	
AD	8.7 x 10 ⁻⁴	1	8.7 x 10 ⁻⁴	2.29	0.20	
BC	0.19	1	0.19	491.6	< 1 x 10 ⁻⁴	
BD	5.5 x 10 ⁻⁴	1	5.5 x 10 ⁻⁴	1.45	0.29	
ABC	0.18	1	0.18	461.3	< 1 x 10 ⁻⁴	
ABD	1.2 x 10 ⁻³	1	1.2 x 10 ⁻³	3.41	0.13	
Residuals	1.5 x 10 ⁻³	4	1.5 x 10 ⁻³	—	—	
Total	3.60	15	3.60	—	—	

df = degree of freedom.

A=Heating temperature (°C)

B=Pyridine concentration (M)

C=Sodium hydroxide concentration (M)

D=Sulfanilic acid concentration (%)

Table 3.2.6: Analysis of variance (ANOVA) for main factors for 2³ factorial design

Source	Sum of squares	df	Mean square	F value	P value Prob > F	The model is significant
Model	2.22	5	0.44	567.9	0.002	
A	1.93	1	1.93	2468.7	4 x 10 ⁻⁴	
B	0.20	1	0.20	250.2	0.004	
C	0.01	1	0.01	18.38	0.05	
AB	0.07	1	0.07	94.57	0.01	
AC	6.1 x 10 ⁻³	1	6.1 x 10 ⁻³	7.81	0.02	
Residuals	1.5 x 10 ⁻³	2	7.8 x 10 ⁻⁴	—	—	
Corrected total	2.22	7	—	—	—	

A= Heating Time (min)

B= Volume of 0.75 M NaOH (mL)

C= Volume of 1.0 % Sulfanilic acid (mL)

Design-Expert® Software

absorbance

● Design points above predicted value

○ Design points below predicted value

1.732

0.227

X1 = A: heating temp

X2 = B: pyridine conc

Actual Factors

C: NaOH conc = 0.75

D: sulfanilic acid = 1.00

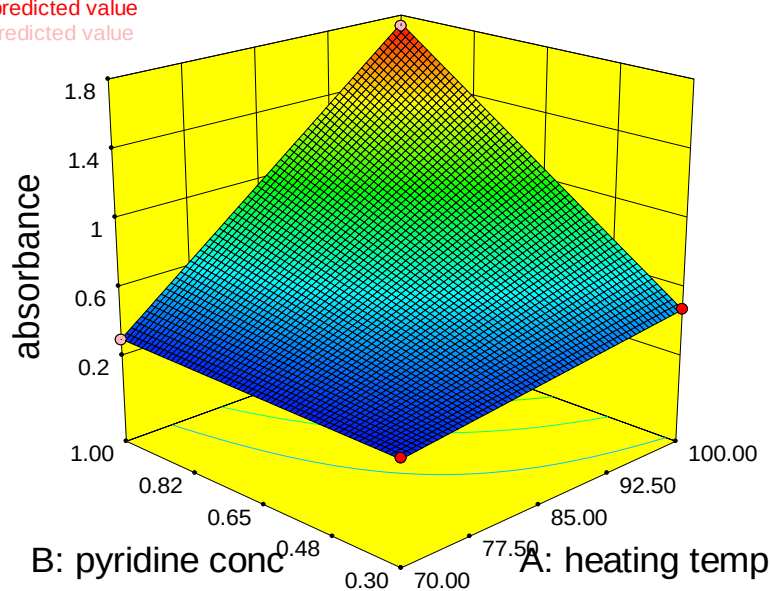


Figure 3.2.13 a: 3-D response plot of absorbance against factor A and B

Design-Expert® Software

absorbance

1.732

0.227

X1 = A: heating temp

X2 = B: pyridine conc

Actual Factors

C: NaOH conc = 0.75

D: sulfanilic acid = 0.99

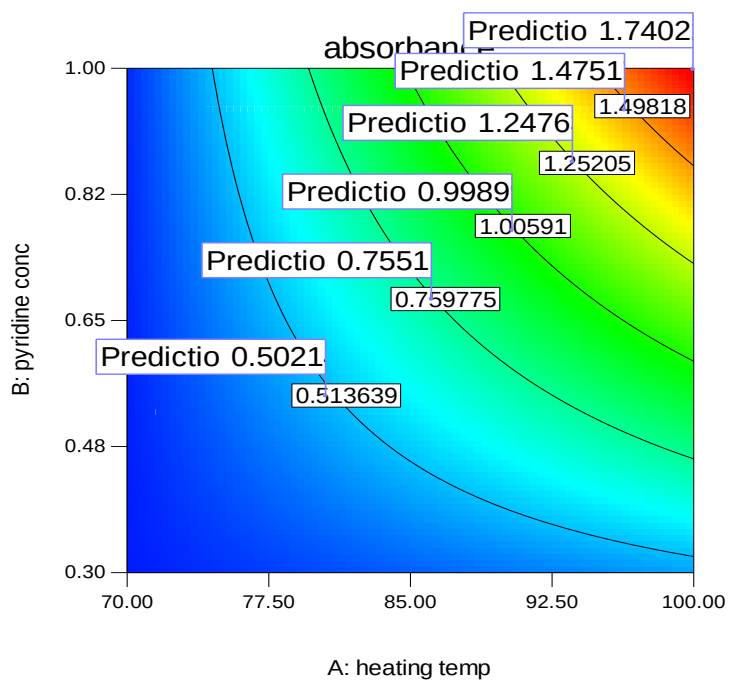


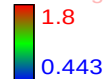
Figure 3.2.13 b: Contour model graph showing predicted and calculated values of response from the design

Design-Expert® Software

absorbance

● Design points above predicted value

○ Design points below predicted value



X1 = A: heating time

X2 = B: vol of 0.75 M (NaOH)

Actual Factor

C: vol of % sulfanilic acid = 2.00

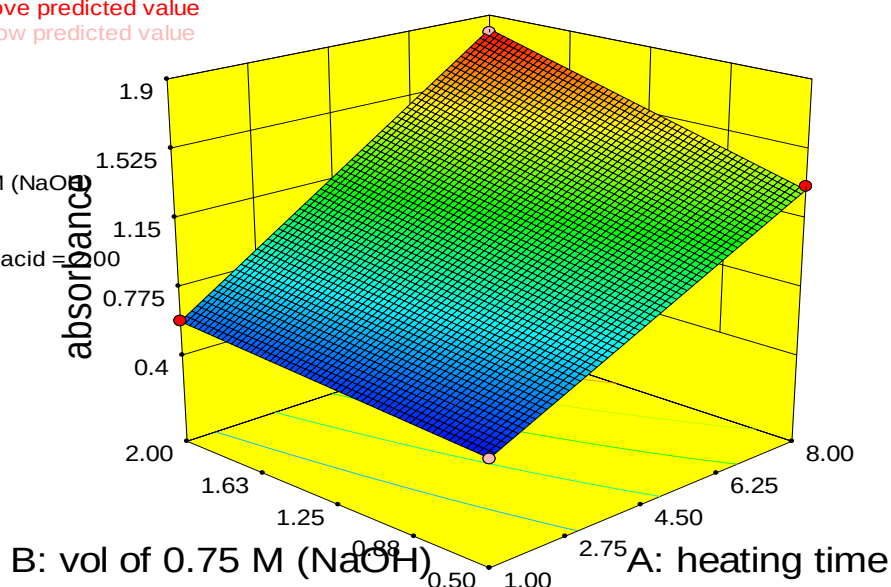


Figure 3.2.14 a: 3-D response plot of absorbance against factor A and B

Design-Expert® Software

absorbance

● Design Points



X1 = A: heating time

X2 = B: vol of 0.75 M (NaOH)

Actual Factor

C: vol of % sulfanilic acid = 2.00

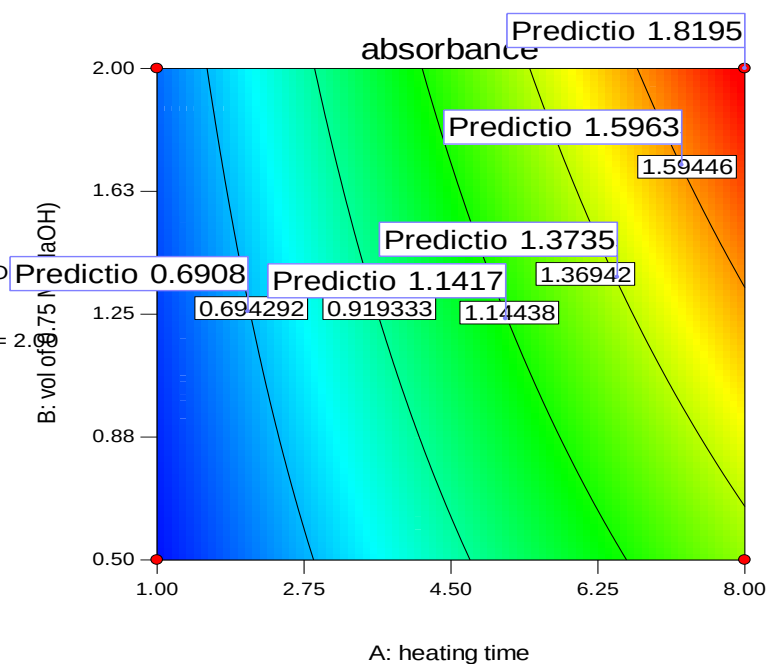


Figure 3.2.14 b: Contour model graph showing predicted and calculated values of response from the design

3.2.3.3 Verification of Beer's law and determination of detection and quantification limit for ametryn determination

Instrument: The same as mentioned before

Reagents: The same as above

Solution: The same as mentioned before

Procedure: From standard stock solution of ametryn ($100.0 \mu\text{g mL}^{-1}$) different volume for $0.1\text{--}20.0 \mu\text{g mL}^{-1}$ were taken in separate 100.0 mL reaction flasks followed by the addition of 0.9 mL of pyridine (79.1 g mol^{-1}) and 2.0 mL of 0.75 M sodium hydroxide solution. These solutions were heated on boiling water bath for 8.0 min . After cooling, 2.0 mL of 1.0% sulfanilic acid was added. The resulting solutions were diluted to 100.0 mL with distilled water. The absorbance of the colored products was measured at 400.0 nm against reagent blank. The results are given in table 3.2.8. A calibration plot of absorbance vs. concentration was constructed and is shown in figure 3.2.15. The molar absorptivity was calculated by using the following formula.

$$\text{Molar absorptivity } (\epsilon) = A/C \times L$$

Where A= absorbance

C= concentration (mol L^{-1})

L= path length (cm)

LOD and LOQ were calculated based on six replicates solution having lowest quantifiable concentration ($\mu\text{g mL}^{-1}$) by the following formula.

Limit of Detection (LOD) (for concentration) = $3 \times S$

Limit of Quantification (LOQ) (for concentration) = $10 \times S$

Where S = Standard deviation

Relative standard deviation (RSD) was calculated by using the following formula.

$$\text{RSD} = \text{Standard deviation} / \text{Mean} \times 100$$

All the statistical values are given in table 3.2.8.

Table 3.2.7: The effect of concentration on absorbance behavior of ametryn herbicide

Conc. of Ametryn ($\mu\text{g mL}^{-1}$)	Absorbance
0.2	0.009
0.4	0.020
0.6	0.026
0.8	0.042
1.0	0.060
2.0	0.089
4.0	0.253
6.0	0.426
8.0	0.774
10	0.813
12	1.032
15	1.291
20	1.742

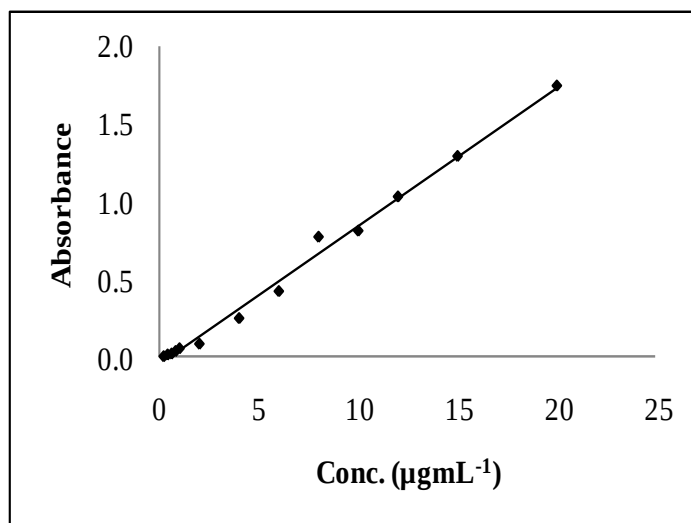


Figure 3.2.15: The effect of concentration on absorbance behavior of ametryn herbicide

Table 3.2.8: Analytical characteristic of the proposed method

Characteristics	Value
λ max (nm)	400
Color	yellow
Linearity range ($\mu\text{g mL}^{-1}$)	0.2-20.0
Slope	0.06
Correlation coefficient (r^2)	0.995
SD	0.009
RSD	11.0
Limit of detection ($\mu\text{g mL}^{-1}$)	0.16
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.54
Molar absorptivity ($\text{L mol}^{-1}\text{cm}^{-1}$)	2.1×10^5

3.2.4 Percent recovery of ametryn herbicide from real samples and commercial formulation

To check the applicability of the method in different matrices, recovery test was performed on control samples, fortified with known concentration of ametryn solution.

Sample extraction procedure

The sugar cane samples under study were first washed properly with cold water and then with hot water in order to remove adhering dust and dirt. Known amounts (60.0 mL) of sugar cane juice was taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then divided into three equal parts. The samples were spiked with different fortified level of ametryn herbicide (4.0, 6.2 and 8.0 $\mu\text{g mL}^{-1}$). All these spiked samples were kept in dark for 24.0 hr at room temperature and afterwards each one was diluted with water up to 50.0 mL. Since ametryn was sprayed in

combination with atrazine on sugarcane field therefore atrazine was removed twice from the sample by addition of 10.0 mL of chloroform. The water extract was then diluted to 100.0 mL with 50.0 mL ethanol. The ethanolic solution was then analyzed by the proposed method.

For commercial formulation, a known amount (0.25 g) was taken and dissolved in water and atrazine was removed twice from the sample by addition of 10.0 mL of chloroform. The water extract was then diluted to 100.0 mL with 50.0 mL ethanol. Known concentration ($3.7 \mu\text{g mL}^{-1}$) was taken and to this was added different concentration of standard solution (3.8, 5.9 and $7.8 \mu\text{g mL}^{-1}$). These solutions were then analyzed by the proposed method.

Results of percent recovery of atrazine in real samples and formulation are given in table 3.2.9. Percent recovery was calculated using the following formula.

% Recovery = $\frac{\text{Total } \mu\text{g found in fortified samples} - \mu\text{g found in sample}}{\mu\text{g of standard added}} \times 100$

Table 3.2.9: Determination of ametryn in fortified real and commercial formulations (n=6)

Real samples/Commercial formulation	Sample conc. taken ($\mu\text{g mL}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Sugar cane	0.7	4.0	4.6	96.3 $\pm$ 0.26
		6.2	6.8	98.4 $\pm$ 0.10
		8.0	8.5	97.5 $\pm$ 0.36
Atrazine + Ametryn	3.7	3.8	7.5	96.1 $\pm$ 0.20
		5.9	9.5	96.6 $\pm$ 0.35
		7.8	11.4	97.5 $\pm$ 0.22

3.2.5 Application of the investigated method for the analysis of ametryn in real samples and commercial formulation

Instrument: The same as mentioned before

Samples: Samples were collected from an agriculture field, where ametryn was sprayed and were first analyzed for the presence of residue. Known amounts (60.0 mL) of sugar cane juice was taken and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then divided into three equal parts and each one was diluted with water up to 50.0 mL. Atrazine was removed twice from the sample by addition of 10.0 mL of chloroform. The water extract was then diluted to 100.0 mL with 50.0 mL ethanol. The ethanolic solution was then analyzed by the proposed method. The results are given in table 3.2.10.

For checking the applicability to commercial formulation, a known amount (0.25 g in 20.0 mL of distilled water) of herbicide (mixture of atrazine and ametryn) were taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then diluted with water up to 100.0 mL. Atrazine was removed twice from the sample by step wise addition of 10.0 mL of chloroform. The water extract was then diluted to 100.0 mL with 50.0 mL ethanol. Known volume of this sample solution was analyzed by the proposed method in triplicate. The results are given in table 3.2.11.

Table 3.2.10 Residue level of ametryn herbicide in real samples

S.NO	Samples	Residue ($\mu\text{g mL}^{-1}$)
1	Sugar cane	0.35 ± 0.07

Table 3.2.11 Concentration of ametryn herbicide in commercial formulation

Commercial formulation	Label Amount ($\mu\text{g g}^{-1}$)	Batch Analysis ($\mu\text{g g}^{-1}$)
Atrazine + Ametryn	0.4	0.39 ± 0.15

3.2.6 Results and discussion

Ametryn was reacted with pyridine in the presence of alkali leading to the formation of quaternary pyridinium hydroxide. This undergoes addition of a hydroxyl group resulting in carbinol base. Glutaconic dialdehyde is formed from carbinol base due to the breaking of heterocyclic ring of the carbinol base in the presence of alkali. Glutaconic dialdehyde is an unstable yellow colored product and can become stable by reacting with sulfanilic acid leading to the formation of a new yellow colored product with maximum absorbance at 400.0 nm. The proposed reaction mechanism is given in figure 3.2. 2.

The effect of various reagent concentrations on the reaction of ametryn for the formation of azo dye was studied. Pyridine solution concentration was varied in the range of 0.1–1.3 M. The absorbance increased when pyridine concentration was increased from 0.1 to 1.1 M (Figure 3.1.4). Beyond this decrease in absorbance was observed with increase in concentration of pyridine solution.

Formation of carbinol base from quaternary pyridinium hydroxide takes place in the presence of alkali. The concentration of sodium hydroxide was varied in the range of 0.25–1.25 M. Maximum absorbance was observed at 0.75 M concentration of sodium hydroxide (Figure 3.1.6). More than 0.75 M sodium hydroxide results in hydrolyzing the carbinol base and destroying the aromaticity of the pyridine ring yielding glutaconic dialdehyde.

Heating time optimization revealed that maximum absorbance of yellow colored product was observed when the solution containing ametryn and pyridine was heated in a boiling water bath for 8 min, further heating lead to decrease in absorbance. This is due to the dissociation of the product beyond optimum heating time.

Sulfanilic acid was used as coupling reagents to stabilize the unstable glutaconic dialdehyde. The effect of sulfanilic acid concentration was investigated in the range of 0.5–2.0 % (Figure 3.1.7). The absorbance increased with sulfanilic acid from 0.5 to 1.0 % and no effect was observed with further increase in sulfanilic acid concentration.

The effect of pH on the yellow colored product formation was studied in the range of 1.4–6.2 and it was found that the final product formed only in acidic medium with sulfanilic acid at pH 4.8–5.5 (Figure 3.2.10).

The effect of time on the stability of yellow dye was investigated up to 1.0 hr. Coupling product with sulfanilic acid was found to be stable for 30.0 min at room temperature.

The concentration vs. absorbance curve was found linear over the concentration range of 0.2–20.0 $\mu\text{g mL}^{-1}$ with sulfanilic acid as a coupling reagent. The linearity of the calibration graph was proved by the high value of the correlation coefficient ($r^2 = 0.995$). The limit of detection was determined by establishing the minimum level at which ametryn can be detected reliably ($3S = 0.16$) using six replicate determinations and the limit of quantification was calculated by establishing the lowest concentration of ametryn that can be measured with acceptable precision and accuracy ($10S = 0.54$) also with six replicate determinations for each coupling reagent. The validity of the method was evaluated by statistical evaluation of the regression lines. The precision of the method was tested by the standard deviation ($SD = 0.01$) and relative standard deviation ($RSD = 11$). The small values of the SD and RSD show low scattering of the point on the calibration curve. The results are summarized in table 3.2.9.

A recovery test was performed on samples fortified with known concentration of ametryn solution. Six replicates of the fortified samples were analyzed using the proposed method. The results are given in table 3.2.10. The average recoveries were found in the range of 96.3–97.5 % from sugar cane samples. A recovery test was also applied to the commercial formulations (Tables 3.2.8). The average recoveries found were in the range of 96.1–97.5 %. The method was also applied for residue determination sugar cane samples. The value for ametryn in sugarcane was found to be $0.35 \pm 0.07 \mu\text{g mL}^{-1}$ (Table 3.2.11). In order to evaluate the analytical applications of the proposed method, it was applied for ametryn determination in commercial formulations. The values obtained were comparable with the label values (Table 3.2.12).

3.3 SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF METRIBUZIN HERBICIDES

3.3.1 Method development strategy for spectrophotometric determination of metribuzin

The chemical structure of metribuzin is given below in figure 3.3.1

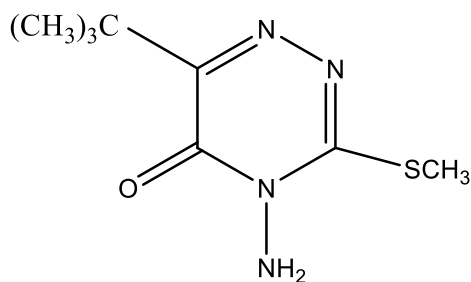


Figure 3.3.1 Chemical structure of metribuzin herbicide

The chemical name of metribuzin is 4-amino-6-*tert*-butyl-3-methylthio-1,2,4-triazin-5(4*H*)-one. Metribuzin has two chemical groups' methylthio and amine attached to the triazine ring with the potential to coordinate with transition metals such as Cu (II). Metribuzin could act as bidentate chelating agent and chelation may take place through both amino and thio groups leading to a five-membered ring with one molecule of metribuzin. Therefore two molecules of metribuzin could react with Cu (II) and form a stable yellow colored extractable complex. The product could be determined spectrophotometrically, from which the amount of metribuzin could be determined. The proposed reaction mechanism is given in figure 3.3.2.

3.3.2 Preliminary investigation of the possibility of complexation of metribuzin with Cu (II)

Preliminary studies were conducted to investigate the possible complexation of metribuzin with copper for the formation of a colored product. Initially high concentration of metribuzin ($1000.0 \mu\text{g mL}^{-1}$), relatively high concentration of copper solution ($1000.0 \mu\text{g mL}^{-1}$) were used to ensure the complexation reaction. The formation of the colored product indicated the possibility of the proposed reaction and the subsequent spectrophotometric determination of metribuzin by this method. Therefore further studies were focused on optimization of various parameters for completion of reaction involved for establishing of the method and are given below.

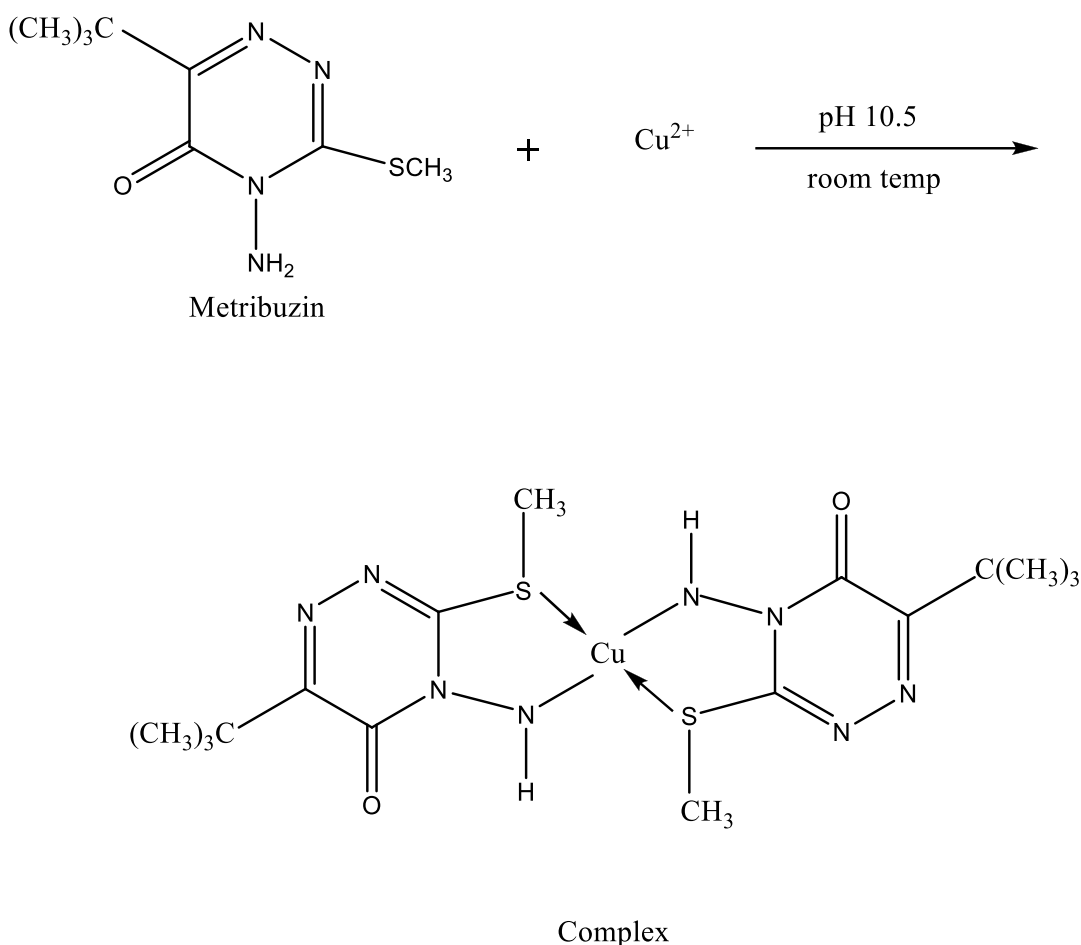


Figure 3.3.2: Proposed reaction mechanism for spectrophotometric determination of metribuzin herbicide

3.3.3 Optimization studies for spectrophotometric determination of metribuzin herbicide using complexation reaction

After the preliminary reaction, it was evident that metribuzin undergo complexation reaction with Cu (II) under basic conditions to form a colored product. For quantitative determination it was important to optimize various parameters involved in the reaction and determination step.

3.3.3.1 Investigation of various parameters for the determination of metribuzin herbicide

Instruments

A UV–Visible spectrophotometer (Unico 2100 United Products Instrument Inc., Dayton, NJ, USA) with 1.0 cm cells was used for all spectral measurements. A pH meter model pH-422 (wissenschaftlich-Technische Werkstätten, W. Germany) was used for pH measurements.

Reagents and chemicals

All chemicals used were of analytical reagent grade or similar purity. Copper sulphate pentahydrate, ammonia, carbon disulphide and chloroform were purchased from Merck (Darmstadt, Germany), whereas distilled ethanol was used throughout the work. Standard reference metribuzin was purchased from Dr. Ehrenstorfer GmbH Germany. Commercial sample containing metribuzin was purchased from the local market.

Solution preparation

- i) **Ammonia solution** (0.2 M): Ammonia solution was prepared by dilution from concentrated ammonia solution (18.0 M).
- ii) **Copper solution** ($1000.0 \mu\text{g mL}^{-1}$): Copper sulphate pentahydrate (0.395 g) was dissolved in 10.0 mL distilled water and diluted to 100.0 mL in volumetric flask.
- iii) **Metribuzin solution** ($1000 \mu\text{g mL}^{-1}$): Metribuzin stock solution was prepared by dissolving 0.1 g of authentic standard metribuzin in 50.0 mL ethanol and diluted with distilled water up to 100.0 mL in volumetric flask. Working standard

solutions of 100.0 and 10.0 $\mu\text{g mL}^{-1}$ solutions were prepared by dilution of the stock solution with distilled water.

Procedure

Metribuzin standard solution of 25.0 $\mu\text{g mL}^{-1}$ was taken in 100.0 mL volumetric flask. Metal solution of 2.0 mL from stock solution (1000.0 $\mu\text{g mL}^{-1}$) and ammonia solution (0.2 M) was added to it followed by addition of 1.0 mL carbon disulphide (the unreacted metribuzin herbicide has no interference ($\lambda_{\text{max}} = 300.0 \text{ nm}$) on the absorbance of the complex formed as it is not soluble in carbon disulphide) and diluted up to the mark with distilled water. The resulting solution was transferred to separatory funnels and 10.0 mL of chloroform was added to it and shaken well for 2.0 min. The two phases were then allowed to separate. The chloroform layer was removed and the absorbance of yellow colored complex extracted was measured with increasing wavelength and each wavelength was calibrated with blank solution. The results are shown in figure 3.3.3.

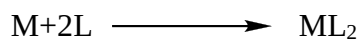
As the proposed reaction requires basic media, therefore the effect of ammonia concentration was studied in the range of 0.05–1.0 M. The results are shown in figure 3.3.5.

Similarly, the effect of pH on the complex formation was also studied in the range of 9.0–11.5 using 0.2 M ammonia solution. The results are shown in figure 3.3.6.

The formula and stability constant of the complex was determined using Jobs' method of continuous variation and is discussed as following.

Following the Job's method of continuous variation different volumes of metribuzin solution ($6 \times 10^{-3} \text{ M}$) were added to six separate conical flasks followed by the addition of 1.0 mL of carbon disulphide (1.0 %) solution, 1.0 mL of ammonia solution (0.2 M) was added to each solution and diluted up to the mark with distilled water. Then different volumes of Cu (II) solution ($6.0 \times 10^{-3} \text{ M}$) were added to the mixtures in such a way that the total volume and total moles of metribuzin and Cu (II) in each mixture remained constant but the mole ratio of these two varied systematically. Each mixture was transferred to a separating funnel and after shaking for three minutes and layers

separation, the lower chloroform layer in each case was collected in a 10.0 mL flask. The absorbance was measured at 340.0 nm against a reagent blank. The absorbance was plotted against the volume ratio of Cu (II) and metribuzin. The results are given in table 3.3.1 and are shown in figure 3.3.4. The empirical formula or the combining ratio of the complex formed was determined to be ML_2 from the theoretical maximum on the curve. The formation constant K_f for the complex was evaluated from measurement of the deviations from the theoretical straight lines and the calculations are given below.



Free Metal (C_M)

$$C_1V_1 = C_2V_2$$

$$6 \times 10^{-3} \times 3 = X \times 10$$

$$X = C_M = 1.8 \times 10^{-3}$$

Free Ligand (C_L)

$$C_1V_1 = C_2V_2$$

$$6 \times 10^{-3} \times 7 = X \times 10$$

$$X = C_L = 4.2 \times 10^{-3}$$

Conc. of the complex [ML_2]

$$[ML_2] = (A_{\text{actual}}/A_{\text{extrapolated}}) \times C_M$$

$$[ML_2] = (1.683/1.710) \times 1.8 \times 10^{-3}$$

$$[ML_2] = 1.7 \times 10^{-3}$$

$$[M] = C_M - [ML_2]$$

$$[M] = 1.0 \times 10^{-4}$$

$$[L] = C_L - [ML_2]$$

$$[L] = 2.5 \times 10^{-3}$$

$$K_f = [ML_2] / [M] [L]^2$$

$$K_f = 2.7 \times 10^6$$

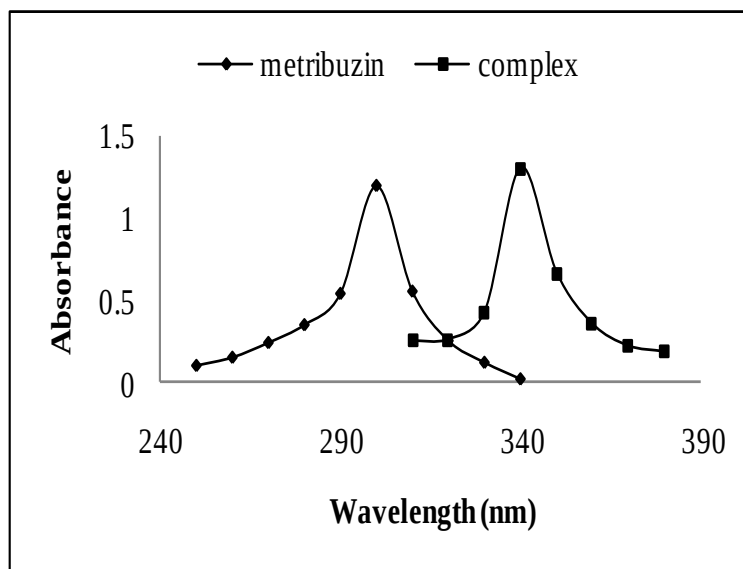


Figure 3.3.3: Investigation of suitable wavelength for the spectrophotometric determination of metribuzin herbicide

Conditions

$\lambda_{\max}$	Varied
Metribuzin solution	50.0 $\mu\text{g mL}^{-1}$
Copper solution (1000.0 $\mu\text{g mL}^{-1}$)	2.0 mL
Ammonia solution	0.2 M

Table 3.3.1: Formula and stability constant determination by Job's method

M:L	Absorbance
9:01	0.432
8:02	0.497
7:03	0.703
6:04	0.943
5:05	1.182
4:06	1.499
3:07	1.683
2:08	1.597
1:09	1.267

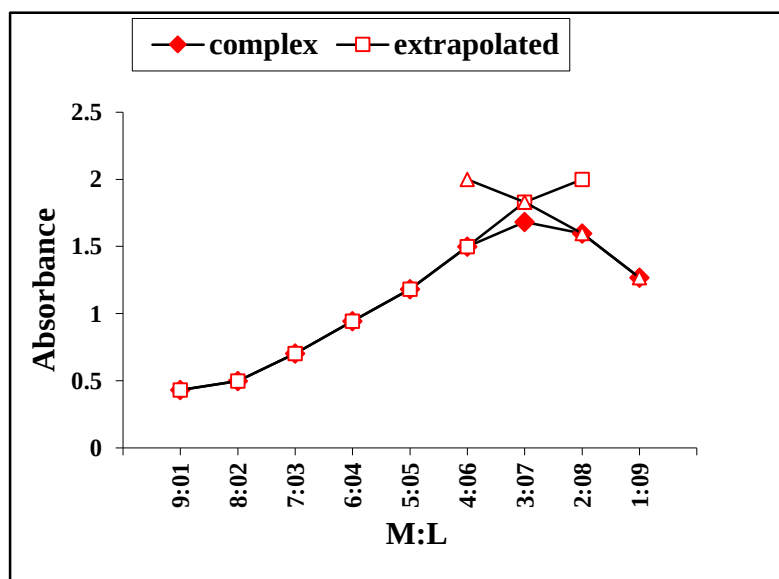


Figure 3.3.4: Formula and stability constant determination by Job's method

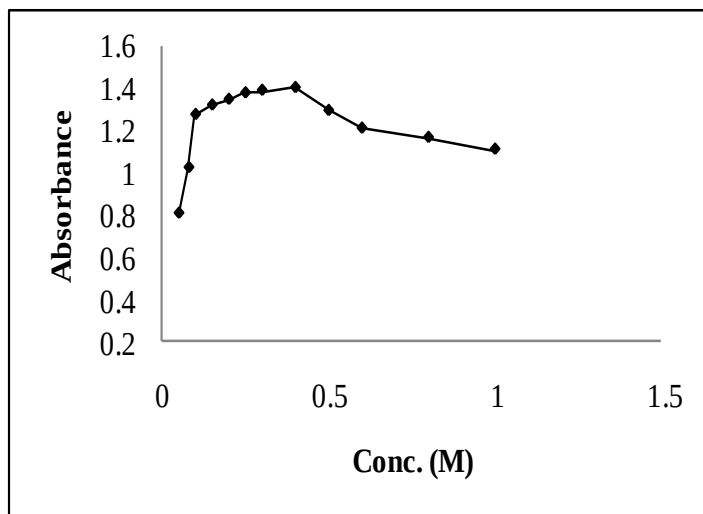


Figure 3.3.5: Effect of ammonia concentration on reaction of metribuzin with Cu (II)

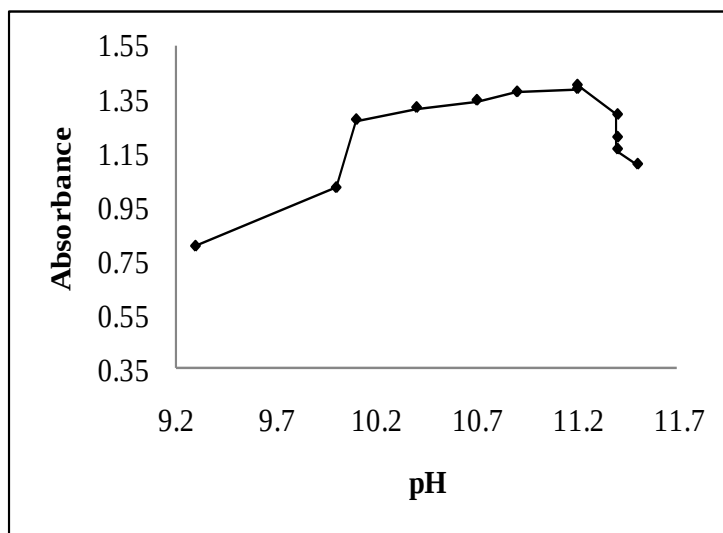


Figure 3.3.6: The effect of pH on reaction of metribuzin with Cu (II)

3.3.3.2 The factorial design to optimize different variables for metribuzin determination

A 2^3 factorial design with a complete repetition performed in one run resulting in a total of eight experiments was chosen for optimization of metribuzin complexation with Cu (II). The optimization design with a threefold repetition for complex formation is given in table 3.3.3. Three factors were selected for optimization of complexation reaction. The factors and their lower (–) and high level (+) are given in table 3.3.3 and are shown in figure. 3.3.8. The pH, metal ion, Cu (II) concentration and ligand (metribuzin) concentration were varied but the total volume was kept constant for all experiment. The effect of changing a factor from a low level to a high level value was studied on absorbance measurement. For maximum complex formation of metribuzin with metal ion, the highest response was found in the eighth run in table 3.3.3. Using the data found in table 3.3.3, the effects of the main factors and their interactions with each other were estimated and the results are given in table 3.3.4. The most calculated effect on the absorbance of complex was seen in pH ($C = 0.47$) and ligand (metribuzin) concentration ($B = 0.34$). The BC interaction (0.27) has positive effect on complex formation while AC (-0.35) and ABC (-0.035) have the same negative effect on the complexation reaction. For statistical confirmation of these effects, the analysis of variance (ANOVA) was carried out and the results are given in table 3.3.5. These results confirmed that B and C are statistically the main factors for complex formation. Note that factor C is significant (0.44) than B (0.23) and the interaction of BC has significant effect (1.0) on complex formation. The optimum conditions for the highest absorbance of metribuzin complex with Cu (II) were seen in the eighth run in table 3.3.3. Figure 3.3.9 represents a contour plot for the response derivations. The line curvature is due to the impact of interactions of the two significant factors which confirm our results.

Table 3.3.2: The 2^3 experimental design

Run	A*	B*	C*	Factor A*	Factor B*	Factor C*	Response Absorbance
1	-1	-1	-1	20	20	0.05	0.201
2	+1	-1	-1	40	20	0.05	0.201
3	-1	+1	-1	20	40	0.05	0.201
4	+1	+1	-1	40	40	0.05	0.341
5	-1	-1	+1	20	20	0.20	0.403
6	+1	-1	+1	40	20	0.20	0.403
7	-1	+1	+1	20	40	0.20	1.008
8	+1	+1	+1	40	40	0.20	1.009

A= metal concentration ($\mu\text{g mL}^{-1}$)B= ligand concentration ($\mu\text{g mL}^{-1}$)

C= pH

Table 3.3.3: Effect list of main factors and their interactions

Error or included in model	Term	Effect	Sum of Square	% Contribution
Error	A*	0.035	0.002	0.302
Model	B*	0.34	0.228	27.73
Model	C*	0.47	0.441	53.64
Error	AB	0.035	0.002	0.302
Error	AC	-0.035	0.002	0.293
Model	BC	0.27	0.143	17.42
Error	ABC	-0.035	0.002	0.293
Lenth's ME 0.197		Lenth's SME 0.472		

A= metal concentration ($\mu\text{g mL}^{-1}$)B= ligand concentration ($\mu\text{g mL}^{-1}$)

C= pH

Table 3.3.4: Analysis of variance (ANOVA) for main factors

source	Sum of squares	df*	Mean square	F value	P value (Prob > F)	This method is significant
Model	0.81	3	0.27	110.59	0.0003	
B*	0.23	1	0.23	93.12	0.0006	
C*	0.44	1	0.44	180.13	0.0002	
BC	1	0.14	58.52	0.0016	—	
Residuals	9.800E-003	4	2.450E-003	—	—	
Cor total	0.82	7	—	—	—	

df = degree of freedom

B = ligand concentration ($\mu\text{g mL}^{-1}$)

C = pH

Design-Expert® Software
absorbance

A: metal conc
B: legand conc
C: pH
■ Positive Effects
■ Negative Effects

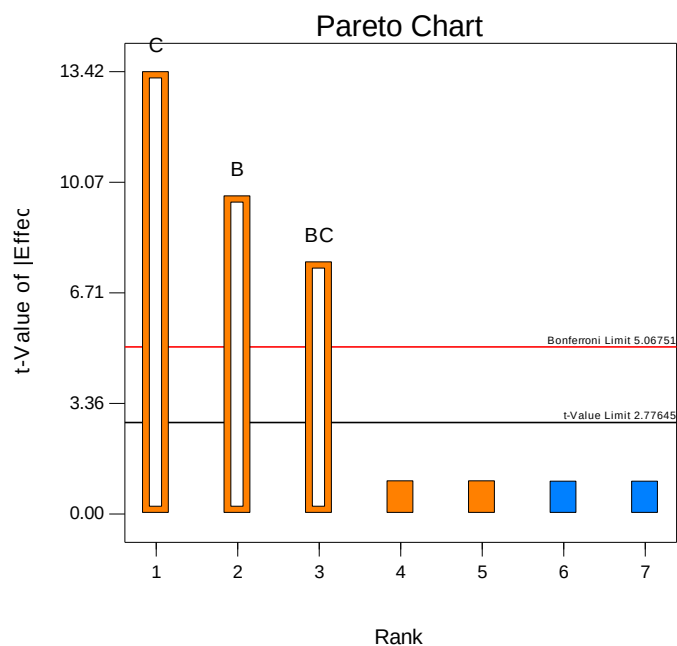


Figure 3.3.7: Standardized Pareto charts for pH and ligand concentration

Design-Expert® Software

absorbance

X1 = B: legand conc

X2 = C: pH

X3 = A: metal conc

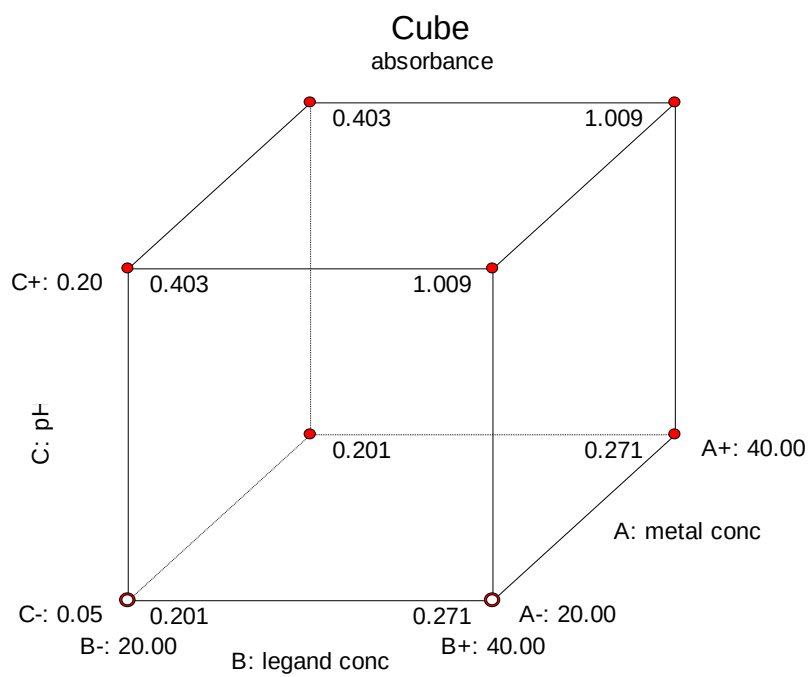


Figure 3.3.8: Cube model graph of the 2^3 factorial design

Design-Expert® Software

absorbance

1.009

0.201

X1 = B: legand conc

X2 = C: pH

Actual Factor

A: metal conc = 30.00

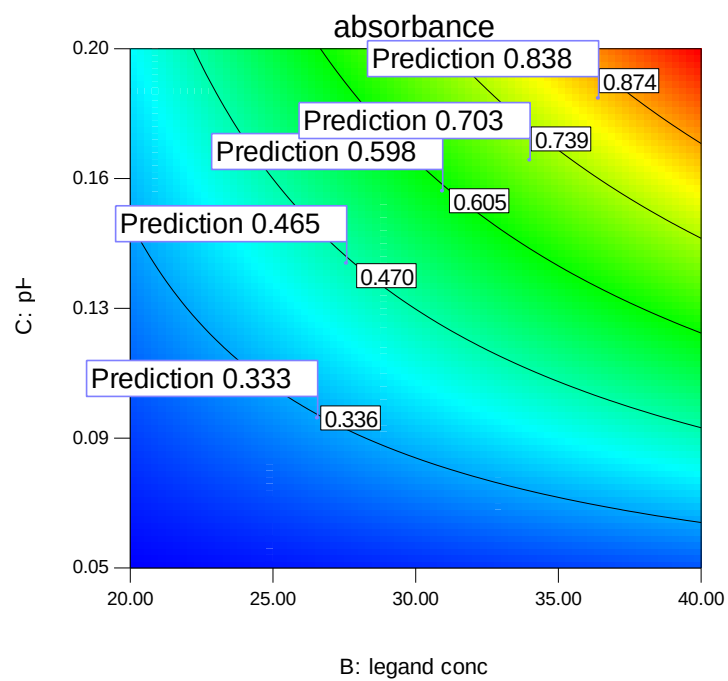


Figure 3.3.9: Contour plot of the predicted and calculated values of response from the design

3.3.3.3 Verification of Beer's law and determination of detection and quantification limit for metribuzin determination

Instrument: The same as mentioned before

Reagents: The same as above

Solution: The same as mentioned before

Procedure:

Metribuzin standard solutions of 0.8–25.0 $\mu\text{g mL}^{-1}$ were taken in ten separate 100.0 mL volumetric flasks. Metal solution of 2.0 mL from stock solution (1000.0 $\mu\text{g mL}^{-1}$) and ammonia solution (0.2 M) was added to each of the solutions followed by addition of 1.0 mL carbon disulphide and diluted up to the mark with distilled water. These solutions were transferred to separatory funnels and 10.0 mL of chloroform was added to it and shaken well for 2.0 min. The two phases were then allowed to separate. The chloroform layer was removed and the absorbance of yellow colored extracted complex was measured at 340.0 nm against reagent blank. The results are given in table 3.3.6. A calibration plot of absorbance vs. concentration was constructed and is shown in figure 3.3.10. The molar absorptivity was calculated by using the following formula.

$$\text{Molar absorptivity } (\epsilon) = A/C \times L$$

Where A= absorbance

C= concentration (mol L^{-1})

L= path length (cm)

LOD and LOQ were calculated based on six replicates solution having lowest quantifiable concentration ($\mu\text{g mL}^{-1}$) by the following formula.

Limit of Detection (LOD) (for concentration) = $3 \times S$

Limit of Quantification (LOQ) (for concentration) = $10 \times S$

Where S = Standard deviation

Relative standard deviation (RSD) was calculated by using the following formula.

$$\text{RSD} = \text{Standard deviation} / \text{Mean} \times 100$$

All the statistical values are given in table 3.3.7.

Table 3.3.5: Investigation of the effect of concentration on absorbance behavior of metribuzin

Conc. of metribuzin ($\mu\text{g mL}^{-1}$)	Absorbance
0.8	0.016
2.0	0.034
4.0	0.073
6.0	0.114
8.0	0.155
10	0.219
12	0.284
15	0.386
10	0.568
25	0.672

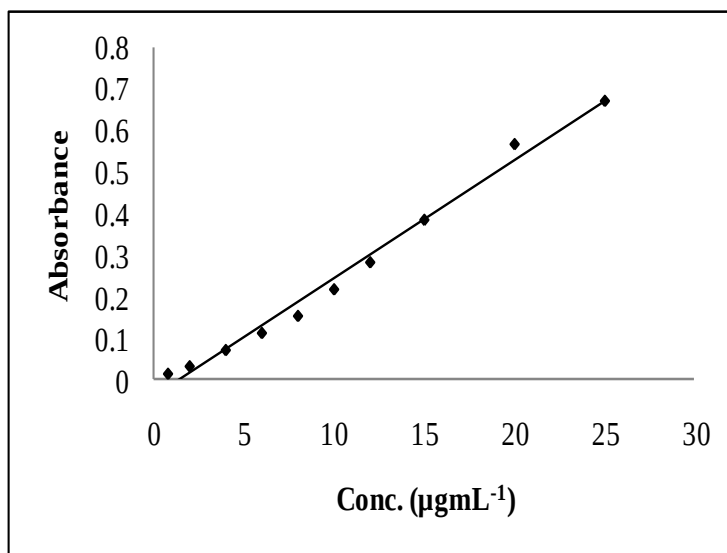


Figure 3.3.10: Investigation of the effect of concentration on absorbance behavior of metribuzin herbicide

Table 3.3.6: Analytical characteristic of the proposed method

Characteristics	Value
λ max (nm)	340
Color	yellow
Linearity range ($\mu\text{g mL}^{-1}$)	0.8-25
Slope	0.047
Correlation coefficient (r^2)	0.998
SD	0.20
RSD	5
Limit of detection ($\mu\text{g mL}^{-1}$)	0.66
Limit of quantification ($\mu\text{g mL}^{-1}$)	2.2
Molar absorptivity ($\text{L mol}^{-1}\text{cm}^{-1}$)	5.67×10^3
Stability constant	2.7×10^6

3.3.4 Percent recovery of metribuzin from real samples and commercial formulation

To check the applicability of the method in different matrices, recovery test was performed on control samples, fortified with known concentration of metribuzin solution.

Sample extraction procedure

The potato samples under study were first washed properly with cold water and then with hot water in order to remove adhering dust and dirt. Metribuzin solution ($4.0 \mu\text{g g}^{-1}$) was added as residue to these potato samples and kept overnight for maximum adsorption. The samples were spiked with different fortified level of metribuzin herbicide. All these spiked samples were kept at room temperature for few hr. Metribuzin was removed on solubility basis by soaking the potatoes (250.0 g) in 50.0 mL chloroform for two hours and then filtered. The filtrate (chloroform) was evaporated and the residue was redissolved in 50.0 mL ethanol. The ethanolic solution was then analyzed by the proposed method.

For checking the applicability to commercial formulations, known amount (0.37 g in 20.0 mL of distilled water) of herbicide metribuzin were taken separately and stirred on magnetic plate for 30.0 min. The homogeneous solution of each sample was then diluted with water up to 100.0 mL. Metribuzin was extracted from the sample by extracted twice with 10.0 mL of chloroform. The combined chloroform extract was evaporated to dryness and the residue was dissolved in 50.0 mL methanol and diluted with distilled water in a 100.0 mL volumetric flask. Known volume of this sample solution was then analyzed by the proposed method in triplicate.

Results of percent recovery of metribuzin in real samples and formulation are given in table 3.3.7. Percent recovery was calculated by using the formula given below.

% Recovery = $\frac{\text{Total } \mu\text{g found in fortified samples} - \mu\text{g found in sample}}{\mu\text{g of standard added}} \times 100$

Table 3.3.7: Determination of metribuzin in fortified real and commercial samples (n=6)

Real samples/Commercial formulation	Sample conc. taken ($\mu\text{g g}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Potatoes	4	10	12.6	86.0 $\pm$ 0.9
		12	15.0	91.7 $\pm$ 0.2
		15	18.1	94.0 $\pm$ 0.4
Metribuzin	10	10	19.8	98.0 $\pm$ 0.7
		12	21.8	98.3 $\pm$ 0.2
		15	25.1	100.7 $\pm$ 0.4

3.3.5 Application of the investigated method for the analysis of metribuzin in real samples and commercial formulation

Instrument: The same as mentioned before

Samples: Potato samples were collected from an agriculture field, where metribuzin was sprayed and were first analyzed for the presence of residue. For residue determination in real samples, metribuzin was removed on solubility basis by soaking the potatoes 250.0 g in 50.0 mL chloroform for two hours and then filtered. The filtrate (chloroform) was evaporated and the residue was redissolved in 50.0 mL ethanol. The ethanolic solution was then analyzed by the proposed method. The results are shown in table 3.3.8.

Active ingredient concentration in commercial formulation was determined by taking known amount (0.14 g) and dissolved in 50.0 mL methanol in a beaker and filtered with Whatman filter (NO:40) paper to remove any suspended particles. This solution was then transferred to 100.0 mL volumetric flask and finally diluted to 100.0 mL with distilled water. Known volume from this solution was taken (0.3 mL) and the color was developed with the procedure mentioned before. The results are shown in table 3.3.9.

Table 3.3.8: Residue level of metribuzin in real samples

S.NO	Samples	Residue ($\mu\text{g g}^{-1}$)
1	Potato	0.15 $\pm$ 0.07

Table 3.3.9: Concentration of metribuzin in commercial formulation

Commercial formulation	Label Amount (gm L^{-1})	Amount found (gm L^{-1})
Metribuzin	0.7	0.68 $\pm$ 0.11

3.3.6 Results and discussion

Metribuzin has two chemical groups' methylthio and amine attached to the triazine ring with the potential to coordinate with transition metals such as Cu (II). Preliminary results confirmed the complexation of metribuzin with copper. The proposed reaction mechanism is given in figure. 3.3.2. Mitribuzin acts as bidentate chelating agent and chelation takes place through both amino and thio groups leading to a five-membered ring with one molecule of metribuzin. Therefore two molecules of metribuzin reacted with Cu (II) and formed a stable yellow colored extractable complex. The resulting complex was extracted in chloroform and was found to have absorption maxima at 340 nm against the reagent blank.

As the proposed reaction requires basic media, therefore the effect of ammonia concentration was studied in the range of 0.05–1.0 M. Maximum absorbance was observed with ammonia concentration in the range of 0.2–0.4 M (Figure 3.3.5). The effect of pH on the complex formation was also studied in the range of 9.0–11.5 using 0.2 M ammonia solution and it was found that maximum complexation achieved at pH 10.5 (Figure 3.3.6).

The composition of the complex was investigated using Job's method of continuous variations (Figure 3.3.4). Maximum absorbance for the complex was observed at a mole ratio of 3:7 which indicated the formation of 1:2 (Cu (II): metribuzin) complex. The

formation constant of the complex was calculated from the data of continuous variations method and was found to be 2.7×10^6 .

The concentration vs. absorbance curve was found linear over the concentration range of 2.0–25.0 $\mu\text{g mL}^{-1}$. The statistical parameters calculated from the calibration graph are given in table 3.3.7. The linearity of calibration graph is reflected by the high value of correlation coefficient ($r^2 = 0.998$). The molar absorptivity of the resulting yellow colored complex was found to be $5.67 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$. The limit of detection (LOD) and quantification (LOQ) were calculated using the minimum concentration at which metribuzin could be detected reliably and it was found to be 0.66 and 2.2 $\mu\text{g mL}^{-1}$ respectively.

A recovery test was performed on control samples fortified with known concentration of metribuzin solution. Similarly the percent recovery from commercial formulations was also determined by standard addition method to check the interferences effect of emulsified solution. Recovery values in commercial formulations were found to be 98.0 % $\pm$ 0.7 to 100.7 % $\pm$ 0.4 and in case of real samples the results of the recovery were found to be in the range of 86.0 % $\pm$ 0.9 to 94.0 % $\pm$ 0.4. The method was also applied for residue determination in potato sample. The value for metribuzin residue in potatoes was found to be $0.15 \pm 0.07 \mu\text{g mL}^{-1}$.

3.4 SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF METRIBUZIN HERBICIDE

3.4.1 Method development strategy for spectrophotometric determination of metribuzin herbicide

The chemical structure of metribuzin is given below in figure 3.4.1

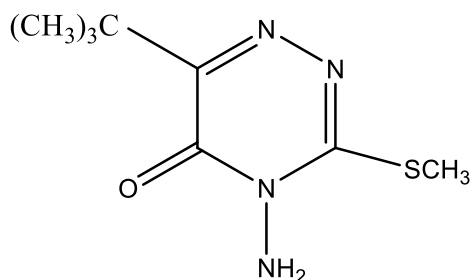
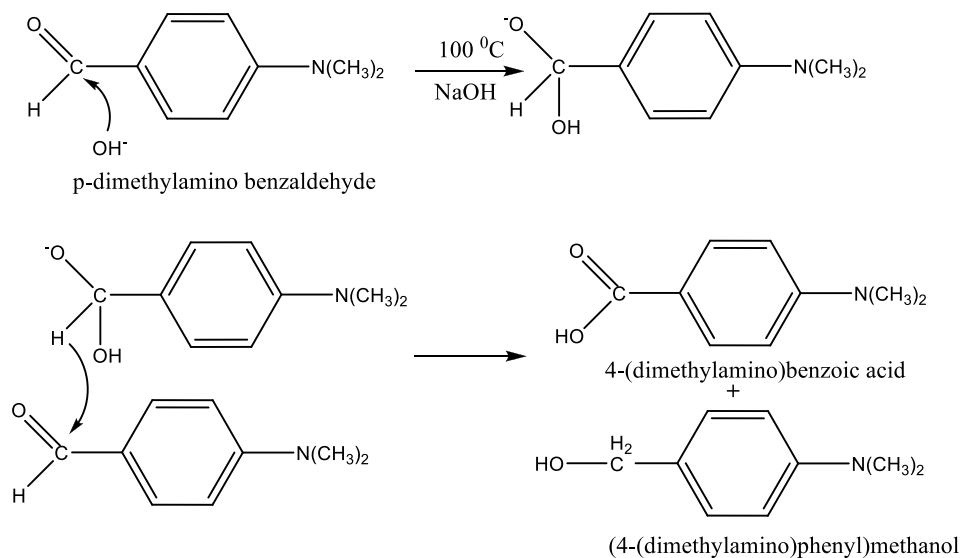


Figure 3.4.1 Chemical structure of metribuzin herbicide

The chemical name of metribuzin is 4-amino-6-*tert*-butyl-3-methylthio-1,2,4-triazin-5(4*H*)-one. *p*-dimethylaminobenzaldehyde (DMAB) can form *p*-dimethylaminobenzoic acid in the presence of sodium hydroxide (Cannizzaro's reaction) at 100.0 °C. It is expected from the structure of metribuzin that amine group attached with triazine ring has the potential to react with *p*-dimethylaminobenzoic acid in acidic media at 100.0 °C and could result in a colored product. The product could be determined spectrophotometrically, from which the amount of metribuzin could be determined. The proposed reaction mechanism is shown in figure 3.4.2.

Step-I



Step-II

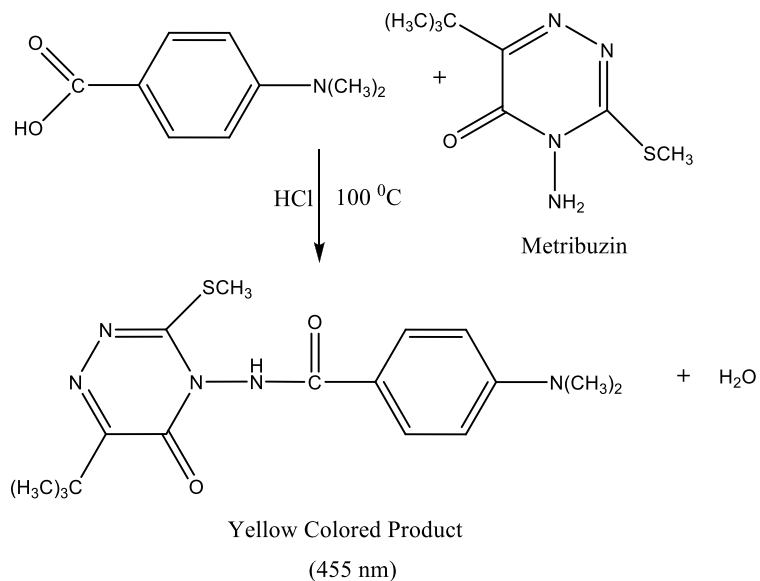


Figure 3.4.2: The proposed reaction mechanism for spectrophotometric determination of metribuzin herbicide

3.4.2 Preliminary investigation of the possibility of Cannizzaro's reaction and coupling of metribuzin with *p*-dimethylaminobenzoic acid

Preliminary studies were conducted to investigate the possibility of cannizzaro's reaction and the subsequent coupling of the resulting *p*-dimethylaminobenzoic acid with metribuzin for the formation of a colored product. Initially high concentration of sodium hydroxide and *p*-dimethylaminobenzaldehyde were used to ensure the cannizzaro's reaction. The resultant *p*-dimethylaminobenzoic acid formed was coupled at 100.0 °C with metribuzin to form the expected colored product. The formation of the colored product indicated the possibility of the reaction and the subsequent spectrophotometric determination of metribuzin by this method. Therefore further studies were focused on optimization of various parameters for establishing of the method and are given below.

3.4.3 Optimization studies for spectrophotometric determination of metribuzin using cannizzaro's reaction

After the preliminary reaction, it was evident that *p*-dimethylaminobenzaldehyde undergo cannizzaro's reaction in the presence of sodium hydroxide and the *p*-dimethylaminobenzoic acid formed in this reaction can couple with metribuzin and results in the formation of a colored product. For quantitative determination it was important to optimize various parameters involved either in the reaction and determination steps.

3.4.3.1 Investigation of various parameters for the determination of metribuzin herbicide

Instruments

A UV–Visible spectrophotometer (Unico 2100 United Products Instrument Inc., Dayton, NJ, USA) with 1.0 cm cells was used for all spectral measurements. A pH meter model pH-422 (wissenschaftlich-Technische Werkstätten, W. Germany) was used for pH measurements.

Reagents and chemicals

All chemicals used were of analytical reagent grade or similar purity. *P*-dimethylaminobenzaldehyde (DMAB) or Ehrlich reagent, sodium hydroxide, hydrochloric acid (37.0 %) and methanol were purchased from (Merck Darmstadt, Germany) and were used throughout the work without further purification. Reference standard of metribuzin was purchased from Dr Ehrenstofer GmbH (Germany). Commercial formulation (Metribuzin 70 WP) containing 70.0 % W/W of active ingredient (Roots international Agro Division) was purchased from the local market.

Solution Preparation

- i) **Metribuzin solution** ($1000.0 \mu\text{g mL}^{-1}$): Metribuzin stock solution ($1000.0 \mu\text{g mL}^{-1}$) was prepared by dissolving 0.1 g of authentic standard metribuzin in 50.0 mL ethanol and diluted with distilled water up to 100.0 mL in volumetric flask. Working standard in the range of 10.0 - $100.0 \mu\text{g mL}^{-1}$ solutions were prepared by dilution of the stock solution with distilled water.
- ii) ***p*-dimethylaminobenzaldehyde solution** (0.04 M): *p*-dimethylaminobenzaldehyde solution was freshly prepared by dissolving 0.6 g in 100.0 mL of methanol.
- iii) **Sodium hydroxide solution** (0.87 M): Sodium hydroxide solution was prepared by dissolving 3.48 g in 100.0 mL of distilled water.

Procedure

Metribuzin solution ($20.0 \mu\text{g mL}^{-1}$) was transferred quantitatively into three separate reaction flasks followed by the addition of 10.0 mL DMAB (0.04 M) and 2.0 mL of sodium hydroxide (0.87 M) solutions and were heated at 100.0°C for 4.0 min on water bath. While on the water bath, the solutions were acidified with 2.8 M hydrochloric acid solution and were heated for 1.0 min again. After cooling, the solutions were diluted to 25.0 mL with distilled water and allowed to stand for 15.0 min. The absorbance of the colored product was measured in the range of 410.0-500.0 nm against reagent blank. The

absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are shown in figure 3.4.3.

For optimization of DMAB concentration, metribuzin solutions ($20.0 \mu\text{g mL}^{-1}$) were transferred quantitatively into nine separate reaction flasks followed by the addition of 5.0 mL DMAB solution having varied molarity (0.0067-0.06 M) and 2.0 mL of sodium hydroxide (0.87 M) solution while the rest of the procedure was kept the same. The absorbance of the colored product was measured and results are shown in figure 3.4.4.

Similarly for finding optimum volume of DMAB, only volume of DMAB (2.0-14.0 mL) was varied, while keeping the rest of the procedure the same and absorbance was measured, the results are shown in figure 3.4.5.

The DMAB is derivatized into *p*-dimethylaminobenzoic acid in basic media. The effect of sodium hydroxide concentration on the reaction was investigated in the range of 0.25-1.0 M. The results are shown in figure 3.4.6.

Similarly for volume optimization of sodium hydroxide, only the volume of 0.87 M sodium hydroxide was varied from 1.0-6.0 mL. The results are shown in figure 3.4.7. The conversion of DMAB into *p*-dimethylaminobenzoic acid via cannizzaro's reaction was studied on boiling water bath from 1.0 to 6 min. The results are shown in figure 3.4.8.

For optimization of hydrochloric acid concentration, to each of the nine reaction flasks different volume of hydrochloric acid (37.0 %) solution was added varying from 0.04-0.6 mL giving a final molar concentration in the range of 0.44-3.0 M after dilution. Color was developed in the same way as mentioned before and absorbance was noted. The results are shown in figure 3.4.9.

For optimization of the heating time of the coupling reaction, the solutions were heated on boiling water bath and heating time was varied from 0.5-4.0 min. The absorbance of the colored solution was measured at the maximum wavelength after following the same procedure. The results are shown in figure 3.4.10.

In order to check the stability of the end product, the absorbance of the final colored product formed was observed after every two hr for three consecutive days.

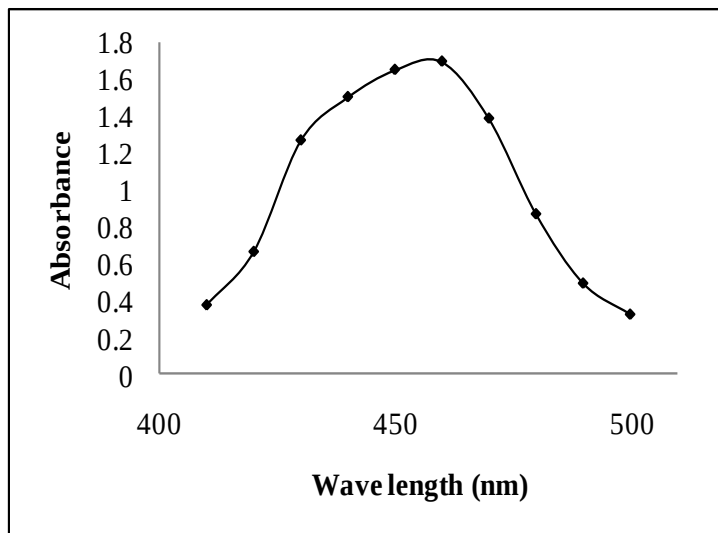


Figure 3.4.3: Investigation of suitable wavelength for the spectrophotometric determination of metribuzin

Conditions

$\lambda_{\max}$	Varied
Metribuzin solution	20.0 $\mu\text{g mL}^{-1}$
HCl solution	2.8 M
NaOH solution (0.87 M)	2.0 mL
<i>p</i> -dimethylaminobenzaldehyde (0.04 M)	10.0 mL

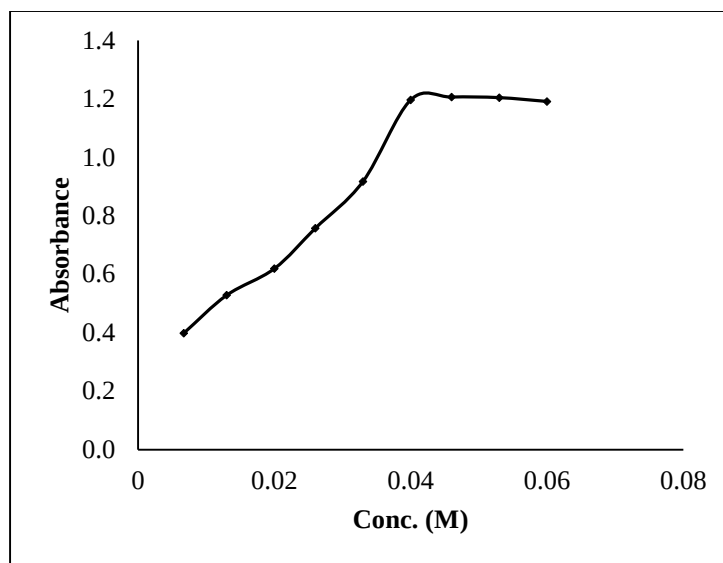


Figure 3.4.4: The effect of DMAB concentration on reaction with metribuzin

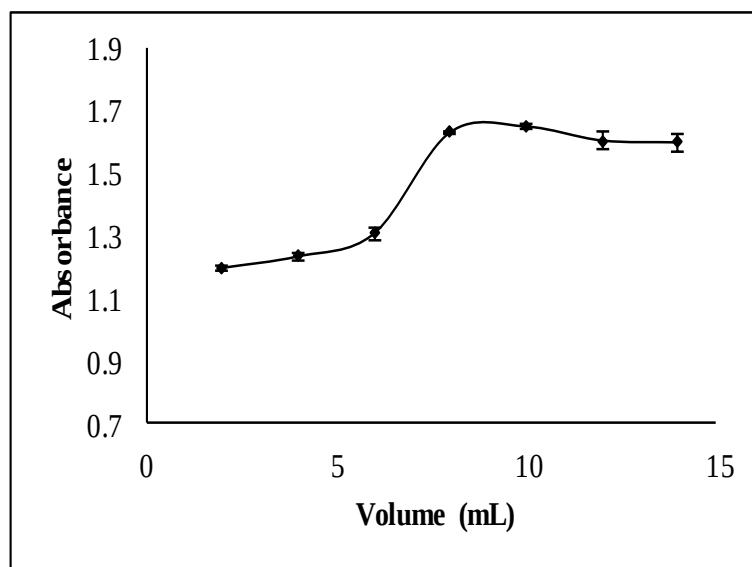


Figure 3.4.5: The effect of DMAB volume on reaction with metribuzin

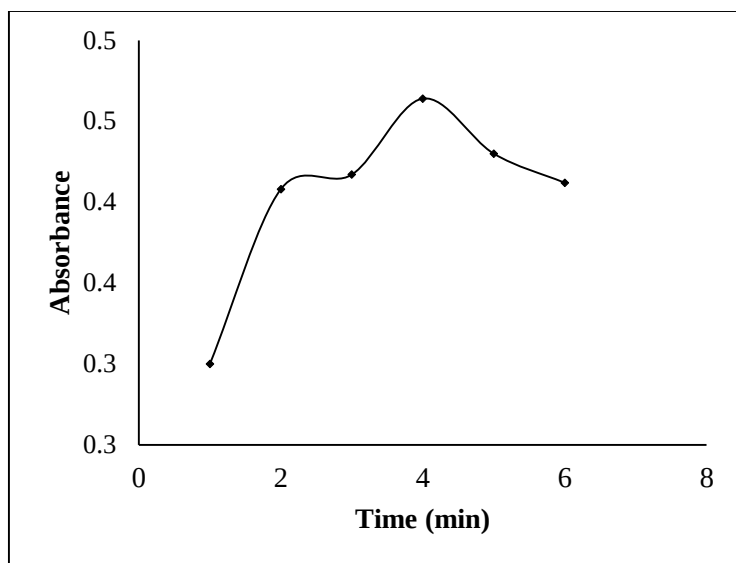


Figure 3.4.6: The effect of heating time on cannizzaro's reaction

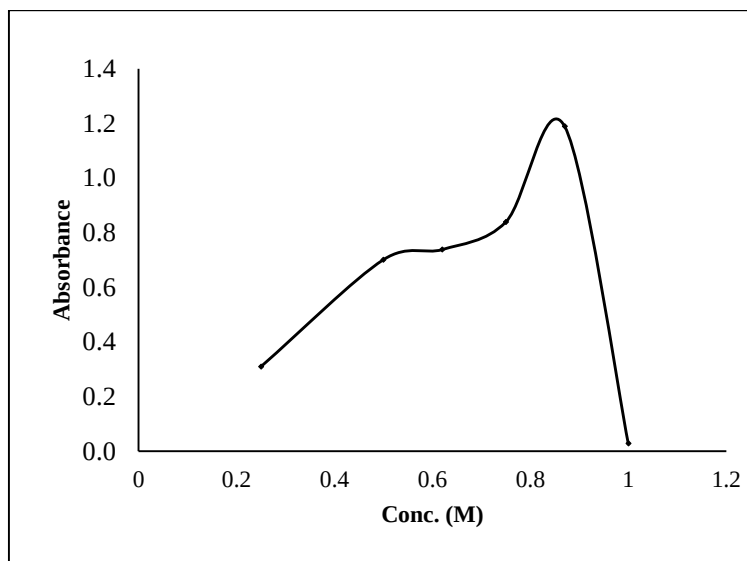


Figure 3.4.7: The effect of sodium hydroxide concentration on reaction of metribuzin

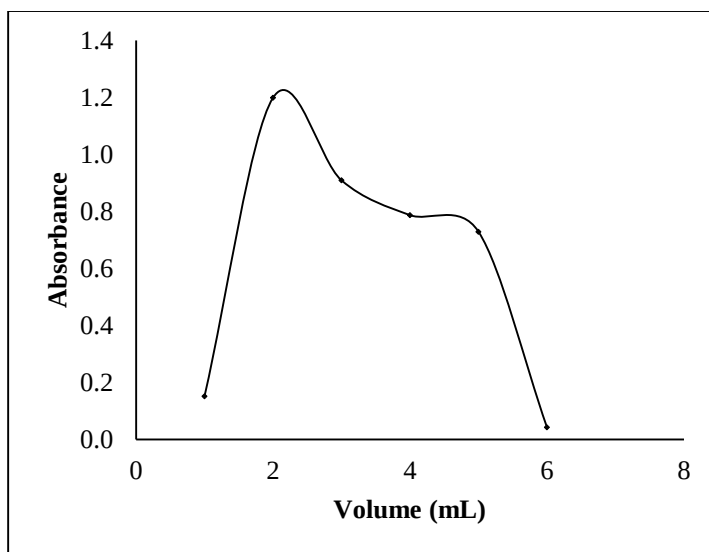


Figure 3.4.8: The effect of sodium hydroxide volume on reaction of metribuzin

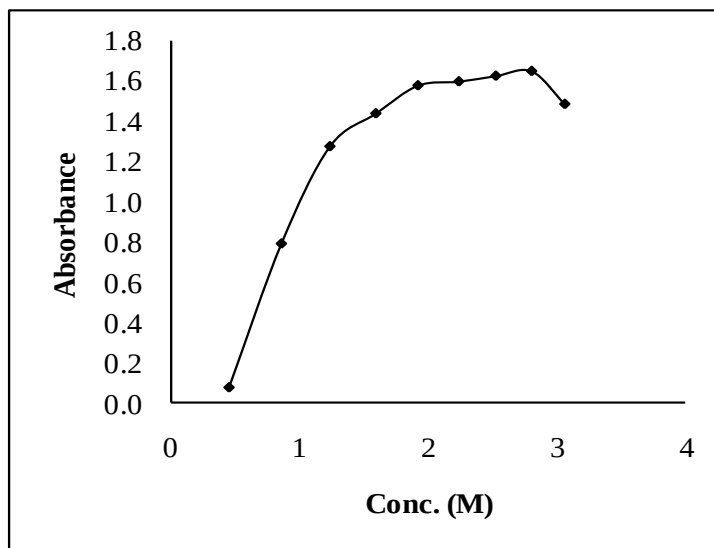


Figure 3.4.9: The effect of hydrochloric acid concentration on reaction of metribuzin

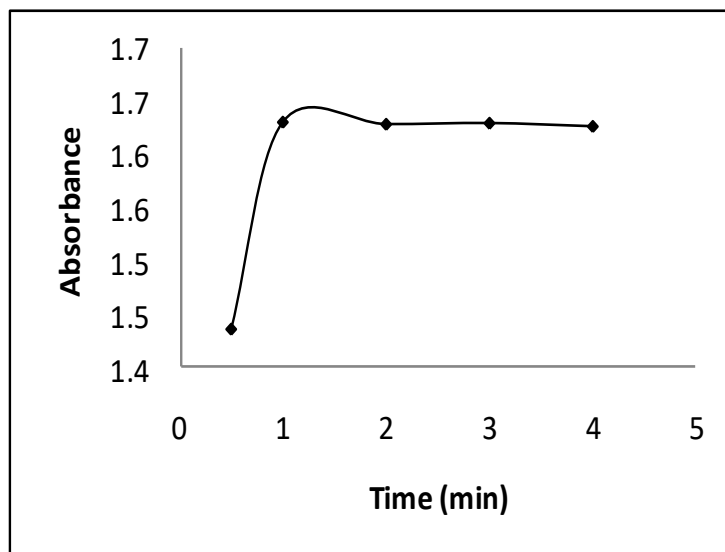


Figure 3.4.10: The effect of heating time on the formation of colored product

3.4.3.2 Application of factorial design for optimization of different variables for metribuzin determination

Optimization for step 1 using 2^3 factorial design

A 2^3 factorial design with a complete repetition performed in one run resulting in a total of 8.0 experiments was chosen for optimization of heating time, concentration of *p*-dimethylaminobenzaldehyde and sodium hydroxide solutions. The effect of changing a factor from a low to a high level value was studied on absorbance measurement. The highest response was found in the eighth run of the table 3.4.1 giving optimum heating time of 4.0 min, *p*-dimethylaminobenzaldehyde and sodium hydroxide concentration of 0.04 M and 2.87 M respectively.

Among the three factors, all showed a positive effect but the most prime impact on the final product formation was found to be the heating time in step 1 (C). Heating time in step 1 showed 44.04 % contribution while sodium hydroxide and DMAB concentration gave 29.55 and 10.03 % contribution. BC (13.55) was significant than AC (2.80) and AB (1.0×10^{-4}) among all possible combinations of interaction of the table 3.4.2.

Figure 3.4.11 shows pareto chart with factors arranged in descending order from most effective to least effective. Effects that are above the bonferroni limit are almost certainly significant (C, B, BC, A and AC).

The analysis of variance (ANOVA) was carried out for 2^3 factorial design, and the results are given in table 3.4.3. The values of sum of square confirm that A, B and C were statistically the main factors for metribuzin determination. Factor C (0.22) was more significant and the interaction of AC (0.014) and BC (0.067) also has a marked effect on colored product formation.

Optimization for step 2 using 2^2 factorial design

A 2^2 factorial design with a complete repetition performed in one run resulting in a total of 4 experiments was chosen for optimization of heating time and concentration of hydrochloric acid solution.

The highest response was found in the fourth run on the table 3.4.4 giving optimum heating time of 1 minute and hydrochloric acid concentration of 2.8 M for the reaction of metribuzin with *p*-dimethylaminobenzoic acid.

Information from Pareto chart shown in figure 3.4.12 and (ANOVA) results given in table 3.4.5 showed that concentration of hydrochloric acid has more impact in the final step of the reaction.

HCl concentration was observed as a key factor with 90.0 % contribution in controlling the formation of maximum final product (Table 3.4.6).

Final equation in terms of actual factors for 2^3 and 2^2 factorial design.

The overall absorbance of final equation in terms of actual factors of 2^3 indicates that all the factors (A, B and C) and some of their interactions (AC and AB) were influencing the absorbance of the final product formation to some extent.

Absorbance (2.54) = 0.622 (DMAB) - 0.05 (conc. of sodium hydroxide) - 0.04 (heating time in step 1) + 1.84 (DMAB $\times$ heating time in step 1) + 0.19 (conc. of sodium hydroxide $\times$ heating time in step 1).

In case of 2^2 factorial design, the equation finish up with a sum of absorbance presenting the degree of participation of the two factors (A and B) in the last step of the reaction.

Absorbance (0.433) = 0.189 (heating time in step 2) + (conc. of hydrochloric acid).

Table 3.4.1: The 2^4 experimental design

Run	A*	B*	C*	A*	B*	C*	Response Absorbance
1	-1	-1	-1	0.01	0.25	1	0.032
2	+1	-1	-1	0.04	0.25	1	0.110
3	-1	+1	-1	0.01	0.87	1	0.123
4	+1	+1	-1	0.04	0.87	1	0.193
5	-1	-1	+1	0.01	0.25	4	0.100
6	+1	-1	+1	0.04	0.25	4	0.335
7	-1	+1	+1	0.01	0.87	4	0.547
8	+1	+1	+1	0.04	0.87	4	0.792

A = DMAB (M)

B = NaOH (M)

C = Heating Time (min)

Table 3.4.2: The effect list of main factors and their interactions for 2⁴ factorial design

Error or included in model	Term	Effect	Sum of square	% Contribution
Model	A*	0.157	0.04	10.03
Model	B*	0.269	0.149	29.55
Model	C*	0.329	0.216	44.04
Model	AB	5.0×10^{-4}	5.0×10^{-7}	1.0×10^{-4}
Model	AC	0.083	0.013	2.803
Model	BC	0.018	0.066	13.55
Model	ABC	0.004	4.0×10^{-5}	0.008
Lenth's ME 0.886 Lenth's SME 2.12				

A = DMAB (M)

B = NaOH (M)

C = Heating Time (min)

Table 3.4.3: ANOVA of the main effects for 2⁴ factorial design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	The method is Significant
Model	0.49	5	0.098	4794.45	2.0×10^{-4}	
A*	0.049	1	0.049	2404.78	4.0×10^{-4}	
B*	0.15	1	0.15	7085.88	1.0×10^{-4}	
C	0.22	1	0.22	10560.10	$<1.0 \times 10^{-4}$	
AC	0.014	1	0.14	672.10	15×10^{-4}	
BC	0.067	1	0.067	3249.39	3.0×10^{-4}	
Residual	4.1×10^{-5}	2	2.05×10^{-5}			
Cor total	0.49	7				

df = degree of freedom

A = DMAB (M)

B = NaOH (M)

C = Heating Time (min)

Table 3.4.4: The 2⁴ experimental design

Run	A*	B*	A*	B*	Response Absorbance
1	-1	-1	0.05	0.44	0.032
2	+1	-1	1.00	0.44	0.210
3	-1	+1	0.05	2.81	0.610
4	+1	+1	1.00	2.81	0.792

A = Heating time (min)

B = HCl (M)

Table 3.4.5: ANOVA of the main effects for 2² factorial design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	The method is significant
Model	0.37	2	0.18	46100.00	0.0033	
A*	0.032	1	0.032	8100.00	0.0071	
B*	0.34	1	0.34	84100.00	0.0022	
Residual	4.0 x 10 ⁻⁶	2	4.0 x 10 ⁻⁶			
Corrected total	0.49	7				

A = Heating time (min)

B = HCl (M)

Table 3.4.6: The effect list of main factors and their interactions for 2² factorial design

Error or included in model	Term	Effect	Sum of square	% Contribution
Model	A*	0.180	0.032	8.785
Model	B*	0.580	0.336	91.21
Model	AB	0.002	4.0 x 10 ⁻⁶	0.001
Lenth's ME 3.430 Lenth's SME 10.137				

A = Heating time (min)

B = HCl (M)

Design-Expert® Software
Absorbance

A: Ehrlich reagent
B: NaOH
C: Heating time in step 1
■ Positive Effects
■ Negative Effects

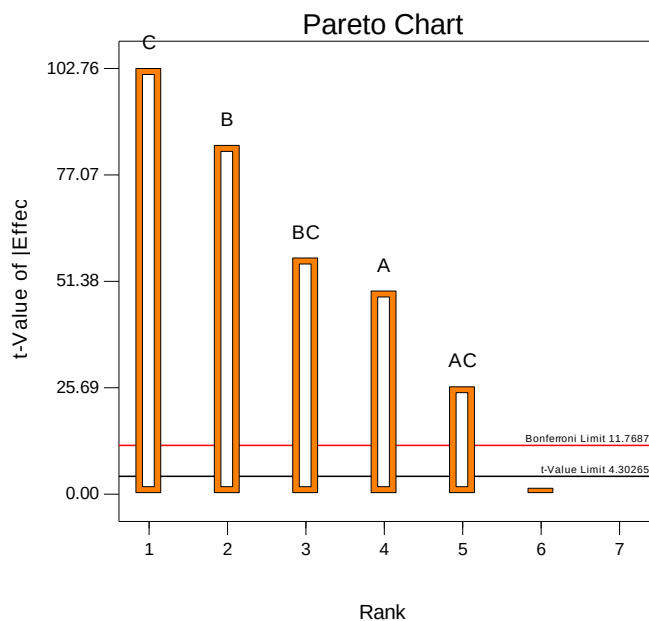


Figure 3.4.11: Pareto chart of effects on the final product formation by factors involved in step 1 of the reaction

Design-Expert® Software
Absorbance

A: Heating time in step 2
B: HCl
■ Positive Effects
■ Negative Effects

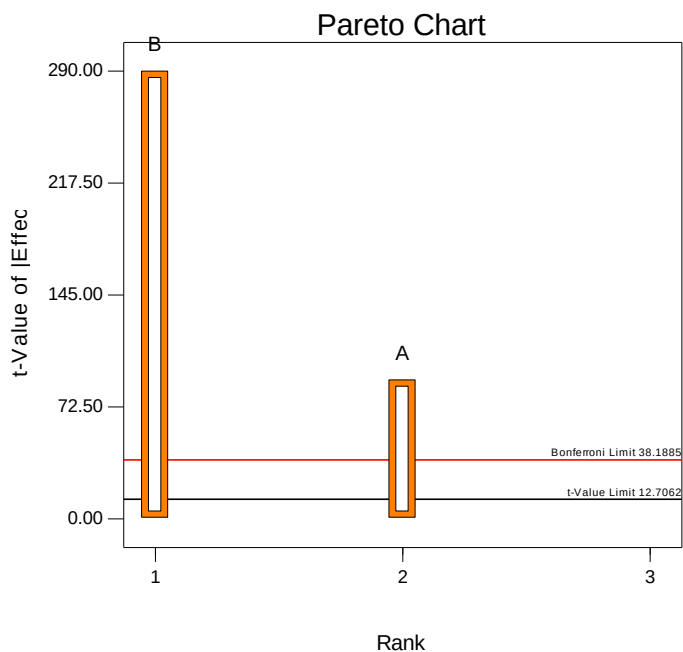


Figure 3.4.12: Pareto chart of effects on the final product formation by factors involved in step 2 of the reaction

3.4.3.3 Verification of Beer's law and determination of detection and quantification limit for metribuzin determination

Instrument: The same as mentioned before

Reagents: The same as above

Solution: The same as mentioned before

Procedure:

From standard stock solution of metribuzin ($100.0 \mu\text{g mL}^{-1}$) different volume for $0.2\text{--}20.0 \mu\text{g mL}^{-1}$ were taken in six separate 25.0 mL reaction flasks followed by the addition of 10.0 mL DMAB (0.04 M) and 2.0 mL of sodium hydroxide (0.87 M) solutions. These solutions were heated at 100.0°C for 4.0 min on water bath followed by addition of 2.8 M hydrochloric acid solution and heated again for 1.0 min on water bath at 100.0°C . After cooling, the solutions were diluted to 25.0 mL with distilled water and allowed to stand for 15.0 min . The absorbance of the colored product was measured at 455.0 nm against reagent blank. The results are given in table 3.4.7. A calibration plot of concentration vs. absorbance was constructed and is shown in figure 3.4.13. The molar absorptivity was calculated by using the following formula.

$$\text{Molar absorptivity } (\epsilon) = A/C \times L$$

Where A= absorbance

C= concentration (mol L^{-1})

L= path length (cm)

LOD and LOQ were calculated based on six replicates solution having lowest quantifiable concentration ($\mu\text{g mL}^{-1}$) using the following formula.

$$\text{Limit of Detection (LOD)} \quad (\text{for concentration}) = 3 \times S$$

$$\text{Limit of Quantification (LOQ)} \quad (\text{for concentration}) = 10 \times S$$

Where S = Standard deviation

Relative standard deviation (RSD) was calculated by using the following formula.

$$\text{RSD} = \text{Standard deviation} / \text{Mean} \times 100$$

All the statistical values are given in table 3.4.8.

Table 3.4.7: The effect of concentration on absorbance behavior of metribuzin

Conc. of metribuzin ($\mu\text{g mL}^{-1}$)	Absorbance
0.2	0.009
0.4	0.020
0.6	0.026
0.8	0.042
1.0	0.060
2.0	0.089
4.0	0.253
6.0	0.426
8.0	0.774
10	0.813
12	1.032
15	1.291
20	1.742

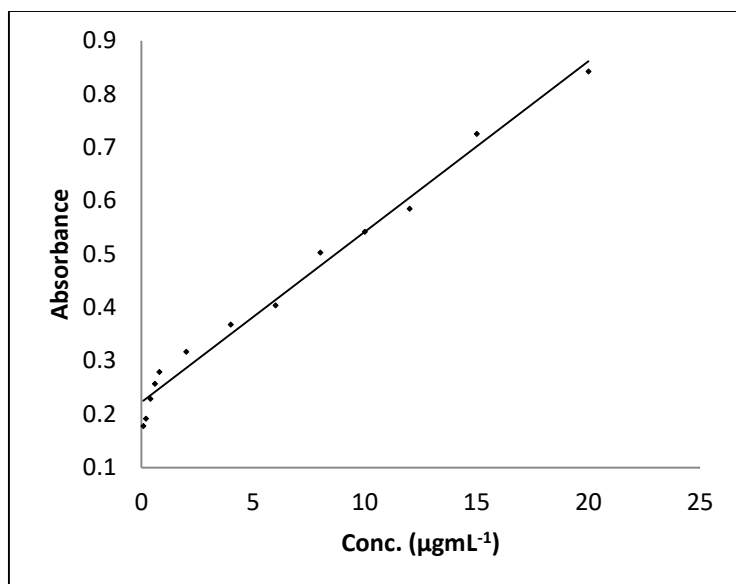


Figure 3.4.13: The effect of concentration on absorbance behavior of metribuzin herbicide

Table 3.4.8: Analytical characteristic of the proposed method

Characteristics	Analysis
λ max (nm)	455
Color	yellow
Calibration curve range (μgmL^{-1})	0.2 - 20
Slope	0.0287
Limit of detection (μgmL^{-1})	0.05
Limit of quantification (μgmL^{-1})	0.2
Molar absorptivity ($\text{Lmol}^{-1}\text{cm}^{-1}$)	8.5×10^6
Relative standard deviation	10.52
Correlation coefficient (r^2)	0.994

3.4.4 Percent recovery of metribuzin from real samples and commercial formulation

To check the applicability of the method in different matrices, recovery test was performed on potato samples, fortified with known concentration of metribuzin solution.

Sample extraction procedure

The potato (250.0 g) sample under study was washed thoroughly with water to remove adhering dust and dirt. Metribuzin was extracted by soaking the potatoes in 250.0 mL chloroform for two hours and then sonicated for 15.0 min. After sonication the sample was filtered. The chloroform filtrate was evaporated at 40.0 °C on water bath and the residue was redissolved in 10.0 mL methanol. Several different concentrations of the standard metribuzin solution (3.0, 3.7, 5.7 $\mu\text{g mL}^{-1}$) were added to the 1.0 mL extract of control samples of potatoes. The methanolic solution was then analyzed by the proposed method. All the analyses were carried out in triplicate.

For checking the applicability of the proposed method to commercial formulation, a known weight (0.142 g) of the commercial formulation was taken and diluted with methanol up to 50.0 mL and was filtered to remove any insoluble substances from it. Known volume of the filtrate (2.2 $\mu\text{g mL}^{-1}$) containing metribuzin was taken in nine separate reaction flasks and to these were added standard solution (3.0, 3.7, 5.7 $\mu\text{g mL}^{-1}$) of metribuzin. These solutions were then analyzed by the proposed method.

Results of percent recovery of metribuzin in real samples and formulation are given in tables 3.4.9. Percent recovery was calculated by using the formula given below.

% Recovery = $\frac{\text{Total } \mu\text{g found in fortified samples} - \mu\text{g found in sample}}{\mu\text{g of standard added}} \times 100$

Table 3.4.9: Determination of metribuzin in fortified real samples and commercial formulation (n=6)

Real samples/Commercial formulation	Sample conc. taken ($\mu\text{g mL}^{-1}$)	Standard conc. added ($\mu\text{g mL}^{-1}$)	Total conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Potatoes	0.33 (residue from real samples)	3.0	3.10	92.3 $\pm$ 0.34
		3.7	3.74	92.2 $\pm$ 0.10
		5.7	5.76	95.3 $\pm$ 0.10
Metribuzin formulation	2.2 (known amount from commercial formulation)	3.0	5.17	99.0 $\pm$ 0.70
		3.7	5.74	95.8 $\pm$ 0.20
		5.7	7.54	93.7 $\pm$ 0.40

3.4.5 Application of the investigated method for the analysis of metribuzin in real samples and commercial formulation

Instrument: The same as mentioned before

Samples: Samples were collected from an agriculture field, where metribuzin was sprayed and were first analyzed for the presence of residue. Sonication method was used for residue determination in real samples, metribuzin was extracted by soaking the potatoes in 250.0 mL chloroform for two hours and then sonicated for 15.0 min at 40.0 Hz frequency. After sonication the sample was filtered. The chloroform filtrate was evaporated at 40.0 °C on water bath and the residue was redissolved in 10.0 mL methanol. The methanolic solution was then analyzed by the proposed method. The results are shown in Table 3.4.10.

For checking the active ingredient of commercial formulations, known amount (0.37 g in 20.0 mL of distilled water) of herbicide metribuzin were taken separately and stirred on magnetic plate for 30.0 min. This homogeneous solution was then diluted with methanol: water solution (1:1) up to 100.0 mL and was filtered using Whatman filter paper (NO:

40). Known amount ($20.0 \mu\text{g mL}^{-1}$) of this sample solution was then analyzed by the proposed method in triplicate. The results are given in table 3.4.11.

Table 3.4.10: Residue level of metribuzin in real samples

S.NO	Samples	Residue ($\mu\text{g g}^{-1}$)
1	Potato	0.33 ± 0.13

Table 3.4.11: Concentration of metribuzin in commercial formulation

Commercial formulation	Label Amount ($\mu\text{g g}^{-1}$)	Amount found ($\mu\text{g g}^{-1}$)
Metribuzin formulation	0.7	0.67 ± 0.17

3.4.6 Results and discussion

This method is based on Cannizzaro's reaction, *p*-dimethylaminobenzaldehyde (DMAB) react with sodium hydroxide at 100.0°C to form a *p*-dimethylaminobenzoic acid. The resultant *p*-dimethylaminobenzoic acid reacts with metribuzin herbicide in acidic media at 100.0°C and form a yellow colored product which could be measured at 455.0 nm . The proposed reaction mechanism is given in figure 3.4.2.

Different experimental variables affecting the formation of colored product were optimized. Such variables were changed and optimized individually while other variables were kept constant. The variables studied were concentration of DMAB, sodium hydroxide, hydrochloric acid and heating time for the reaction.

The concentration of DMAB was varied from 0.0067 – 0.06 M . The absorbance increased up to 0.040 M solution (Figure 3.4.4). Beyond this no change in absorbance was observed with increase in molar concentration of DMAB.

The DMAB is derivatized into *p*-dimethylaminobenzoic in alkaline media. The effect of concentration of sodium hydroxide on the reaction was studied and maximum reaction product was formed at 0.87 M (Figure 3.4.7).

The conversion of DMAB into *p*-dimethylaminobenzoic acid via cannizzaro's reaction was studied from 1.0 to 6.0 min. Maximum formation of *p*-dimethylaminobenzoic acid was observed with 4 min heating at 100.0 °C on a water bath (Figure 3.4.6).

The effect of acidity on the formation of yellow product was studied. The results are shown in figure 3.4.9. With increase in concentration of hydrochloric acid solution increase in absorbance was observed from 0.44 to 3.0 M. It was found that 2.8 M hydrochloric acid solution was enough for adjustment of acidity of solution desired for the reaction.

Similarly the heating time for the reaction of *P*-dimethylaminobenzoic acid with metribuzin was studied from 30.0 sec to 4.0 min at 100.0 °C. Increase in product concentration was observed with 1 min heating and no effect with further increase in heating time (Figure 3.4.10).

To check the stability of the final product, effect of time was studied up to 3.0 days (72.0 hr) with time interval of 2.0 hr. The colored reaction product was found to be stable for 2.0 days (44.0 hr).

The concentration vs. absorbance curve was found linear over the concentration range of 0.2 – 20.0 µgmL⁻¹. The most important optical characteristics and quantitative parameters, such as the slope, intercept, correlation coefficient obtained by the linear squares treatment of the calibration graph is given in the table 3.4.8. Under the specified reaction conditions, the linearity of calibration line was proved by the high value of correlation coefficient ($r^2 = 0.994$). The molar absorptivity of the resulting yellow colored product was found to be $2.1 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$.

The limit of detection was found to be 0.05 µgm⁻¹ as 3S and limit of quantification of 0.2 µgmL⁻¹ as 10S of the standard deviation and were computed by determination of six replicate respectively.

A recovery test was performed on potato samples fortified with known concentration of metribuzin solution. Three replicates of the fortified samples were analyzed using the proposed method. Similarly the percent recovery from commercial formulations was also

determined by standard addition method to check the interferences effect of emulsified solution. Recovery values in commercial formulations were found to be $93.7 \% \pm 0.7$ to $99.0 \% \pm 0.7$ and in case of real samples the results of the recovery were found to be in the range of $92.3 \% \pm 0.4$ to $95.3 \% \pm 0.1$.

The method was also applied for residue determination in potato samples and commercial formulation. The values for metribuzin in potato sample were found to be $0.33 \pm 0.13 \mu\text{gg}^{-1}$. The value obtained for commercial formulation was comparable with the label value.

3.5 DETERMINATION OF SELECTED TRIAZINE GROUP OF HERBICIDES IN SOIL SAMPLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The herbicides selected from triazine group for HPLC studies were atrazine, ametryn, metribuzin and terbutryn. The chemical structures of these herbicides are given below in figure 3.5.1.

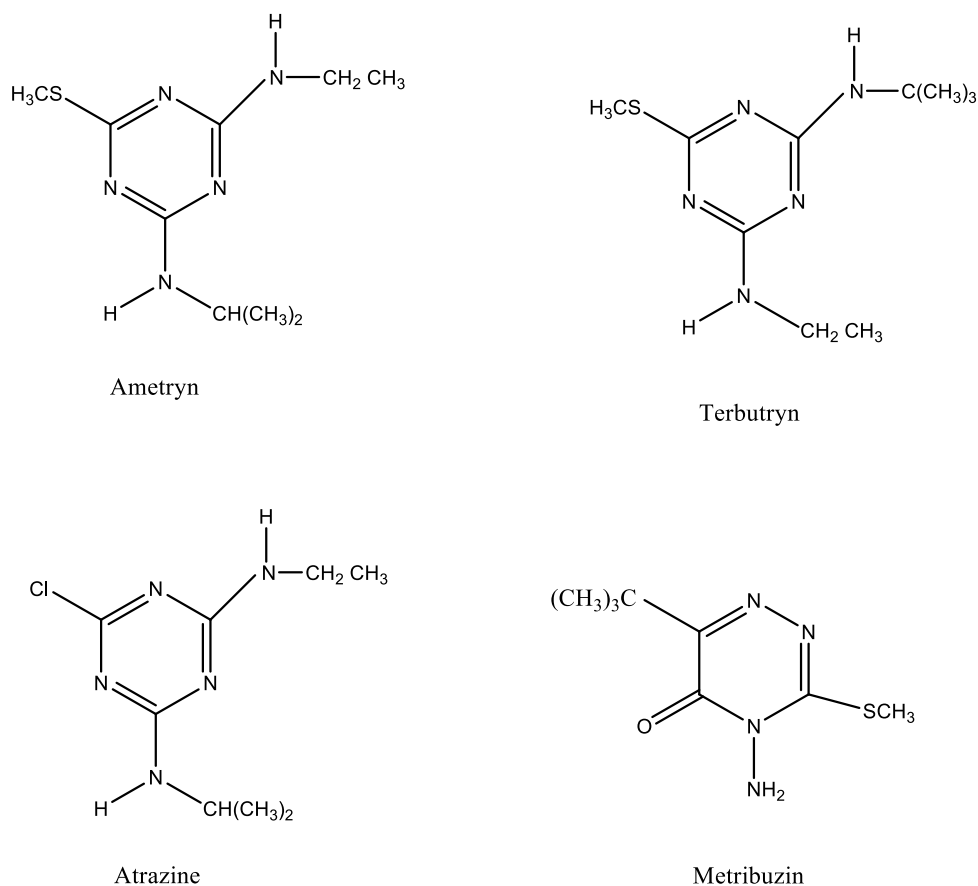


Figure 3.5.1 Chemical structure of selected triazine herbicides

As triazine herbicides are soluble in polar solvents, therefore reverse phase assembly was selected for its determination.

3.5.1 Investigation of suitable parameters for the HPLC determination of selected triazine herbicides

Instrument

An Acme 9000 Series HPLC equipped with SP930 isocratic pump, UV730D detector Young Lin (Korea) was used. All separations were carried out on HiQ SiL (C₁₈ column HS 4.6 mm Ø x 150 mm KYATECH, Japan). A KEN ST/SS25 KENWOOD (China) microwave with temperature control system was used for closed-vessel microwave-assisted extraction of triazine herbicides. A ks300 KUM SUNG ultrasonic (Korea) sonicator was used as degasser. Centrifugation was carried out on a CL international clinical centrifuge (U.S.A).

Reagents and chemicals

All chemicals used were of analytical grade or similar. HPLC grade methanol was purchased from Merck (Darmstadt, Germany), ethyl acetate from BDH (Poole, England) and acetonitrile from Scharlau Chemie (Italy, European Union). Atrazine, metribuzin, ametryn and terbutryn standards were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany).

Standard triazine solution (1000 µg mL⁻¹)

Stock solutions (1000.0 µg mL⁻¹) of all triazine herbicides were prepared by dissolving 0.01 g of standard herbicides in 10.0 mL methanol. Working standard of 100.0 µg mL⁻¹ and lower concentration solution were prepared by dilution of the stock solution with methanol.

Optimization of HPLC parameters

All physical parameters such as wavelength, flow rate, solvent elution mode and time for elution and chemical parameters as solvents and composition of solvents for isocratic elution were optimized for the HPLC determination of selected herbicides.

The choice of wavelength is based on where the compounds of interest have the best response and interfering compounds have the lowest response. A wavelength of 254 nm was used for HPLC analysis.

For optimization of flow rate of mobile phase, flow rate was varied in the range of 0.7 to 1.0 mLmin⁻¹ and 1.0 mLmin⁻¹ was chosen as optimum.

All the optimized parameters are presented in table 3.5.1 and were used for subsequent analysis. A mobile phase flow rate in the range of 0.7—1.0 mLmin⁻¹ was evaluated using methanol: water in the ratio 60:40 and 70:30 for the separation of four triazine herbicides. There was no considerable difference in the resolution of the atrazine and ametryn peak in chromatograms obtained for all conditions mentioned above. A complete resolution of triazines mixture was achieved using 1.0 mLmin⁻¹ flow rate and mobile phase ratio of 70:30 in acceptable time of 11.0 min (Figure 3.5.2). The retention times for metribuzin, atrazine, ametryn and terbutryn are 3.68, 4.73, 6.38 and 9.50 min, respectively.

Table 3.5.1: Optimum HPLC conditions for analysis of selected triazine herbicides using reverse phase mode

Parameters	Values
Eluent used (methanol: water)	70: 30
Flow rate (mLmin ⁻¹)	1.0
Injection volume (μL)	20.0
Wavelength (nm)	254.0

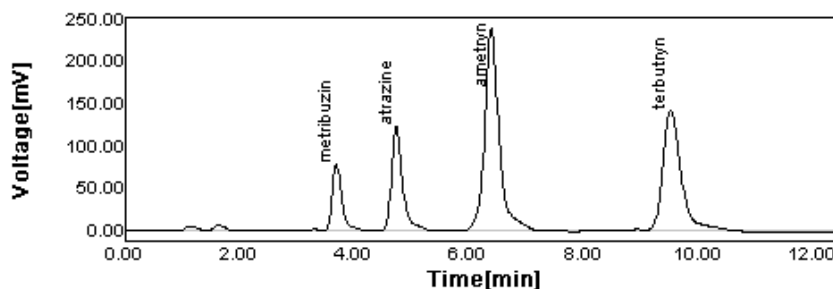


Figure 3.5.2 HPLC chromatogram of metribuzin, atrazine, ametryn and terbutryn

3.5.2 Construction of calibration plot for the determination of selected triazine herbicides using reverse phase HPLC

Instrument: The same as mentioned before

Reagents: The same as mentioned before

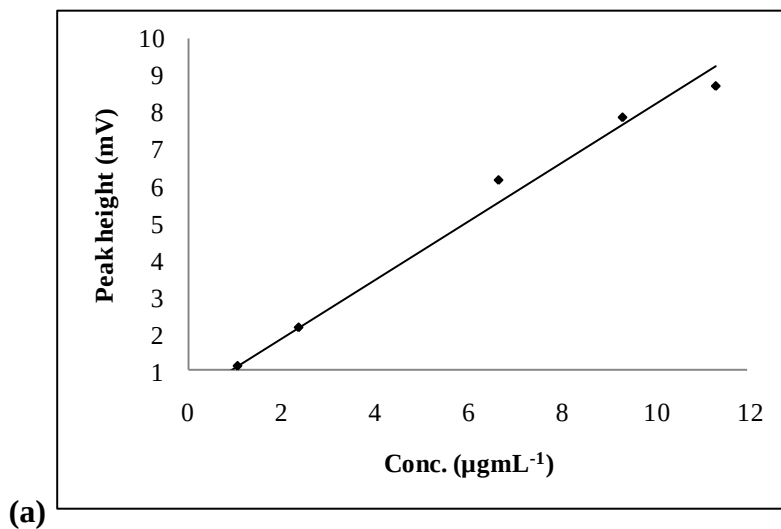
Solution: In different concentration range

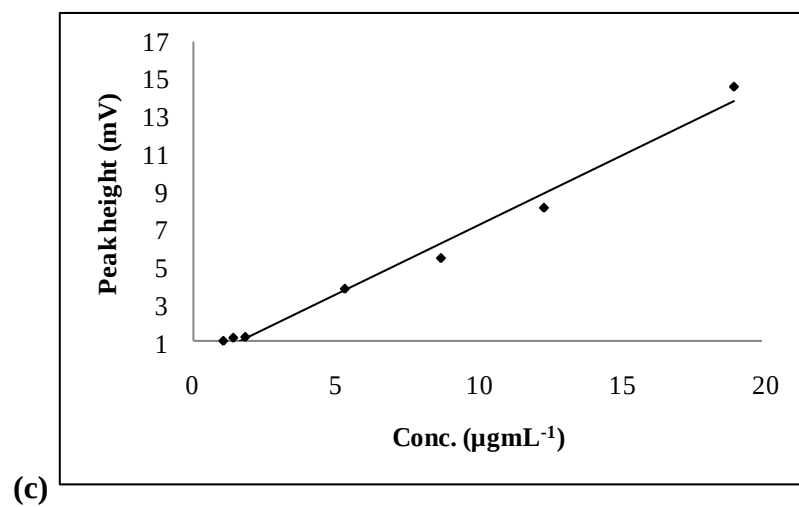
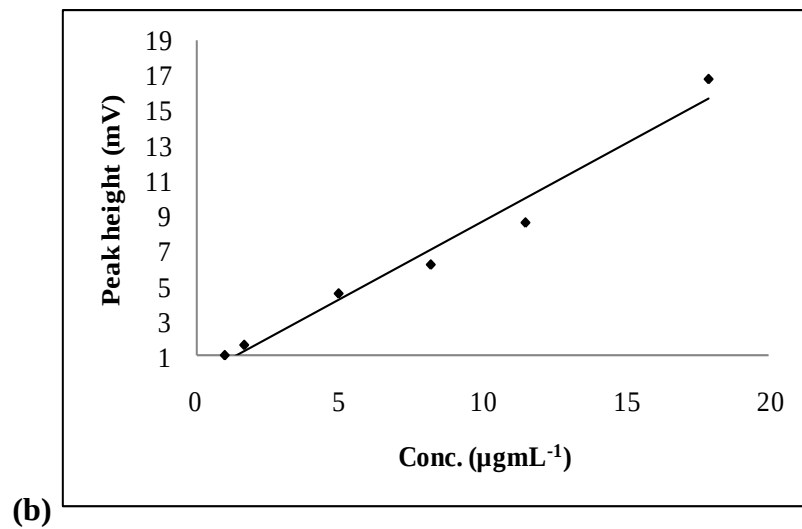
Procedure

From (1000.0 $\mu\text{g mL}^{-1}$) standard stock solution of atrazine, ametryn, metribuzin and terbutryn, working standard solutions were prepared in the range of 0.6-19.0 $\mu\text{g mL}^{-1}$ using methanol for dilution. HPLC system was equilibrated with the mobile phase prior to analysis. After equilibration solutions were injected one by one and the peak height was noted for each concentration. Accuracy and precision of the method were determined with three replicates. Calibration curves range established using HPLC for metribuzin, atrazine, ametryn and terbutryn are 1.0—19.0 $\mu\text{g mL}^{-1}$, 0.9—18.0 $\mu\text{g mL}^{-1}$, 0.6—11.0 $\mu\text{g mL}^{-1}$ and 0.7—11.0 $\mu\text{g mL}^{-1}$ respectively. A calibration plot of peak height vs. concentration was constructed for the four triazines and is shown in figure 3.5.3. Analytical characteristics for HPLC determination were calculated and the results are given in table 3.5.2. The limits of detection (LODs) and limits of quantification (LOQs) were calculated for minimum concentration ($\mu\text{g mL}^{-1}$) for which a proper peak was observed. The LODs of metribuzin, atrazine, ametryn and terbutryn were found to be 0.30, 0.24, 0.16 and 0.20 $\mu\text{g mL}^{-1}$ while LOQs are 1.0, 0.8, 0.5, 0.6 $\mu\text{g mL}^{-1}$ respectively.

Table 3.5.2: Characteristics for HPLC-UV determination of selected triazine herbicides

Analysis	Characteristics			
	Metribuzin	Atrazine	Ametryn	Terbutryn
Working concentration range ($\mu\text{g mL}^{-1}$)	1.0—19.0	0.9—18.0	0.6—11.0	0.7—11.0
Limit of detection ($\mu\text{g mL}^{-1}$)	0.30	0.24	0.16	0.20
Limit of quantification ($\mu\text{g mL}^{-1}$)	1.00	0.82	0.54	0.60
Standard deviation	0.10	0.08	0.06	0.05
Relative standard deviation	6.32	6.43	7.60	6.90
Regression equation	$1.40 X + 0.00$	$1.135 X + 0.00$	$0.185 X + 0.00$	$1.183 X + 0.00$
Correlation Coefficient (r^2)	0.9829	0.9860	0.9645	0.9871





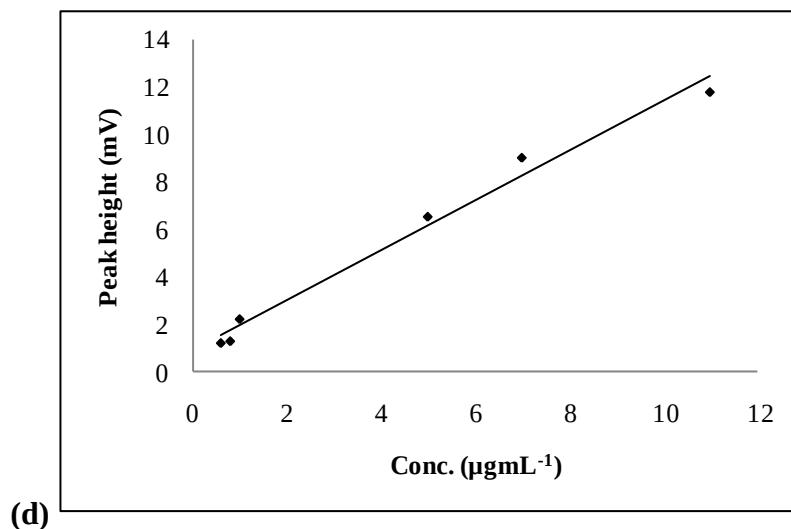


Figure 3.5.3 Calibration plot for (a) terbutryn, (b) atrazine, (c) metribuzin and (d) ametryn using reverse phase HPLC

3.5.3 Optimization of extraction procedure for determination of selected triazine herbicides using reverse phase HPLC

After optimization of instrumental and physical parameters it was important to optimize extraction procedure for selected triazine herbicides. For this purpose the effects of various experimental parameters of microwave assisted extraction (MAE) were studied. Parameters, such as solvent type and volume, extraction time and irradiation energy were optimized.

Preliminary experiments were performed to select the optimum operation conditions for the microwave-assisted solvent extraction of the four triazine herbicides.

The triazine herbicides are highly soluble in methanol, acetonitrile and ethylacetate. Organic solvents such as methanol, acetonitrile and ethylacetate were first studied for extraction of each triazine herbicide separately at 80.0 % of 850.0 W output of microwave for 4.0 min using 30.0 mL of solvent in triplicate at three different concentrations of triazine herbicides in control samples. The results are given in table 3.5.3 and are shown in figure 3.5.4. MAE for the mixture of the four triazines from soil samples was performed using acetonitrile: ethyl acetate (Table 3.5.6) and (Figure 3.5.4) and acetonitrile: ethyl acetate: methanol (Table 3.5.5) and (Figure 3.5.6).

To find out the effect of irradiation energy on the extraction efficiency, extractions were carried out at 10.0, 30.0, 50.0 and 80.0 % of 850.0 W output of microwave respectively. The results are given in table 3.5.6 and are shown in figure 3.5.7.

Extraction time optimizations were carried out at 80.0 % of 850.0 W for 1.0, 2.0, 3.0, 4.0 min using acetonitrile: ethylacetate: methanol as the extraction solvent. Under the condition of changing the extraction time, the results of the MAE recoveries of four triazines from soil samples are given in table 3.5.7 and are shown in figure 3.5.8.

For optimization of extraction volume, four different volumes of extractant solvent, 20.0, 30.0, 40.0 and 50.0 mL were studied respectively. The results are given in table 3.5.8 and are shown in figure 3.5.9.

In order to find the most significant variables for MAE, Plackett-Burman design was built resulting in 12 experiments. Experimental design calculations were performed by using the software Stat-Ease Design-Expert trial Version 7.1.

The analyzed factors influencing the extraction efficiency were solvent volume, irradiation energy and equilibration time. These factors were evaluated at two levels each with a complete repetition performed in one run (Table 3.5.9). In each case, the % recovery of metribuzin, atrazine, ametryn and terbutryn were evaluated.

The effects of the main factors and their interactions with each other were estimated for Plackett–Burman design. The results are given in table 3.5.10 and shown in figure 3.5.10.

Table 3.5.3: The effect of solvent type on recoveries of selected triazine herbicides (n=3)

Solvents	Recovery (%)			
	Atrazine	Ametryn	Metribuzin	Terbutryn
Methanol	86±0.31	59±0.47	79±0.25	67±0.13
Ethylacetate	71±0.24	79±0.55	85±0.63	80±0.19
Acetonitrile	65±0.27	88±0.21	73±0.11	87±0.20

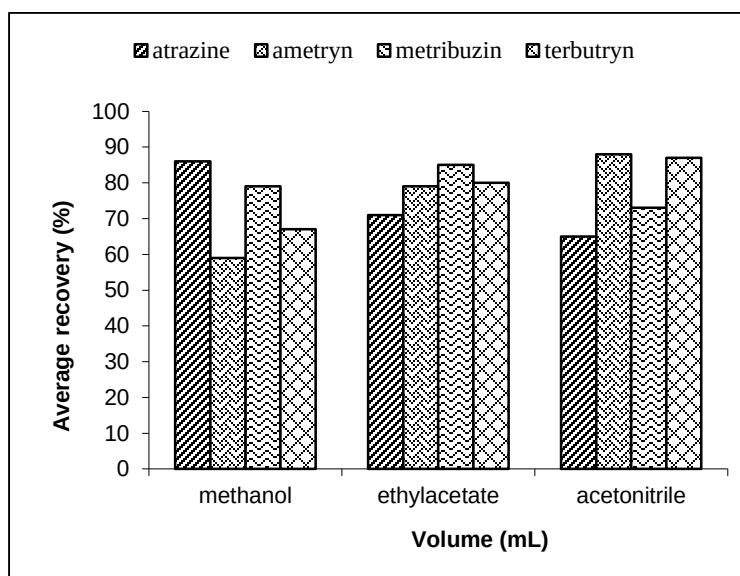


Figure 3.5.4 The effect of solvent type on recoveries of selected triazine herbicides (n=3)

Irradiation energy = 80.0 % of 850.0 W
Solvent volume = 30.0 mL, Time = 4.0 min

Table 3.5.4: The effect of solvent volume ratio on extraction average recoveries (%) of selected triazine herbicides with acetonitrile: ethylacetate (v/v) (n=3)

Acetonitrile: ethylacetate (mL)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
2:8	82±0.17	74±0.09	82±0.28	72±0.23
4:6	82±0.42	70±0.16	85±0.33	90±0.37
6:4	91±0.36	88±0.11	89±0.21	85±0.37
8:2	65±0.28	74±0.13	70±0.25	75±0.22

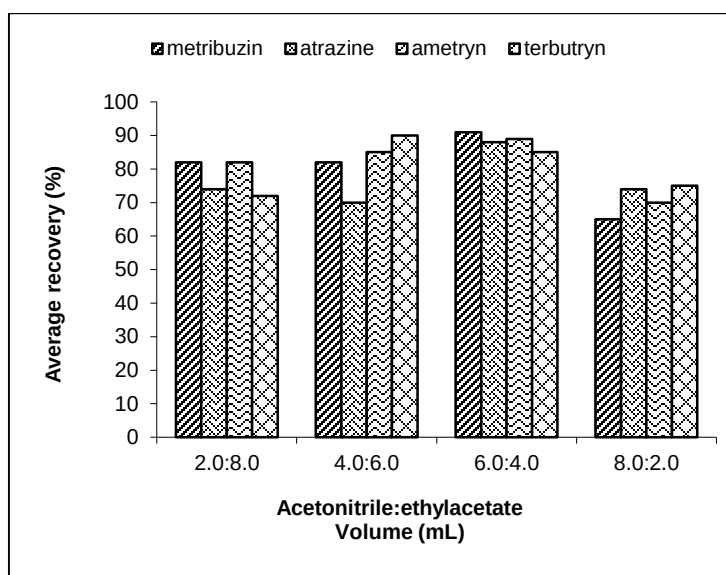


Figure 3.5.5 The effect of solvent volume ratio on extraction average recoveries (%) of selected triazine herbicides with acetonitrile: ethylacetate (v/v) (n=3)

Irradiation energy = 80.0 % of 850.0 W

Solvent volume = 30.0 mL, Time = 4.0 min

Table 3.5.5: The effect of solvent volume ratio on extraction average % recoveries of selected triazine herbicides with acetonitrile:ethylacetate:methanol (v/v) (n=3)

Acetonitrile:ethylacetate:methanol (mL)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
1.6 : 1.0 : 7.4	34±0.34	100±0.62	95±0.25	35±0.26
2.4 : 3.0 : 3.6	56±0.38	98±0.46	96±0.23	37±0.19
3.0 : 2.0 : 5.0	91±0.42	95±0.33	89±0.31	90±0.32
4.0 : 2.6 : 3.4	82±0.55	71±0.29	85±0.35	90±0.27

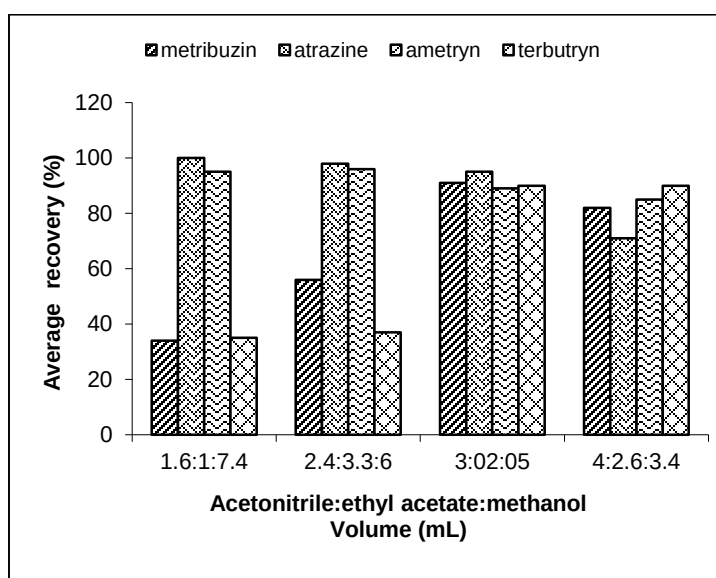


Figure 3.5.6 The effect of solvent volume ratio on extraction average % recoveries of selected triazine herbicides with acetonitrile:ethylacetate:methanol (v/v) (n=3)

Irradiation energy = 80.0 % of 850.0 W
Solvent volume = 30.0 mL, Time = 4.0 min

Table 3.5.6: The effect of irradiation temperature on recoveries of selected triazine herbicides (n=3)

Irradiation temperature (% of 850 W energy)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
10	17±0.22	18±0.39	25±0.52	20±0.24
30	15±0.41	18±0.41	30±0.43	25±0.41
50	43±0.52	44±0.49	57±0.37	45±0.32
80	91±0.42	95±0.33	89±0.31	90±0.32

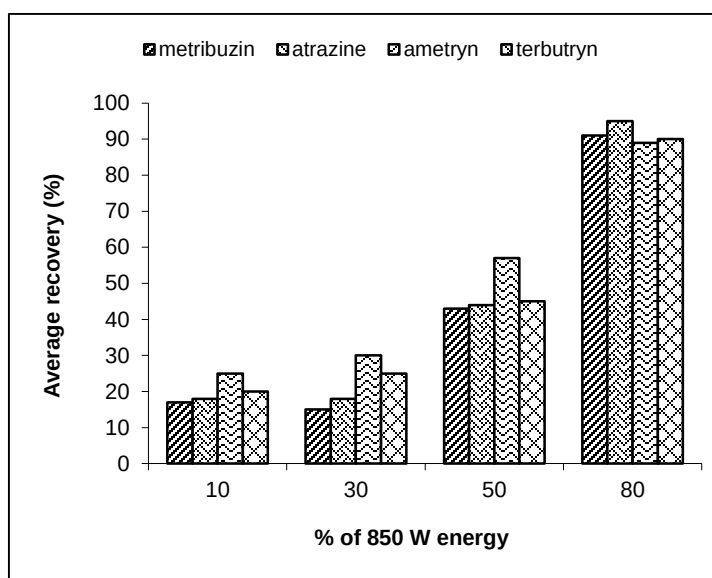


Figure 3.5.7 The effect of irradiation temperature on recoveries of selected triazine herbicides (n=3)

Extracting solvent = Acetonitrile:ethylacetate:methanol (3:2:5)

Solvent volume = 30.0 mL, Time = 4.0 min

Table 3.5.7: The effect of extraction time on recoveries of selected triazine herbicides (n=3)

Irradiation time (min)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
1	40±0.26	37±0.36	42±0.28	42±0.19
2	39±0.29	64±0.31	64±0.30	35±0.21
3	60±0.33	59±0.35	65±0.39	62±0.22
4	91±0.42	95±0.33	89±0.31	90±0.32

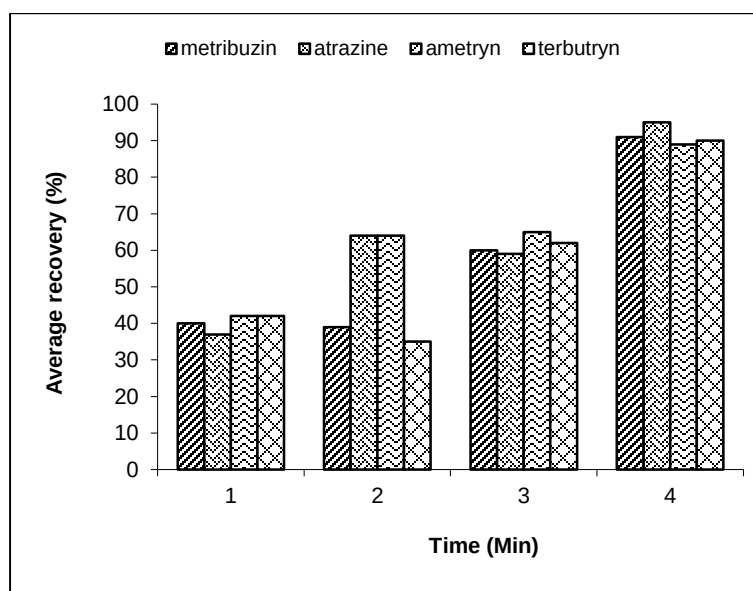


Figure 3.5.8 The effect of extraction time on recoveries of selected triazine herbicides (n=3)

Extracting solvent = Acetonitrile:ethylacetate:methanol (3:2:5)

Irradiation energy = 80 % of 850 W, Solvent volume = 30 mL

Table 3.5.8: The effect of extractant volume on recoveries of selected triazine herbicides (n=3)

Extraction volume (mL)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
20	65±0.52	74±0.46	70±0.18	57±0.22
30	82±0.63	70±0.37	85±0.25	90±0.29
40	91±0.42	95±0.33	89±0.31	90±0.32
50	95±0.55	92±0.44	96±0.31	95±0.39

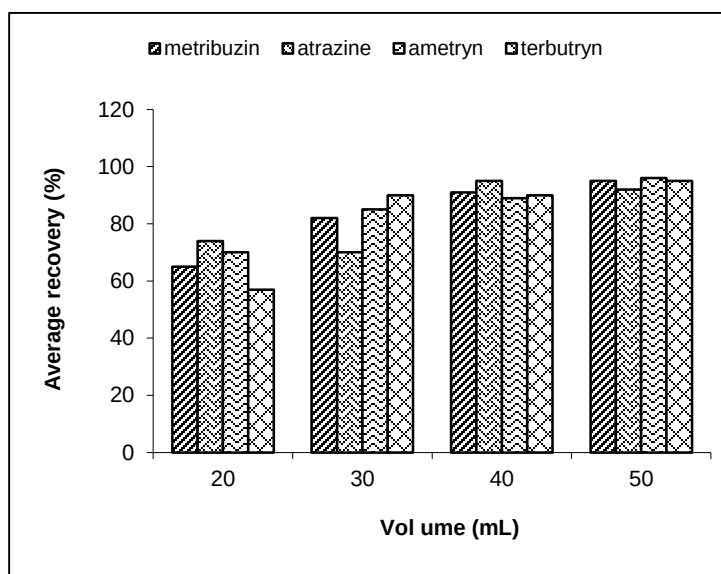


Figure 3.5.9 The effect of extractant volume on recoveries of selected triazine herbicides (n=3)

Extracting solvent = Acetonitrile:ethylacetate:methanol (3:2:5)

Irradiation energy = 80.0 % of 850.0 W, Time = 4.0 min

Table 3.5.9: Plackett–Burman design built for factor selection

Std Run	A*	B*	C*	% Recovery			
				Metribuzin	Atrazine	Ametryn	Terbutryn
1	50	3.00	20	43	44	57	45
2	10	3.00	30	17	18	25	20
3	50	1.00	30	40	37	42	42
4	10	3.00	20	15	16	23	18
5	10	1.00	30	12	13	20	15
6	10	1.00	20	11	12	18	13
7	50	1.00	20	39	36	41	41
8	50	3.00	20	43	44	57	45
9	50	3.00	30	60	59	65	62
10	10	3.00	30	17	18	25	20
11	50	1.00	30	40	37	42	42
12	10	1.00	20	11	12	18	13

A=Temperature of microwave oven (% of 850 W)

B= Extraction time in microwave (min)

C=Volume of extraction solvent (mL)

Table 3.5.10: Effect list of main factors and their interactions

Require in model or error	Term	% Contribution			
		Metribuzin	Atrazine	Ametryn	Terbutryn
Model	A*	86.60	82.58	80.02	85.80
Model	B*	5.84	9.17	13.56	6.45
Model	C*	2.36	2.17	0.95	2.70
Model	AB	1.20	2.66	4.75	1.09
Model	AC	1.20	1.01	0.14	1.09
Model	BC	1.55	1.35	0.27	1.42
Model	ABC	1.20	1.01	0.27	1.42

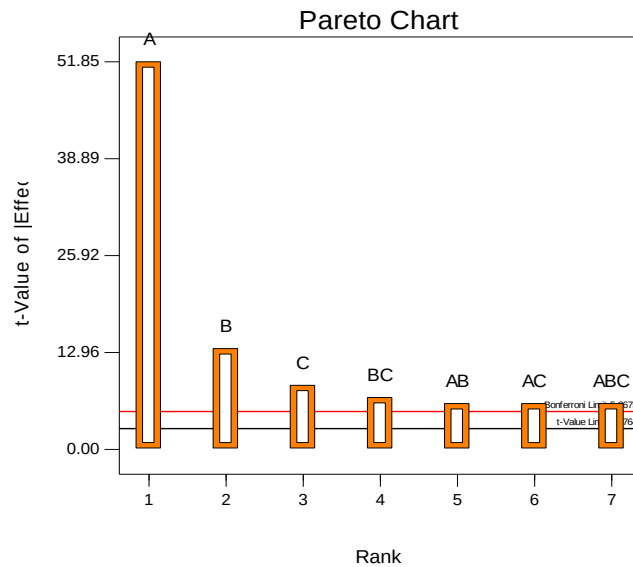
A=Temperature of microwave oven (% of 850 W)

B= Extraction time in microwave (min)

C=Volume of extraction solvent (mL)

Design-Expert® Software
metribuzin

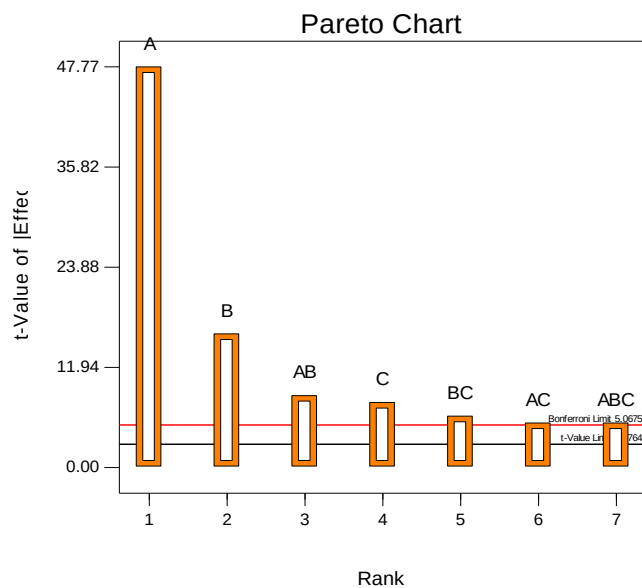
A: temperature
B: time
C: solvent volume
■ Positive Effects
■ Negative Effects



(a)

Design-Expert® Software
atrazine

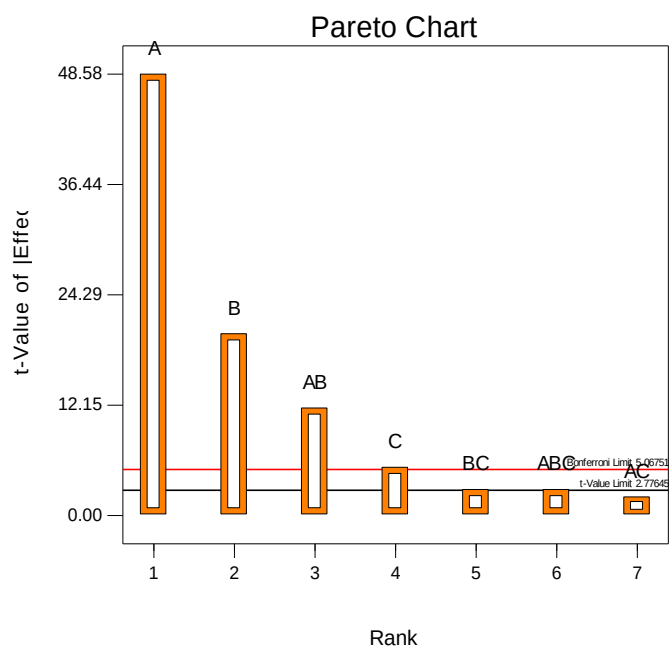
A: temperature
B: time
C: solvent volume
■ Positive Effects
■ Negative Effects



(b)

Design-Expert® Software
ametryn

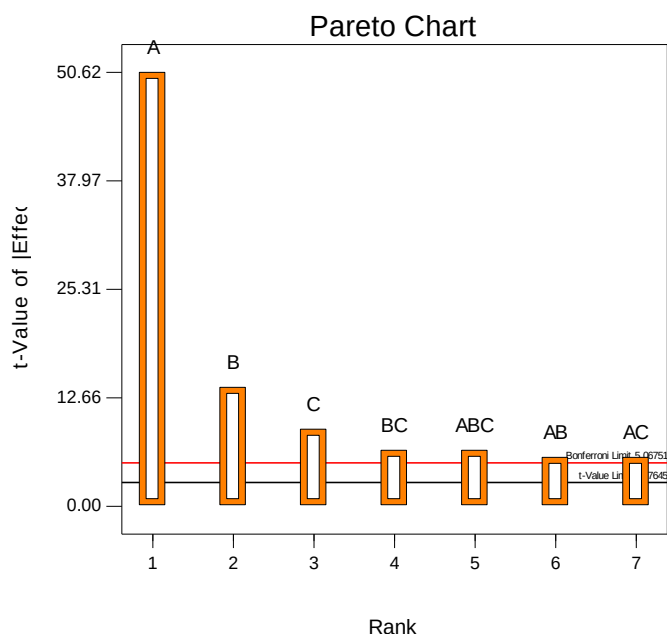
A: temperature
B: time
C: solvent volume
■ Positive Effects
■ Negative Effects



(c)

Design-Expert® Software
terbutryn

A: temperature
B: time
C: solvent volume
■ Positive Effects
■ Negative Effects



(d)

Figure 3.5.10 Standardized Pareto charts for screening metribuzin (a), atrazine (b), ametryn (c) and terbutryn (d) for the determination of the main factors affecting the MAE

3.5.4 Application of the investigated method for the analysis of selected triazine herbicides in real sample

Instrument: The same as mentioned before

Sample collection, preparation and extraction

Surface soil was collected from a corn field. Soil sample (20.0 g) was air dried and homogenized using pestle and mortar. The homogenized sample was passed through a steel mesh of size 355.0 μm . There were no detectable levels of target analyte except atrazine (concentration was below the detection limit of the method) in the soil before spiking.

The soil samples (3.0 g) were transferred into nine separate closed extraction vessel. Three sets of soil samples (3.0 g) were spiked with different fortified level (6.0, 12.0 and 18.0 $\mu\text{g mL}^{-1}$) of herbicides solution mixture (atrazine, metribuzin, ametryn and terbutryn). All these spiked samples were kept in dark for 24 hr at room temperature. To these samples were added 50.0 mL of acetonitrile: ethylacetate: methanol (3:2:5) and

were mechanically shaken vigorously. Extraction in microwave was performed for 4 min at 80 % of 850 W magnetron outputs of microwave. After extraction the vessel was removed from the microwave and allowed to cool down to room temperature for about 10 min. The extract was filtered and the residue was washed three times with 3.0 mL of methanol. To reduce the volume of the extract, it was allowed to evaporate at room temperature for an hour followed by centrifugation ($1790 \times g$) for 15.0 min and used for HPLC analysis. Percent recovery was calculated using the following formula.

$$\% \text{ Recovery} = \mu\text{g found} / \mu\text{g added} \times 100$$

The results are given in table 3.5.11 and are shown in figure 3.5.11 to 3.5.17.

Table 3.5.11: Percent recovery of selected triazine herbicides from soil sample (n=3)

Analyte	Conc. added (μgmL^{-1})	Conc. found (μgmL^{-1})	% Recovery
Metribuzin	6.00	5.78	96.3 $\pm$ 0.23
	12.0	10.7	89.9 $\pm$ 0.10
	18.0	17.2	95.6 $\pm$ 0.08
Atrazine	6.00	5.54	92.3 $\pm$ 0.08
	12.0	10.1	84.2 $\pm$ 0.13
	18.0	16.0	88.9 $\pm$ 0.09
Ametryn	6.00	5.65	94.2 $\pm$ 0.16
	12.0	10.6	88.8 $\pm$ 0.11
	18.0	16.3	90.6 $\pm$ 0.12
Terbutryn	6.00	6.37	106.2 $\pm$ 0.25
	12.0	11.3	94.2 $\pm$ 0.08
	18.0	15.0	83.3 $\pm$ 0.12

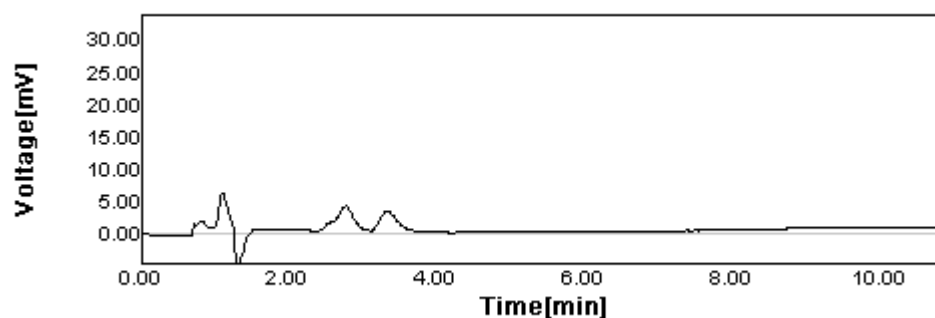


Figure 3.5.11 Chromatogram of the extracts from unspiked soil sample using reverse phase HPLC

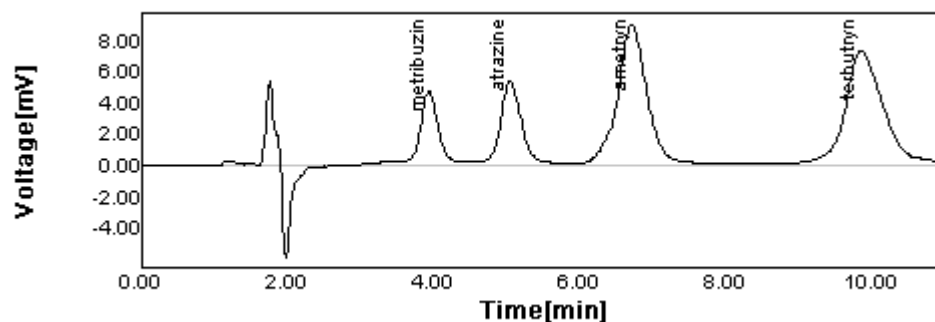


Figure 3.5.12 Chromatogram of standard triazine herbicides ($6.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

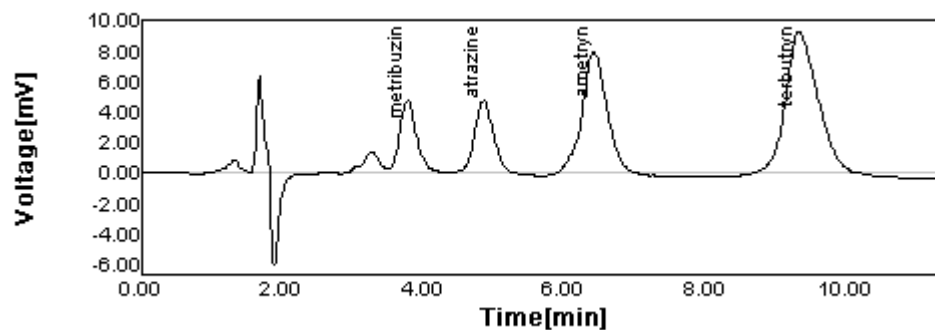


Figure 3.5.13 Chromatogram for $6.0 \mu\text{g mL}^{-1}$ fortified soil sample using reverse phase HPLC

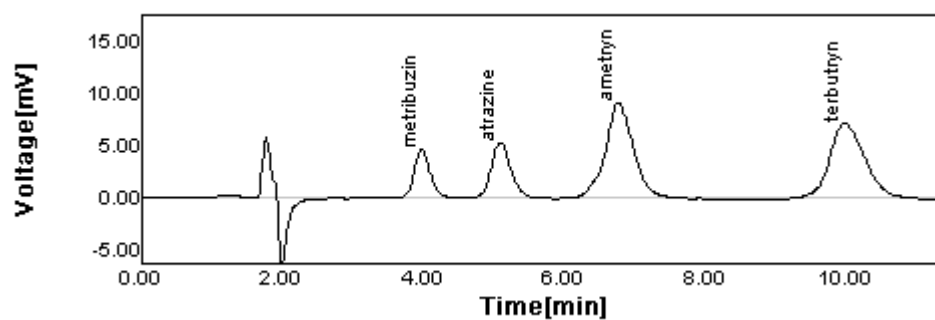


Figure 3.5.14 Chromatogram of standard triazine herbicides ($12.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

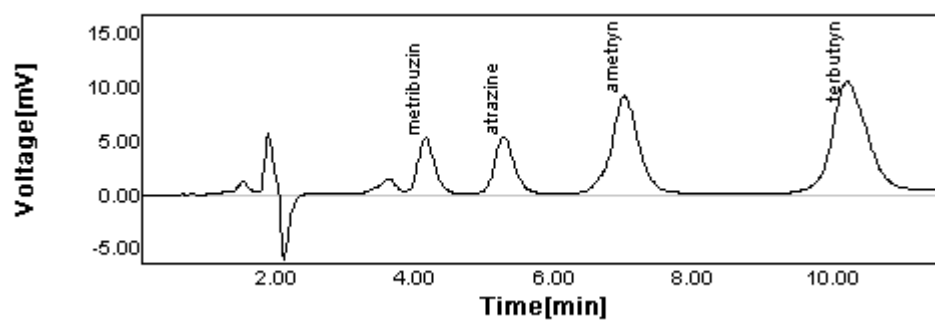


Figure 3.5.15 Chromatogram for $12.0 \mu\text{g mL}^{-1}$ -fortified soil sample using reverse phase HPLC

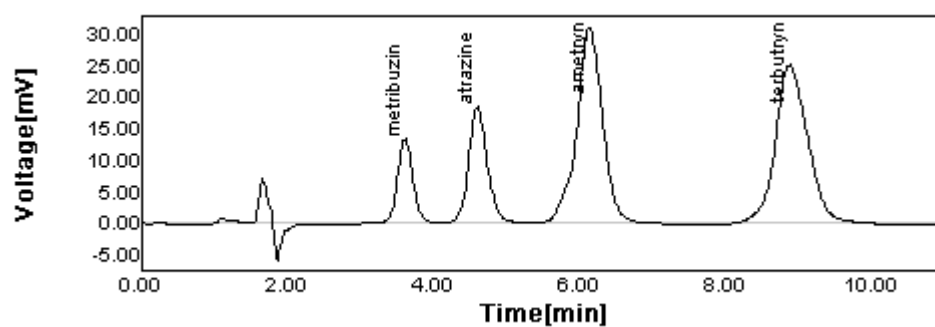


Figure 3.5.16 Chromatogram of selected standard triazine herbicides ($18.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

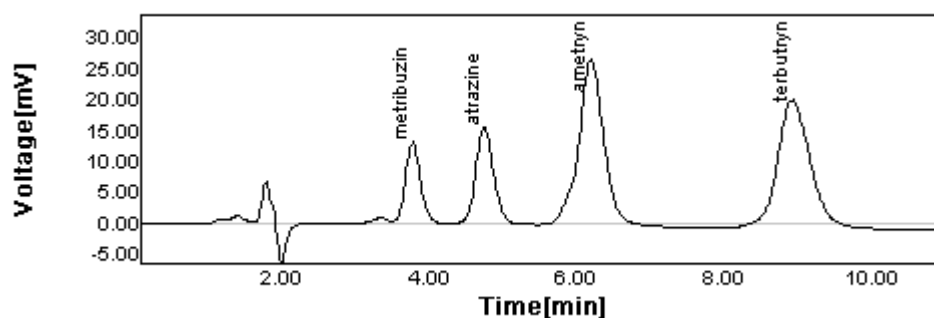


Figure 3.5.17 Chromatogram for 18.0 $\mu\text{g mL}^{-1}$ fortified soil sample using reverse phase HPLC

3.5.5 Results and discussion

High performance liquid chromatographic method with UV detection was developed for the determination of selected herbicides (atrazine, metribuzin, ametryn and terbutryn) in soil samples. The proposed method was based on microwave-assisted extraction (MAE) of soil samples for 4.0 min at 800.0 of 850.0 W magnetron out puts in the presence of mixture of solvents (methanol: acetonitrile: ethylacetate). A reverse phase HPLC with HiQ SiL C₁₈ column was used. Sample injection volume was 20.0 μL and the column was maintained at room temperature of about 25.0 °C. The mobile phase used was methanol and water (70: 30, v/v) and flow rate was kept at 1.0 mL min^{-1} . The absorbance was measured at 254.0 nm.

The other related important factors influencing the MAE efficiency, such as the solvent type and its volume, irradiation energy and time of irradiation were optimized in detail.

Organic solvents such as methanol, acetonitrile and ethyl acetate were first studied for extraction of each triazine herbicide separately at 80.0 % of 850.0 W output of microwave for 4.0 min using 30.0 mL of solvent in triplicate at three different concentrations of triazine herbicides in control samples and the average recoveries found are given in figure 3.5.4. Atrazine was found to be well extracted in methanol with average recoveries of 86.0 %. Similarly it was observed that metribuzin is 85.0 % recovered in ethyl acetate as compared to 79.0 % and 59.0 % average recovery in methanol and acetonitrile respectively. Acetonitrile was found to be more effective as an

extraction solvent for ametryn and terbutryn with average % recovery of 88.0 % and 87.0 % respectively.

MAE for the mixture of the four triazines from soil samples was performed using different solvent composition like acetonitrile:ethylacetate (Figure 3.5.5) and acetonitrile: ethylacetate: methanol (Figure 3.5.6). With 6:4 combination of acetonitrile: ethylacetate, average recovery of metribuzin has improved significantly by 6.0 %, where as that of atrazine, ametryn and terbutryn remained unchanged. The recoveries of all four triazine herbicides were at their highest level (metribuzin, atrazine, ametryn and terbutryn was 91.0 %, 95.0 %, 89.0 % and 90.0 % respectively) when methanol was introduced as an extraction solvent along with acetonitrile and ethyl acetate.

To find out the effect of irradiation energy on the extraction efficiency, extractions were carried out at 10.0, 30.0, 50.0 and 80.0 % of 850.0 W output of microwave respectively. The extraction recoveries are shown in figure 3.5.7. The recoveries increased clearly as the irradiation energy for extraction increased up to 80.0 % of 850.0 W. Metribuzin, atrazine, ametryn and terbutryn showed significant increase in % recoveries (from 43.0, 44.0, 57.0 and 45.0 to 91.0, 95.0, 89.0 and 90.0 respectively) when the irradiation energy was changed from 50.0 to 80.0 % of 850.0 W.

Extraction time optimizations were carried out at 80.0 % of 850.0 W for 1.0, 2.0, 3.0, 4.0 min using acetonitrile: ethylacetate: methanol as the extraction solvent. Under the condition of changing the extraction time, the results of the MAE recoveries of four triazines from soil samples are shown in figure 3.5.8. When the extraction times were increased from 1.0 min to 4.0 min the recoveries of all four triazine herbicides increased.

Four different volumes of extractant solvent, 20.0, 30.0, 40.0 and 50.0 mL were studied respectively (Figure 3.5.9). The recoveries of metribuzin, atrazine, ametryn and terbutryn increased when the solvent volume was increased from 20.0 mL to 30.0 mL and then 40.0 mL. Small increase in recoveries was observed when the extractant volume was increased up to 50.0 mL, except for atrazine where decrease in the recovery was observed. Thus, the optimum volume of extractant solvent was used as 50.0 mL.

A Plackett-Burman factorial design was used as a screening method in order to select the variables that influence MAE extraction.

The effects of the main factors and their interactions with each other were estimated for Plackett–Burman design. The results are shown in figure 3.5.10. Among the three screened factors, all showed a positive effect but the most pronounced effect on the % recovery of selected herbicide was found to be microwave irradiation energy (A) and equilibration time (B). Irradiation energy showed higher % contribution for MAE extraction of metribuzin (86.60 %) than for atrazine (82.58 %), ametryn (80.02 %) and terbutryn (85.80 %). Time has prime impact on ametryn recovery (13.56 %) that is more than its effect on all others. AB (4.75) is significant for ametryn, AC (1.20) and BC (1.55) for metribuzin among all possible combinations of interaction.

The soil sample collected from corn field was analyzed. There were no detectable levels of the target analytes except for atrazine (which was below the detection limit of the method) in the soil before spiking. Recoveries of $89.9 \% \pm 0.10$ — $96.3 \% \pm 0.23$ for metribuzin, $84.2 \% \pm 0.13$ — $92.3 \% \pm 0.08$ for atrazine, $88.8 \% \pm 0.11$ — $94.2 \% \pm 0.16$ for ametryn and $83.3 \% \pm 0.12$ — $106.2 \% \pm 0.25$ for terbutryn for spiked samples indicated that the present method provides good recoveries and reasonable precision for triazines.

3.6. DETERMINATION OF SELECTED TRIAZINE HERBICIDES IN CORN SAMPLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The triazine herbicides selected for this study were atrazine, ametryn, metribuzin and terbutryn.

Instrument: The same as mentioned in part V of Chap III

Reagents: The same as mentioned in part V of Chap III

Standard triazine herbicide solution: All solutions were prepared in the same way as mentioned in part V of Chap III

Optimized HPLC Conditions: All physical parameters such as wavelength, flow rate, solvent elution mode, and time for elution and chemical parameters like solvents and composition of solvents for isocratic elution were optimized for the HPLC determination of triazine group of herbicides following the same procedure as mentioned in part V of Chapter III. All the optimized parameters are presented in table 3.6.1 and were used for subsequent analysis. Representative HPLC chromatogram of metribuzin, atrazine, ametryn and terbutryn is shown in figure 3.6.1. Analytical characteristics for HPLC-UV determination of selected triazine herbicides is given in table 3.6.2.

Table 3.6.1: Optimum HPLC conditions for analysis of selected triazine herbicides using reverse phase mode

Parameters	Values
Eluent used (acetonitrile: water: methanol)	70: 20: 10
Flow rate (mLmin ⁻¹)	1.0
Injection volume (μL)	20.0
Wavelength (nm)	254.0

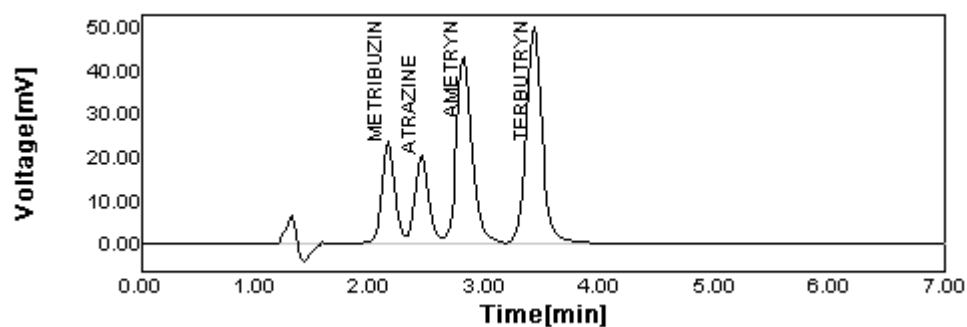


Figure 3.6.1 HPLC chromatogram of metribuzin, atrazine, ametryn and terbutryn

Table 3.6.2: Characteristics for HPLC-UV determination of selected triazines

Analysis	Characteristics			
	Metribuzin	Atrazine	Ametryn	Terbutryn
Working concentration range ($\mu\text{g mL}^{-1}$)	0.4—10	0.4—10	0.4—10	0.4—10
Limit of detection ($\mu\text{g mL}^{-1}$)	0.048	0.081	0.096	0.048
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.16	0.27	0.32	0.36
Standard deviation	0.016	0.027	0.032	0.036
Relative standard deviation	4.13	6.48	10.10	10.09
Regression equation	$0.45 X + 0.00$	$0.40 X + 0.00$	$0.20 X + 0.00$	$0.22 X + 0.00$
Correlation Coefficient (r^2)	0.9752	0.9863	0.9955	0.9986

3.6.1 Optimization of extraction procedure for determination of selected triazine herbicides using reverse phase HPLC

Three different extraction procedures that is microwave-assisted extraction (MAE); ultrasonic-assisted extraction (UAE) and shake-flask were chosen for extraction of triazine herbicides from corn samples followed by high-performance liquid chromatographic determination.

After optimization of instrumental and physical parameters of HPLC, it was important to optimize extraction procedure for the extraction of selected triazine herbicides by microwave-assisted extraction; sonication and mechanical shaking. For this purpose the effects of various experimental parameters of these techniques were investigated.

The effect of various experimental parameters of microwave-assisted extraction steps like irradiation energy, irradiation time, and solvent type were optimized. Preliminary experiments were performed to select the optimum operation conditions for the microwave-assisted solvent extraction of selected triazine herbicides.

The triazine herbicides are highly soluble in methanol, acetonitrile and ethylacetate. Organic solvents such as methanol, acetonitrile, and ethylacetate were first studied for extraction of triazine herbicide separately and then with solvent combination of n-hexane: ethylacetate 1:9 at 100.0 % of 850.0 W output of microwave for 2.0 min using 10.0 mL of solvent in triplicate at three different concentrations of triazine herbicides in control samples. The results are given in table 3.6.3 and are shown in figure 3.6.2.

The effect of irradiation energy on the extraction efficiency of triazine herbicides from corn sample was studied at 10.0, 30.0, 50.0, 80.0 and 100.0 % of 850.0 W of magnetron power output of microwave respectively. The results are given in table 3.6.4 and are shown in figure 3.6.3.

The optimization of irradiation time on extraction efficiency was carried out at 100.0 % of 850.0 W for 1.0, 1.5, 2.0, 2.5 min. Under the condition of changing the extraction time, the results of the MAE recoveries of four triazines are given in table 3.6.5 and are shown in figure 3.6.4.

The effect of sonication on extraction efficiency of triazine herbicides from the corn sample was investigated for 5.0, 10.0 and 15.0 min using 40.0 Hz frequency. The results are given in table 3.6.6 and are shown in figure 3.6.5.

The effect of time on extraction of selected herbicides using orbital shaker was studied for 10.0, 20.0, 40.0 and 60.0 min at room temperature at 120.0 rpm. The results are given in table 3.6.7 and are shown in figure 3.6.6.

Table 3.6.3: The effect of solvent type on recoveries of triazines (n=3)

Solvents	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
Methanol	23±0.23	27±0.19	27±0.06	42±0.16
Acetonitrile	42±0.15	52±0.12	49±0.09	64±0.19
Ethylacetate	75±0.23	73±0.18	75±0.11	76±0.18
n-hexane: ethylacetate	86±0.23	86±0.26	90±0.19	83±0.20

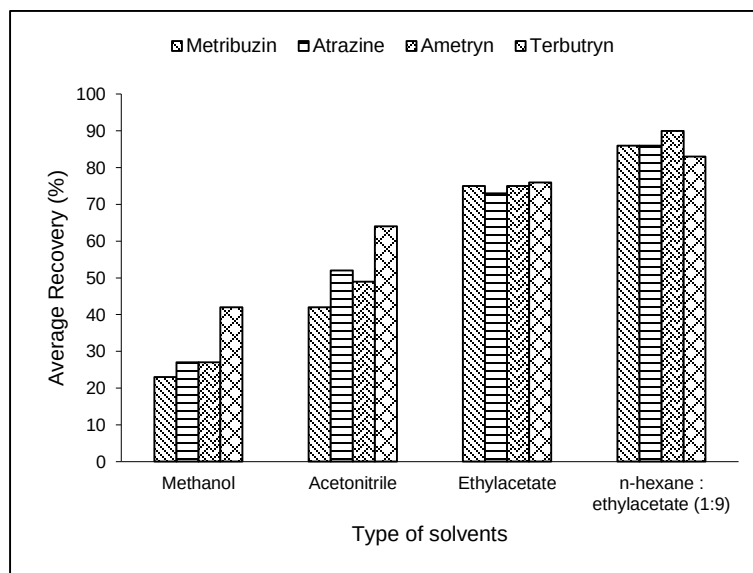


Figure 3.6.2 The effect of solvent type on recoveries of triazines (n=3)

Irradiation energy = 100 % of 850 W, Solvent volume = 10.0 mL,
Time = 2 min

Table 3.6.4: The effect of irradiation energy on recoveries of triazines (n=3)

Irradiation temperature (% of 850 watt energy)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
10	53±0.31	44±0.17	46±0.55	47±0.14
30	62±0.42	63±0.09	55±0.25	59±0.39
50	71±0.23	73±0.27	69±0.39	68±0.16
80	81±0.67	78±0.33	77±0.11	75±0.08

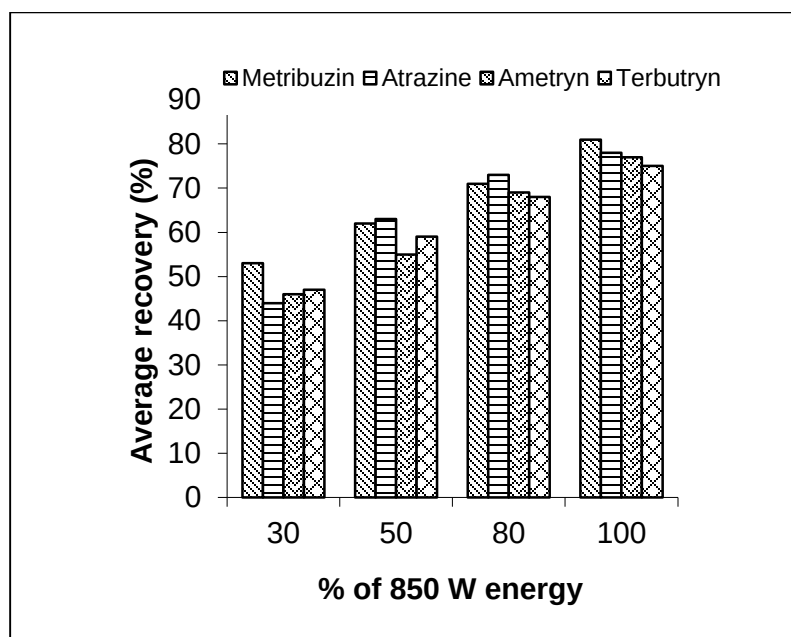


Figure 3.6.3 The effect of irradiation energy on recoveries of triazines (n=3)

Extracting solvent = ethylacetate: n-hexane (9:1), Solvent volume = 10.0 mL
Time = 2.0 min

Table 3.6.5: The effect of irradiation time on recoveries of triazines using MAE method (n=3)

Irradiation time (min)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
1.0	47±0.30	36±0.36	35±0.28	37±0.34
1.5	77±0.20	63±0.15	58±0.11	69±0.23
2.0	84±0.57	84±0.45	77±0.44	78±0.32
2.5	68±0.52	52±0.49	58±0.41	82±0.95

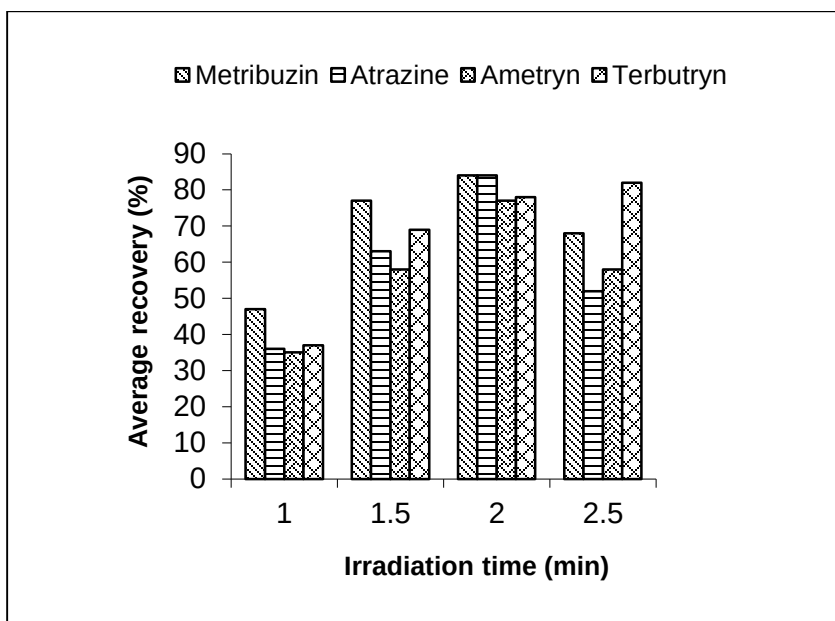


Figure 3.6.4 The effect of irradiation time on recoveries of triazines using MAE method (n=3)

Extracting solvent = ethylacetate: n-hexane (9:1),

Irradiation energy = 100.0 % of 850.0 W, Solvent volume = 10.0 mL

Table 3.6.6: The effect of sonication time on extraction recoveries of triazines using ultrasonic assisted extraction method (n=3)

Sonication time (min)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
5	48±0.39	58±0.37	40±0.41	41±0.33
10	62±0.60	61±0.48	61±0.25	64±0.19
15	85±0.93	80±0.37	74±0.65	71±0.44

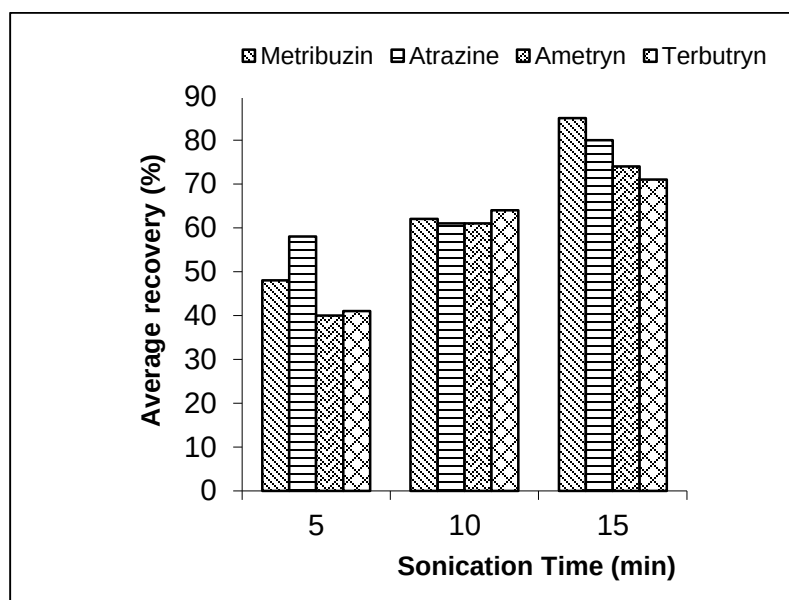


Figure 3.6.5 The effect of sonication time on extraction recoveries of triazines using ultrasonic assisted extraction method (n=3)

Extracting solvent = ethylacetate: n-hexane (9:1), Solvent volume = 20.0 mL

Table 3.6.7: The effect of shake-flask time on extraction of triazines using shake-flask method (n=3)

Time for Shake-flask time (min)	Recovery (%)			
	Metribuzin	Atrazine	Ametryn	Terbutryn
10	38±0.20	41±0.28	36±0.30	36±0.34
20	57±0.70	54±0.68	49±0.23	47±0.50
40	61±0.39	59±0.54	53±0.55	49±0.67
60	78±0.07	73±0.10	69±0.09	68±0.08

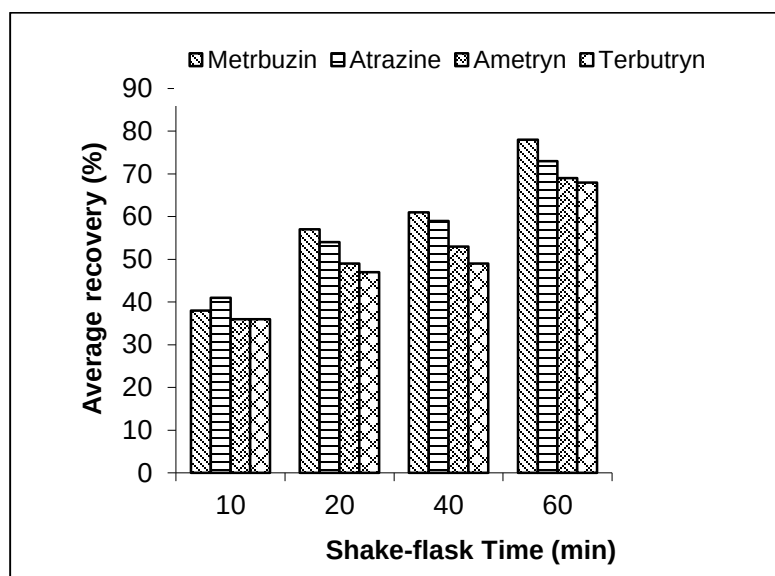


Figure 3.6.6 The effect of shake-flask time on extraction of triazines using shake-flask method (n=3)

Extracting solvent = ethylacetate: n-hexane (9:1), Solvent volume = 20.0 mL

3.6.2 Comparison of extraction time for the analysis of selected triazine herbicides in control corn sample using different extraction techniques

Instrument: The same as mentioned before

Sample collection, preparation and extraction

Ripe corn samples were collected from an agriculture field. Corn sample of (250.0 g) was washed with acetonitrile, n-hexane and methanol and dried in open air at room temperature. There were no detectable levels of target analyte in the corn samples before spiking. Corn samples (1.0 g) were spiked with different fortified level (4.0, 6.0 and 8.0 $\mu\text{g g}^{-1}$) of herbicides solution mixture (atrazine, metribuzin, ametryn and terbutryn) separately in triplicate. All these spiked samples were kept for 24 hr at room temperature.

The triazines were extracted from the corn samples by the following methods. The results in terms of comparison of time are given in table 3.6.7.

Extraction from corn

Microwave assisted extraction

A known weight (1.0 g) of a dried corn sample was taken in a closed extraction vessel containing 10.0 mL of ethylacetate: n-hexane (9:1) and placed in the microwave. Extraction was performed for 2.0 min at 100.0 % of 850.0 W magnetron outputs of microwave. After extraction the vessel was allowed to cool down to room temperature. The solvent was carefully decanted and corn sample was washed with the same solvent. Combined solvent was centrifuged for 10.0 min at 200.0 rpm for separation of fine particles. The supernatant was separated and evaporated in a rotary evaporator at 40.0 °C to dryness. The residue was dissolved in 1.0 mL of acetonitrile for HPLC analysis.

Ultrasonic assisted extraction

A known weight (1.0 g) of dried corn sample was sonicated for 15.0 min with 20.0 mL of ethylacetate: n-hexane (9:1) in an ultrasonic bath (frequency 40.0 Hz). The extract was filtered and residue was washed three times with acetonitrile: n-hexane (9:1) solvent. The

filtrate was evaporated using rotary evaporator at 40.0 °C to dryness and the residue was dissolved in 1.0 mL of acetonitrile for HPLC analysis.

Shake-flask extraction

A known weight (1.0 g) of dried corn sample was shaken using orbital shaker at 120.0 rpm in an Erlenmeyer flask for 60.0 min in 20.0 mL of ethylacetate: n-hexane (9:1). The extract was filtered and the filtrate was dried at 40.0 °C using rotary evaporator. The residue was dissolved in 1.0 mL acetonitrile and used for HPLC analysis.

Table 3.6.8: The effect of time on percent recovery of triazine using MAE, UAE and shake-flask method

Extraction Technique	Time (min)	Recovery (%)			
		Metribuzin	Atrazine	Ametryn	Terbutryn
MAE	1.0	47.5±0.30	36.7±0.36	35.9±0.28	37.8±0.34
	1.5	77.9±0.20	63.3±0.15	58.4±0.11	69.7±0.23
	2.0	84.3±0.57	84.7±0.45	77.5±0.44	78.8±0.32
	2.5	67.5±0.52	52.4±0.49	58.6±0.41	81.6±0.95
Shake-flask	10	38.4±0.20	41.6±0.28	36.7±0.30	36.6±0.34
	20	57.4±0.70	54.7±0.68	49.5±0.23	47.9±0.50
	40	61.8±0.39	59.9±0.54	53.9±0.55	49.5±0.67
	60	78.2±0.07	73.1±0.10	69.5±0.09	68.6±0.08
UAE	05	48.3±0.39	58.6±0.37	40.9±0.41	41.6±0.33
	10	62.9±0.60	61.6±0.48	61.3±0.25	64.5±0.19
	15	85.5±0.93	80.1±0.37	74.9±0.65	71.6±0.44

3.6.3 Application of the investigated method for the analysis of selected triazine herbicides in corn sample

Instrument: The same as mentioned before

Sample collection, preparation and extraction

Ripe corn samples were collected from an agriculture field. Corn sample of (250.0 g) were dried in open air at room temperature. There were no detectable levels of target analyte in the corn samples before spiking.

Extraction from corn

Microwave assisted extraction

Known weights (1.0 g) of a dried corn sample were taken in nine separate beakers and spiked with different concentration (4.0, 6.0 and 8.0 $\mu\text{g g}^{-1}$) of herbicides solution mixture (atrazine, metribuzin, ametryn and terbutryn) in triplicate. All these spiked samples were kept for 24.0 hr at room temperature. These samples were then transferred to a closed extraction vessel containing 10.0 mL of ethylacetate: n-hexane (9:1) and placed in the microwave. Extraction was performed for 2.0 min at 100.0 % of 850.0 W magnetron outputs of microwave. After extraction the vessel was allowed to cool down to room temperature. The solvent was carefully decanted and corn sample was washed with the same solvent. Combined solvent was centrifuged for 10.0 min at 2000.0 rpm for separation of fine particles. The supernatant was separated and evaporated in a rotary evaporator at 40.0 °C to dryness. The residue was dissolved in 1.0 mL of acetonitrile for HPLC analysis. The results are given in table 3.6.7 and are shown in figure 3.6.7-3.6.13.

Table 3.6.9: Percent recovery of selected triazine herbicides from corn sample (n=3)

Analyte	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Metribuzin	4.0	3.95	98.8 $\pm$ 0.12
	6.0	4.78	79.7 $\pm$ 0.13
	8.0	6.63	82.9 $\pm$ 0.25
Atrazine	4.0	3.77	94.3 $\pm$ 0.28
	6.0	4.83	80.5 $\pm$ 0.15
	8.0	6.36	79.5 $\pm$ 0.17
Ametryn	4.0	3.76	94.0 $\pm$ 0.20
	6.0	4.74	79.0 $\pm$ 0.17
	8.0	6.28	78.5 $\pm$ 0.10
Terbutryn	4.0	3.65	91.3 $\pm$ 0.16
	6.0	5.63	93.8 $\pm$ 0.22
	8.0	7.68	96.0 $\pm$ 0.14

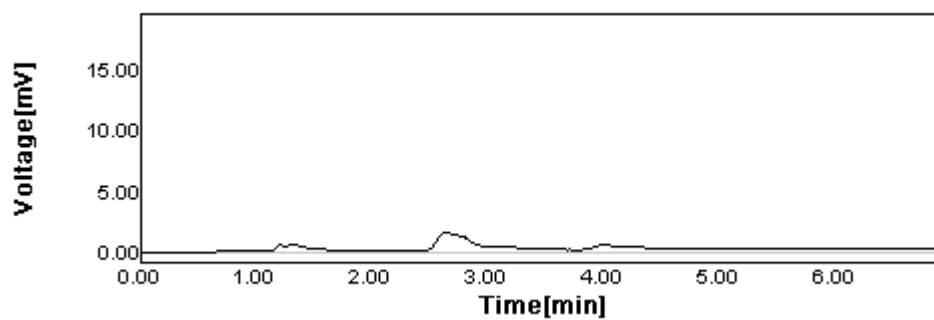


Figure 3.6.7 Chromatogram of the extracts from unspiked soil sample using reverse phase HPLC

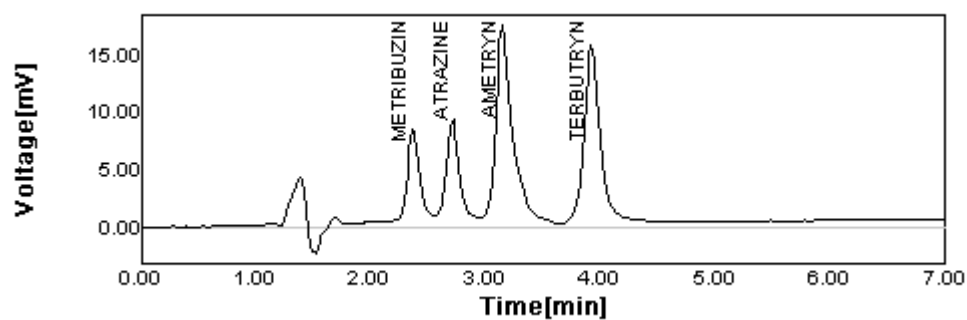


Figure 3.6.8 Chromatogram of standard triazine herbicides ($4.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

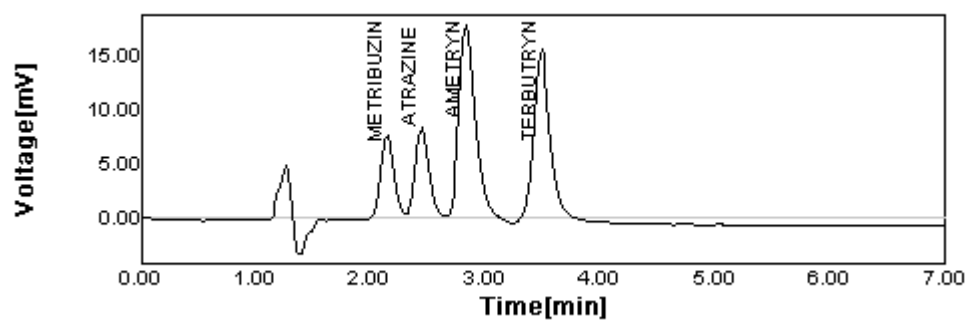


Figure 3.6.9 Chromatogram of fortified corn sample ($4.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

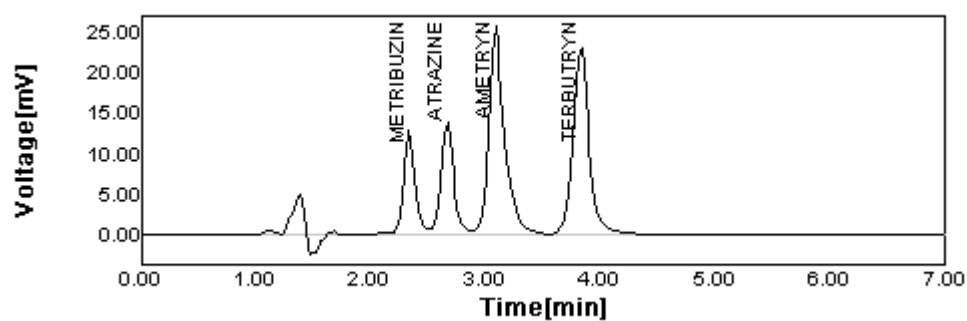


Figure 3.6.10 Chromatogram of standard triazine herbicides ($6.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

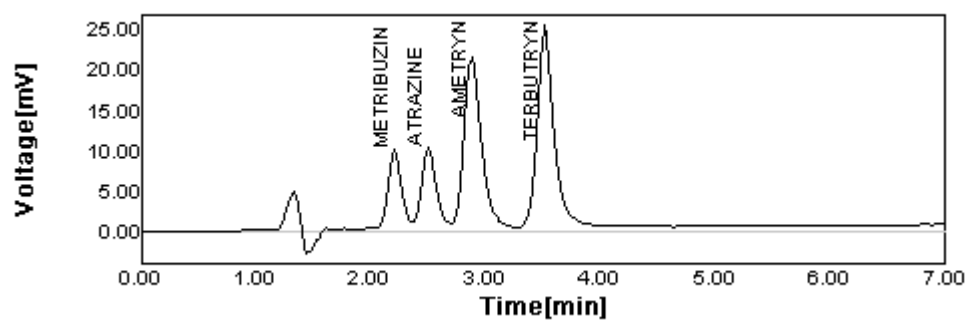


Figure 3.6.11 Chromatogram of fortified corn sample ($6.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

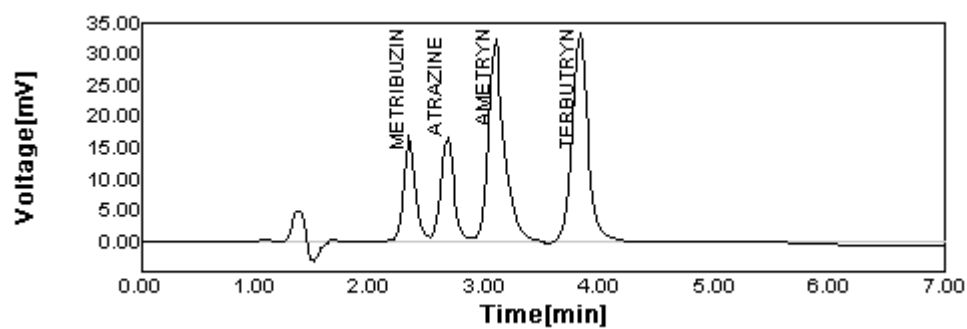


Figure 3.6.12 Chromatogram of standard triazine herbicides ($8.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

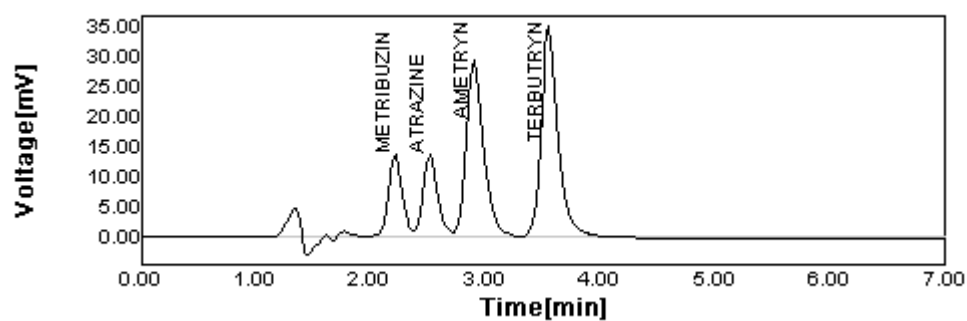


Figure 3.6.13 Chromatogram of fortified corn sample ($8.0 \mu\text{g mL}^{-1}$) using reverse phase HPLC

3.6.4 Results and discussion

Three different extraction procedure, microwave assisted extraction (MAE), ultrasonic assisted extraction (UAE) and shake-flask traditional extraction methods were investigated for the extraction of selected herbicides and the results of extraction were compared at optimum conditions. Maximum recoveries were achieved with 2.0 min of MAE in comparison to 15.0 min of UAE and 60.0 min of shaking-flask at three different spiked levels of control corn samples. The proposed MAE method allows extraction of triazine herbicides in a small volume of solvent and faster than the other extraction methods.

Extraction time for sonication and mechanical shaking and related important factors influencing MAE efficiency, such as the solvent type, irradiation energy and irradiation time were optimized in detail.

Organic solvents such as methanol, acetonitrile and ethylacetate were first studied for extraction of selected triazine herbicide collectively at 100.0 % of 850.0 W output of microwave for 2.0 min using 10.0 mL of solvent in triplicate at three different concentrations of triazine herbicides in control samples (Figure 3.6.2). It was observed that all herbicides are well extracted in ethyl acetate as compared to methanol and acetonitrile. Average recovery (%) of metribuzin, atrazine, ametryn and terbutryn were 75.0 %, 73.0 %, 75.0 % and 76.0 % respectively in ethyl acetate.

MAE for mixture of four triazines from corn samples was performed using ethylacetate: n-hexane (9:1). Average recovery (%) of selected triazines has improved significantly in ethylacetate: n-hexane (9:1) (Figure 3.6.2). The recoveries of selected triazine herbicides were at their highest at optimum conditions of MAE (metribuzin, atrazine, ametryn and terbutryn was 86.0 %, 86.0 %, 90.0 % and 83.0 % respectively).

To find out the effect of irradiation energy on the extraction efficiency, extractions were carried out at 10.0, 30.0, 50.0, 80.0 and 100.0 % of 850.0 W output of microwave respectively. The extraction recoveries are shown in figure 3.6.3. The recoveries increased clearly as the irradiation energy for extraction increased up to 100.0 % of 850.0 W. Metribuzin, atrazine, ametryn and terbutryn showed increase in % recoveries (from

71.0 %, 73.0 %, 69.0 % and 68.0 % to 81.0 %, 78.0 %, 77.0 % and 75.0 % respectively) when the irradiation energy was changed from 80.0 to 100.0 % of 850.0 W.

Extraction time optimizations were carried out at 100.0 % of 850.0 W for 1.0, 1.5, 2.0, 2.5 min. Under the condition of changing the extraction time, the results of the MAE recoveries of four triazines are shown in figure 3.6.4. When the extraction times were increased from 1.0 min to 2.0 minutes the recoveries of all four triazine herbicides increased. Average recovery (%) of metribuzin, atrazine, ametryn and terbutryn at 2.0 min were 84.0 %, 84.0 %, 77.0 % and 78.0 % respectively.

Sonication of the corn sample was done for 5.0, 10.0 and 15.0 min at 40.0 °C. It was found that maximum extraction was achieved with 15.0 min of sonication as shown (average recovery (%) of metribuzin, atrazine, ametryn and terbutryn 85.0 %, 80.0 %, 74.0 % and 71.0 % respectively) in figure 3.6.5.

Shake-flask extraction was performed for 10.0, 20.0, 40.0 and 60.0 min at room temperature. It was observed that increasing the time from 10.0 to 60.0 min shows increase in average recoveries (%) of the selected triazines (Figure 3.6.6). Average recovery (%) of metribuzin, atrazine, ametryn and terbutryn at 60.0 min were 78.0 %, 73.0 %, 69.0 % and 68.0 % respectively.

The statistical parameters calculated from the calibration graph are given in the table 3.6.2. Linearity of calibration graphs was proved by the high value of correlation coefficient (r^2). The limits of detection (LODs) and the limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 3 and 10 times, respectively.

Based on the above observations, it was decided to use MAE optimized extraction procedure for obtaining triazines extracts from corn samples. Fresh corn samples were analyzed using MAE giving recoveries (%) for metribuzin, atrazine, ametryn and terbutryn in the range of 79.7 % $\pm$ 0.13—98.8 % $\pm$ 0.12, 79.5 % $\pm$ 0.17—94.3 % $\pm$ 0.28, 78.5 % $\pm$ 0.10—94.0 % $\pm$ 0.20 and 91.3 % $\pm$ 0.16—96.0 % $\pm$ 0.14 respectively.

3.7 DETERMINATION OF SELECTED TRIAZINE HERBICIDES IN WATER AND SUGAR CANE SAMPLES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The triazine herbicides selected for this study were atrazine, ametryn, metribuzin and terbutryn.

Instrument: The same as mentioned in part V of Chap III

Reagents: The same as mentioned in part V of Chap III

Standard triazine herbicide solution: All solutions were prepared in the same way as mentioned in part V of Chap III

Optimized HPLC Conditions: All physical parameters such as wavelength, flow rate, solvent elution mode, and time for elution and chemical parameters like solvents and composition of solvents for isocratic elution were optimized for the HPLC determination of triazine group of herbicides following the same procedure as mentioned in part V of Chapter III. All the optimized parameters are presented in table 3.7.1 and were used for subsequent analysis. Representative HPLC chromatogram of metribuzin, atrazine, ametryn and terbutryn is shown in figure 3.7.2. Analytical characteristics for HPLC-UV determination of selected triazine herbicides is given in table 3.7.2.

Table 3.7.1 Optimum conditions for determination of selected triazine herbicides using reverse phase HPLC

Parameters	Values
Eluent used (acetonitrile: water: methanol)	70: 20: 10
Flow rate (mLmin ⁻¹)	1.1
Injection volume (μL)	20.0
Wavelength (nm)	254.0

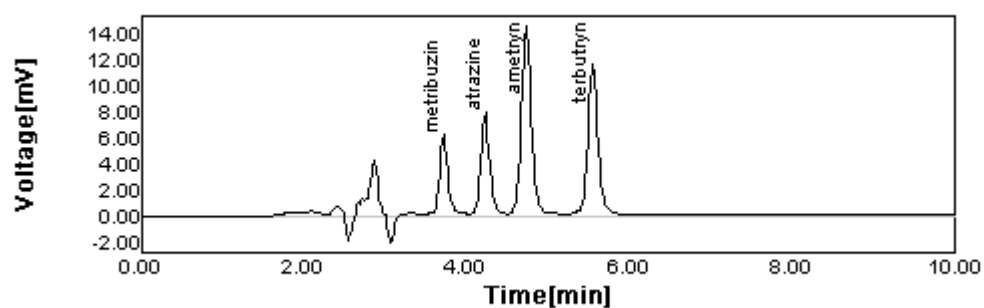


Figure 3.7.1 HPLC chromatogram of metribuzin, atrazine, ametryn and terbutryn

Table 3.7.2: Analytical characteristics for HPLC-UV determination of selected triazine herbicides

Analysis	Characteristics			
	Metribuzin	Atrazine	Ametryn	Terbutryn
Working concentration range ($\mu\text{g mL}^{-1}$)	0.4—12.0	0.4—12.0	0.4—12.0	0.4—12.0
Limit of detection ($\mu\text{g mL}^{-1}$)	0.86	0.97	0.85	0.99
Limit of quantification ($\mu\text{g mL}^{-1}$)	2.87	3.25	2.86	3.30
Standard deviation	0.28	0.32	0.28	0.33
Regression equation	$0.680 X + 0.343$	$0.554 X + 0.186$	$0.33 X + 0.047$	$0.422 X + 0.016$
Correlation Coefficient (r^2)	0.9953	0.9950	0.9955	0.9952

3.7.1 Preconcentration of selected triazine herbicide and its determination by reverse phase HPLC

Herbicides at time are present at low concentration in samples of environmental importance. Therefore to bring it to the limit of quantification for toxicological determination, preconcentration is needed. A low cost biosorbent (mulberry wood sawdust) has been investigated for preconcentration of selected triazine herbicides followed by HPLC separation and quantification.

Characterization of mulberry wood sawdust

Sawdust was characterized using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX).

Specific surface area determination

Sorption capacity of sorbent is related to its surface area. The analysis of surface area, average pore width, micropore volume and pore diameter of sawdust was performed with Quantachrome instrument, surface area & pore size analyzer (NOVA 2200e) (version 2.1). The analysis was carried out using nitrogen gas adsorption method. The results of these analyses are given in table 3.7.3.

SEM analysis

Morphology of the sawdust (chemically treated and non-treated sawdust) was analyzed by Scanning Electronic Microscopy (SEM). The structures were examined at 1000X and 2000X magnifications as can be observed from the SEM photographs (Figure 3.7.3).

EDX analysis

Energy Dispersive X-ray (EDX) study was conducted for chemically treated and untreated sawdust. EDX results are given in table 3.7.4 and are shown in figure 3.7.4.

Preparation of sorbent

Sawdust of locally available mulberry wood was passed through sieve of mesh size 355.0 μm to get relatively uniform particle size. Sawdust was washed with water to remove

dust and dirt. After washing, it was soaked in 1.0 M sodium hydroxide solution and kept overnight. Sawdust treated with sodium hydroxide was washed thoroughly with distilled water until the pH of wash solution became neutral. Washed sawdust dried at 60.0 °C (in oven till constant weight was obtained) was stored in a bottle for further use.

Sorption procedure

Sorption studies of selected triazine herbicides were carried out using chemically treated sawdust in column for separation and preconcentration while temperature affect on sorption of selected triazine herbicides was studied in batch method.

a. Column method

Sorption studies for the selected triazine herbicides were carried out using a 5.0 mL borosilicate glass column. The column was packed with 0.3 g of chemically treated sawdust. The column was equilibrated with 10.0 mL of water before loading it with solution of selected triazine herbicides. Aqueous solution (10.0 mL) containing metribuzin, atrazine, ametryn and terbutryn ($4.0 \mu\text{g mL}^{-1}$ of each in a solution mixture) were loaded on sawdust column and was equilibrated for 6.0 min. After equilibration time, the solutions were collected and filtered using glass syringe fitted with nylon membrane filters (pore size $0.45 \mu\text{m}$) and analyzed using reverse phase HPLC.

b. Sorption procedure for thermodynamic study

For thermodynamics studies, 10.0 mL (2.0 , 3.0 and $4.0 \mu\text{g mL}^{-1}$) solution mixture of selected triazine herbicides were added to 0.3 g of chemically treated saw dust in a 100 mL glass beaker and was placed on a water bath for 15.0 min. The temperature of water bath was varied in the range of 60.0-80.0 °C. These solutions while hot were centrifuged at 2500 rpm ($1790 \times g$) for 5.0 min and the supernatant was filtered using glass syringe fitted with nylon membrane filters (pore size $0.45 \mu\text{m}$) and analyzed using reverse phase HPLC. All analyses were carried out in triplicate.

The amount of herbicides sorbed at equilibrium per unit mass of biosorbent, q_e (mg g^{-1}) and the percentage sorbed were determined using equation (1) and (2).

$$q_e = \frac{(C_i - C_e)}{m} V_s \quad (1)$$

$$\%_{ads} = \frac{(C_i - C_e)}{(C_i)} 100 \quad (2)$$

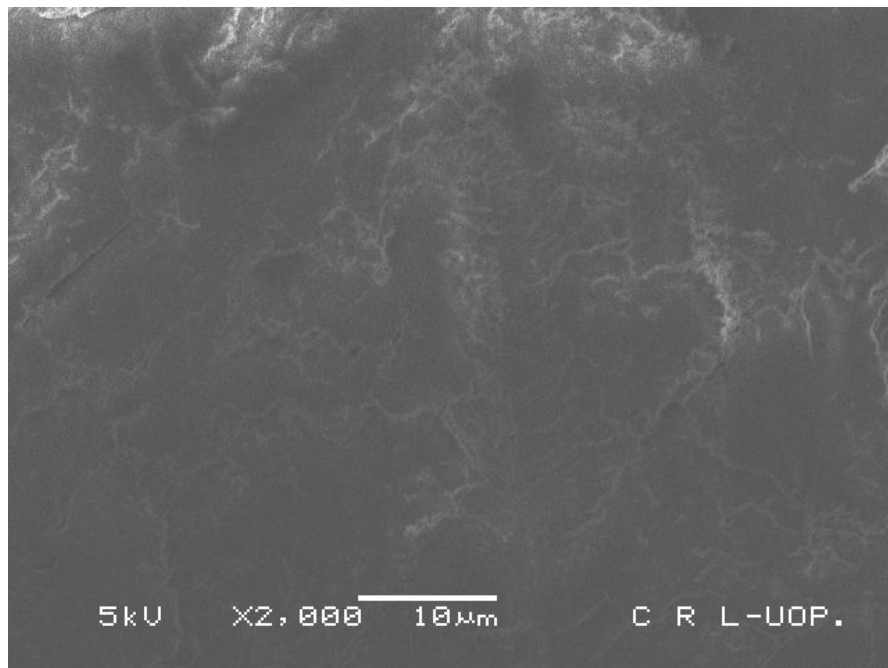
Where C_i ($\mu\text{g mL}^{-1}$) is the initial concentration of the herbicides added to sawdust, C_e ($\mu\text{g mL}^{-1}$) is the concentration of the herbicides at equilibrium after sorption, V (mL) is the volume of the herbicide solution and m (g) is the mass of the sawdust used for sorption.

Table 3.7.3: Surface area and pore size of chemically treated sawdust

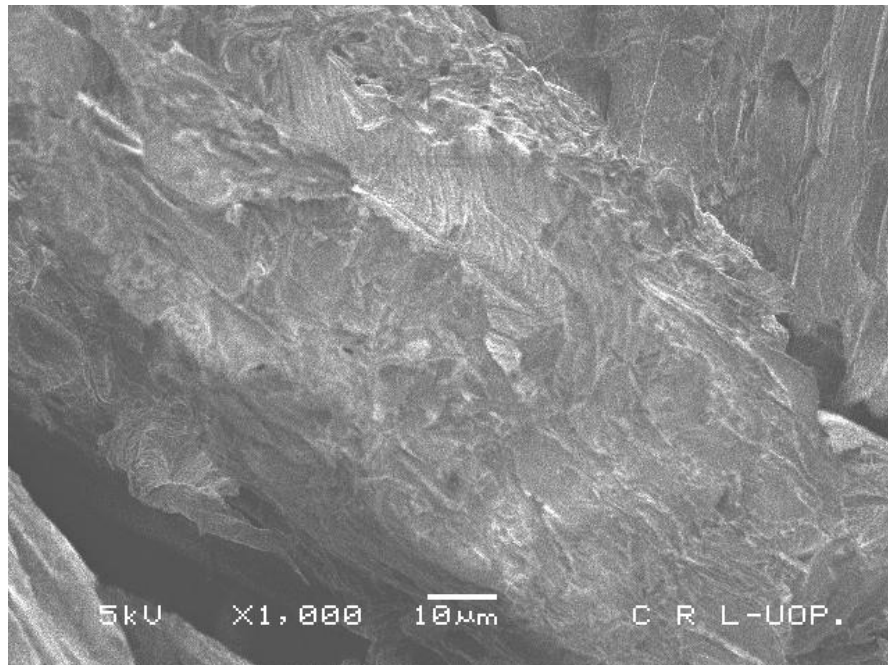
Adsorbent	BET m^2/g	Langmuir- m^2g^{-1}	DR- m^2g^{-1}	BJH- m^2g^{-1}	DR-method		BJH- method	
					Average Pore Width (Å)	Micropore Volume (cc/g)	Pore Diameter (Å)	Pore Volume (cc/g)
Chemically treated Sawdust	53.1	0.00	133.9	106.8	143.3	0.04	129.6	0.12

Table 3.7.4: Elemental analysis of chemically treated and untreated sawdust

Adsorbent	Element content (weight fraction %)		
	Carbon	Oxygen	Calcium
Untreated sawdust	46.76	51.56	1.58
Chemically treated sawdust	51.62	48.38	0.00

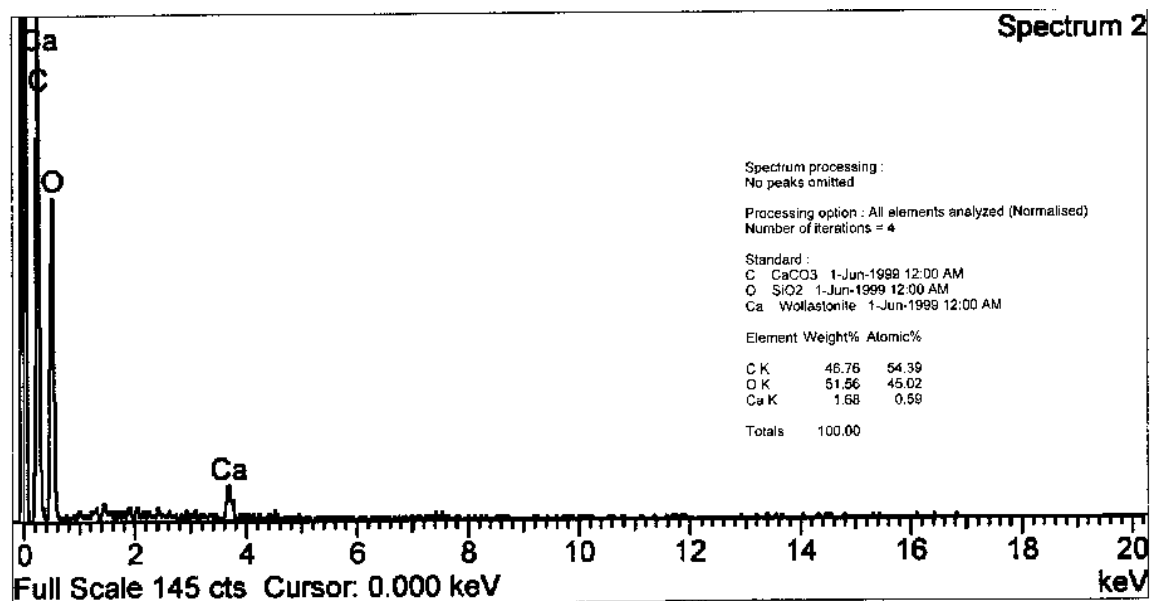


(a)

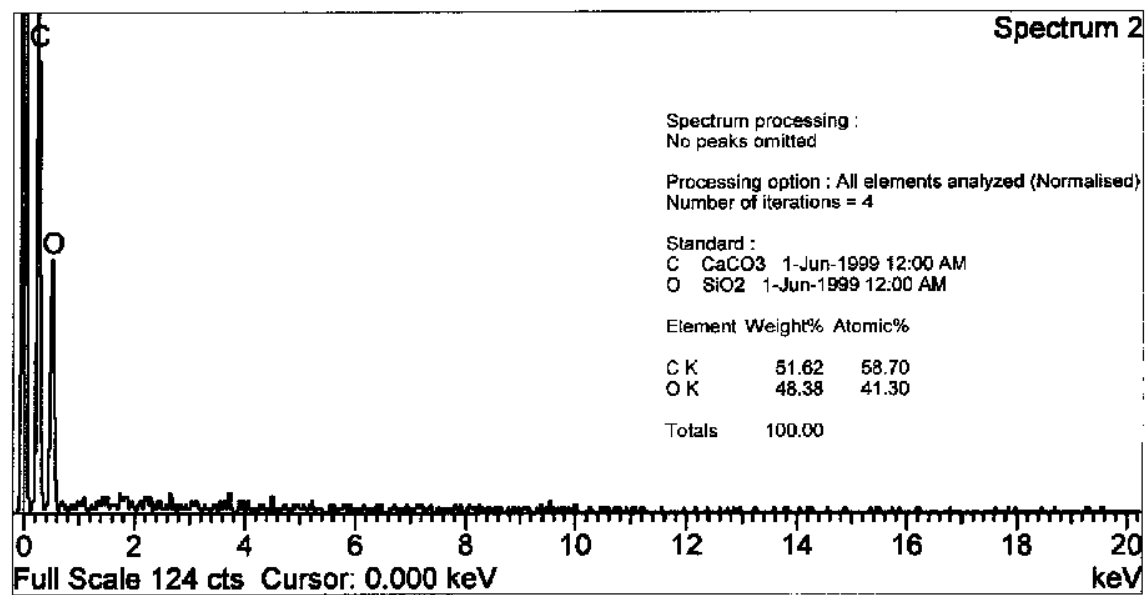


(b)

Figure 3.7.2 SEM image of (a) untreated sawdust and (b) chemically treated sawdust



(a)



(b)

Figure 3.7.3 EDX graph of (a) untreated Sawdust and (b) chemically treated Sawdust

3.7.2 Optimization of sorption parameters for preconcentration of selected triazine herbicides

Experiments were performed to investigate optimum parameters for sorption of the selected triazine herbicides on sawdust.

Amount of sorbent

The optimum amount of chemically treated sawdust giving maximum sorption of the selected triazine herbicide (8.0 $\mu\text{g mL}^{-1}$ solution mixture) was investigated. For this purpose, amount of sawdust introduced into the column was varied in the range of 0.03-0.50 g and the results are given in table 3.7.5 and are shown in figure 3.7.5.

Thermodynamics study

The effect of temperature on the sorption of selected triazine herbicides was studied using 10 mL solution mixture of each herbicide (2.0, 3.0 and 4.0 $\mu\text{g mL}^{-1}$). The different concentration of herbicide mixture solution was added to 0.3 g of sorbent and heated on a thermostatic water bath for 15.0 min at 60.0, 70.0 and 80.0 °C. After heating, all the solutions were filtered and the filtrate was analyzed for residual concentration of herbicides using reverse phase HPLC. The results are given in table 3.7.6 and shown in figure 3.7.6.

Thermodynamics parameters were calculated for determination of sorption process. The change in energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) for sorption of selected triazine herbicides on sawdust were calculated by using the following equations.

$$K_D = \frac{q_e}{C_e} \quad (4)$$

$$\Delta G^\circ = -RT \ln K_D \quad (5)$$

$$\ln K_D = -\frac{G^\circ}{RT} = \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (6)$$

Where,

K_D = distribution constant

q_e = amount of herbicide sorbed at equilibrium

C_e = equilibrium concentration of the herbicide

The values for K_D , ΔG° , ΔH° and ΔS° were calculated and the results are given in table 3.7.7.

Kinetic Study

Kinetic study of the selected triazine herbicides was carried out on a glass column packed with 0.3 g of sawdust. Different solution (10.0 mL) containing concentrations of metribuzin ($4.5 \mu\text{g mL}^{-1}$), atrazine ($6.0 \mu\text{g mL}^{-1}$), ametryn ($6.0 \mu\text{g mL}^{-1}$) and terbutryn ($4.0 \mu\text{g mL}^{-1}$) were used for this study. The equilibration time was studied at 3, 6, 9, 12 and 15 min. The results are given in table 3.7.8 and shown in figure 3.7.7.

The kinetic modeling of sorption of target herbicides on sawdust was studied using pseudo-first-order and pseudo-second-order models. The equation for pseudo-first-order is expressed as follow:

$$\log (q_e - q_t) = \log q_e - \frac{K_1 t}{2.303} \quad (7)$$

Where q_e is the amount of herbicide sorbed (mg g^{-1}) at equilibrium, q_t is the amount of herbicides sorbed (mg g^{-1}) at any given time (min), K_1 is the pseudo-first-order reaction rate constant for sorption (min^{-1}). The equation for pseudo-second-order is as:

$$\frac{t}{q_t} = \frac{t}{q_e} + \frac{1}{K_2 q_e^2} \quad (8)$$

Where q_e is the amount of herbicide sorbed (mg g^{-1}) at equilibrium, q_t is the amount of herbicides sorbed (mg g^{-1}) at any given time (min), K_2 is the pseudo-second-order reaction rate constant for sorption ($\text{g mg}^{-1} \text{min}^{-1}$).

Kinetic parameters calculated for the herbicides sorption on sawdust using pseudo-first-order and pseudo-second-order reaction equation are given in the table 3.7.9, while the pseudo second order kinetics of the studied herbicides are shown in figure 3.7.8.

Isotherms studies

The sorption isotherms of the herbicides on sawdust were studied by the two most commonly used isotherms models Freundlich and Langmuir isotherms. The different

parameters calculated from the slope and intercept of Freundlich and Langmuir isotherms for the selected triazine herbicides are given in the table 3.7.10 and 3.7.11.

The Freundlich isotherm is used for non-ideal sorption on heterogeneous surfaces and is expressed by the following equation:

$$q_e = K_F C_e^{\frac{1}{n}} \quad (9)$$

The logarithmic form of Freundlich equation is given as:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (10)$$

Where K_F is the Freundlich adsorption isotherm constant (mgg^{-1}), $1/n$ (gL^{-1}) is a measure of the sorption intensity and q_e is the amount of herbicide sorbed (mgg^{-1}) and C_e is the equilibrium concentration of herbicide ($\mu\text{g mL}^{-1}$).

The Langmuir isotherm is used for monolayer sorption on a homogeneous surface and expressed by the following equation:

$$q_e = \frac{K_L C_e}{1 + a_L C_e} \quad (11)$$

In a linear form, it is expressed as follows:

$$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L C_e}{K_L} \quad (12)$$

Where C_e is the equilibrium concentration ($\mu\text{g mL}^{-1}$), q_e is the amount of solute sorbed per gram of sorbent; K_L and a_L are the Langmuir sorption isotherm constants and are related to the maximum capacity (Lg^{-1}) and bonding strength (Lmg^{-1}) respectively. The theoretical monolayer capacity is Q_0 and is numerically equal to K_L/a_L . The isotherms are shown in figures 3.7.11 and 3.7.12.

Desorption Procedure

For the desorption studies, chemically treated saw dust was loaded with the 10.0 mL solution mixture of selected triazine herbicides (2.0 and 4.0 $\mu\text{g mL}^{-1}$ solution of metribuzin, atrazine and ametryn and 3.0 and 5.0 $\mu\text{g mL}^{-1}$ of terbutryn) in 5.0 mL

borosilicate glass column. The column was equilibrated with 10.0 mL of water before loading it with solution of selected triazine herbicide. The solutions were allowed to equilibrate for 6 min. The sorbed herbicides were eluted with methanol. Elutes were collected from the column and were filtered using glass syringe equipped with nylon membrane filters (pore size 0.45 μm), followed by separation and determined by HPLC. The sawdust column was regenerated after each sample analysis by washing with 2 x 10.0 mL of aqueous solution of methanol (1:1 methanol: water) followed 10 mL of water. Desorption percentage ($\%_{\text{des}}$) of the selected triazine herbicides released by sawdust is given by equation (3).

$$\%_{\text{des}} = \frac{q_e \cdot m_c}{C_{\text{ed}} \cdot V_{\text{sd}}} 100 \quad (13)$$

Where q_e (mgg^{-1}) is the amount of the selected herbicides in one gram of sawdust, m_c (g) is the mass of the sawdust, C_{ed} ($\mu\text{g mL}^{-1}$) is the equilibrium concentration of the selected triazine herbicides in solution after desorption and V_{sd} is the volume of the desorption solution.

Recoveries for separation of triazine herbicides sorbed on sawdust column was studied using different aqueous solutions of methanol as an eluent and the results are shown in the figure 3.7.12. Different concentrations (2.0 and 4.0 $\mu\text{g mL}^{-1}$ of metribuzin, atrazine and ametryn and 3.0 and 5.0 $\mu\text{g mL}^{-1}$ of terbutryn in a solution mixture) of the herbicides were loaded onto the column. The results are given in table 3.7.12 and shown in figure 3.7.11.

To obtain high enrichment of the herbicides, the recoveries of metribuzin, atrazine, ametryn and terbutryn were investigated for different volumes of the solution mixture of selected herbicide (30.0, 50.0, 100.0, 200.0 and 300.0) and the results are given in table 3.7.13 and are shown in the figure 3.7.12.

Table 3.7.5: Optimization of the amount of sawdust for sorption of selected triazine herbicides

Analyte	Weight (g)	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Sorption (%)
Metribuzin	0.03	8	6.34	20.8 $\pm$ 0.09
	0.05	8	2.34	70.8 $\pm$ 0.07
	0.1	8	1.44	82.0 $\pm$ 0.10
	0.15	8	1.49	81.4 $\pm$ 0.03
	0.2	8	1.37	82.9 $\pm$ 0.05
	0.3	8	0	100.0 $\pm$ 0.23
	0.5	8	0	100.0 $\pm$ 0.10
Atrazine	0.03	8	6.66	16.8 $\pm$ 0.08
	0.05	8	3.74	53.3 $\pm$ 0.16
	0.1	8	2.12	73.5 $\pm$ 0.34
	0.15	8	2.29	71.4 $\pm$ 0.10
	0.2	8	1.35	83.4 $\pm$ 0.05
	0.3	8	0	100.0 $\pm$ 0.07
	0.5	8	0	100.0 $\pm$ 0.31
Ametryn	0.03	8	6.29	21.4 $\pm$ 0.11
	0.05	8	4.69	41.4 $\pm$ 0.05
	0.1	8	3.73	53.4 $\pm$ 0.13
	0.15	8	3.85	51.9 $\pm$ 0.06
	0.2	8	1.79	77.6 $\pm$ 0.25
	0.3	8	0	100.0 $\pm$ 0.12
	0.5	8	0	100.0 $\pm$ 0.10
Terbutryn	0.03	8	6.06	24.3 $\pm$ 0.06
	0.05	8	5.36	33.0 $\pm$ 0.04
	0.1	8	4.81	39.9 $\pm$ 0.07
	0.15	8	4.96	38.0 $\pm$ 0.03
	0.2	8	2.84	64.5 $\pm$ 0.06
	0.3	8	0	100.0 $\pm$ 0.17
	0.5	8	0	100.0 $\pm$ 0.10

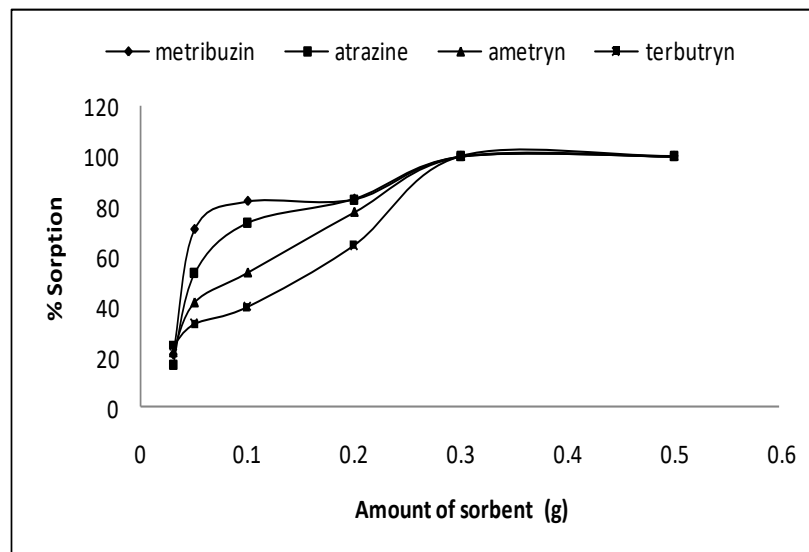


Figure 3.7.4 The effect of sawdust amount on sorption of selected triazine herbicides

Table 3.7.6: The effect of temperature on sorption of selected triazine herbicides on sawdust (n=3).

Analyte	Temperature (°C)	Recovery (%)
Metribuzin	60	76.3±0.27
	70	72.8±0.19
	80	62.0±0.36
Atrazine	60	86.5±0.24
	70	84.6±0.12
	80	58.5±0.16
Ametryn	60	89.3±0.26
	70	89.1±0.25
	80	60.9±0.27
Terbutryn	60	96.6±0.20
	70	97.7±0.32
	80	86.5±0.14

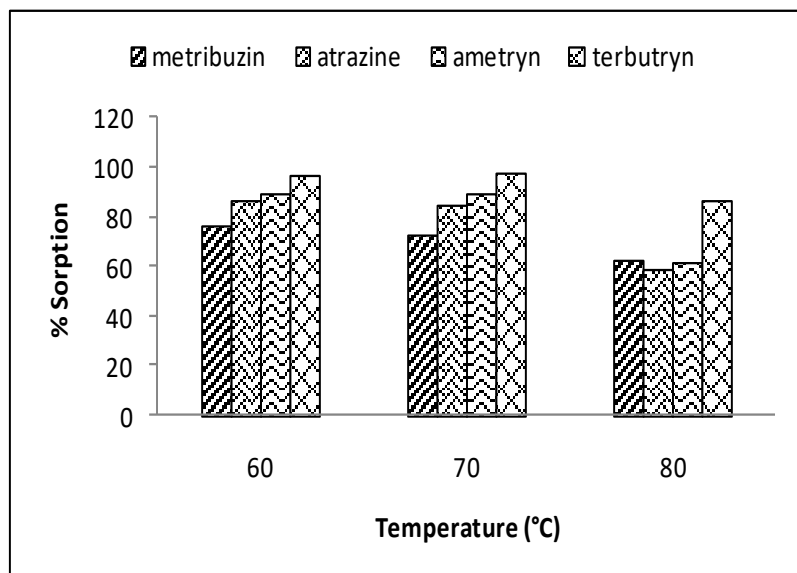


Figure 3.7.5 The effect of temperature on sorption of selected triazine herbicides on sawdust (n=3)

Table 3.7.7: Thermodynamics parameter for sorbed selected triazine herbicides on sawdust

Analyte	ΔG° (KJmol ⁻¹)			ΔH° (kJmol ⁻¹)	ΔS° (kJK ⁻¹ mol ⁻¹)
	333 K	343 K	353 K		
Metribuzin	-12.33	-12.08	-12.10	-16.18	-11.97 x 10 ⁻³
Atrazine	-15.16	-14.96	-12.85	-53.28	-11.35 x 10 ⁻²
Ametryn	-15.16	-14.76	-12.92	-53.28	-11.27 x 10 ⁻²
Terbutryn	-18.68	-18.29	-15.06	-8.1 x 10 ⁻⁴	0.016

Table 3.7.8: Effect of equilibration time on sorption of selected triazine herbicides on sawdust (n=3)

Analyte	Time (min)	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Sorption (%)
Metribuzin	3	4.5	3.74	83.3 $\pm$ 0.03
	6	4.5	4.27	94.9 $\pm$ 0.07
	9	4.5	4.19	93.1 $\pm$ 0.10
	12	4.5	4.26	94.7 $\pm$ 0.11
	15	4.5	3.81	84.8 $\pm$ 0.16
Atrazine	3	6.0	5.33	88.8 $\pm$ 0.06
	6	6.0	6.17	102.8 $\pm$ 0.19
	9	6.0	6.00	100.0 $\pm$ 0.24
	12	6.0	5.34	89.0 $\pm$ 0.21
	15	6.0	4.72	78.8 $\pm$ 0.08
Ametryn	3	6.0	5.25	87.6 $\pm$ 0.13
	6	6.0	5.73	95.5 $\pm$ 0.14
	9	6.0	5.58	93.0 $\pm$ 0.27
	12	6.0	5.42	90.3 $\pm$ 0.09
	15	6.0	5.01	83.5 $\pm$ 0.32
Terbutryn	3	4.0	3.27	81.9 $\pm$ 0.15
	6	4.0	4.05	101.2 $\pm$ 0.33
	9	4.0	3.94	98.5 $\pm$ 0.08
	12	4.0	3.93	98.3 $\pm$ 0.09
	15	4.0	3.45	86.3 $\pm$ 0.17

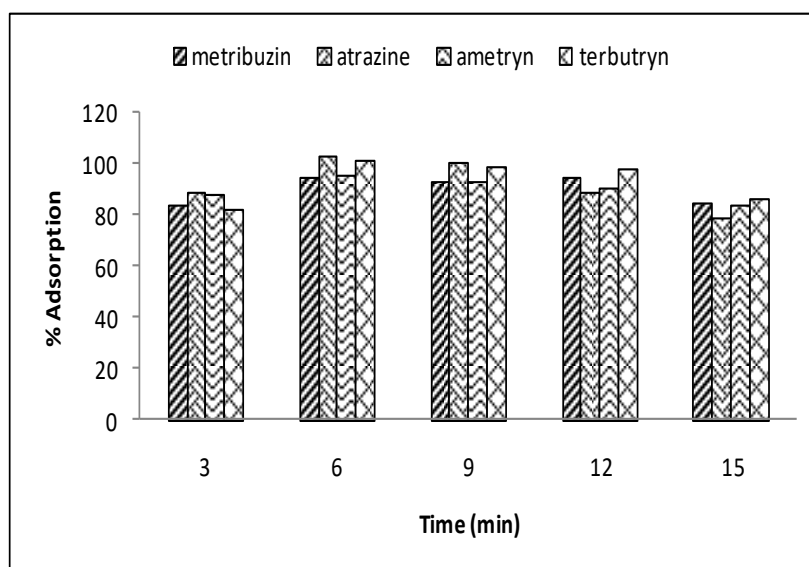
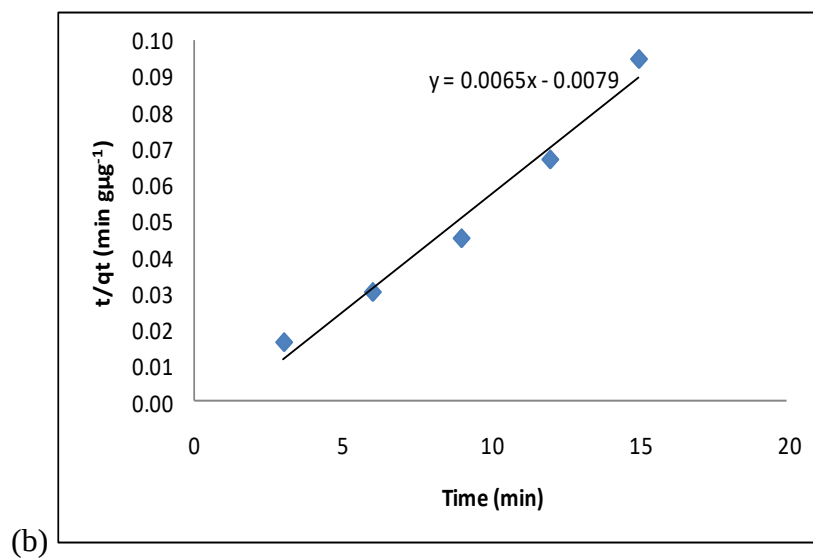
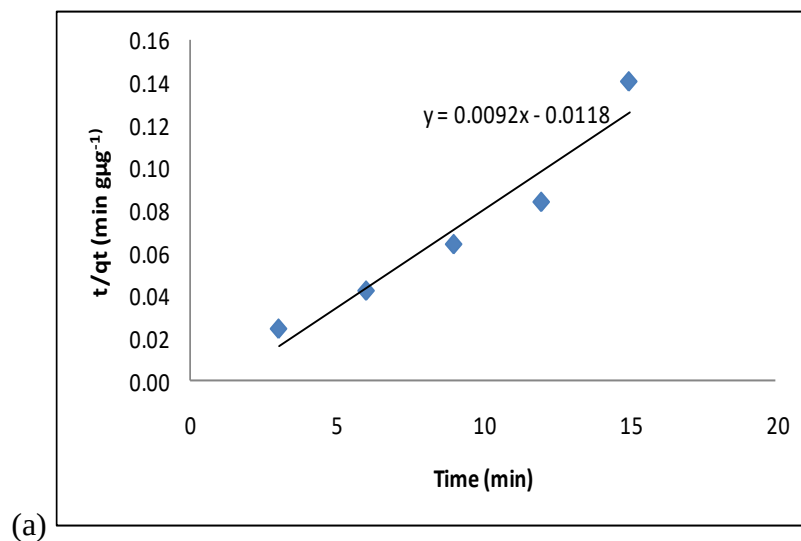


Figure 3.7.6 Effect of equilibration time on sorption of selected triazine herbicides on sawdust (n=3)

Table 3.7.9: Kinetic parameters for the sorption of selected triazine herbicides

Analyte	Pseudo-first order			Pseudo-second order		
	K_1 (min^{-1})	q_e (mgg^{-1})	r^2	K_2 ($\text{g mg}^{-1}\text{min}^{-1}$)	q_e (mgg^{-1})	r^2
Metribuzin	-8.79	1.03	0.145	0.0150	84.74	0.932
Atrazine	-0.43	1.96	0.142	0.0096	126.58	0.976
Ametryn	-0.13	2.84	0.141	0.0007	526.00	0.978
Terbutryn	-0.02	3.96	0.0008	0.0013	294.00	0.997



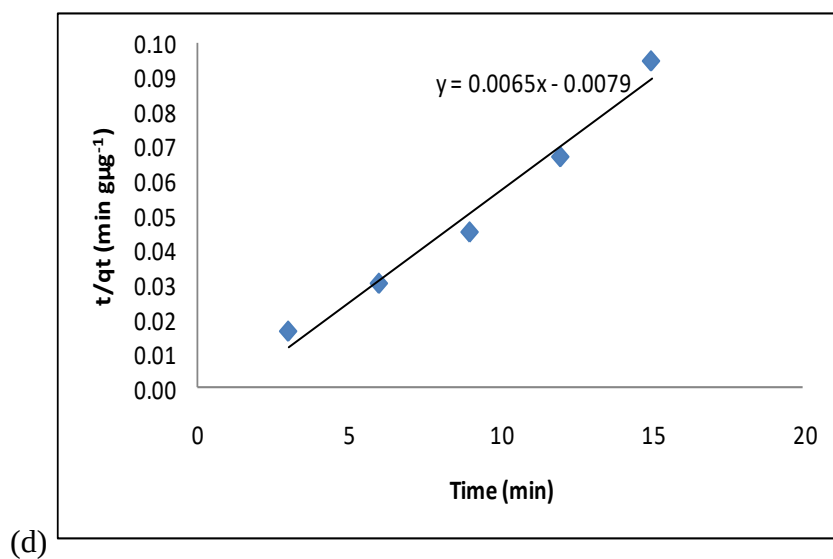
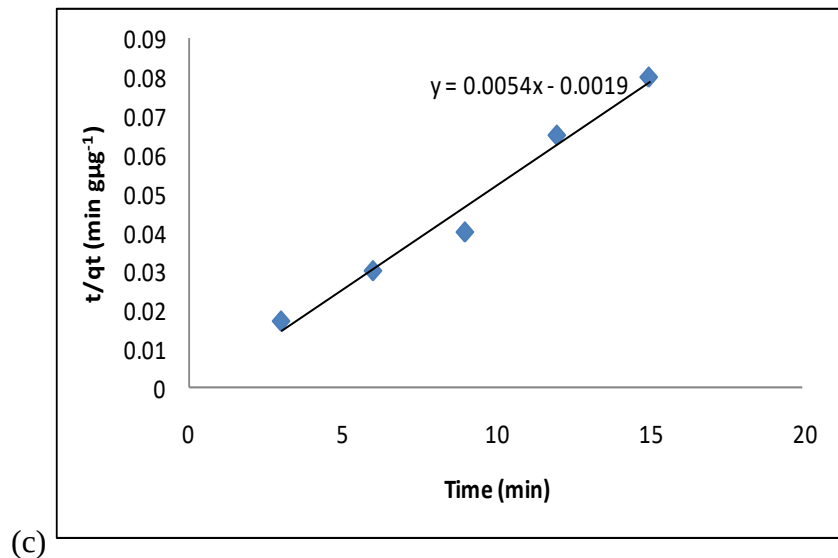


Figure 3.7.7 Pseudo-second kinetics of the selected triazine herbicides on sawdust, (a) metribuzin, (b) atrazine, (c) ametryn and (d) terbutryn

Table 3.7.10: Freundlich parameters for the sorption of selected triazine herbicides on sawdust

Analyte	r^2	Adsorption Intensity (n)	K_f (mgg⁻¹)
Metribuzin	0.976	0.149	2.24
Atrazine	0.944	0.121	2.23
Ametryn	0.960	0.124	2.29
Terbutryn	0.989	0.182	2.38

Table 3.7.11: Langmuir parameters for the sorption of selected triazine herbicides on sawdust

Analyte	K_L(Lg⁻¹)	Q_o (mgg⁻¹)	a_L(Lmg⁻¹)	r^2
Metribuzin	6.761	3.952	1.710	0.990
Atrazine	7.087	3.360	2.109	0.989
Ametryn	2.039	4.411	0.462	0.992
Terbutryn	11.682	4.215	2.771	0.996

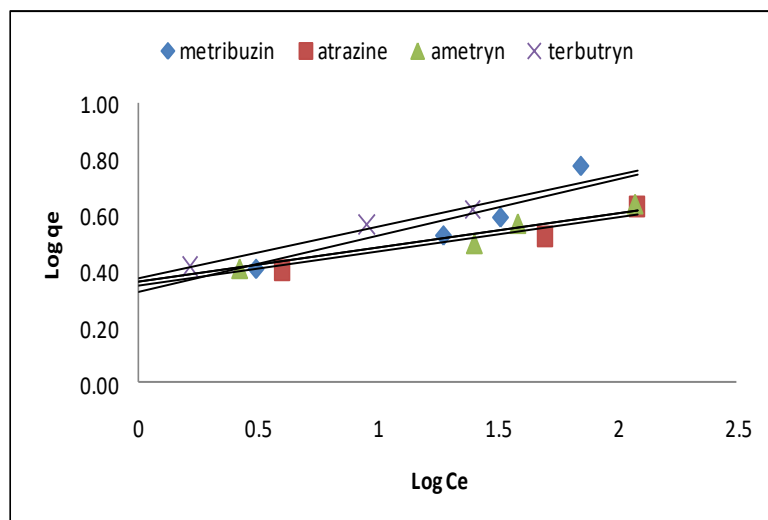


Figure 3.7.8 Linear form of Freundlich isotherm for sorption of selected triazine herbicides on sawdust

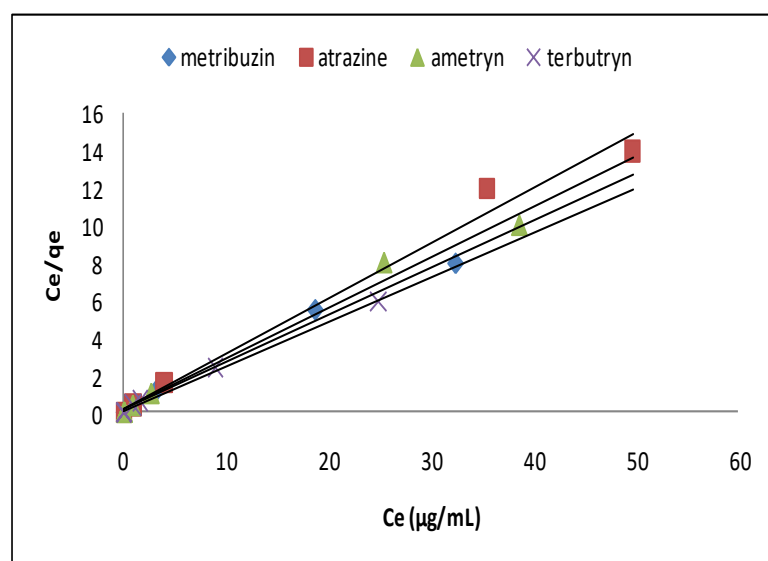


Figure 3.7.9 Langmuir isotherm for sorption of selected triazine herbicides on sawdust

Table 3.7.12: Effect of aqueous methanol solution on the recovery (%) of selected triazine herbicides on sawdust

Analyte	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)		
			Aqueous solution of methanol (%)		
			5	20	100
Metribuzin	2	2.00	100.0 $\pm$ 0.08	0	0
	4	3.83	95.75 $\pm$ 0.09	0	0
Atrazine	2	1.65	82.50 $\pm$ 0.07	0	0
	4	3.25	81.25 $\pm$ 0.11	0	0
Ametryn	2	1.71	0	105.0 $\pm$ 0.25	0
	4	4.27	0	85.50 $\pm$ 0.32	0
terbutryn	3	2.65	0	0	88.33 $\pm$ 0.15
	5	5.09	0	0	101.8 $\pm$ 0.23

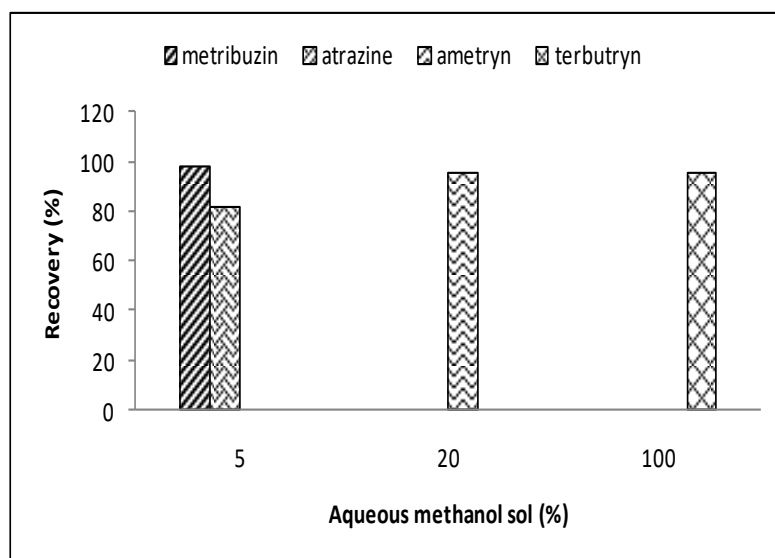


Figure 3.7.10 Effect of aqueous methanol solution on the recovery (%) of selected triazine herbicides on sawdust (n=3)

Table 3.7.13: Effect of sample volume on sorption of selected triazine herbicides on sawdust (n=3)

Analyte	Volume (mL)	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Sorption (%)
Metribuzin	30	3	2.77	92.3 $\pm$ 0.10
	50	3	2.45	81.7 $\pm$ 0.13
	100	3	2.53	84.3 $\pm$ 0.09
	200	3	0.38	12.7 $\pm$ 0.04
	300	3	0.27	09.0 $\pm$ 0.07
Atrazine	30	3	2.78	92.7 $\pm$ 0.12
	50	3	2.62	87.3 $\pm$ 0.14
	100	3	2.70	90.0 $\pm$ 0.06
	200	3	0.63	21.0 $\pm$ 0.05
	300	3	0.84	21.0 $\pm$ 0.19
Ametryn	30	3	2.83	94.3 $\pm$ 0.21
	50	3	2.84	94.7 $\pm$ 0.16
	100	3	2.88	96.0 $\pm$ 0.18
	200	4	4.02	100.0 $\pm$ 0.09
	300	3	2.94	73.5 $\pm$ 0.13
Terbutryn	30	3	3.01	100.0 $\pm$ 0.08
	50	3	3.0	100.0 $\pm$ 0.15
	100	3	2.98	99.3 $\pm$ 0.21
	200	4	4.25	106.2 $\pm$ 0.19
	300	4	3.92	98.0 $\pm$ 0.11

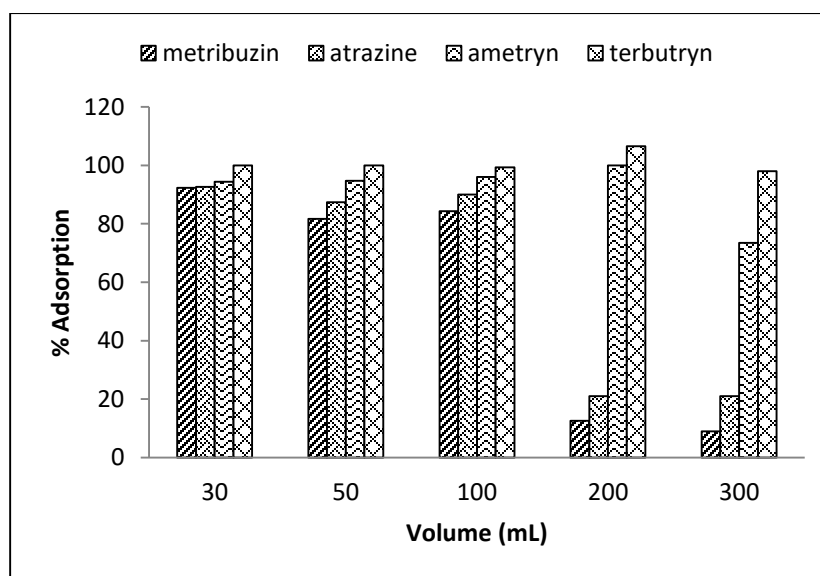


Figure 3.7.11 Effect of sample volume on sorption of selected triazine herbicides on sawdust (n=3)

3.7.3 Application of the investigated method for the analysis of selected triazine herbicides in real sample

Instrument: The same as mentioned before

Column preparation for preconcentration of samples

Sorption studies for the samples (water and sugarcane) were carried out using a 5.0 mL glass column. The column was packed with known amount (0.3 g) of the chemically treated sawdust. The column was equilibrated with 10.0 mL of water before loading it with sample of selected triazine herbicide.

Sample collection, preparation and extraction

The proposed method was evaluated by analyzing water samples (500.0 mL) and a sugarcane juice sample (250.0 mL diluted with distilled water to 500.0 mL). The water samples were collected from river Jhelum and from a selected tube well. The water samples were filtered and centrifuged to remove any suspended particles. The sugarcane was collected from field and was washed thoroughly with water to remove dust and dirt. Sugarcane juice was obtained by crushing the sugarcane in the sugarcane crushing machine locally available in the market and was filtered and centrifuged to remove any suspended particles.

The water samples (500.0 mL) were passed through the column in five attempts (100.0 mL each time) and the column was eluted with methanol (10.0 mL) each time. These extracts were combined and the volume of the combined extract (50.0 mL) was reduced to 10.0 mL by evaporating the solution on water bath at 40.0 °C and then analyzed by reverse phase HPLC. There were no detectable levels of target analyte in these samples. The water sample from river Jhelum (10.0 mL) was taken in nine separate beakers and was spiked with different concentration of selected triazine herbicides (atrazine (4.0, 5.0 and 7.0 $\mu\text{g mL}^{-1}$), metribuzin (4.0, 5.0 and 7.0 $\mu\text{g mL}^{-1}$), ametryn (4.0, 5.0 and 9.0 $\mu\text{g mL}^{-1}$) and terbutryn (4.0, 5.0 and 9.0 $\mu\text{g mL}^{-1}$). The water sample of the tube well was taken in six separate beakers and were spiked with solution mixture (10.0 mL) of selected herbicides (5.0 and 9.0 $\mu\text{g mL}^{-1}$ of atrazine, metribuzin, ametryn and terbutryn). These samples were loaded on the column and were eluted with methanol (10.0 mL). The elutes

were collected and filtered using glass syringe equipped with nylon membrane filters (pore size 0.45 μm) and determined using reverse phase HPLC. Results of these analyses are given in table 3.7.14.

Sugarcane sample (500.0 mL after dilution with distilled water) was passed through the column in five attempts (100.0 mL each time) and the column was eluted with methanol (10.0 mL) each time. These extract were combined and volume of the combined extract (50.0 mL) was reduced to 10.0 mL by evaporating the solution on water bath at 40.0 $^{\circ}\text{C}$ and then analyzed by reverse phase HPLC. There were no detectable levels of target analyte in these samples. Sugarcane juice (1.0 mL) was taken from the concentrated juice obtained from the sugarcane in the first place and were diluted to 20.0 mL with water in six separate beakers and were spiked with different concentration of selected triazine herbicides (atrazine (5.0 and 6.0 $\mu\text{g mL}^{-1}$), metribuzin (5.0 and 6.0 $\mu\text{g mL}^{-1}$), ametryn (6.0 and 8.0 $\mu\text{g mL}^{-1}$) and terbutryn (6.0 and 8.0 $\mu\text{g mL}^{-1}$). The elutes were collected and filtered using glass syringe equipped with nylon membrane filters (pore size 0.45 μm) and determined using HPLC. Results of these analyses are given in table 3.7.14.

Typical chromatogram obtained for water samples and sugarcane juice sample are shown in figure 13-22.

Table 3.7.14: Determination of selected triazine herbicides in water and sugarcane samples with the proposed method (n=3)

Analyte	Samples	Conc. added ($\mu\text{g mL}^{-1}$)	Conc. found ($\mu\text{g mL}^{-1}$)	Recovery (%)
Metribuzin	Water sample 1*	4.0	3.72	93.0 ± 0.60
		5.0	4.36	87.2 ± 0.51
		7.0	6.25	89.3 ± 0.16
	Water sample 2*	5.0	4.63	92.6 ± 0.13
		9.0	8.26	91.8 ± 0.05
	Sugarcane	5.0	3.38	67.6 ± 0.11
		6.0	4.55	75.8 ± 1.08
Atrazine	Water sample 1	4.0	3.74	93.5 ± 0.34
		5.0	4.21	84.2 ± 0.53
		7.0	6.62	94.6 ± 0.13
	Water sample 2	5.0	4.69	93.8 ± 0.21
		9.0	8.12	90.2 ± 0.13
	Sugarcane	5.0	3.92	78.4 ± 0.24
		6.0	4.93	82.2 ± 1.30
Ametryn	Water sample 1	4.0	3.98	99.5 ± 0.11
		5.0	4.64	92.8 ± 0.28
		9.0	8.24	91.6 ± 0.22
	Water sample 2	5.0	4.71	94.2 ± 0.46
		9.0	8.11	90.1 ± 0.16
	Sugarcane	6.0	5.05	84.7 ± 0.06
		8.0	6.44	80.5 ± 1.87
Terbutryn	Water sample 1	4.5	4.26	94.7 ± 0.29
		5.0	4.73	94.6 ± 0.12
		9.0	8.98	99.8 ± 0.25
	Water sample 2	5.0	4.96	99.2 ± 0.71
		9.0	8.54	94.9 ± 0.10
	Sugarcane	6.0	5.48	91.3 ± 0.07
		8.0	6.87	85.9 ± 2.08

Water sample 1 = Water from river Jhelum

Water sample 2 = Water from selected tube well

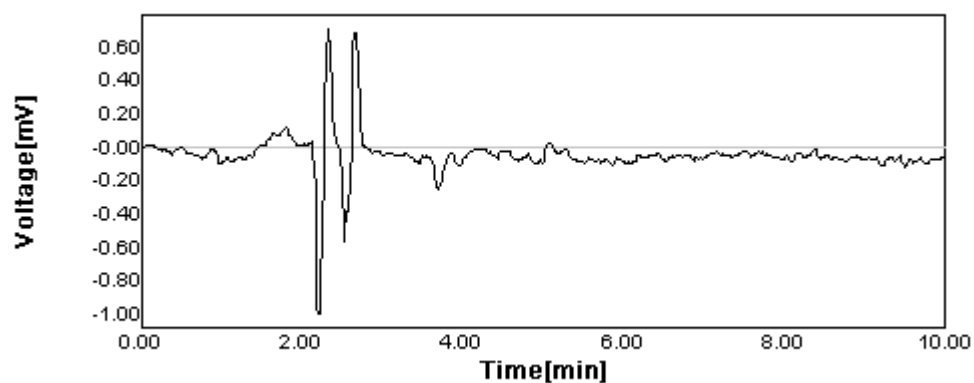


Figure 3.7.12 Chromatogram of unspiked water sample 1 using reverse phase HPLC

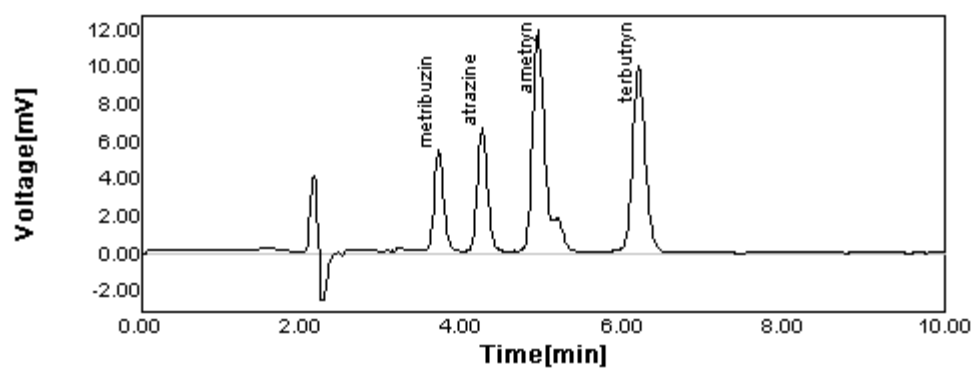


Figure 3.7.13 Chromatogram for 3.0 $\mu\text{g mL}^{-1}$ spiked water sample 1 using reverse phase HPLC

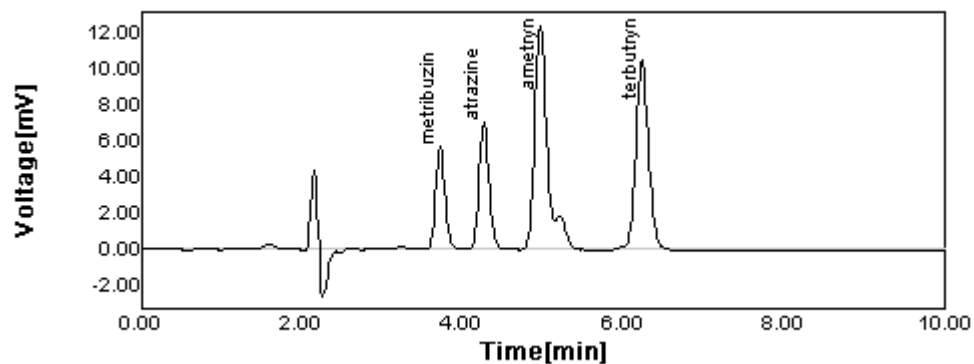


Figure 3.7.14 Chromatogram for 5.0 $\mu\text{g mL}^{-1}$ spiked water sample 1 using reverse phase HPLC

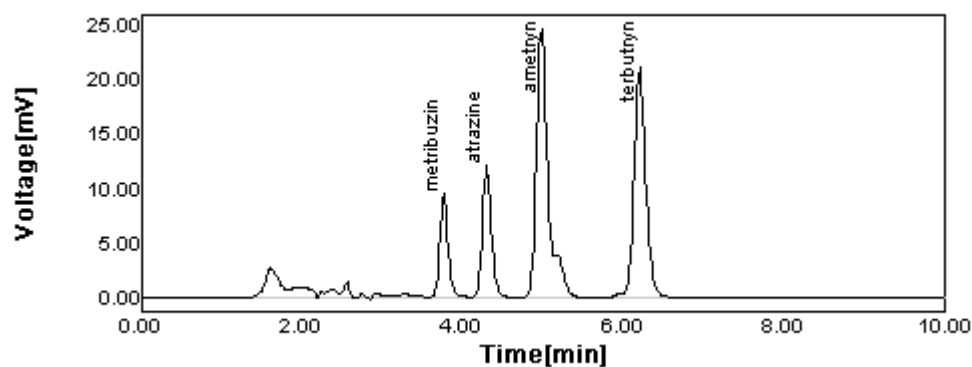


Figure 3.7.15 Chromatogram for 7.0 $\mu\text{g mL}^{-1}$ spiked water sample 1 using reverse phase HPLC

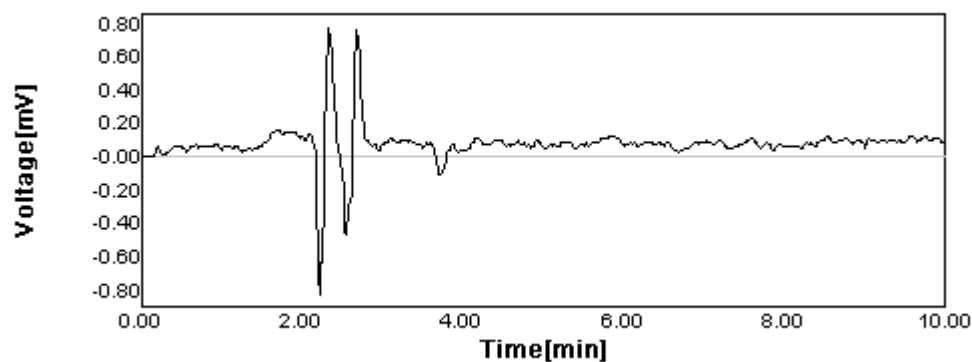


Figure 3.7.16 Chromatogram of unspiked water sample 2 using reverse phase HPLC

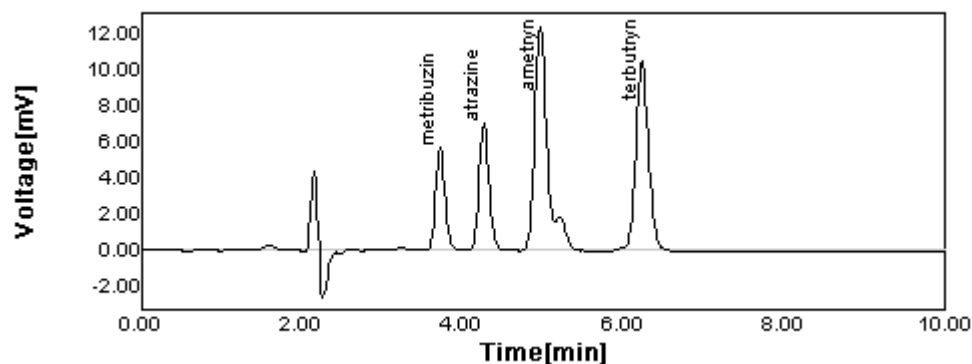


Figure 3.7.17 Chromatogram for 5.0 $\mu\text{g mL}^{-1}$ spiked water sample 2 using reverse phase HPLC

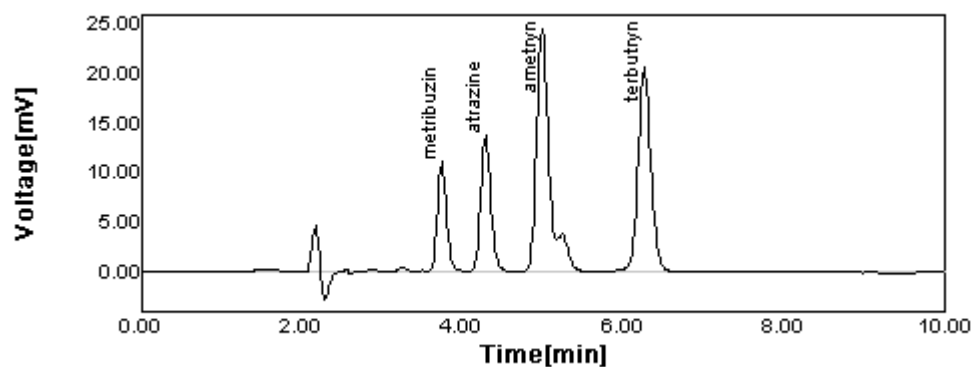


Figure 3.7.18 Chromatogram for 9.0 $\mu\text{g mL}^{-1}$ spiked water sample 2 using reverse phase HPLC

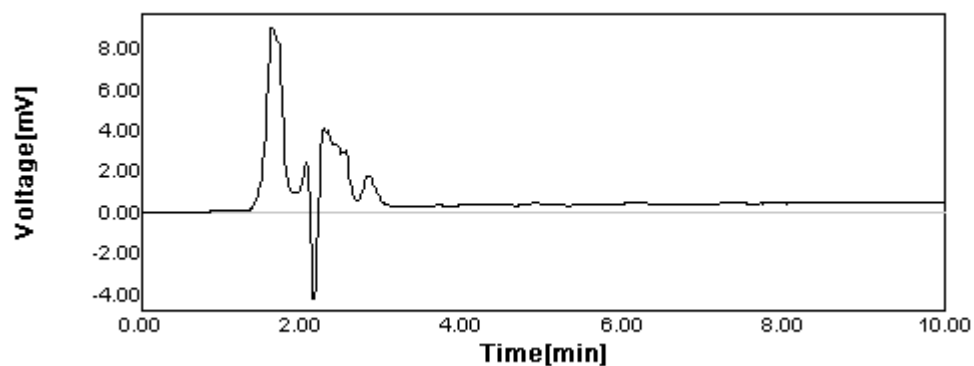


Figure 3.7.19 Chromatogram for unspiked sugarcane juice sample using reverse phase HPLC

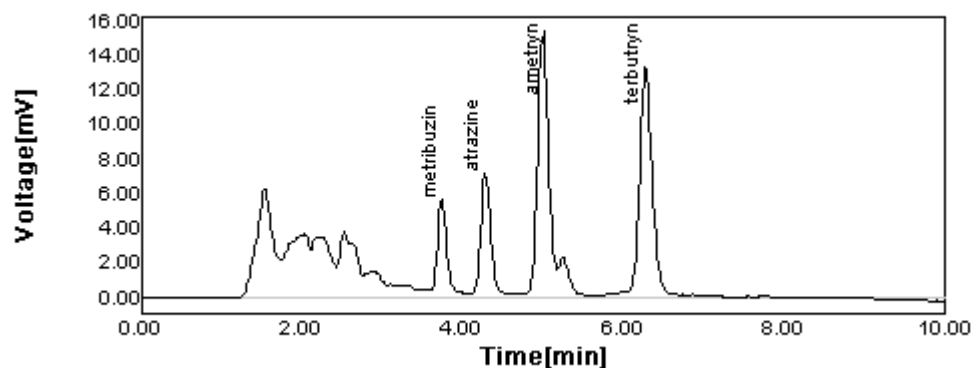


Figure 3.7.20 Chromatogram for 5.0 $\mu\text{g mL}^{-1}$ spiked sugarcane juice sample using reverse phase HPLC

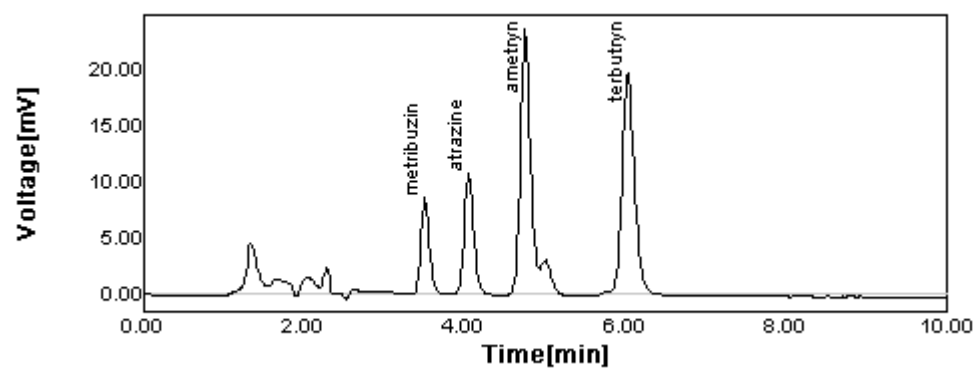


Figure 3.7.21 Chromatogram for 6.0 $\mu\text{g mL}^{-1}$ spiked sugarcane juice sample using reverse phase HPLC

3.7.4 Results and discussion

A low cost biosorbent (mulberry wood sawdust) has been investigated for the preconcentration and separation of selected triazine herbicides. Sawdust was characterized using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX).

SEM analysis showed that the chemically treated sawdust has larger pore size than the untreated sawdust (Figure 3.7.3). The EDX analysis showed that the untreated sawdust contained calcium, carbon and oxygen whereas calcium was removed from chemically treated sawdust (Figure 3.7.4).

Sorption (%) of the selected triazines increased significantly with the increasing amount of biosorbent. It was found that 0.3 g of sawdust was enough to give 100.0 % sorption of selected triazine herbicide ($8.0 \mu\text{g mL}^{-1}$). The results are given in table 3.7.5 and are shown in figure 3.7.5.

The temperature effect of the sorption of selected herbicide on sawdust was studied at 60.0, 70.0 and 80.0 °C and the results are given in table 3.7.6 and are shown in figure 3.7.6. It was observed that with increase in temperature, there is a slight decrease in sorption of all triazine herbicides on sawdust. The decrease in sorption can be correlated with increase in solubility with increase in temperature. Further increase in temperature decreased the interaction of the selected triazine with the surface of sawdust particles. The order of solubility of the triazine herbicides in water is metribuzin > atrazine > ametryn > terbutryn. Since metribuzin is more soluble in water, desorption is more pronounced with increase in temperature. At 60.0 °C, % sorption of 76.0, 86.0, 89.0 and 96.0 % was observed for metribuzin, atrazine, ametryn and terbutryn respectively. Further decrease in % sorption was observed at 70.0 and 80.0 °C for metribuzin, atrazine and ametryn herbicides.

The values for K_D , ΔG° , ΔH° and ΔS° were calculated. The negative value obtained for ΔH° indicated an exothermic sorption process. The exothermic sorption process could be due to increase in the rate of diffusion and mobility of selected triazine herbicides from external boundary layer into the internal pores of the sorbent due to increase in temperature. The positive value of ΔS° for terbutryn showed more affinity of sawdust for

the herbicide as compared to the negative value of ΔS° for metribuzin, atrazine and ametryn. Whereas the negative value of ΔG° for the selected triazine herbicides showed a spontaneous sorption process (Table 3.7.7).

The kinetic study of metribuzin, atrazine, ametryn and terbutryn were carried out on 0.3 g of sawdust column using 10.0 mL mixture of (4.5, 6.0, 6.0 and 4.0 $\mu\text{g mL}^{-1}$) metribuzin, atrazine, ametryn and terbutryn respectively. The equilibration time was studied at 3.0, 6.0, 9.0, 12.0 and 15.0 min. The results are given in table 3.7.8 and figure 3.7.7. Sorption of the triazine herbicides increased when the equilibration time was increased from 3.0 to 6.0 min. It was observed that % sorption remained the same at 9.0 and 12.0 min of equilibration time. With further increase in the total equilibration time, decrease in the sorption was observed because of diffusion of these herbicides back in methanol.

Sorption studies revealed that the experimental data fits well to the second order kinetics with correlation coefficient (r^2) values of 0.932, 0.976, 0.978 and 0.999 for metribuzin, atrazine, ametryn and terbutryn respectively (Figure 3.7.8). Kinetic parameters were calculated for the herbicides sorption on sawdust using pseudo-first-order and pseudo-second-order reaction equation (Table 3.7.9).

The sorption isotherms of the herbicides on sawdust were studied by the two most commonly used isotherms models Freundlich and Langmuir isotherms (Figure 3.7.9 and 3.7.10). The Freundlich isotherm is used for non-ideal sorption on heterogeneous surfaces. Different parameters calculated from the slope and intercept of Freundlich and Langmuir isotherms for selected triazine herbicides are given in the table 3.7.7 and 3.7.8. The sorption data of the selected triazine herbicides studied best fit the Langmuir isotherm with sorption capacity (Q_0) of 3.952, 3.360, 4.411 and 4.215 mg g^{-1} for metribuzin, atrazine, ametryn and terbutryn respectively.

Separation of triazine herbicides under study was achieved on the sawdust column using different aqueous solutions of methanol as shown in the figure 3.7.11. Different concentrations of the herbicides were loaded onto the column given in table 3.7.12. Metribuzin and atrazine were eluted completely with 5.0 % of methanol solution and ametryn and terbutryn were left onto the column as such. Ametryn was eluted with 20 %

and terbutryn with 100 % of methanol solution. Elutes collected were diluted to 10 mL with methanol and analyzed by HPLC.

To obtain high enrichment of the herbicides, the recoveries of metribuzin, atrazine, ametryn and terbutryn were investigated for different volumes of the solution as shown in the figure 3.7.12. The sorption of metribuzin, atrazine, ametryn and terbutryn were more than 80.0 % up to 100.0 mL of volume of solution passed through the sawdust column. When the volume used was more than 100.0 mL, the recoveries of metribuzin and atrazine dropped to 9.0 and 21.0 % respectively for 300.0 mL of herbicide solution whereas the ametryn and terbutryn had recoveries more than 90.0 %.

The proposed method was evaluated by analyzing water samples and sugarcane sample collected. The water samples spiked with different concentration of the four herbicides are given in the table 3.7.14. The recoveries (%) from water samples (river Jhelum) obtained for metribuzin, atrazine, ametryn and terbutryn were 93.0, 94.0, 99.0 and 99.0 % respectively. Similarly the recoveries (%) from tube well water obtained for metribuzin, atrazine, ametryn and terbutryn were 92.0, 93.0, 94.0 and 99.0 % respectively. Recoveries (%) from sugarcane samples were 75.0, 82.0, 84.0 and 91.0 for metribuzin, atrazine, ametryn and terbutryn respectively.

3.8 SYNTHESIS AND CHARACTERIZATION OF AMETRYN MOLECULARLY IMPRINTED POLYMER (MIP) AND IT'S APPLICATION FOR BINDING CAPACITY OF AMETRYN HERBICIDE

In this work MIP for group selective extraction of triazine class of herbicides are synthesized. Three different functional monomers methacrylic acid, maleimide and 4-vinyl pyridine were used to prepare ametryn MIP's. The general reaction mechanism is shown in figure 3.8.1.

In previous studies carried out by Ye's group, they found that reducing the cross-linking density using technical grade divinylbenzene-55 (DVB-55) resulted in monodispersed beads with relatively larger particle size useful to be used as HPLC stationary phase [1, 2]. In this work, using methacrylic acid (MAA) as functional monomer, DVB-55 as a cross linker and methylethyl ketone (MEK): heptane (30:70) as the reaction solvent, ametryn imprinted polymer was synthesized.

In order to study the approach of multiple hydrogen bonding interaction between the template ametryn and the functional monomer, maleimide was used in combination with DVB-55 and with a reaction solvent MEK. In another experiment MIP's were synthesized using DVB-55 and maleimide in reaction solvent MEK: heptane (70:30). The interaction between template and maleimide was believed to be based on the idea of three point hydrogen bond interaction of donor-acceptor-donor on the alternating maleimide-DVB polymer chain as shown in the figure 3.8.2. Using the same model of interactions between the alternating copolymer of styrene and maleimide with copolymer of styrene and 2,4-diamino-6-vinyl-1,3,5-triazine, Meijer adopted this strategy of triple hydrogen bond interaction to get highly compatible heterogeneous polymers [3].

Based on the idea of nucleic acid' base pairs as reported earlier by Haginaka, synthesis of uniformly sized ametryn (pka 3.8) imprinted polymer was done using 4-vinylpyridine (4-VPY) (pka 5.62) in acetonitrile using DVB-55 as a cross linker. Haginaka reported synthesis of ametryn-imprinted polymer using 4-VPY with ethylene glycol dimethacrylate (EDMA) as a cross linker by multi-step swelling and polymerization [4].

This study was also carried out to investigate potential ability of the maleimide to be used as functional monomer for preparation of new MIP's as solid phase extraction material for triazine herbicides. Based on the better binding capacity of the MIP's prepared using MAA as functional monomer as compared to maleimide and 4-vinyl pyridine, these were further investigated for the selectivity of other triazines namely atrazine, simazine, propazine, prometryn and terbutryn.

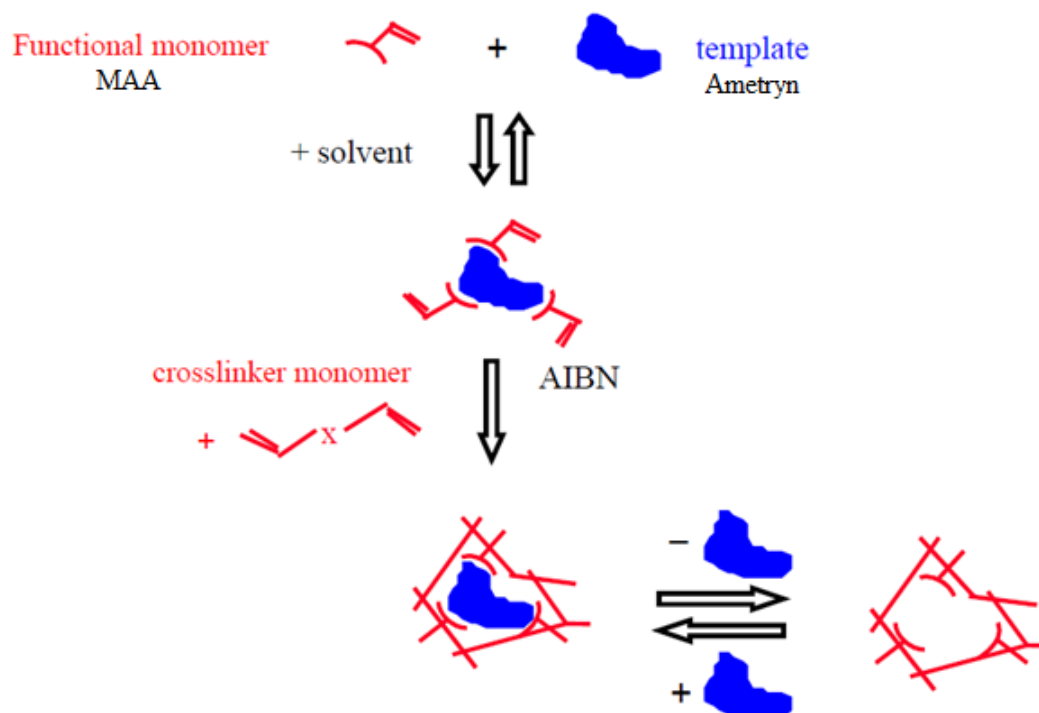


Figure 3.8.1 General reaction mechanism for synthesis of ametryn molecularly imprinting polymer

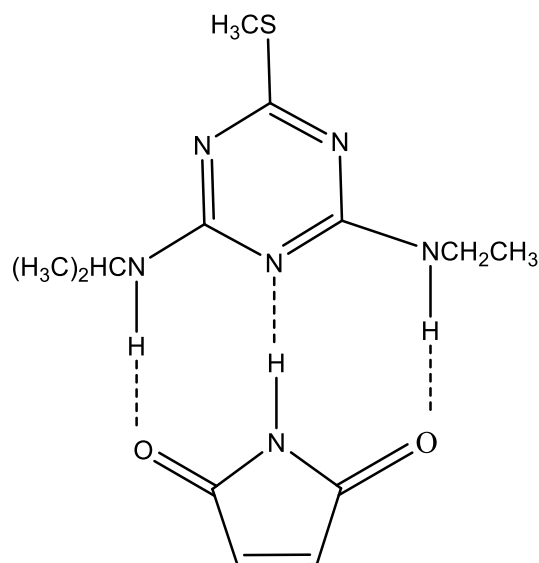


Figure 3.8.2 Proposed three point hydrogen bond interactions (donor-acceptor-donor) between ametryn and maleimide molecule

3.8.1 Investigation of suitable parameters for the HPLC determination of selected triazine herbicides

Instrument

Scanning electron microscopy (SEM) imaging was done on a JEOL JSM-6700F Field Emission Scanning Electron Microscope (Tokyo, Japan). Polymer microspheres were sputter coated with gold prior to the SEM measurement. A Lachrom Merck Hitachi HPLC equipped with L-7100 gradient pump, diode array detector L-7455, solvent degasser L-7612 and auto sampler L-7200 was used. All HPLC analysis was carried out on Lichrospher RP-18 column, 5 μm , Merck, Darmstadt Germany. Stovall hybridization oven (Greensboro, NC, USA) and Branso 3200 (USA) ultrasonic cleanser was used for polymer preparation and sonication respectively. Centrifugation was carried out on a Biofuge pico (Kendro Laboratory, Germany). Determination of yield (%) of the particles formed was done gravimetrically.

Reagents and chemicals

All chemicals used were of analytical grade or similar purity. Ametryn (98.8 %), atrazine (99.2 %), terbutryn (99.0 %), divinylbenzene (DVB, technical grade, 55.0 %, mixture of

isomers), maleimide (99.0 %), heptane (anhydrous 99.0 %), methylethyl ketone (MEK), 4-vinylpyridine (4VPY, 95.0 %) and 2,2'-azobisisobutyronitrile (AIBN, 98.0 %) were purchased from Sigma-Aldrich. DVB-55 was passed through the aluminium oxide column to remove the inhibitor, p-tert-butylcatechol. Prometryn (98.8 %), propazine (99.3 %), simazine (99.0 %) and acetonitrile (HPLC grade) were purchased from Fluka. Acetic acid (glacial, 100.0 %) was obtained from Merck. Methacrylic acid (MAA, 98.5 %) was purchased from ACROS (Geel, Belgium). Solvents like MEK, heptane and acetonitrile were used as received. Ultra pure deionized water was used for HPLC mobile phase.

Standard triazine solution (1000 $\mu\text{g mL}^{-1}$)

Working standard of 100.0 $\mu\text{g mL}^{-1}$ and 0.4-10.0 $\mu\text{g mL}^{-1}$ solution were prepared in the same way as mentioned in Part V of chapter III.

Optimization of HPLC parameters

All physical parameters such as wavelength, flow rate, solvent elution mode, and time for elution and chemical parameters like solvents and composition of solvents for isocratic elution were optimized for the HPLC determination of triazine group of herbicides following the same procedure as mentioned in part V of Chapter III. All the optimized parameters are presented in table 3.8.1 and were used for subsequent analysis. The characteristics for HPLC-UV determination of selected triazines were calculated from this and the results are summarized in table 3.8.2.

Table 3.8.1 Optimum conditions for determination of selected triazine herbicides using reverse phase HPLC

Parameters	Values
Eluent used (acetonitrile: water)	70: 30
Flow rate (mL min^{-1})	1.0
Injection volume (μL)	10.0
Wavelength (nm)	254.0

Table 3.8.2 Characteristics for HPLC-UV determination of selected triazines

Analysis	Characteristics					
	Atrazine	Propazine	Simazine	Prometryn	Ametryn	Terbutryn
Working concentration range ($\mu\text{g mL}^{-1}$)	2.0—10	2.0—10	2.0—10	2.0—10	0.4—10	2.0—10
Limit of detection ($\mu\text{g mL}^{-1}$)	0.45	0.60	0.33	0.69	0.57	0.45
Limit of quantification ($\mu\text{g mL}^{-1}$)	1.5	2.0	1.1	2.3	1.9	1.5
Standard deviation	0.15	0.20	0.11	0.23	0.19	0.15
Relative standard deviation	7.5	10.0	5.7	11.7	9.5	7.6
Regression equation	$1015 x + 5202.8$	$763.15 x + 4462.3$	$1200.9 x + 7072.8$	$913.23 x + 4891.1$	$2902.8 x + 9937.5$	$1380.7 x + 5238.7$
Correlation Coefficient (r^2)	0.9983	0.9983	0.9939	0.9928	0.9985	0.9946

3.8.2 Synthesis of ametryn imprinted polymer for the determination of ametryn herbicide using reverse phase HPLC

Polymerization Procedure

Precipitation polymerization was used for the synthesis of molecularly imprinted microspheres under the condition given in table 3.8.3. Ametryn being the template molecule was dissolved in preparation solvent (40.0 mL) in a 150 mm x 25 mm borosilicate glass tube equipped with screw cap. The functional monomer (MAA, maleimide and 4-VPY), the cross-linking monomer (DVB) and the initiator (AIBN) were added to it. These solutions were then purged with a gentle flow of nitrogen gas for 5 min

and finally sealed under N₂ flow. Polymerization was carried out in hybridization oven by fixing the borosilicate glass tubes horizontally between the clips of rotating wheel and were rotated at a speed of 20 rpm. Temperature was gradually increased from 20.0 to 60.0 °C for the first 20 min and thereafter kept constant at 60.0 °C for 24.0 hr for MIP₁ (synthesized using functional monomer MAA) microsphere formation. For synthesis of MIP₂ (synthesized using functional monomer maleimide) and MIP₃ (synthesized using functional monomer 4-VPY) microsphere, temperature was gradually increased from 20.0 to 70.0 °C for 20.0 min and thereafter kept constant at 70.0 °C. After the polymerization process was completed, particles were separated and collected from the supernatant by centrifugation. The template was removed from the ametryn MIP's by batch mode solvent extraction using methanol containing 10.0 % acetic acid (v/v) until no ametryn was detected in washing solvent. These solutions were analyzed by using UV spectrometric measurement. Microspheres free of template were washed with methanol and acetone and dried in a vacuum chamber. The non imprinted reference polymers were also prepared under the same conditions except for adding the template to the reaction mixture.

Saturation experiment

A known weight (10.0 mg) of a well dried polymer was taken in a microcentrifuge tubes (2.5 mL) and to this was added a known amount (10.0 µgmL⁻¹ in acetonitrile) of target herbicide. These microcentrifuge tubes were incubated at room temperature. After giving it 24.0 hr of maximum binding time, the supernatant was separated from the particles by centrifuging at 5000 rpm for 10.0 min. The supernatant was filtered with 13.0 mm syringe filter w/0.2 µm PTFE membrane and was analyzed using HPLC. The amount of analyte bound to the polymer was calculated by subtraction of free fraction detected in the supernatant from total amount added to polymer. The data obtained was the mean values of triplicate measurements.

Table 3.8.3 Synthesis of ametryn imprinted polymer

Polymer	Template (mmol)	Functional monomer (mmol)	DVB-55 (mmol)	Agitation	Size (μm)	Yield (%)	Reaction temp (°C)	Solvent volume (mL)
MIP ₁	0.53	MAA (1.31)	5.25	yes	1.6-2.5	35	60	40
MIP ₂	0.53	Maleimide (0.53)	0.53	yes	1.0	25	70	25
MIP ₃	0.53	4-VPY (1.31)	5.25	yes	1.0	35	70	40

Effect of percentage of cross linking monomer and solvent composition on the polymer morphology

The solvent system used with the each of the functional monomer, the characterization, binding capacity and molecular recognition studies of the polymers synthesized is discussed in detail as following.

Two different commercially available forms of DVB, DVB-55 and DVB-80 were used as cross linker for MIP₁ particle formation.

The solvent effect was studied for the synthesis of MIP₁ (MAA/DVB-55) polymer using neat acetonitrile and also by using varied ratio of two solvents (MEK and heptane). The results are given in table 3.8.4. The solvent system was varied from 20:80 to 35:65 ratio of MEK: heptane.

The reaction solvent MEK: heptane was also used for MIP₂ polymer synthesis (maleimide/ DVB-55) the results are given in table 3.8.5. Polymers were synthesized using different ratios of the two solvents. The polymer formation with 4-vinylpyridine was done only in acetonitrile.

Table 3.8.4: The effect of reaction solvent on the polymer (MAA/DVB) morphology

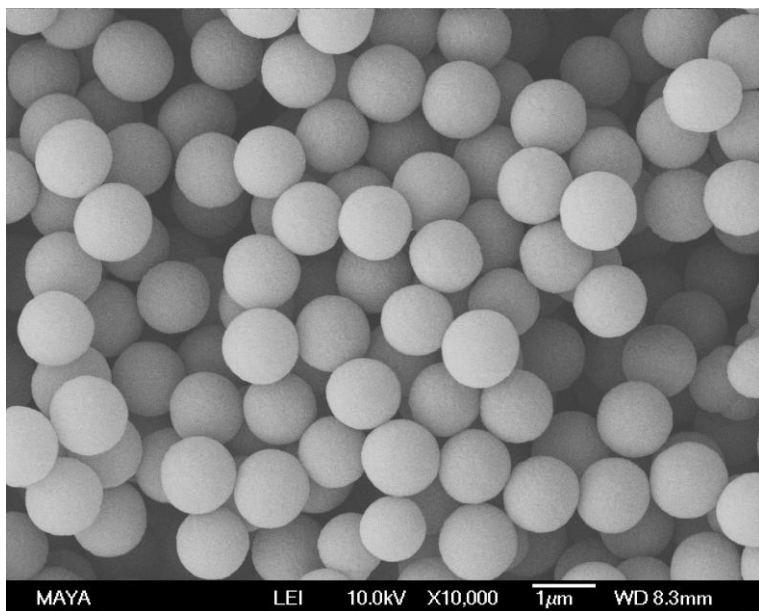
Experiment	Reaction solvent MEK: heptane	Observations
1	20:80	highly polydispersed, coagulum
2	22:78	highly polydispersed, coagulum
3	25:75	Slightly polydispersed
4	30:70	monodispersed particles
5	35:65	No polymer
6	Pure acetonitrile	Slightly polydispersed

Table 3.8.5: The effect of reaction solvent on the polymer (maleimide/DVB) morphology

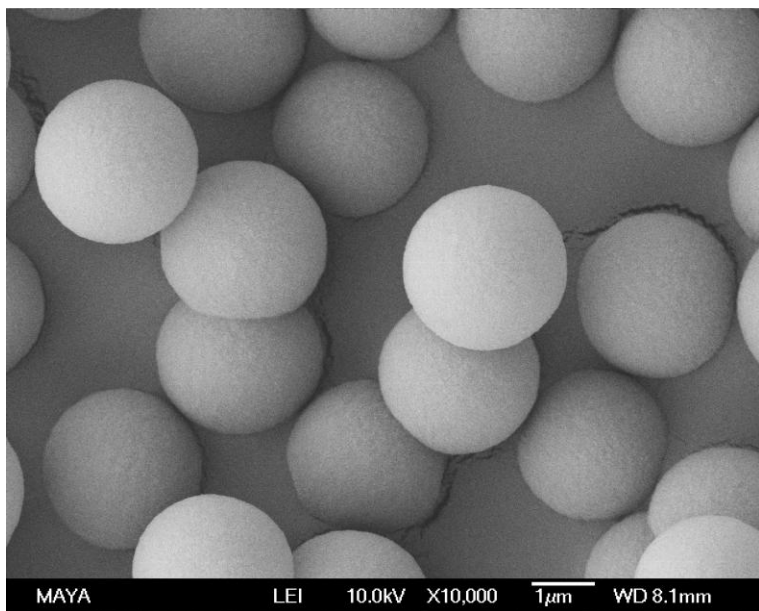
Experiment	Reaction solvent MEK: heptane	Observations
1	40:60	Gels
2	50:50	Gels
3	60:40	Gels
4	70:30	monodispersed particles
5	80:20	monodispersed particles
6	90:10	monodispersed particles
7	Pure MEK	monodispersed particles
8	Pure acetonitrile	monodispersed particles

SEM Characterization of the polymers

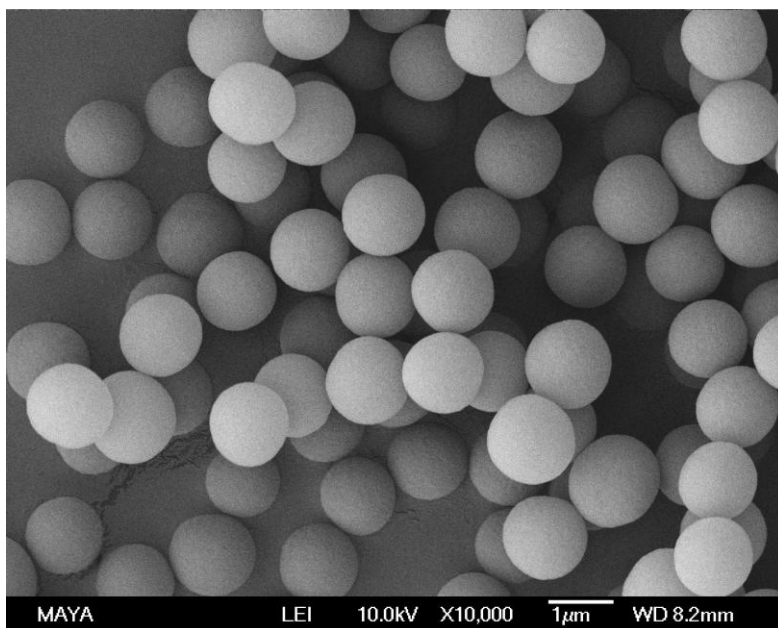
The approximate yield (%) of MIP₁ (MAA/DVB) polymer was 35.0 %, MIP₂ (maleimide/DVB) and MIP₃ (4-VPY/DVB) was 25.0 % and 35.0 % respectively. Use of DVB-55 resulted in larger particles in micron-range. The SEM study of the synthesized polymers (MIP's and NIP's) was carried out by sputtering the microsphere with gold particles and the images are shown in the figure 3.8.3.



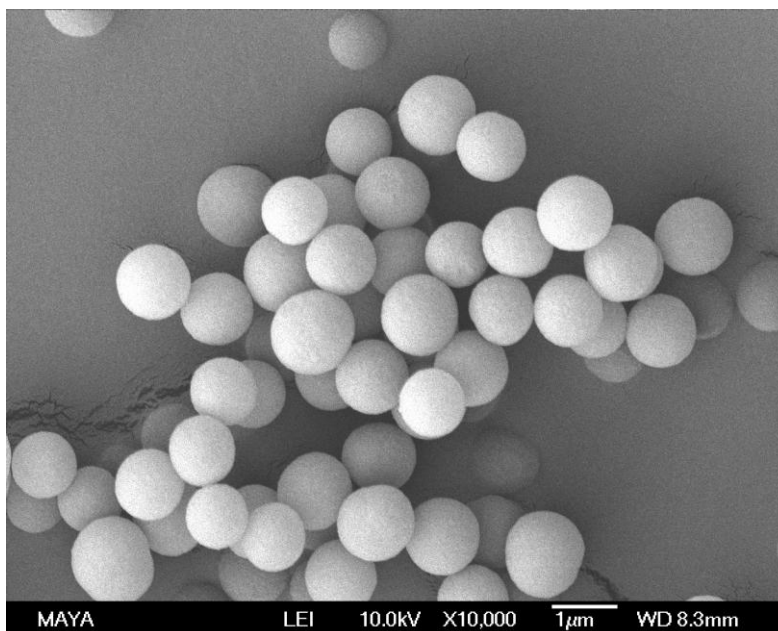
(a)



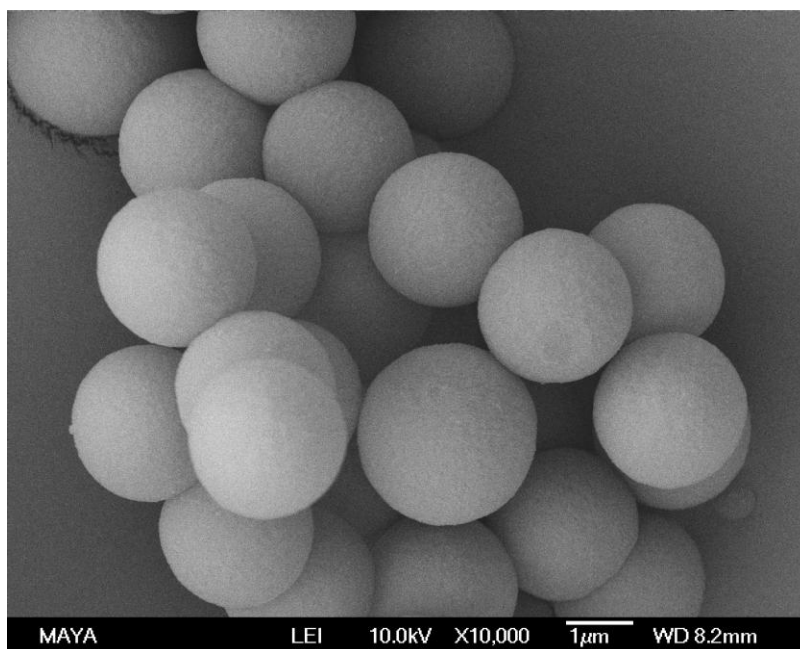
(b)



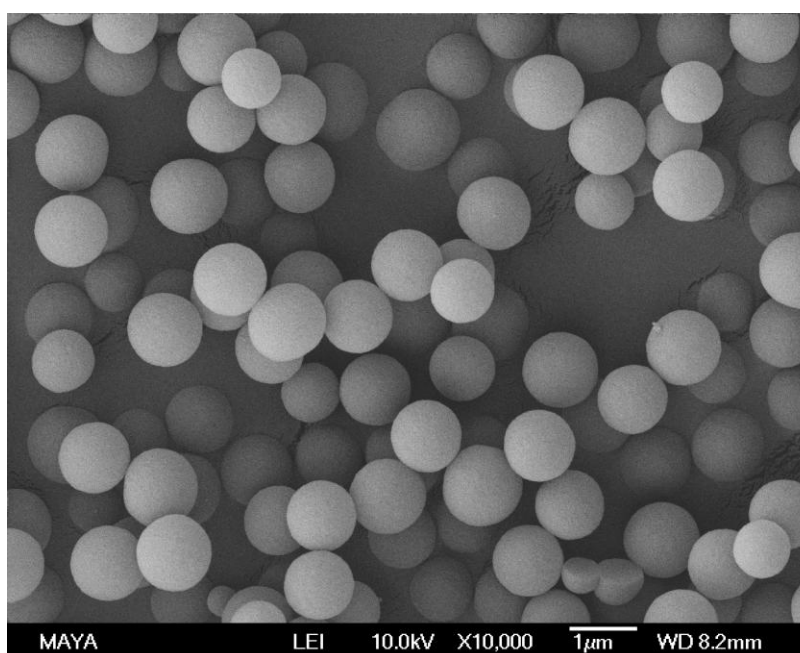
(c)



(d)



(e)



(f)

Figure 3.8.3 Scanning electron micrographs of (a) Mip Maleimide/DVB, (b) Mip MAA/DVB, (c) Mip 4-vinylpyridine/DVB, (d) Nip Maleimide/DVB, (e) Nip MAA/DVB, (f) Nip 4-vinylpyridine/DVB

Study of the binding capacity of the polymers for ametryn

The specific binding sites provided by the imprinted particles were determined by measuring the difference in the ametryn uptake by the imprinted and non-imprinted particles of the three types of polymer synthesized. The binding capacities of MIP₁ and MIP₂ polymers prepared using MAA and maleimide were checked in aqueous and in pure acetonitrile and also in solvent mixtures of aqueous and acetonitrile, the results are given in tables (3.8.6 and 3.8.7) and shown in figure (3.8.4 and 3.8.5). The binding capacity of the polymer (synthesized using maleimide in solvent mixture of MEK: Heptane 70: 30) was also performed in different mixture of solvents, the results are given in table 3.8.8 and shown in figure 3.8.6. The binding experiments for all polymers carried out in acetonitrile are compared; the results shown in figure 3.8.7.

Table 3.8.6: Effect of solvent type on binding of ametryn to MAA/DVB based polymer (n=3)

Polymer	Acetonitrile	Aqueous	(ACN:Water) 20:80	(ACN:Water) 30:70	(ACN:Water) 50:50	(ACN:Water) 80:20
MIP	83.3±0.08	93.2±0.10	98.0±0.28	100.0±0.15	71.4±0.18	27.0±0.04
NIP	47.3±0.09	94.7±0.05	80.9±0.32	85.1±0.07	63.0±0.52	37.0±0.29

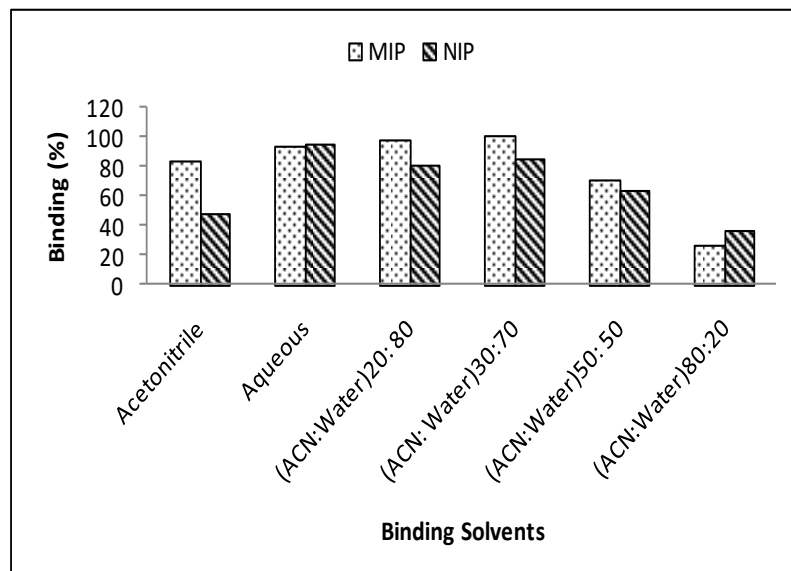


Figure 3.8.4 Effect of solvent type on binding of ametryn to MAA/DVB based polymer (n=3)

Table 3.8.7: Effect of solvent type on binding of ametryn to maleimide (synthesized in pure MEK) based polymer (n=3)

Polymer	Acetonitrile	Aqueous	(ACN:Water) 20:80	(ACN:Water) 30:70	(ACN:Water) 50:50
MIP	7.8±0.14	80.6±0.01	72.4±1.24	26.2±0.26	8.5±1.19
NIP	0.4±0.01	77.0±0.21	54.5±0.09	12.6±0.36	6.9±0.72

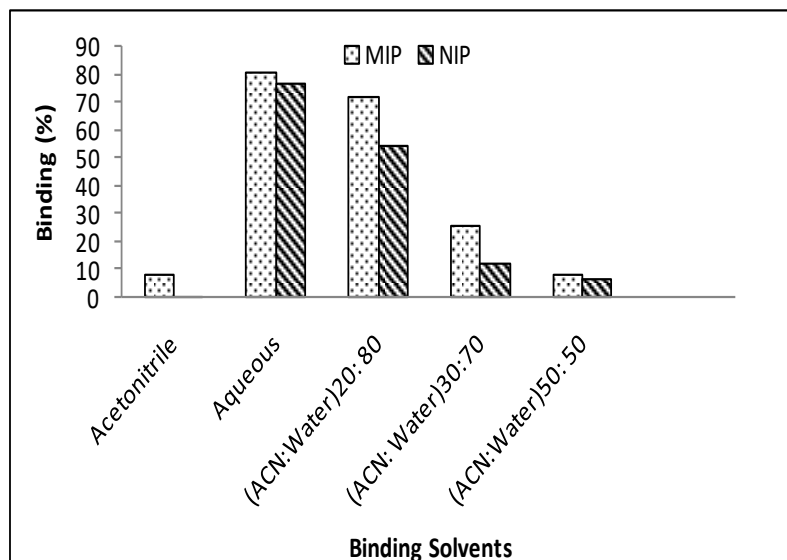


Figure 3.8.5 Effect of solvent type on binding of ametryn to maleimide (synthesized in pure MEK) based polymer (n=3)

Table 3.8.8: Effect of solvent type on binding of ametryn on maleimide (synthesized in 70:30 MEK: heptane) based polymer (n=3)

Polymer	1 % Acetic acid in ACN	3 % Acetic acid in ACN	Acetonitrile	Aqueous	ACN:Water (20:80)	ACN:Water (50:50)	ACN:Water (80:20)
MIP	26.9±0.44	28.2±0.41	31.0±0.44	98.0±0.19	55.3±0.18	26.4±0.28	27.6±1.04
NIP	27.1±0.52	28.9±0.11	34.8±0.11	97.3±0.45	56.8±0.64	28.5±0.1	42.3±0.53

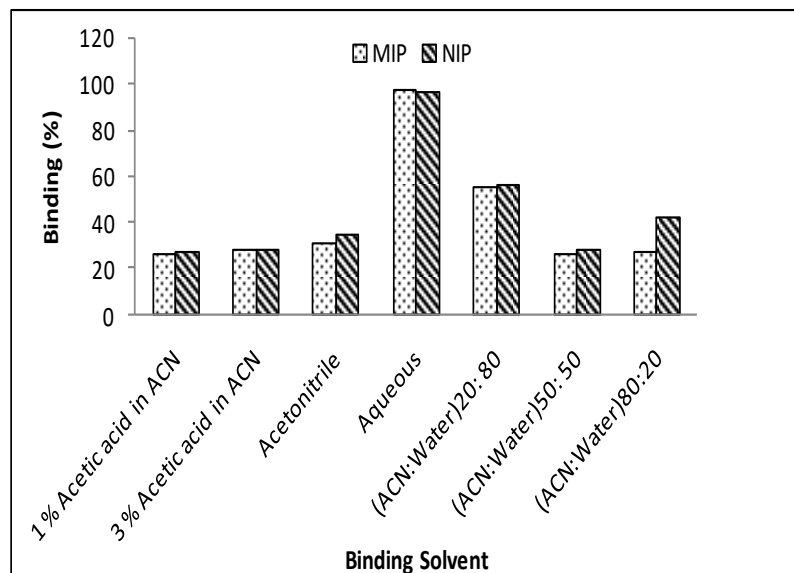


Figure 3.8.6 Effect of solvent type on binding of ametryn on maleimide (synthesized in 70:30 MEK: heptane) based polymer (n=3).

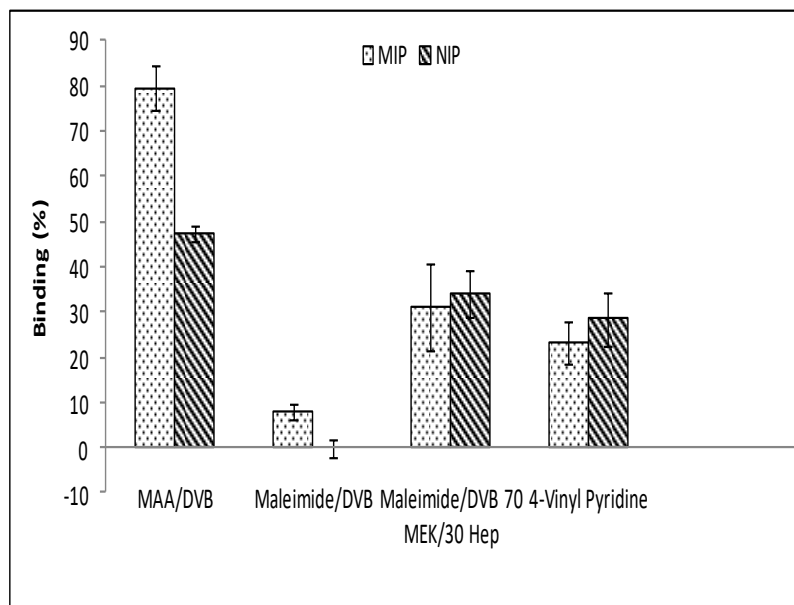


Figure 3.8.7 Binding capacity of different polymer for ametryn herbicide in acetonitrile

Molecular recognition and selectivity studies of the MAA- based polymer

In order to verify the specific binding capacity of ametryn, the molecular recognition studies were performed at different concentration of the ametryn standard solutions (2.0, 4.0, 6.0, 8.0 and 10.0 $\mu\text{g mL}^{-1}$). The standard solution of ametryn was added to both MIP's and NIP's. The results are shown in figure 3.8.8. Selectivity experiments of MIP₁ polymer were performed for other members of the triazine class of herbicides. The experiments were done by adding 10.0 $\mu\text{g mL}^{-1}$ solution of each triazine herbicide (atrazine, simazine, prometryn, propazine, terbutryn) to known amount of polymer (10.0 mg). The results are shown in figure 3.8.9.

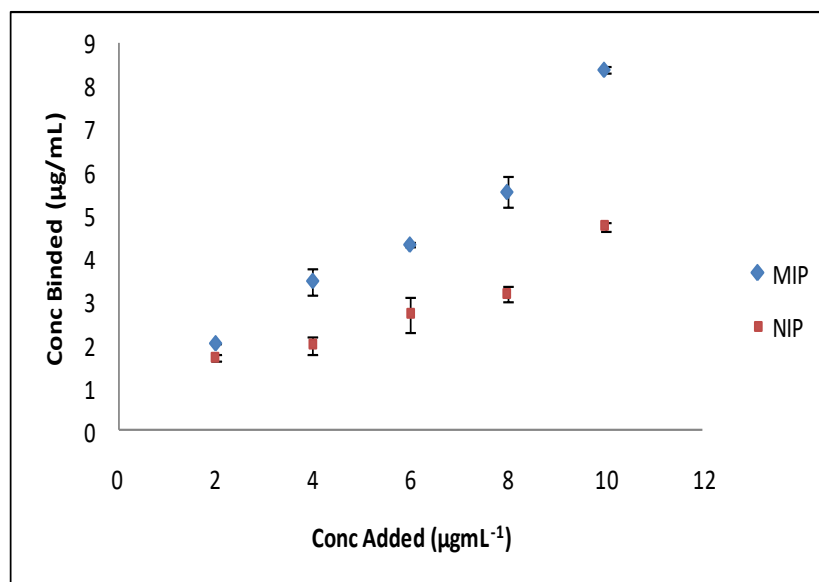


Figure 3.8.8 Molecular recognition studies of MAA/DVB polymer at different concentration of ametryn

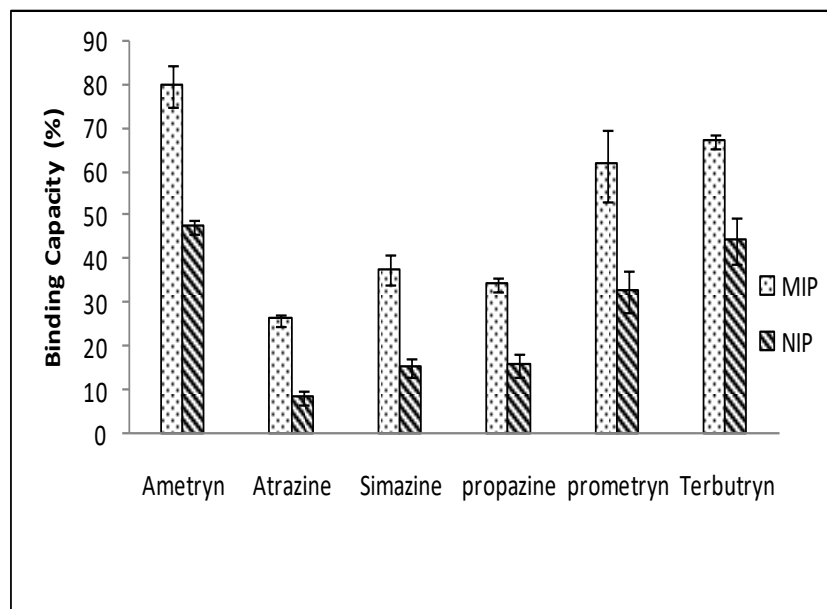


Figure 3.8.9 Binding capacity of MAA/DVB polymer for selected triazine herbicides

3.8.3 Result and Discussion

In previous studies conducted by Lei Ye's molecularly imprinted polymer group [1], they found synthetic conditions for obtaining polymer with controllable size in the range of 130.0 nm to 2.4 μm using propranolol as template molecule. The size of MIP beads was altered by varying composition of the cross-linking monomer DVB-55 and trimethylolpropane trimethacrylate (TRIM). The polymer was synthesized in acetonitrile using MAA as functional monomer and ethylene glycol dimethacrylate (EDMA) as a cross linker. The beads obtained were monodispersed and had excellent recognition property. In this work, three different functional monomers MAA, maleimide and 4-VPY were used to obtain monodispersed ametryn imprinted polymers with narrow size distribution range from 1.0-2.5 μm . All the binding experiments were carried out in polar organic solvent acetonitrile. The solvent combination of MEK: heptane has been optimized in this study to reduce the polydispersity of the MIP₁ polymer (using functional monomer MAA). The solvent condition was also screened for synthesis of MIP₂ polymer (using functional monomer maleimide). The solvent condition used for MIP₂ was selected because of the better solubility of the maleimide in the mixture of solvents. Acetonitrile was used as a solvent for synthesis of MIP₃ polymer (using 4-vinyl pyridine).

The use of DVB-80 resulted in polymer formation with small percentage of coagulum as well as high degree of polydispersity. The particles formed with DVB-55 were not very different from the one formed by using DVB-80 but had the advantage of being softer in morphology. Therefore DVB-55 was used for polymer synthesis.

The effect of reaction solvent was studied for the synthesis of MIP₁ (MAA/DVB-55) polymer. It was observed that polymer formed was polydispersed at low MEK to heptane ratio (20:80). As the MEK ratio was increased from 20:80 to 25:75, the polymer formed was slightly polydispersed. The microsphere formed with 30:70 of MEK: heptane appeared almost monodispersed. MEK: heptane ratio of 35:65 resulted in no polymer formation which was probably due to the greater solubility of the dissolved polymer in this solvent system. By using neat acetonitrile (and DVB-55 as cross linker) there was

observed an increase in the particle size with high degree of polydispersity and coagulum. The results are given in table 3.8.4.

The effect of reaction solvent was also studied for the synthesis of MIP₁ (maleimide/DVB-55) polymer. It was noticed that gel formation took place at lower MEK to heptane ratio; it was observed that low solubility of maleimide in heptane was the reason for the gel formation. When the ratio of MEK to heptane was increased from 60:40 to 70:30, uniform monodispersed particles were formed. By further decreasing the amount of heptane till neat MEK was used, it was observed that the particles formed are also monodispersed with narrow size distribution but at the same time resulted in reduced polymer yield. The same effect of decreased polymer yield was also observed when pure acetonitrile was used though the particles obtained were also uniform in shape and size and was monodispersed.

The polymer formation with 4-vinylpyridine was done only in acetonitrile and the polymer formed were found to be monodispersed with narrow size distribution range.

The SEM of the three types of polymer prepared was done by sputtering the microsphere with gold particles and the collection of images are shown in the figure 3.8.4. The MIP₁ (MAA/DVB) particle average diameters was 1.5-2.5 μm which in future could be used as stationary phase for HPLC analysis. These particles were monodispersed with uniform shape. Compared to the MIP₁ particles, the particles of the MIP₂ (maleimide/DVB) were smaller in size approximately of 1.0 μm as is shown in the SEM image. These particles were highly monodispersed with no sign of aggregation and with high degree of uniformity. Uniform monodispersed particles with narrow particle size of 1.0 μm were obtained for MIP₃ polymer (4-VPY/DVB system).

It was observed that binding capacity (35.0 % and 25.0 %) of polymers (synthesized with maleimide in 70:30 MEK: heptane and 4-vinylpyridine respectively) was approximately same to that of their non-imprinted polymer. It was because of the fact that the major contribution in the binding was by non-specific binding sites present in these particles. While MIP₂ polymer (using maleimide) prepared just in pure MEK showed 5.0 % binding and the non-specific binding from non-imprinted polymer was negligible.

Binding results of the MIP₁ polymer showed that the imprinted polymer is binding approximately twice (80.0 %) to that of the non-imprinted polymer (47.0 %). In MIP₂ polymer, the specific multiple hydrogen bond interaction (donor-acceptor-donor) between the template and the functional monomer is one dimensional on the alternating units of the polymer. Whereas the hydrogen bond interactions available on the functional monomer in the MIP₁ polymer is three dimensional and this polymer was further used for the molecular recognition studies of target molecule. It was observed from binding experiment that MIP₁ polymer had a high selectivity and greater binding capacity for the ametryn herbicide.

Selectivity experiments of MIP₁ polymer were performed for other members of the triazine class of herbicides. The results showed that the binding capacity was more for the methylthio triazine herbicides as compared to chloro triazines. These observations suggested that the molecular imprinting cavities match the closely related homolog of ametryn among the triazine group and the smaller molecules could not be held strongly by these cavities. Figure 3.8.10 shows the binding capacities of imprinted and non-imprinted polymer for atrazine, simazine, propazine, prometryn and terbutryn are (26, 37, 33, 61, 66 %) and (8, 14, 15, 32, 44 %) respectively.

3.8.4 References

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Conclusion

The aim of this work was to develop fast, sensitive, selective and inexpensive spectrophotometric methods for identification, speciation and quantification of triazine group of herbicides. Efficient extraction procedures were also investigated for the HPLC analysis of selected triazine herbicides. In the present work, eight new methods/procedures have been developed for determination of selected triazine herbicides in real samples.

The first method described is a spectrophotometric determination of atrazine herbicide. The method is based on coupling with sulfanilic acid or aniline leading to the formation of colored product which is stable at room temperature for 24.0 hr. The method does not involve any cleanup procedures as it is free from interference of a large number of foreign species. The method can be suitably adopted for routine analysis to check the purity of atrazine in their formulations and residue determination in real samples.

The second proposed method is a spectrophotometric method for determination of ametryn herbicide. The method can be used for separation and determination of ametryn residue in sugarcane samples as well as commercial herbicide formulations using solvent extraction method.

The third method described is for the spectrophotometric determination of metribuzin in commercial formulations and residue determination in vegetable samples. The optimum conditions for highest absorption due to maximum complexation reaction found experimentally were confirmed using an experimental design. The primary effective factors responsible for the maximum absorbance of complex were found to be metribuzin concentration and pH for complexation reaction.

The fourth method is for determination of metribuzin herbicide and is based on the reaction of p-dimethylaminobenzaldehyde (DMAB) with sodium hydroxide via Cannizzaro's reaction at 100.0 °C to form a p-dimethylaminobenzoic acid. The proposed method has been successfully applied for the analysis of commercial formulation and potato sample. The recoveries of the method were found to be in the range of 92.16 % ±

0.06 to 96.66 % $\pm$ 0.18. Two level factorial designs of 23 and 22 were used to optimize all parameters, determine the influence of different parameters and their interactions on the final product formation.

The fifth method describes a microwave assisted extraction (MAE) with extraction solvent acetonitrile/ethylacetate/methanol (3:2:5) followed by HPLC separation for recovery of atrazine, ametryn, metribuzin and terbutryn from soil sample. MAE followed by HPLC separation provided high recoveries (83.33 % $\pm$ 0.12 – 96.33 $\pm$ 0.23), minimum matrix effects and good separation. The results obtained for recovery, precision and accuracy show that the proposed method could be successfully used for identification, determination and quantification of these triazines in soil samples.

The sixth method is based on comparison of microwave assisted extraction (MAE), ultrasonic assisted extraction (UAE) and shake flask extraction procedure for the determination of triazine herbicides from corn sample were compared. Validation of the extraction procedures was studied by standard addition technique. The results indicated that MAE was superior to UAE and shake-flask methods in terms of short extraction time, low solvent use and high extraction efficiency with good reproducibility.

The seventh method described is about the removal efficiency of selected triazine herbicides using sawdust as sorbent. The method may be used for preconcentration as well as decontamination of triazine herbicides. The kinetic of adsorption were studied which shows that the equilibration time for deriving the adsorption isotherm is 6.0 min. The experimental results show that the ametryn and terbutryn can be completely separated on a saw dust using different aqueous solution of methanol. The developed method was successfully applied for the removal of selected herbicides from various aqueous and sugarcane sample.

The eight proposed method is for the preparation of molecularly imprinted polymers (MIPs) for ametryn herbicide using divinyl benzene (DVB) as a cross linker and methacrylic acid (MAA), maleimide and 4-vinyl pyridine as a functional monomer. Ametryn herbicide was better held by polymer prepared using MAA functional monomer. Uniform monodispersed particles with narrow size distribution were obtained

using maleimide as functional monomer. MAA polymer also showed good binding selectivity for chloro triazine, atrazine, simazine, propazine and methylthio triazine prometryn and terbutryn. The use of DVB as cross linker resulted in polymer formation with particle size between 1.0-2.5 μm .