

**DEVELOPMENT OF NEW METHODS FOR
DETERMINATION AND IDENTIFICATION
OF PESTICIDES IN REAL SAMPLE**

By

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**INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR**

DECEMBER 2006

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DETERMINATION AND IDENTIFICATION OF
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DISSERTATION

*SUBMITTED TO THE UNIVERSITY
OF PESHAWAR IN PARTIAL FULFILMENT OF
THE REQUIRMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSPHY IN CHEMISTRY*

INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR

DECEMBER 2006

Dated 8/11/08 Controller of Examination UOP.

**INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR**

It is recommended that the dissertation prepared by
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Identification of Pesticides in Real Sample"** be accepted as fulfilling this part of the
requirements for the Degree of
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To
My Parents

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ACKNOWLEDMENT

All the praise for almighty Allah who enabled and encouraged me to complete this work. He has the knowledge of known and unknown and equipped his humble creature with mental facility.

I feel great pleasure and honor in expressing my sincere gratitude to my supervisor, Prof. Dr. Rasul Jan for his valuable guidance, constant help and encouragement throughout this work. I will always remain grateful to him for the liberty he graciously granted me to encroach upon his precious time.

Words cannot express the nature and extent of my deepest appreciation for my co-supervisor Dr. Jasmine Shah. It was she, who was always there in the hour of need. I want to thanks her for her kind behavior, valuable advices, timely suggestions and constant encouragement all time.

I would like to express my appreciation to my parents for their love, prays and financial support. Special thanks to my brothers and sisters for their friendly and supporting attitude during my studies.

I want to special thanks to my best friend Fatima Malik without whom life seemed to be incomplete. Her encouraging attitude made me to cope up the troubles, which I had faced during the study. I feel proud to have such a nice friend whose fragrance of love will always blossom my yesterday, my day and my tomorrow.

Thanks to all of the former and present co-workers Mir Azam Khan, Dr Hamayun, Romana, fazal Mabood, fozia, Shahida, Kashif, Salman, Hussain and Rahim. They always extended their cooperation for research work relationship in the Analytical Section of this institute. Thanks to Laboratory assistant Mr. Israr Khan and Mr. Zahir Shah for cooperation through out this project.

Moral support and good wishes from all my colleagues and faculty members of this institute are most appreciated.

Nadia Bashir

SUMMARY

A variety of analytical methods are available for the analysis of herbicides in environmental samples. Most of these methods require well equipped laboratories, sophisticated and expensive instrumentation and technical manpower. These methods cannot be used on routine basis in developing countries like Pakistan. On the other hand lack of awareness among the people increases the random and inadequate use of herbicides in these countries. To protect environment and safeguard human health, there is an urgent need for the availability of such methods, which could be routinely used in these countries for monitoring herbicides content in soil, water and food products.

Accordingly the objective of this work was to investigate simple, rapid and economical spectrophotometric methods for analysis of various acidic herbicides. The combination of spectrophotometric method with a simple flow injection analyzer makes the methods more versatile due to simplicity and speed. Acidic herbicides studied are fluroxypyr, acetochlor, 2,4-dichlorophenoxy acetic acid (2,4-D), bromoxynil and chloramben.

The first chapter of the dissertation is introduction and covers an account of the acidic herbicides selected for this work. It includes information regarding physical, chemical, and toxicological aspects of fluroxypyr, acetochlor, 2,4-D, bromoxynil and chloramben.

The second chapter covers a thorough review of the relevant literature reported for the determination of these herbicides. This chapter includes chromatographic, spectrophotometric, enzyme link immunosorbant analysis, flow injection and other analytical techniques used for the determination of acidic herbicides in environmental and real samples. The method has been discussed with emphasis on scope, type of sample, reagent used, brief procedure, linearity range, detection limit and percent recoveries.

The third and last chapter contain experimental work with the results and discussion of the various methods investigated and its application for the analysis of active ingredients in formulation and residue level determination in real samples both by batch and online methods. This chapter is sub divided into five parts. Part I of this chapter deal with the method development strategy and proposed chemical reaction for spectrophotometric determination of fluroxypyr. The method is based on direct exploitation of primary aromatic amine group for the formation of diazotized reagent

followed by coupling with β -naphthol to form azo dye. Chemical and physical parameters were optimized for batch and online methods. The limit of detection (LOD) and limit of quantification (LOQ) for the investigated methods were found to be 0.05ppm and 0.17ppm for online and 0.073 ppm & 0.246ppm for batch method respectively. Molar absorptivity was found to be $1.5 \times 10^4 \text{L.mol}^{-1}.\text{cm}^{-1}$ for online analysis and $2.19 \times 10^4 \text{L.mol}^{-1}.\text{cm}^{-1}$ for batch analysis. The method was successfully applied for the determination of fluroxypyr herbicide in commercial formulation and real samples (wheat & maize) using both batch and online version. The result of both batch and online method was also compared. For online method, a sample through put of 60 samples/ hr was achieved.

Part II of this chapter deals with method development for acetochlor. It includes method development strategy, proposed chemical reaction and parameters optimization with results and discussion. Acetochlor herbicide was hydrolyzed in acidic media by heating and the hydrolyzed product was used as coupling reagent for the formation of azo dye. The azo dye formed was measured at 400nm. A linear relationship between concentration and absorbance was observed in the range (9ppb-10ppm) investigated. Molar absorptivity was $4.86 \times 10^4 \text{L.mol}^{-1}.\text{cm}^{-1}$ and $5.37 \times 10^4 \text{L.mol}^{-1}.\text{cm}^{-1}$ for online and batch method respectively. The relative standard deviation (RSD) was found to be 1.55% and 3.6% for the online and batch analysis respectively. The developed method was successfully applied for the determination of acetochlor in commercial formulation and real samples. The active ingredient found was in close agreement with the label value. The residue level found in maize grains was ranging from $1.35 \pm 0.13 \mu\text{g/g}$ to $1.37 \pm 0.09 \mu\text{g/g}$ which is much higher than the New Zealand (NZ) food and safety authority MRLs 2005 of $0.02 \mu\text{g/g}$. Percent recoveries in samples varied from 93-95.5%. The agreements between the label quantity and good percent recoveries has shown the potentialities of the method for the determination of acetochlor in formulation and real samples.

Part III of this chapter deals with method development for phenoxy acetic acid herbicides and its determination in commercial formulation and in real samples. The method is based on base hydrolysis of phenoxy acetic acid herbicides into their corresponding phenols followed by coupling of the hydrolyzed product (phenol) with diazotized sulfanilic acid to form an azo dye. LOD and LOQ were calculated using authentic standard of 2,4- D herbicide and was found to be 0.084 ppm & 0.28 ppm for batch and 0.13 ppm & 0.42 ppm for online analysis. The validity of investigated

method was checked for online and batch analysis. Percent recoveries in samples varied from 90-95.3%. The method was successfully applied for the determination of active ingredient in commercial formulation of MCPA & 2, 4-D herbicides and for residue determination in real samples. The residue level in the analyzed wheat and maize samples for 2,4-D varied from $0.87 \pm 0.15 \mu\text{g/g}$ to $6.02 \pm 0.42 \mu\text{g/g}$ and $0.92 \pm 0.22 \mu\text{g/g}$ to $5.1 \pm 0.28 \mu\text{g/g}$ respectively. The residue level found were much higher than the NZ food and safety authority MRLs 2005 for cereal grains, which is $0.05 \mu\text{g/g}$. Molar absorptivity was calculated and found to be $1.09 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$ and $2.39 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$ for batch and online method respectively. The online method has a sampling frequency of 90 samples/hr.

Part IV of chapter III deals with the investigation of a new spectrophotometric method for the analysis of bromoxynil herbicide using two different coupling reagents (aniline & β -naphthol). The method is based on reduction of bromoxynil in to its corresponding amine at room temperature followed by the diazotization of hydrolyzed product. The diazotized product was coupled with two different coupling reagents β -naphthol and aniline for the formation of azo dye which gives absorption peaks at 430nm & 500nm respectively. Both coupling reagents were used for determination of bromoxynil in commercial formulations and in real samples. The sensitivity was greater, when aniline was used as coupling reagent. For bromoxynil- aniline couple LOD and LOQ were found to be 6.0 ppb and 22.0 ppb for batch analysis and 9.0 ppb and 30.0 ppb for online analysis respectively. The RSD was calculated for the online and batch analysis using aniline as coupling reagent and was found to be 0.32% and 3.6%. Both batch and online methods were successfully applied for the determination of active ingredient in formulation and real samples and the results of both methods were comparable. The residue Level found in wheat and maize grain were varied from $70.9 \pm 0.05 \mu\text{g/g}$ - $96.6 \pm 0.01 \mu\text{g/g}$. The residue levels found were much higher in grains than US EPA MRLs 2004, which are 0.05ppm and 0.3ppm for wheat and maize grains, respectively and NZ food safety authority 2005, which is 0.02ppm for cereal grains. Online method has a sampling frequency of 80 sample/hr.

Part V of this chapter deals with method development for determination of chloramben in real samples. The method is based on direct exploitation of primary aromatic amine to form a diazotized product and subsequently formation of azo dye. Chemical and physical parameters were optimized for batch and online analysis. The

method was validated for determination of chloramben in real samples. The LOD and LOQ were calculated and found to be 0.01 ppm and 0.04 ppm for online analysis and 0.05 ppm and 0.17 ppm for batch analysis respectively. The RSD was calculated for both batch and online analysis and was found to be 10.6% and 8.3% respectively. Molar absorptivity was calculated and found to be $2.45 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ and $2.64 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ for batch and online method respectively. By using the online method a sample through put of 90 samples/ hr was achieved. The values for chloramben found in tomato, corn and cornflour was 0.12 ± 0.110 ppm, 0.10 ± 0.33 ppm, 0.09 ± 0.07 ppm for online and 0.13 ± 0.130 ppm, 0.103 ± 0.09 ppm, 0.1 ± 0.22 ppm for batch analysis. Percent recovery was checked and was found to be in the range of 88%-96.0%.

Introduction

Introduction:⁽¹⁻⁷⁾

Pakistan is an agro based country. Agriculture is the largest sector in the economy of Pakistan and accounts for 24% of gross domestic product (GDP). Government figures show that average growth in this sector during the past five years has been 4.6% per annum and in 1998, a growth rate of 5.9% was a major contributor to the overall GDP growth rate of 5.4%. Approximately 70% of Pakistan's foreign exchange earnings come from the sale of agricultural products (such as cotton, rice, fruits and vegetables etc) and this has stimulated demand for the provision of raw materials, equipments and services to agro-based industries, mainly in the cotton textile industry which is the largest industrial sub-sector in the country. This sector is also the largest source of employment and accounts for over 50% of Pakistan's labour force. Nearly two million new jobs have been added since 1995 of which 70% are estimated to be in rural areas.

Cotton, rice, wheat, sugar cane, maize are major crops of Pakistan. To increase the fertility of soil and to increase the production rate of crops, farmers use several techniques. Among these are mechanical agriculture, extensive use of fertilizers and insecticides. Similarly the trends for eliminating unwanted plants have changed from manual towards chemical one and the use of herbicides is on the rise. Pesticide use began in Pakistan in the 1950s for locust control. In 1954, the government imported formulated pesticides amounting to 254 tones. This was the beginning of the pesticide business in the country. Until 1980, the Plant Protection Department was responsible for pesticide imports and their distribution in the country through the national agricultural extension network. Most of the pesticide imports were used for aerial spraying to control locust, pests of sugarcane, cotton, rice, tobacco and fruit crops. The cost of pesticides was subsidized and aerial spraying was free of charge. Pesticides were privatized in 1980 and since then there has been a steady increase in pesticides import and consumption. As a result, consumption of pesticides in Pakistan has increased from 665 tones in 1980 to 14,773 tones in 1990 and 61,229 tones in 2000 worth about 7.7 billion rupees. Unfortunately, the widespread and inappropriate use of pesticides has resulted in complicating pest's problems. Excessive and inappropriate pesticide use has disturbed the agro-ecosystem and killed non-target and environment-friendly organisms, including birds apart from leading to other environmental problems. Results from a four year

testing programme on Pakistani fruit and vegetables have shown that maximum residue limits (MRLs) are regularly exceeded. A comparative assessment of organophosphate (OPs), organochlorine (OCs) and synthetic pyrethroid insecticide residue level in fruit and vegetables in Pakistan are shown in Table 1.

Table 1: Comparative assessment of pesticide residue level in fruit and vegetables in Pakistan ⁽¹⁾

Area/Province	A	B	C
Karachi (Sindh)	250	93	45
North West Frontier Province	154	54	22
Islamabad	96	48	11
Quetta/Pishin (Baluchistan)	50	19	11

A = Number of samples, B = Number contaminated, C = Number above MRL

An important group of anthropogenic pollutants of soil are herbicides, which can also be detected in water and some microorganisms. Herbicides presence in the environment results from their widespread use on a global scale (annually approximately 2.5million ton). The behavior of herbicides in soil and water is regulated by herbicidal properties, soil characteristics and climatic conditions. It is a function of acid or base strength, water solubility, volatility, soil retention and persistence. Pesticide has basic, acidic or nonionizable properties. This determines its ability to exist in the soil, water or be retained onto soil solids. Thus, the acid or base strength of both the herbicide and the soil determines potential herbicide movement and plant uptake. In general, herbicides having pH close to the pH of the soil are strongly retained and are not subject to runoff, erosion and/or leaching. In contrast, herbicides having pH much different to that of the soil are less strongly retained and are subject to runoff, erosion and/or leaching. Also, they are more available for plant uptake than strongly retained herbicides. A Classification scheme for organic herbicide behavior in soils is given in table 2.

Table 2: Classification scheme for organic herbicide behavior in soils².

Herbicide Family	Commonly Used Herbicides
Nonionic	
Dinitroanilines	Treflan/Prowl/Sonalan
Thiocarbamates	Sutan+/Eptam/Eradicane
Chloroacetamides	Lasso/Dual/Frontier/Harness/Surpass/Ramrod
Substituted ureas	Lorox/Karmex/Linnex
Acidic	
Phenoxy acetic acid	2,4-D/MCPA/Banvel/Tordon/Stinger
Benzoic Acid	chloramben
Carboxylic Acid(Pyridinoxy,PicolinicAcids)	Fluroxypyr/ picloram/ triclopyr/ clopyralid
Nitriles	Bromoxynil/ Dichlobenil
Chloroacetanilide	Acetochlor/Butachlor,
Sulfonylureas	Accent/Beacon/Ally/Harmony/Pinnacle/Peak/ Glean/Amber/Finesse/Battalion/Permit
Imidazolinones	Pursuit/Arsenal/Assert
Organic phosphorus	Roundup/Touchdown/Ignite
Basic	
Triazines	atrazine/Bladex/Evik/Milogard/Igran/Sencor/Lexone
Dipyridiniums	Cyclone/Gramoxone Extra/Diquat

Acidic herbicides are widely used in agriculture and forestry; they are also applied to clean up sport areas, railway, highways, cemeteries and also to control seaweed and other vegetation in marshy areas, water bodies and drainage ditches.

Due to relatively good solubility in water, acidic herbicides easily enter different water bodies and other environmental systems. If their degradation rate is not high enough, they can have harmful effects on soil micro flora, can enter in to food chain and then the human body. Acidic herbicides are toxic for many living organisms, although none

belongs to a group of very toxic pollutants. Some are mutagenic, teratogenic, and carcinogenic. Some have detrimental effects on mammals causing pyrexia, nausea, hypotonia, coma, metabolic acidosis, convulsion, cytoskeletal perturbation and renal damage. Phenolic herbicides, for example, dinitrophenol damage liver parenchyma, kidneys, nerve muscle, and mucous membrane and cause skin and mucosa irritation. Phenoxy acids are dispersed in the environment due to their persistence, polar nature and water solubility and their residues and transformation products are present in the several matrices like water, soil, cereals and other vegetable products. Therefore, understanding and predicting the environmental fate of herbicides is critically important. Environmental fate is determined by the individual fate processes of transformation and transport. Chemical and physical processes are primary determinants of transformation and transport of herbicides applied to soils and plants. Transformation determines what herbicides are degraded to in the environment and how quickly, while transport determines where herbicides move in the environment and how quickly. Jointly, these processes affect how much of a pesticide and its metabolites (degradation products) are present in the environment, where and for how long. Herbicide fate also varies in response to changes in environmental conditions and application management practices (e.g., spray drift, volatilization), therefore it is important to understand these variables as well.

Unfortunately, because of less effective legislation, lack of awareness of harmful effects, and technical knowledge, pesticide usage has not been properly regulated. Therefore, it is very important that pesticides content especially herbicides be regularly monitored in soil, water, and food products. A variety of methods are available for the determination of pesticides residue in soil, water, fruits, vegetables and other food products. However, most of these methods use expensive instrumentation and are aimed for technologically advanced countries.

The aim of the present work was to investigate such spectrophotometric method, which should be compatible in reproducibility, sensitivity and speed with the existing one and should be simple and cheap enough to be adopted on routine basis in our country.

The strategy for analytical method development is mostly based on exploitation of physico-chemical and other properties of the selected compound. In the following

sections physico-chemical and toxicity information for the selected herbicides fluroxypyr(Starane), Acetochlor, 2,4-D, Bromoxynil and chloramben are given below.

1.1: FLUROXYPYR ⁽⁸⁻¹³⁾

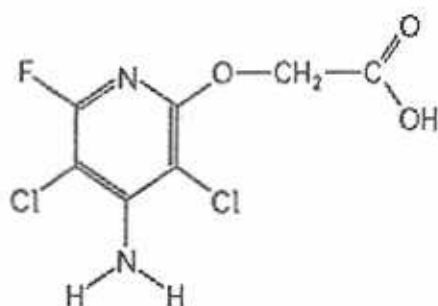
Starane is a common name for the herbicide fluroxypyr (4-amino-3,5-dichloro-6-fluro-2-pyridyloxy acetic acid). Fluroxypyr belongs to the Pyridines group of chemicals. Fluroxypyr herbicide is a selective post emergence product. It is registered as a spray treatment for the control of a wide range of broadleaf weeds in fallow, lucerne, maize, millets, pastures, poppies, sorghum, sugar cane, sweet corn and winter cereals. Also for the control of woody weeds in agricultural non-crop areas, commercial and industrial areas, forests, pastures and rights-of-way. Fluroxypyr works with a mode of action where the weed cannot grow due to disruption of plant cell growth. Fluroxypyr induces auxin-type responses in susceptible annual and perennial broadleaf weeds (auxin being a type of plant growth hormone). Once absorbed into the plant, it accumulates in growing tissues to higher concentrations than the native auxin does, and degrades more slowly. Plant growth is disrupted by the deregulation of cellular growth process following binding of fluroxypyr to plant cell auxin receptor sites. Fluroxypyr also interferes with the plant's ability to metabolize nitrogen and produce enzymes. When a plant's strict growth regulation is disrupted in this fashion, plant growth becomes disorganized, disrupting key metabolic process and results in plant death.

1.1.1 Physical properties:

Chemical Name: 4-amino-dichloro-6-fluoro-pyridyl-2-oxyacetic acid

Chemical Family: Pyridinoxy acid

Chemical structure:



Molecular Formula: C₇H₅O₃N₂FCl₂

Molecular Weight: 255.0g

Water Solubility: 136µg/L at 25 °C

Solubility in Other Solvents: alcohol v.s.; ether s.; acetone v.s.

Melting Point: 57.5 °C

1.1.2 Toxicology Effects

Acute Toxicity: Acute oral toxicity for technical fluroxypyr in rats is LD₅₀ > 5000 mg/kg in males and females. Acute Dermal Toxicity in rats is LD₅₀ > 2000 mg/kg in males and females. Acute Inhalation Toxicity in rats is LC₅₀ > 2 mg/L in males and females.

Subchronic Toxicity: In a 90-day feeding study in rats, significant nephrotoxicity and deaths were observed at 1000 and 1500 mg/kg/day in both sexes, and in males at 750 mg/kg/day. Death was due to renal papillary necrosis. Signs of ill health, emaciation, decreased food intake, increased kidney weight, and decreased renal function was also observed. Histological changes were observed in the adrenals in both sexes at 1000 and 1500 mg/kg/day. In males the non observable effective level (NOEL) for this study is 80 mg/kg/day. In females the NOEL is 750 mg/kg/day. In a 90-day feeding study in mice, no significant effects were observed at any dose level.

Chronic Toxicity/Carcinogenicity: In the combined chronic toxicity/carcinogenicity study in rats, there was no apparent treatment related increase in any tumor type in either sex at the doses tested [with the exception of an increased incidence of parafollicular cell adenomas (single only) in males at 500 mg/kg/day]. The NOEL is 100 mg/kg/day. Deaths occurred at 1000 mg/kg/day in males within the first 90 days on test (2 by day 28 and 3 more by day 56). In the carcinogenicity study in mice, there were no adverse effects on survival or clinical signs in either sex. There was no apparent treatment-related increase in the incidence of any tumor type in either sex. In a one-year chronic feeding study in dogs, no adverse effects were observed at any dose level. No abnormalities in hematology, clinical chemistry or urinalysis. No abnormal findings were made at necropsy, nor were there any significant changes in food consumption or body weight. The NOEL for this study is 150 mg/kg/day, the highest dose level tested.

Reproductive Toxicity: In a two generation reproduction study in rats, treatment-related deaths due to renal failure occurred in both sexes at the high dose in both generations. The effects observed increased progressively with time of exposure. There were increases in kidney weight with corresponding gross and microscopic findings (papillary atrophy, edema, necrosis, hyperplasia of the pelvic epithelium, degeneration/regeneration of the tubular epithelium, tubulo-interstitial nephritis, and dilatation of the tubules) at the high-dose level in both sexes (both generations) and to a lesser degree in the mid dose males (second generation). The NOEL for maternal/paternal toxicity is 500/100 mg/kg/day. The reproductive NOEL for maternal/paternal is 1000/750mg/kg/day, the highest dose tested.

Mutagenicity: A total of nine studies were available for review; five with the acetic acid form of fluroxypyr and four with the fluroxypyr methylheptyl ester. The studies on the methylheptyl ester were submitted to support the bridging of data between the two forms of fluroxypyr since the ester is rapidly hydrolyzed to the acid. The available studies indicate that fluroxypyr was not mutagenic in bacteria. By contrast, a mutagenic response was uncovered in the forward gene mutation assay with mouse lymphoma cells but not with Chinese hamster ovary (CHO) cells. There was also no evidence of clastogenicity *in vivo* using CHO cells. Hence the marginal positive result seen in the mouse lymphoma assay was not confirmed in a second mammalian cell line and the test material was

negative for unscheduled DNA synthesis (UDS) *in vitro*. Fluroxypyr methylheptyl ester was also not mutagenic in bacteria or clastogenic either *in vitro* or *in vivo*.

1.1.3 Ecological Effects:

Effects on birds and Aquatic organisms: Fluroxypyr is practically non-toxic to the mallard duck and the bobwhite quail on an acute basis ($LD_{50} > 2000$ mg/kg) and practically non-toxic to the mallard duck and the bobwhite quail on a sub acute basis (5-day $LC_{50} > 5000$ ppm). It is slightly toxic to small mammals ($LD_{50} = 880$ mg/kg) and practically non-toxic to honey bees ($LD_{50} > 25$ g/bee).

Fluroxypyr is slightly toxic to the bluegill sunfish (96-hour $LC_{50} > 14.3$ mg/L) and slightly toxic to practically non-toxic to the rainbow trout (96-hour LC_{50} ranges from 13.4 mg/L to > 100 mg/L). It is also practically non-toxic to *Daphnia magna* (48-hour $EC_{50} > 100$ mg/L) and is slightly toxic to the silverside (96-hour $LC_{50} = 40$ mg/L). Fluroxypyr acid is highly toxic to the eastern oyster (96-hour $LC_{50}/EC_{50} = 0.068$ mg/L); fluroxypyr 1-methylheptyl ester is slightly toxic to the eastern oyster (96-hour $LC_{50}/EC_{50} = 51$ mg/L). It is practically non-toxic to the grass shrimp (96-hour $LC_{50}/EC_{50} > 120$ mg/L).

1.1.4 Environmental fate:

Groundwater contamination: Available data indicate that fluroxypyr acid is mobile to very mobile, However, dissipation by hydrolysis and microbial degradation reduced persistence and limited downward transport (i.e., leaching).

1.2: Acetochlor ⁽¹⁴⁻²¹⁾

Acetochlor is used for control of most annual grasses and certain broadleaf weeds and yellow nutsedge. Tolerant crops include cabbage, citrus, coffee, corn (all types), cotton, green peas, maize, onion, orchards, peanuts, potatoes, soybeans, sugar beets, sugarcane, sunflower, and vineyards. Acetochlor is applied preemergence; preplant incorporated and is compatible with most other pesticides and fluid fertilizers when used at recommended rates. Usually 0.3-0.6 inches of rainfall activate the product if it occurs within 7-10 days.

1.2.1 Physical Properties:

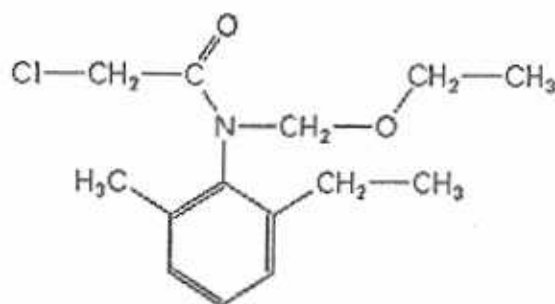
Appearance: Technical acetochlor is an oily liquid at room temperature, light amber to violet in color.

Chemical name: 2-chloro-N- (ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide.

Empirical Formula: $C_{14}H_{20}ClNO_2$

Molecular Weight: 269.8g

Structural Formula:



Chemical class: Chloro acetanilide herbicides

Solubility: In water 233 mg/l at 25°C. Soluble in diethyl ether, acetone, benzene, chloroform, ethanol, ethyl acetate, and toluene.

Specific gravity: 1.100 at 30 °C, 1.136 at 20°C.

Formulations: 90% E.C., 50% E.C.

Stability: Although acetochlor is stable under normal temperatures and pressures, thermal decomposition products may include toxic oxides of nitrogen and carbon and toxic and corrosive fumes of chlorides.

1.2.2 Toxicological Effects

Acute Toxicity : Acetochlor is considered moderately toxic by ingestion. The acute oral LD50 of technical acetochlor when administered to bobwhite quail was 1590 mg/kg. For rats acute LD50 is 2953mg/kg. Acute toxicology places technical acetochlor in toxicity category II for eye irritation, toxicity category III for acute oral, acute dermal, and acute inhalation. Technical acetochlor is in toxicity category IV for primary skin irritation and is considered a skin sensitizer.

Chronic Toxicity: A three week dermal study with rats showed that fed dosages of 0.1, 1.0, 10, or 100 mg/kg/day resulted in minimum to mild skin irritation after 21 days. Signs of systemic toxicity were not apparent at any level. Higher doses were not possible because of severe dermal toxicity at higher doses. In a 90-day chronic toxicity study in rats, no significant toxicological effects were observed at dietary levels of 800 ppm. The

4-hour inhalation LC50 for technical acetochlor in rabbits was 1.5 mg/L. Rabbit eye irritation was substantial but temporary; skin irritation was considered severe.

Reproductive Effects: A two-generation reproduction study with rats showed that fed dosages of 0, 30.4, 74.1, and 324.5 mg/kg/day (males) or 0, 44.9, 130.1, and 441.5 mg/kg/day (females), the reproductive NOEL was 30.4 mg/kg/day for males and 44.9 mg/kg/day for females based on decreased body weight gain of F2b pups at 74.1 mg/kg/day for males and 130.1 mg/kg/day for females.

Teratogenic Effects: A study with rats showed that fed dosages of 0, 40, 150, and 600 mg/kg/day, the NOEL was 150 mg/kg/day based on increased resorptions, post-implantation loss, and decrease in mean fetal weight at 600 mg/kg/day, the highest dose tested. The maternal toxicity for this study was 150 mg/kg/day based on animals sacrificed approaching death, clinical observations, and decreased body weight gain at 600 mg/kg/day, the highest dose tested. In a developmental study with rabbits fed dosages of 0, 30, 100, and 300 mg/kg/day, acetochlor did not induce either maternal or developmental toxicity up to 300 mg/kg/day, the highest dose tested.

Mutagenic Effects: -In mutagenicity study acetochlor induced a reproducible, positive, mutagenic response in a strain of *Salmonella typhimurium* with metabolic activation at 100 ug/plate. Significant increases in number of revertant colonies were not induced in four other strains.

Carcinogenic Effects: A two year chronic feeding/carcinogenicity study with rats showed that fed dosages of 0, 0.8, 7.9, and 79.6 mg/kg/day, carcinogenic effects noted at 79.6 mg/kg/day, the highest dose tested, included a significant increase in nasal epithelial adenomas and thyroid follicular cell adenomas in both sexes at 79.6 mg/kg/day.

Organ Toxicity: One year feeding study with dogs showed that fed dosages of 0, 2, 10, and 50 mg/kg/day, the NOEL was 2 mg/kg/day based on increased salivation, ornithine carbamyl transferase, and triglyceride values accompanied by decreased blood glucose levels and liver glycogen levels at 10 mg/kg/day. Interstitial nephritis, tubular degeneration of the testes and hypospermia were reported.

1.2.3 Ecological effects:

Effects on Birds, Aquatic and other organisms : The eight-day dietary LC50 in mallard ducks and bobwhite quail was greater than 5620 ppm. The 96-hour LC50 in rainbow trout exposed to technical acetochlor was 0.45 mg/L and 1.30 mg/L in bluegills sunfish. The 48-hour EC50 in *Daphnia magna* was 16.00 mg/L. Acetochlor is considered moderately toxic to honeybees.

1.2.4 Environmental Fate

Breakdown of Chemical in Soil and Groundwater: Acetochlor is adsorbed by soil colloids and leaches very little. Low soil moisture has little influence on efficiency. The main method of degradation is microbial breakdown. Acetochlor's average persistence at recommended rates is 8 to 12 weeks, but may vary depending on soil type and climatic conditions. It is very active on high organic matter soils. Photoproducts are identified as 2-ethyl-6-methylaniline; N,N-diethylaniline; 4,8-dimethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 2-oxo-N-(2-ethyl-6-methylphenyl)-N-(ethoxymethyl) acetamide; N-(ethoxymethyl)-2'-ethyl-6'-methylformanilide; 1-hydroxyacetyl-2-ethoxyl-7-thylindole; 8-ethyl-1-ethoxymethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 4,8-dimethyl-1-ethoxymethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 2-hydroxy-2'-ethyl-6'-methyl-N-(ethoxymethyl) acetanilide and a compound related to acetochlor.

Breakdown of Acetochlor in Vegetation: Acetochlor is absorbed mainly by germinating plant shoots, and secondly by roots. It translocates throughout the plant, with higher concentrations in vegetative parts rather than in reproductive parts. Acetochlor appears to inhibit protein synthesis in susceptible plants.

1.3: 2,4-Dichloroacetic acid ⁽²²⁻²⁵⁾

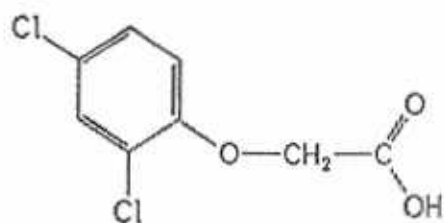
2,4-Dichloroacetic acid (2,4-D) is a herbicide of the phenoxy family used for selective control of broadleaf weeds. 2,4-D is a synthetic auxin herbicide, which causes disruption of plant hormone responses. Plant injuries include growth and reproduction abnormalities, especially on new growth. Symptoms may appear on young growth almost immediately after application, but death may not occur for several weeks. 2,4-D formulations are typically applied as broadcast, banded, or directed (spray or wiper) applications during dormancy or preplant, preharvest, preemergence, emergence, post emergence, or post harvest using ground or aerial equipment. Registered forms of 2,4-D

include 2,4-D acid, 2,4-D dimethylamine salt (DMAS), 2,4-D isopropyl acid (IPA), 2,4-D triisopropyl acid (TIPA), 2,4-D ethylhexyl ester (EHE), 2,4-D butoxy ethyl ester (BEE), 2,4-D diethyl amine (DEA), 2,4-D isopropyl ester (IPE), and 2,4-D sodium salt. The product "Agent Orange", used extensively throughout Vietnam, was about 50% 2,4-D. However, the controversies associated with the use of Agent Orange were associated with a contaminant (dioxin) in the 2,4,5-T component of the defoliant.

1.3.1 Physical Properties:

Appearance: 2,4-D is a white powder.

Chemical structure:



Chemical Name: (2,4-dichlorophenoxy)acetic acid

Molecular Weight: 221.04g

Chemical class: Phenoxy acid herbicides

Water Solubility: 900 mg/L at 25 °C

Solubility in Other Solvents: ethanol v.s.; diethyl ether v.s.; toluene s.; xylene s.

Melting Point: 140.5 °C

Vapor Pressure: 0.02 mPa at 25 °C (acid)

1.3.2 Toxicological Effects:

Acute toxicity: The acid form is of slight to moderate toxicity. The oral LD50 of 2,4-D ranges from 375 to 666 mg/kg in the rat, 370 mg/kg in mice, and from less than 320 to 1000 mg/kg in guinea pigs. The dermal LD50 values are 1500 mg/kg in rats and 1400 mg/kg in rabbits, respectively. In humans, prolonged breathing of 2,4-D causes coughing.

burning, dizziness, and temporary loss of muscle coordination. Other symptoms of poisoning can be fatigue and weakness with possible nausea. On rare occasions following high levels of exposure, there can be inflammation of the nerve endings with muscular effects.

Chronic toxicity: Rats given high amounts, 50 mg/kg/day, of 2,4-D in the diet for 2 years showed no adverse effects. Dogs fed lower amounts in their food for 2 years died, probably because dogs do not excrete organic acids efficiently.

Reproductive effects: High levels of 2,4-D (about 50 mg/kg/day) administered orally to pregnant rats did not cause any adverse effects on birth weights or litter size. Higher doses (188 mg/kg/day) resulted in fetuses with abdominal cavity bleeding and increased mortality. DNA synthesis in the testes was significantly inhibited when mice were fed with large amounts (200 mg/kg/day) of 2,4-D. These evidence suggests that if 2,4-D causes reproductive effects in animals, this only occurs at very high doses. Thus reproductive problems associated with 2,4-D are unlikely in humans under normal circumstances.

Teratogenic effects: 2,4-D may cause birth defects at high doses. Rats fed with 150 mg/kg/day on days 6 to 15 of pregnancy had offspring with increased skeletal abnormalities, such as delayed bone development and wavy ribs. This suggests that 2,4-D exposure is unlikely to be teratogenic in humans at expected exposure levels.

Mutagenic effects: 2,4-D has been very extensively tested and was found to be non mutagenic in most systems. 2,4-D did not damage DNA in human lung cells. However, in one study, significant effects occurred in chromosomes in cultured human cells at low exposure levels. The data suggest that 2,4-D is not mutagenic or has low mutagenic potential.

Carcinogenic effects: 2,4-D fed to rats for 2 years caused an increase in malignant tumors. Female mice given a single injection of 2,4-D developed cancer (reticulum-cell sarcomas). Another study in rodents showed a low incidence of brain tumors at moderate exposure levels (45 mg/kg/day) over a lifetime. However, a number of questions have

been raised about the validity of this evidence and thus about the carcinogenic potential of 2,4-D. In humans, a variety of studies give conflicting results. Several studies suggest an association of 2,4-D exposure with cancer. An increased occurrence of non-Hodgkin's lymphoma was found among a Kansas and Nebraska farm population associated with the spraying of 2,4-D. Other studies carried out in New Zealand, Washington, New York, Australia, and on Vietnam veterans from the U.S. were all negative. There remains considerable controversy about the methods used in the various studies and their results. Thus, the carcinogenic status of 2,4-D is not yet clear.

Organ toxicity: Most symptoms of 2,4-D exposure disappear within a few days, but there is a report of liver dysfunction from long-term exposure.

Fate in humans and animals: The absorption of 2,4-D is almost complete in mammals after ingestion and nearly the entire dose is excreted in the urine. The compound is readily absorbed through the skin and lungs. The half-life is between 10 and 20 hours in living organisms. There is no evidence that 2,4-D accumulates to significant level in mammals or in other organisms. Between 6 and 8 hours after doses of 1 mg/kg, peak concentrations of 2,4-D were found in the blood, liver, kidney, lungs and spleen of rats. There were lower levels in muscle and brain. After 24 hours, there were no detectable tissue residues. Only traces of the compound have been found in the milk of lactating animals for 6 days following exposure. 2,4-D passes through the placenta in pigs and rats. In rats, about 20% was detected in the uterus, placenta, fetus, and amniotic fluid. Chickens given moderate amounts of 2,4-D in drinking water from birth to maturity had very low levels of the compound in eggs.

1.3.3 Ecological Effects:

Effects on birds, aquatic and other organisms : 2,4-D is slightly toxic to wildfowl and slightly to moderately toxic to birds. The LD50 is 1000 mg/kg in mallards, 272 mg/kg in pheasants, and 668 mg/kg in quail and pigeons.

Some formulations of 2,4-D are highly toxic to fish while others are less so. For example, the LC50 ranges between 1.0 and 100 mg/L in cutthroat trout, depending on the

formulation used. Channel catfish had less than 10% mortality when exposed to 10 mg/L for 48 hours. Green sunfish, when exposed to 110 mg/L for 41 hours, showed no effect on swimming response. Limited studies indicate a half-life of less than 2 days in fish and oysters. Concentrations of 10 mg/L for 85 days did not adversely affect the survival of adult dungeness crabs. For immature crabs, the 96-hour LC50 is greater than 10 mg/L, indicating that 2,4-D is only slightly toxic. Brown shrimp showed a small increase in mortality at exposures of 2 mg/L for 48 hours.

Moderate doses of 2,4-D severely impaired honeybee's brood production. At lower levels of exposure, exposed bees lived significantly longer than the controls. The honeybee LD50 is 0.0115 mg/bee.

1.3.4 Environmental Fate

Breakdown in soil and groundwater: 2,4-D has low soil persistence. The half-life in soil is less than 7 days. Soil microbes are primarily responsible for its disappearance. Rate of breakdown depends upon temperature, moisture, organic matter, and other soil characteristics, which affect microbial activity. Despite its short half-life in soil and in aquatic environments, the compound has been detected in groundwater supplies in at least five States in USA and in Canada. Very low concentrations have also been detected in surface waters throughout the USA.

Breakdown in water: In aquatic environments, microorganisms readily degrade 2,4-D. Rates of breakdown increase with increased nutrients, sediment load, and dissolved organic carbon. Under oxygenated conditions the half-life is 1 week to several weeks.

Breakdown in vegetation: 2,4-D interferes with normal plant growth processes. Uptake of the compound is through leaves, stems, and roots. Plant roots absorb polar (salt) forms of 2,4-D and Leaves absorb nonpolar (ester) forms of 2,4-D most readily. The esters of 2,4-D tends to resist washing from plants and are rapidly converted to the acid by plants. Breakdown in plants is by a variety of biological and chemical pathways. studies have shown complexes with protein or amino acids, oxidation of the acetate moiety and of the ring, hydrolysis to the free phenol, and combination with glycosides. 2,4-D is toxic to most broad leaf crops, especially cotton, tomatoes, beets, and fruit trees .

1.4: Bromoxynil ⁽²⁶⁻³⁰⁾

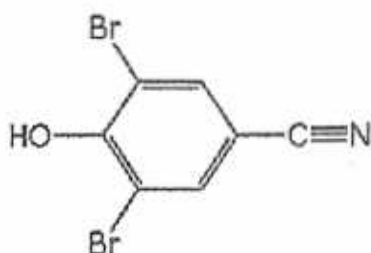
Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) is a herbicide used for post-emergent control of annual broadleaf weeds by inhibiting photosynthesis in the target plants. It has been used to control weeds in cereal, corn, sorghum, onions, flax, mint, turf and on non-cropland. It is available as an emulsifiable concentrate and a suspension concentrate. This herbicide is also present in mixed formulations with other herbicides including MCPA, linuron, dicamba, mecoprop, and 2,4-D. Commercial bromoxynil is usually formulated as the octanoate ester, potassium salt or butyrate ester. Bromoxynil is a Restricted Use Pesticide (RUP), and is not registered for homeowner use. RUPs may be purchased and used only by certified applicators.

1.4.1 Physical Properties:

Appearance: Bromoxynil is a colorless crystalline solid at room temperature.

Chemical Name: 3,5-dibromo-4-hydroxybenzonitrile

Chemical structure:



Molecular Weight: 276.93 g

Chemical class: Nitrile herbicides

Water Solubility: 130 mg/L at 25 °C

Solubility in Other Solvents: methanol v.s.; ethanol v.s.; acetone v.s.; cyclohexane v.s.; tetrahydrofuran v.s.; all at 25 °C.

Melting Point: 194-195 °C

1.4.2 Toxicological Effects

Acute toxicity: Technical bromoxynil has an oral LD50 of 190 mg/kg in rats, an LD50 of 260 mg/kg in rabbits, and an LD50 of 63 mg/kg in guinea pigs, indicating moderate acute toxicity. The dermal LD50 of bromoxynil is greater than 2000 mg/kg in rabbits. The compound is a slight eye irritant but it is not a skin irritant in rabbits. However, when in contact with abraded skin, bromoxynil may produce a mild irritation.

Chronic toxicity: Studies have shown that bromoxynil has no effect on rats when a dietary doses of 15 and 50 mg/kg/day were given for 90 days. Doses up to 5 mg/kg/day for 2 years had no impact on blood chemistry or urine.

Reproductive effects: No changes in reproduction were noted in female rats with a fed dosage of 15 mg/kg/day of bromoxynil over three generations. This suggests that bromoxynil does not cause reproductive effects.

Teratogenic effects: Bromoxynil is a suspected teratogen. The compound produced birth defects in rats at oral doses above 35 mg/kg. Toxic effects included abnormal rib formation and reduced fetal weight. Newborn rabbits had birth defects when bromoxynil was administered to pregnant mothers at doses above 30 mg/kg. In the rabbit, birth defects included changes in bone formation in the skull and hydrocephaly.

Mutagenic effects: Studies have shown that bromoxynil is not a mutagen.

Carcinogenic effects: Rats with a fed dosage of bromoxynil at low levels of 5 mg/kg and below did not develop any cancer related effects .

Fate in humans and animals: No bromoxynil was present in the milk or feces of cows after 9 days exposure to low doses of the herbicide. Less than 20% of the compound was excreted in urine as the parent compound.

1.4.3 Ecological Effects:

Effects on birds: Bromoxynil is highly toxic to pheasants (LD50 of 50 mg/kg) and is moderately toxic to hens (LD50 of 240 mg/kg), quail (LD50 of 100 mg/kg), and mallard ducks (LD50 of 200 mg/kg).

Effects on aquatic and other organisms: Bromoxynil is highly toxic to moderately toxic to freshwater fish; the potassium salt of bromoxynil has an LC50 of 5.0 mg/L in harlequin fish, 0.46 mg/L in goldfish, and 0.063 mg/L in catfish. Bromoxynil has an LC50 of 0.05 mg/L in rainbow trout. It is not toxic to bees.

1.4.3 Environmental Fate:

Breakdown in soil and groundwater: Bromoxynil has a low persistence in soil. In sandy soil, the half-life is about 10 days. Degradation in clay was slower, with half of the bromoxynil degraded to its metabolites in about a 2-week period at 25 °C. The persistence of the compound is also slightly longer in peat field soils than in the sandy soils. Microbes and sunlight break down bromoxynil in the environment.

The evidence suggests that, while bromoxynil is broken down by some soil bacteria, it may inhibit the action of other bacteria that promote the formation of nitrite by a process called nitrification. Bromoxynil's potential to leach to groundwater is low; surface runoff potential is low, and potential for loss on eroded soil is intermediate. Bromoxynil has moderate volatility and the potential for loss to the atmosphere is moderate.

Breakdown in vegetation: The herbicide works by disrupting the plants ability to produce energy for cell-related activities. It is not readily translocated throughout the plant once it has been absorbed. The oil soluble amine and esters have the capacity to resist removal from foliage by rain, the sodium salt does not, since it is water soluble. It may be hydrolyzed to benzoic acid in plants.

Atmospheric Fate: Bromoxynil is discharged into the atmosphere as an aerosol during spraying operations. It is removed from the air by gravitational settling. Due to its very low vapor pressure, bromoxynil should not occur as a vapor in the atmosphere.

Bromoxynil-containing aerosols will undergo photolysis, the rate of which will depend on the solar irradiance and cloud cover. In the laboratory, the photolysis half-life of bromoxynil solutions irradiated with a 100 watt xenon-mercury lamp that was filtered to remove radiation less than 300 nm, was less than 40 min

1.5: Chloramben ⁽³¹⁻³⁴⁾

Chloramben is a selective, preemergence benzoic acid herbicide that is primarily applied to soil for the control of annual grass and broadleaved weed seedlings. It is mostly preemergence used on soybeans, dry beans, peanuts, sunflowers, corn, pumpkins, cucumbers, cotton and post-transplanting on sweet potatoes, tomatoes, peppers, hardwood trees, shrubs, some conifers and various ornamental plants. Chloramben inhibits seedling root development and causes plants to bend and die as they emerge from the soil.

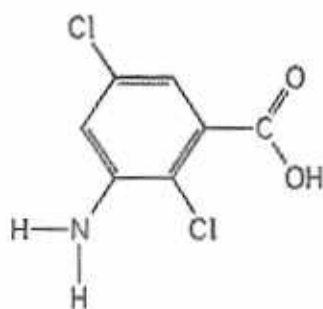
1.5.1 Physical Properties

Appearance: Chloramben is a colorless and odorless crystalline solid .

Chemical Name: 3-amino-2,5-dichlorobenzoic acid

Chemical class: Benzoic acid herbicide

Chemical structure:



Molecular Weight: 206.03g

Water Solubility: 700 mg/L at 25 °C

Solubility in Other Solvents: alcohol v.s.; ether s.; acetone v.s.

Melting Point: 200-201 °C

Adsorption Coefficient: 15 estimated (salts at pH < 5)

1.5.3 Toxicological Effects:

Acute toxicity: The oral LD50 for chloramben in rats is 3500 mg/kg and in mice is 3725 mg/kg. Chloramben irritates throat, lungs, skin, and eyes. However, chloramben is not known to move through the skin. On rats, a low dose skin application caused mild irritation that subsided within 24 hours. The dermal LD50 for rabbits is 3136 mg/kg and for albino rats, it is greater than 3160 mg/kg. This indicates that chloramben has about the same toxicity when applied to the skin as when ingested. The major health concern for humans is dermatitis resulting from skin exposure to chloramben.

Chronic toxicity: In long-term tests on rats, mice, and dogs, no chloramben-related effects on body weight, body function, and organs were observed, even at very high concentrations.

Reproductive effects: No effects on fertility, survival of the fetus, or on nursing ability were found in a long-term study of rats fed with large daily doses of chloramben. This data suggest that chloramben does not cause reproductive toxicity.

Teratogenic effects: When pregnant rabbits were given high doses of chloramben during the sensitive period of gestation, no developmental defects were seen in the fetuses. Pregnant rats fed with lower doses of chloramben during the sensitive period of gestation were not affected, but fetal deaths increased and fetal skeletal development was not complete. At even lower doses, no increase in fetal deaths was seen, but the bones were not fully developed. No changes were seen at the lowest doses. This study indicates that chloramben is unlikely to cause teratogenic effects at expected exposure levels.

Mutagenic effects: Most bacterial and mammalian cell assays of chloramben indicate that it is not toxic to genetic material. However, mutagenicity was seen in one test using CHO cells. These data suggest chloramben shows slight or no mutagenic potential.

Carcinogenic effects: Rats given high daily doses of chloramben for 80 weeks did not show carcinogenicity. But mice given similar doses (up to 300 mg/kg) each day for 80 weeks developed cancerous tumors. Thus, the carcinogenic status of chloramben is not clear.

Organ toxicity: Long-term animal studies did not show evidence of chronic injury to the organs examined.

Fate in humans and animals: Several animal studies have shown that once ingested, chloramben is rapidly excreted in the urine and feces, with no residues found in tissues. Very small amounts of chloramben fed daily to lactating cows left the body via urine (88%) and the feces (5%) within 4 days. No residues were found in the milk. Similarly, dogs fed with very small amounts showed rapid chloramben excretion, with no residues found in the tissues. Chloramben is quickly absorbed from the gastrointestinal tract of female rats. About 97% of an oral dose is absorbed and then excreted through the urine and expired air. In rats fed a single dose, 70% of that recovered 24 hours later from the urine was the original compound. Only 0.6% of the dose remained in the body after 3 to 4 days.

1.5.3 Ecological Effects

Effects on birds: The LC50 of chloramben is 4640 mg/l in mallard ducks, indicating that the compound is practically nontoxic to this species.

Effects on aquatic and other organisms: Chloramben is neither toxic to fish nor bees.

1.5.4 Environmental Fate

Breakdown in soil and groundwater: Chloramben is active in the soil. It is moderately persistent. The wetter the soil, the greater the toxicity of this compound to target plants. Rainfall or irrigation within 10 to 14 days of application is needed for chloramben to be effective against weeds. Leaching is greatest in sandy soil. As the organic matter content of the soil increases, binding of chloramben also increases, and leaching decreases. Volatility & soil movement are more dependent on form of chloramben applied than on

soil type. Aluminum & ammonium salts were observed to leach easily as compared to other forms. Methyl & butoxyethyl esters resisted leaching, while amide was intermediate with respect to leaching. Like most preemergence herbicides, the activity is nearly the same on muck soils as on mineral soils. Biodegradation in nonsterile soils may occur via decarboxylation; however, the rate of microbial degradation is expected to depend on the organic content, temperature, and moisture content. On soil surfaces, some chloramben may photo degrade. In general, chloramben activity lasts for about 6 to 8 weeks in soil. This compound does not evaporate appreciably.

Breakdown in water: Chloramben readily degrades in water exposed to sunlight.

Breakdown in vegetation: Chloramben reacts with plant constituents to form a stable and nontoxic product in both susceptible and tolerant plants. The ease of formation of this metabolite seems to be the reason the herbicide is not totally degraded within the plant. When soil-applied, plant roots are the primary site of absorption and chloramben action. Roots of resistant species, like soybeans, can absorb large amounts of the compound, but translocate very little of it to the above-ground portions of the plant. In contrast, the susceptible barley plant translocates a significant amount into its leaves, and thus dies.

1.6: INSTRUMENTATION

The instruments used for method development and its subsequent application to the real samples for these selected herbicide was online UV-spectrophotometric technique. A brief description of online analysis (FIA) is given below.

1.6.1: FLOW INJECTION ANALYSIS ⁽³⁵⁻⁴⁰⁾

The manual handling of solutions (known as "beaker chemistry") remains the Achilles Heel of modern analytical instrumentation. It is currently being replaced by flow injection analysis (FIA), which is computer compatible and allows automated handling of sample and reagent solutions with a strict control of reaction conditions. FIA was first described in Denmark by Ruzicka and Hansen in 1975. Since then the technique has grown into a discipline covered by six monographs and more than 15,000 research

papers. The scope of the method grew from serial assay of samples to a tool for enhancement of performance of spectroscopic and electrochemical instruments. Most recently FI has found application in biology for study of live cells by fluorescence microscopy and flow cytometry. Other fields include monitoring of chemical processes in real time, biotechnology, and immunoassays including antibody/antigen reactions. FIA is an automated, continuous flow approach to perform chemical analysis, based on injecting a small, well-defined volume of sample into a continuously flowing carrier stream to which appropriate auxiliary reagent streams can be added (see figure 1), whereby a concentration gradient of the sample is created. All of the virtually innumerable, concentrations represented in the gradient can potentially be exploited for the readout. Thus, in contrast to conventional continuous flow procedures (and all batch methods), FIA does not rely on complete mixing of sample and reagent(s) (physical homogenization). Combined with the inherent exact timing of all events it is neither necessary to wait until all chemical reactions have proceeded to equilibrium (chemical homogenization). These facts, which allow transient signals to be used as the readout, do not only permit the procedures to be accomplished within a very short time (typically in less than 30 s), but have opened new and novel avenues to perform an array of chemical analytical assays, which are either very difficult or in many cases directly impossible to implement by traditional means. Thus, in FIA it is feasible to base the assay on the measurement of metastable (intermediary) compounds, which exhibit particularly interesting analytical characteristics; to exploit detection principles relying on bio- or chemiluminescence; to take advantage of the reaction rates of the individually occurring reactions in methods comprising a series of reactions in order to favour the species of interest (kinetic discrimination schemes); and to perform the so-called stopped-flow measurements, where a sample/reagent segment is stopped within the detector, and where the signal recorded directly can be related to the concentration (an approach especially valuable in enzymatic assays).

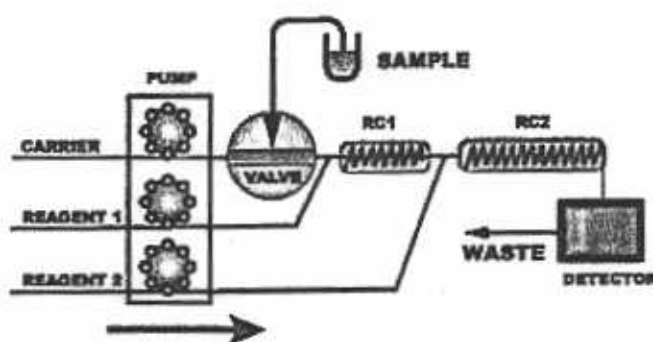
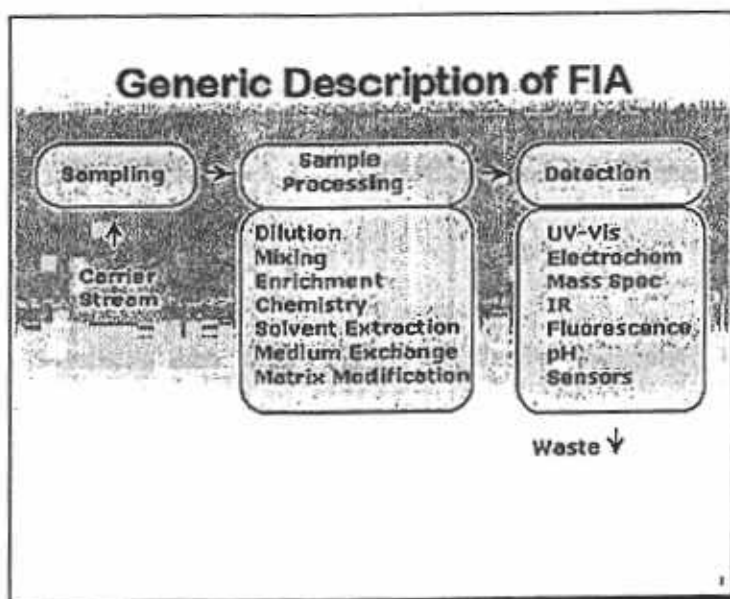


Figure 1: FI manifolds.

Principle of Flow Injection Analysis

In its simplest form, the sample zone is injected into a flowing carrier stream of reagent. As the injected zone moves downstream, the sample solution disperses into the reagent, causing the product to form. Mixing with the reagent in the flowing stream occurs mainly by diffusion-controlled processes and a chemical reaction occurs. A flow through detector placed downstream records the desired physical parameter such as absorbance, fluorescence or electrode potential.

The schematic diagram of FIA is shown below (figure 2). This schematic groups the FIA process into three stages to help visualize how the technique performs a method or analysis.



First is sampling, where the sample is measured out and injected into the flowing carrier stream (thus, the name Flow Injection Analysis). This step is generally performed with a sample injection valve. The second stage is what we call sample processing. The purpose of this step is to transform the analyte into a species that can be measured by the detector and manipulate its concentration into a range that is compatible with the detector, using one or more of the indicated processes. The third stage is detection where the analyte, or a derivative of it, generates a signal peak that is used to quantify the species being determined. As indicated, a large variety of detectors can be used in FIA.

Basic component of an FIA system

The modern Flow Injection Analysis system usually consists of a high quality multichannel peristaltic pump, an injection valve, a coiled reactor, a detector such as a photometer equipped with a flow cell, and an autosampler. Additional components may include a flow through heater to increase the speed of chemical reactions, columns for sample reduction, debubblers, and filters for particulate removal.

Propulsion unit and Injection system

A peristaltic pump is used to propel the stream. The injector may be a loop injector valve as used in high performance liquid chromatography. A bypass loop allows passage of carrier when the injection valve is in the load position. The sample loop is installed across opposite ports of a six-port injection valve. With the valve in load position, the sample stream flows through the sample loop to waste, and reagents are passed through pump to Reactor. The valve is then switched to inject position, and reagent stream is connected with sample loop (figure 3). The concentrated analyte zone flows downstream, merging with a reagent stream for reaction and then detection.

The injected sample volumes may be between 1 and 200 μL (typically 25 μL), which in turn requires no more than 0.5ml of reagent per sampling cycle. This makes FIA a simple, microchemical technique that is capable of having a high sampling rate and minimum sample and reagent consumption.

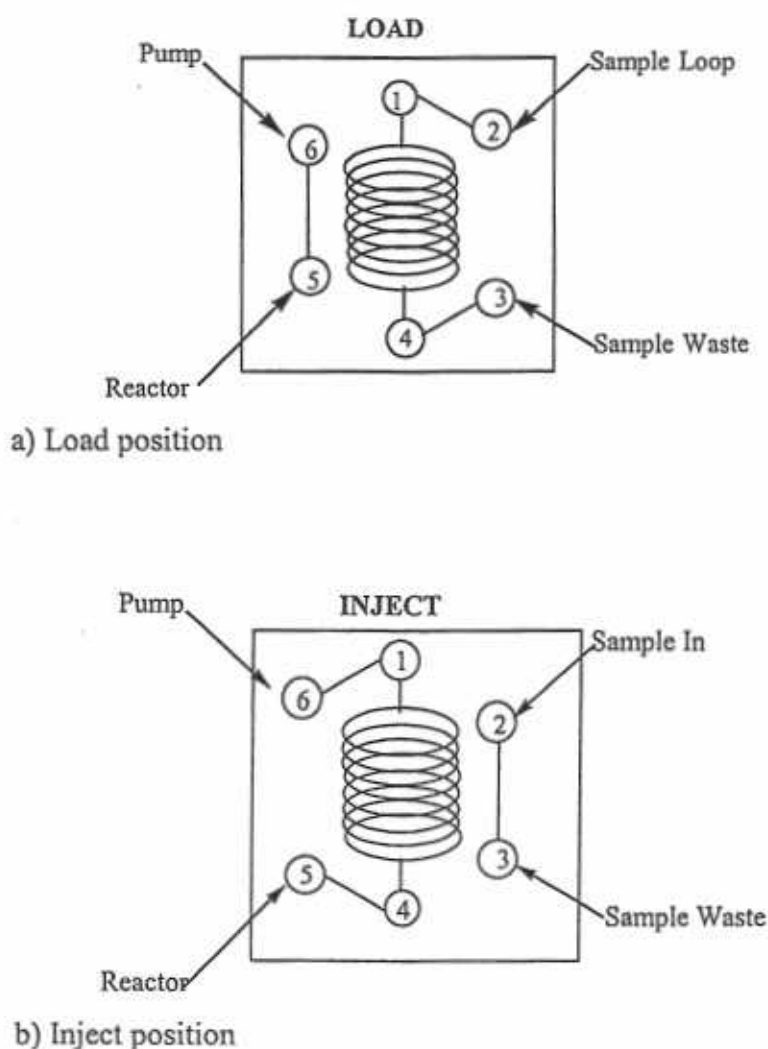


Figure 3: A Six-Port Injection Valve

Flow through cell and Detector

The detection of the FIA signal can be done with any instrument that allows the measuring of a transitory signal and that fulfils the following conditions:

- 1- Small volume measuring cell;
- 2- Linear and quick response for a wide domain of concentrations;
- 3- High sensitivity;
- 4- Low background noise.

Each detector used in FIA systems should be designed to monitor as closely as possible the events occurring in the measuring system. For pH measurements, or in conductimetry, or for simple atomic absorption, when the original sample composition

is to be measured, the sample must be transported through the FIA channel and in to the flow cell in an undiluted form in a highly reproducible manner. For other types of determination, such as spectrophotometry the analyte has to be converted to a compound measurable by a given detector. The most often used commercial detectors are flow through cuvetts with a geometry that fits conventional spectrophotometers (fig4). Usually with a path length of 10mm and a volume of a few to 50-60 μ L. Spectrophotometric detectors are principle detector of each commercial FIA instrumentation.



Figure 4: A commercial flow through cuvette for conventional spectrophotometers.

Systems for transport and reaction.

Two types of tubes used in the FIA systems can be distinguished, as follows:

- a) Flexible tubes for the peristaltic pump with the internal diameter determining the flow rate of the fluid, the material from which these tubes are made (PVC, modified PVC, silicone rubber) depends on the type of fluid involved in the FIA determination and they must be changed periodically;
- b) The tubes for the connections between the component parts of the FIA systems and the reactors are generally made from Teflon, PVC or polyethylene (PET) and the internal diameter varies between 0.3 - 2 mm.

Connectors

The connections between the tubes are made with connectors of different types and shapes that must have a minimum dead space so that the dispersion and reproducibility are not affected. To increase the selectivity, the FIA can be coupled with determination devices for diffusion separation, dialysis, liquid-liquid extraction, filling reactors, mixing chambers (when a very good mixing of the sample with the reagent is desired).

Recording systems

Any recorder may be coupled with an FIA system. Ruzicka and Hansen recommend the use of recorders with variable paper feed speed. The use of a computer coupled with an FIA system is getting more and more common and practically it appears in all published work about this technique today.

Features of the FIA Techniques

The general aspects of the FIA methods as compared with the manual techniques and those with continuous flow are presented in the following paragraphs, without going into extensive details.

Sensitivity. For the FIA methods, the sensitivity is higher due to the physical dispersion or dilution in the flow on one side and the short reaction time on the other side, which leads to the obtaining of partial reaction yields. In the FIA methods with additional chemical processes, the two effects are overlapping, thus, if the experimental conditions are changed to favour the chemical yield, the dispersion increases and vice versa and for this reason, in each FIA assembly those operating conditions must be chosen that lead to the best sensitivity. These effects do not appear in the case of the kinetic methods by stopped-flow.

Selectivity. The FIA methods present generally a better selectivity than the corresponding manual techniques. In many cases, the effect of the interferences is avoided or greatly decreased because the unwanted parallel reactions are not produced or are in the beginning phase in the short residence time that the sample has in the reaction tube. The effect of the foreign species on the recorded signals can be greatly reduced by altering the experimental conditions and the FIA assembly.

Reproducibility and precision. Despite the fact that FIA is a method that is based on system in which the equilibrium is not reached, which means an additional perturbation factor, still a very good reproducibility is obtained even in the case of very small concentrations of the analytes. The precision achieved with this technique is perfectly compatible with the one obtained in the conventional analysis procedures.

Speed. One of the most significant features of the FIA is its speed (expressed by the number of sample that can be analyzed in the time unit) sample throughput, which is 10 times larger than in the case of the sequential flow analysis (SFA) and much larger than for classical methods.

Simplicity. Its principle is simple, the use of easy assemblies and the ease of handling make FIA an attractive technique, as compared with some new analytical methods that are based on expensive instrumentation.

Economy. From an economic point of view, FIA has two qualities regarding:

- a) Consumption of reagents and sample which is from 2 to 15 time lower than in SFA and of 10 up to 100 time lower than for the conventional techniques. This aspect is very important when the reagents are expensive (enzymes) and/or the available sample volume is very small.
- b) Low cost instrumentation in the case of "home-made" FIA assemblies and for the complete analysers, already commercially available from different companies, which represent a positive aspect of this technique, being 5 to 10 time cheaper than the SFA analysers.

Adaptability. Due to its modular character, the replacing of any component with another is done in a very simple manner. For example, the same assembly may be adapted for a certain analysis, its optimization being a simple and fast procedure.

The possibility of using some measuring instruments that is available usually in any laboratory with medium equipment, to construct an FIA assembly together with its adaptability to all types of liquid phase determinations, starting from the most simple ones, without chemical reaction, to the most complex, with dilution or pre-concentration, with the execution of separations, preparation of the reagents, their dilution to the adequate concentrations for the analysis method, execution of differential kinetic

determinations that use inert or active reactors, represent maybe the most important aspects that confirm the quality of this technique.

Dispersion in FIA

To design any FIA system rationally, it is important to know

- (a) How much the original sample diluted on its way towards the detector
- (b) How much time has elapsed between the sample injections and read out.

For this purpose the dispersion coefficient D has been defined as the ratio of concentrations of sample material before and after the dispersion process has taken place in that element of fluid that yields the analytical readout. Usually the measuring is done on the peak maximum, and D is given by the relationship.

$$D = C^0/C^{\max}$$

Where C^0 is the original concentration of injected sample solution and $C^{\max}$ is the concentration after dispersion which corresponds to the maximum of recorded curve.

After injection, during its transport to the detector, the sample is dispersed in the flow. The dispersion of the sample takes place due to the transport by convection and its diffusion. In the case of FIA a laminar flow takes place that generates a parabolic profile of the flow velocities of the different liquid segments. The transport by convection is the leading force at the beginning of the sample transport after injection and has the tendency of scattering the sample along the whole tube (Figure.5 b). The transport by diffusion of the sample may be axial or radial (from the flow direction). The transport by axial diffusion, that appears due to the concentration gradient from the upstream and downstream parts of the injected zone contribute an insignificant part to the total dispersion of the sample.

The transport by radial diffusion perpendicular to the flow direction, contributes as an essential part to the maintaining of the integrity of the sample during its transport to the detector. Due to the radial diffusion the front part of the sample tends to reduce its transport velocity, the molecules from the centre of the tube diffusing towards the wall of the tube due to the concentration gradient. The reverse situation happens for the molecules from the back zone of the sample that diffuse from the tube wall to its centre, moving faster. In this manner the dispersion of the injected sample in the flow is reduced

and the injected sample maintains its integrity. (Figure 5 c). The zone dispersed in the flow has the shape of an asymmetrical peak.

After a sufficiently long transport time (hundreds of seconds) the profile of the dispersed zone tends to become symmetrical, taking the aspect of a Gaussian type curve (Figure 5d).

FIA is worked in such conditions that the profile of the peak is the one presented in the Figure.5c.

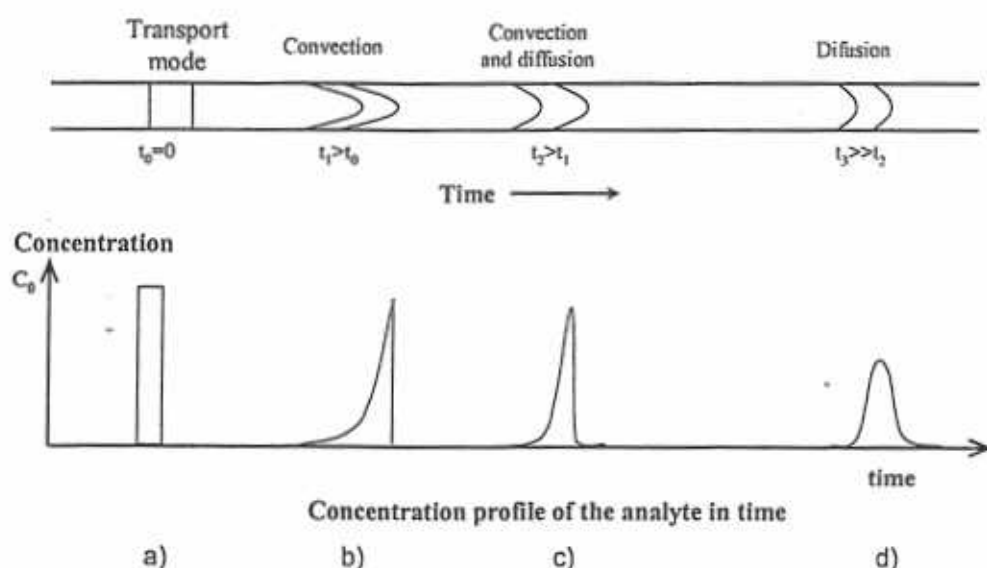


Figure 5: Dispersion of the sample in the flow (up) and the corresponding concentration profile (low) at different time moments. a) In the injection moment; b) immediately after injection; c) at some tens of seconds after injection; d) after a longer time since injection (hundreds of seconds).

The majority of researchers use the classification of the FIA system by the value of the dispersion coefficient proposed by Ruzicka and Hansen, as follows:

- FIA systems with limited dispersion ($D < 3$) that are used when it directly measure one of the parameters of the analyte;
- FIA systems medium dispersion ($3 < D < 10$) which are used when a chemical reaction takes place in the reactor;
- FIA systems with large dispersion ($D > 10$) that are used when a higher degree of mixing between the sample and the reagent is desired.

Factors affecting dispersion coefficient.

The dispersion in an FIA system may be reduced by

- 1) Using short reaction tubes (Figure.6 a)
- 2) Decreasing the volume of the sample (Figure 6 b)
- 3) Increasing the flow rate of carrier and
- 4) Using tubes with small internal diameters.

The increase of the dispersion leads to a longer recovery time to the base line (which decreases the number of samples that can be analyzed in the time unit); decreasing of the sensitivity of the analytical method; dilution of the injected samples.

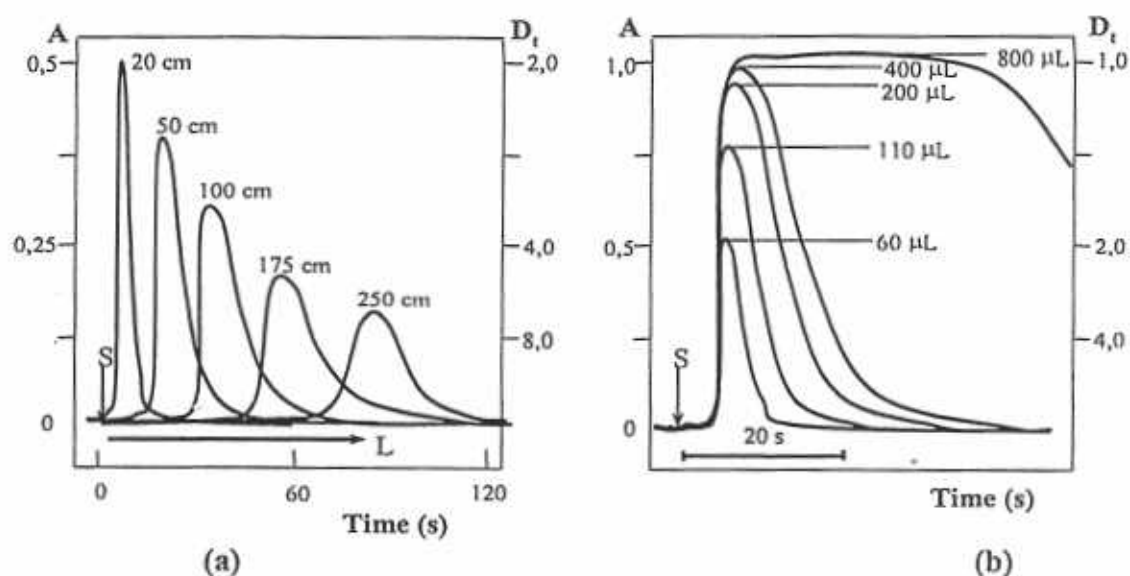


Figure 6. (a) Influence of the tube length on the dispersion. Volume of the injected sample and flow rate is constant (b) Effect of the sample volume on the dispersion. The tube length was 20cm and flow rate was constant.

Thus upon increasing the tube length one obtains a series of curves, the height of which decreases with the increase of tube length (fig 6 a) it means the dispersion of the sample zone increases with the distance travelled, and this band broadening will eventually result in a loss of sensitivity and lower sampling rate.

When increasing volumes of a dye solution are injected, a series of curves will be recorded (fig6b), all starting from same point of injection S, where the height of individual

peaks will increase until an upper limit "steady state" has been reached. At this final level the recorded absorbance will correspond to the concentration of undiluted dye C^0 and $D = 1$. Since the concept of steady state is not used in FIA, therefore limited volume of sample is required.

As conclusion, the dispersion may be easily modified to obtain the suitable conditions for the proposed analytical determination.

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Literature Review

LITERATURE REVIEW

Morrissey et.al (1) developed a simplified procedure for the determination of 2,4-D in soils. Soil samples were separated by supercritical fluid chromatography after extraction without derivatization and without the use of column chromatography for cleanup. Interferences in the chromatographic separation were eliminated by using a tunably selective ion mobility detector. An atmospheric pressure ion formed by the free acid was selectively monitored so the detector could monitor 2,4-D in the presence of other electron-capturing compounds. For a randomly chosen soil sample, the level of 2,4-D detected was estimated at 500 ppb.

Flanagan et.al (2) developed a simple high-performance liquid-chromatographic assay (HPLC) for eight chlorophenoxy (2,4-D and related compounds) and two benzonitrile (bromoxynil and ioxynil) herbicides to aid in the diagnosis of acute poisoning. The method was capable of resolving the chlorophenoxy/benzonitrile mixtures (2,4-D/MCPP, 2,4-D/DCPP, 2,4-D/ioxynil, 2,4-D/MCPP/DCPP, 2,4-D/2,4,5-T, and MCPP/ioxynil) encountered in the U.K. The limit of detection (LOD) at 240 nm was 20 mg/L (10 mg/L for bromoxynil and ioxynil). Intra-assay and inter assay CVs were less than 5% and less than 8%, respectively, for all analytes.

Bergstrom (3) studied the mobility and decomposition of the herbicide fluroxypyr under field conditions in a sandy and clay soils. Leachate was collected in lysimeters with undisturbed soil (sand) and in tile-drained plots (clay). Fluroxypyr levels above the LOD in soil (5mg/kg dry soil) were never found below the topsoil (0.2m) in the clay profile, while, in the sandy profile, levels just above the LOD were occasionally in deeper soil layers.

Cline et.al. (4) characterized the eleven phenols and 2,4-D compounds that may be found in body fluids of humans exposed to pesticides. He derivatized the compound with pentafluorobenzyl bromide and characterized by gas chromatography (GC) with electron capture detection (ECD). These derivatives were further characterized by positive and negative chemical ionization mass spectrometry (MS), nuclear magnetic resonance

spectroscopy (NMR), and gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR). Negative chemical ionization mass spectra of all derivatives have an anionic base peak derived from the parent analyte. In the positive mode the nonchlorinated derivatives have base peaks indicative of the analyte, while chlorinated derivatives are cleaved to give the pentafluorobenzyl cation as base peak. The possibility was explored that ortho-substituted phenols might be formed as by products in these derivatizations.

Suzuki and Watanabe (5) described HPLC method for phenoxy acid herbicides using precolumn derivatization with 9-anthryldiazomethane (ADAM). The phenoxy acid herbicides investigated were (2,4-D, (4-chloro-2-methylphenoxy)acetic acid, 2-(4-chloro-2-methylphenoxy)propionic acid and (4-chloro-2-methylphenoxy)butyric acid. These herbicides reacted with ADAM under mild conditions and were converted into the corresponding fluorescent derivatives. The ADAM derivatives were separated by reversed-phase HPLC and determined using a fluorescence detector. The LOD were about 500 pg per injection. For the application of ADAM to the determination of these herbicides in ground waters, the recoveries were more than 93% and the average relative standard deviation (RSD) was 6.0% at 0.5 $\mu\text{g/L}$.

Tonogai et. al (6) described a method for the determination of six kinds of post-harvest pesticides (DP, OPP, TBZ, 2,4-D, Imazalil and Benomyl) in citrus fruits and fruits juices by using GC-MS (SIM) and HPLC with fluorescent detector. DP for 16 samples, OPP for 27 samples, TBZ for 12 samples, 2,4-D for 10 samples, imazalil for 20 samples and Benomyl for 4 samples were detected in total 32 samples.

Balinova (7) developed a multiresidue analytical method based on C18 solid-phase extraction (SPE) and one-run HPLC determination for the analysis of eleven acidic, neutral and weak basic herbicides in drinking water. Bentazone, 2,4-D, MCPA, fluazifop-acid, metoxuron, monolinuron, metobromuron, diuron, linuron, atrazine and simazine were determined simultaneously in a single run on a C18 HPLC column. Reanalysis of the sample extracts on a second cyano column were used to confirm the identity of the neutral and basic compounds. The LOD, defined as four times the baseline noise, varied

between 0.01 $\mu\text{g/L}$ and 0.1 $\mu\text{g/L}$ depending on the compound, the detection sensitivity of the instrument and the type of HPLC column used.

Oliveira and Palermo-Neto (8) developed a method for determination of 2,4-D in the serum and brain tissue of rats acutely intoxicated with the dimethylamine salt of 2,4-D. After extraction with ethyl ether, 2,4-D derivatization was performed using 2-chloroethanol and BCI3. The average recovery values found for serum and brain tissue were 98.5 ± 4.8 and 93.3 ± 7.5 , respectively. The sensitivity limit of the method was 250 ng/mL for serum and 300 ng/g for brain tissue.

A multi-residue method for the determination of eight polar acidic herbicides (MCPA, MCPB, mecoprop, 2,4-D, dichlorprop, bentazone, dicamba and dikegulac) in surface water was developed by Vink et.al (9). The method involves an off-line solid-phase extraction (SPE) procedure prior to instrumental analysis. The herbicides were isolated on an SPEC C₁₈ column and derivatized with pentafluorobenzyl bromide. After clean-up of the obtained solution on a disposable silica gel cartridge, the herbicide derivatives were determined by capillary gas chromatography with ECD and confirmed by capillary gas chromatography with mass selective detection using negative chemical ionization. The LOD for the herbicides were in the range of 0.02–0.05 $\mu\text{g/L}$ in surface water. The average recovery was 93%.

Rimmer et.al.(10) described a method for the determination of phenoxy acid herbicides in vegetation. Macerated samples were extracted with acetone and followed by filtration and acidification. The herbicides were partitioned into dichloromethane and cleaned-up using high-resolution gel permeation chromatography before undergoing rapid and efficient methylation using trimethylsilyldiazomethane. The resultant methyl esters were then selectively and sensitively analyzed by GC with mass selective detection. The method has been applied to grass samples spiked with four phenoxy acid herbicides; 2,4-D, dichlorprop, MCPA and mecoprop. Recoveries around 100% and reproducibilities of 5% ($n=4$) were achieved at two spiking levels, ~ 700 and $\sim 140 \mu\text{g/kg}$.

Gabriel et.al (11) described simultaneous HPLC determination of bromoxynil, ioxynil and dichlobenil, three aryl nitrile herbicides, and their metabolic products in soil extracts and microbiological media. LODs ranged from 0.56 to 3.97 ppb. Slight modification of the mobile phase composition allowed determination of 13 other aromatic nitriles.

Sanchez et.al (12) described a fluorescence enzyme-linked immunosorbent assay (ELISA) for the quantification of (+/-)-2-(2, 4-dichlorophenoxy) propionic methyl ester (dichlorprop methyl ester). Antibodies for dichlorprop methyl ester were produced by immunizing rabbits with a conjugate of dichlorprop methyl ester with bovine serum albumin. The ELISA procedure was selective for structurally similar compounds or usually found in formulation pesticides. Concentrations for 50% displacement curves were dichlorprop, 83.59 $\mu\text{g/ml}$, and 2,4,5-T, 388.23 $\mu\text{g/ml}$; triclopyr, ioxynil, bentazone, and MCPA had no response.

Apra et.al (13) compared two methods for the quantitative analysis of 2,4-D and 2-methyl-4-chlorophenoxyacetic acid (MCPA) in urine. The first was HPLC method using a C8 column with ion suppression and diode array detection. The LOD of the method was 15 $\mu\text{g/L}$ for both compounds. The percentage coefficient of variation of the whole analysis evaluated at a concentration of 125.0 $\mu\text{g/L}$ was 6.2% for 2,4-D and 6.8% for MCPA. The mean recovery of analysis was 81% for 2,4-D and 85% for MCPA. The second was a GC method in which the compounds were first derivatized with pentafluorobenzylbromide to pentafluorobenzyl esters, which were determined with a slightly polar capillary column and ECD. This method had a LOD of 1 $\mu\text{g/L}$ for both compounds and a percentage coefficient of variation of the whole analysis, evaluated at a concentration of 30.0 $\mu\text{g/L}$, of 8% for 2,4-D, and of 5.5% for MCPA. The mean recovery was 87% for 2,4-D and 94% for MCPA. The low LOD made the second method suitable for assaying the two herbicides in the general population.

Zheng et.al (14) described a HPLC method for the separation and determination of metsulfuron, bensulfuron and acetochlor in the composite herbicide "Nongjiayi". Chromatographic conditions were Shim-Pack CLC-C8 (150 mm x 6 mm i.d., 5 microm), mobile phase of methanol-water (60:40, V/V) and UV detector at 240 nm. Quantification

was carried out with the three external standards. The average recovery was 98.81% with RSD=0.80% for metsulfuron, 99.10% with RSD=0.78% for bensulfuron and 99.72% with RSD=0.69% for acetochlor. The linear range of this method was 0.10-0.40 μg ($r=0.9994$) for metsulfuron, 0.40-1.60 μg ($r=0.9995$) for bensulfuron and 10-40 μg ($r=0.9987$) for acetochlor.

Ferrer et.al (15) combined SPE with HPLC/high-flow pneumatically assisted electrospray mass spectrometry (HPLC/ ESP/MS) for the trace analysis of oxanilic (OA) and sulfonic acids (SA) of acetochlor, alachlor, and metolachlor. The metabolites were separated chromatographically using reversed-phase HPLC and analyzed by negative-ion MS using electrospray ionization in selected ion mode. Quantitation limits (LOQ) were 0.01 $\mu\text{g/L}$ for both the OA and SA based on a 100-mL water sample.

Trau et.al (16) developed an amperometric flow injection immunoanalysis (FIIA) system based on an immunoreactor with immobilized bio components on a silica surface for the determination of the herbicide 2,4-D. In the antigen coating mode the hapten was immobilized and monoclonal primary antibody against 2,4-D together with alkaline phosphatase (AP)-labelled secondary antibody were used as sensing elements in a titration assay. In the antibody coating mode a biotinylated monoclonal antibody was immobilized on the surface of the immunoreactor and a 2,4-D-AP-conjugate was used for detection. For electrochemical measurements p-aminophenol enzymatically generated from p-aminophenyl phosphate was oxidized at a carbon working electrode at +150 mV versus Ag/AgCl. The system enabled the determination of 2,4-D in drinking water samples in the range from 0.2 to 70 $\mu\text{g/L}$. The whole system was computer controlled with a measuring time of 12 min for one determination.

Gerdes et.al (17) developed a method for the determination of the herbicide 2,4-D. Advantage was taken of the cross-reactivity of a monoclonal anti-2,4-D antibody toward MCPA. MCPA was conjugated with bovine serum albumin (BSA), immobilized on the surface of a microtiter plate, and saturated with the anti-2,4-D antibody. Due to the low affinity of the antibody toward MCPA (cross-reactivity of approximately 30%), the addition of 2,4-D resulted in a displacement of the antibody. Remaining antibodies were subsequently detected using a peroxidase-labeled goat anti-mouse antibody. The LOD

was as low as 0.1 $\mu\text{g/L}$ for 2,4-D, which complies with the European Union Drinking Water Directives. When 2,4-D-BSA was used instead of MCPA-BSA conjugates, no significant displacement of bound antibody was observed.

A simple method was developed by Pavlova et.al. (18) for the preparation of a highly active streptavidin-peroxidase conjugate that enhances fourfold the sensitivity of bioassays. Using this conjugate, a quantitative assay of simazine and 2,4-D (competitive ELISA) and a non instrumental express method for simultaneous detection of these pesticides (dot-immuno filtration assay) were developed. The LOD of the pesticides was 3,4 and 2 ng/ml and the duration of the assays is 1.5 h and 5 min for ELISA and dot-immunofiltration, respectively.

Two specific immunoassays for the detection of 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) herbicides were developed by Medyantseva et.al (19). These methods were based on a specific immunoreaction of pesticides with antibodies followed by electrochemical detection of unbounded pesticides by its inhibition effect on the amperometric cholinesterase biosensor. The total time of assay was less than 20 min. The antibodies were used in solution or immobilised on the nitrocellulose membrane. The immobilised antibodies could be regenerated several times and were stable for at least 30 days. The dynamic ranges of detection of 2,4-D and 2,4,5-T are 1×10^{-11} - $5 \times 10^{-7}\text{M}$ and 5×10^{-11} - $5 \times 10^{-7}\text{M}$, respectively. The developed method was applied for 2,4-D determination in milk without sample pre-treatment.

Hu et.al (20) used Capillary LC/Frit-FAB-MS for the determination of 24 thermolabile and polar pesticides including nine neutral/basic and fifteen acidic ones, which were not amenable to routine GC/MS. High sensitivity was obtained for neutral/basic pesticides and asulam by adopting a mobile phase composed of CH_3CN and 0.1% acetic acid, while for the other acidic pesticides, a mobile phase composed of CH_3CN and 5 mM di-n-butylammonium acetate (pH=6.7) was effective. The LOD of the pesticides were in the range of 10300 $\mu\text{g/L}$, except for benazolin whose limit was 1000 $\mu\text{g/L}$. Environmental samples (1 L) were taken from raw water for water supply and applied to analysis after concentration by using SPE method.

A simple and sensitive GC and GC-MS procedure was developed by Miki et.al (21) for fluoroacetic acid (FA) and phenoxy acid herbicides (PAHs) via triphasal extractive pentafluorobenzoylation. The LOD were 0.10-0.20 $\mu\text{g}/\text{mL}$ by GC-ECD and 0.42-0.50 $\mu\text{g}/\text{mL}$ by full-scan GC-MS. PAHs were also detectable in the same manner within the LOD of 0.05-0.10 $\mu\text{g}/\text{mL}$ by GC-ECD and 0.13-0.25 $\mu\text{g}/\text{mL}$ by full-scan GC-MS. Urine and serum which both contained 2,4-D could also be analyzed by GC-MS after the triphasal pentafluorobenzoylation. The LOD was 0.20 $\mu\text{g}/\text{mL}$ in the full-scan mode and 10 ng/mL in the selected ion monitoring mode both for the urine and serum.

An analytical method was presented by Vargo (22) which permits qualitative and quantitative analysis of sulfonic acid degradates of three chloroacetanilide herbicides (acetochlor, alachlor, and metolachlor) and one chloroacetamide herbicide (dimethenamid) in groundwater at trace levels with determination by LC/MS/MS. Excellent linearity was obtained over a calibration range from 0.25 to 10 ng injected on-column, with all linear correlation coefficients exceeding 0.999. Method performance for this analytical procedure was validated by analyzing groundwater samples fortified at levels of 0.1, 1, and 50 ppb. The average recovery at each fortification level for each analyte exceeded 89%. Excellent method precision was demonstrated with percent relative standard deviations of less than 10% for all analytes at all fortification levels.

A solid-phase microextraction (SPME)-GC procedure was reported by Natangelo et.al (23) for the analysis of four selected pesticides (propanil, acetochlor, myclobutanil and fenoxycarb) in water samples. MS was used and two different instruments, a quadrupole MS system and an ion trap operating in the MS-MS mode, were compared. A Carbowax-divinylbenzene SPME fiber was used. Specificity with these two instruments was comparable in the analysis of ground water samples. Recovery tests were made to assess the applicability of the SPME procedure in the quantitative analysis of contaminated groundwaters.

Guo et.al (24) determined Bentazon and 2,4-D butyl(the active ingredients of Cao Di) by reversed phase HPLC simultaneously using methanol/acetonitrile/citrate buffer(pH 3.1) as a mobile phase on Shim-pack clc-CN column. Results showed that the linear

correlation and recovery of 2,4-D butyl were 1.000 and $(100.2 \pm 0.8)\%$ respectively, and those of bentazon were 1.000 and $(98.6 \pm 0.9)\%$ respectively. The coefficients of variation for bentazon and 2,4-D butyl was 0.37%.

Beeson et.al (25) developed an isotope dilution HPLC/MS/MS method for quantifying the urinary metabolites of the pesticides atrazine, malathion, and 2,4-D. Urine samples were extracted with an organic solvent, and the organic fraction was concentrated. The concentrate was then analyzed using HPLC/MS/MS. The LOD for the metabolites were less than $0.5 \mu\text{g/L}$ in 10 mL of urine, with a high degree of accuracy and precision.

Hogendoorn et.al (26) observed that the coupled-column (LC-LC) configuration consisting of a $3 \mu\text{m}$ C18 column ($50 \times 4.6 \text{ mm I.D.}$) as the first column and a $5 \mu\text{m}$ C18 semi-permeable-surface (SPS) column ($150 \times 4.6 \text{ mm I.D.}$) as the second column appeared to be successful for the screening of acidic pesticides in surface water samples. In comparison to LC-LC employing two C18 columns, the combination of C18/SPS-C18 significantly decreased the baseline deviation caused by the hump of the co-extracted humic substances when using UV detection (217 nm). The developed LC-LC procedure allowed the simultaneous determination of the target analytes bentazone and bromoxynil in uncleaned extracts of surface water samples to a level of $0.05 \mu\text{g/l}$ in less than 15 min. In combination with a simple SPE step (200 ml of water on a 500 mg C18-bonded silica) the analytical procedure provides a high sample throughput. Weighted regression of the recovery data showed that the method provides overall recoveries of 95 and 100% for bentazone and bromoxynil, respectively, with corresponding intra-laboratory reproducibilities of 10 and 11%, respectively.

Tessier and Clark (27) reported hapten design in the development of two competitive enzyme-linked immunosorbent assays (cELISA) for the detection of 2-chloro-2',6'-diethylacetanilide (CDA) and 2-hydroxy-2',6'-diethylacetanilide (HAD). The acetanilide compounds CDA and HDA are environmental degradative products of the chloroacetanilide herbicide alachlor. SPE of CDA and HDA residues from aqueous samples results in a 1000-fold concentration factor, resulting in an effective LOD of 15 pg/mL for both assays. The specificity of the cELISAs required preservation of the degree of substitution of the acetanilide moiety in the hapten design. The hapten synthesis

strategies are suitable for metabolites of other chloroacetanilide herbicides (i.e., acetochlor, butachlor, metolachlor, and propachlor).

A new label--laccase from the fungus *Coriolus hirsutus*--was applied by Zherdev et.al(28) for solid-phase enzyme-linked immunosorbent assays of the pesticide 2,4-D. Two proposed assays were based on (1) competitive binding of antibody-laccase conjugate with immobilized 2,4-D-protein conjugate and 2,4-D in tested sample, and (2) competition of 2,4-D and 2,4-D-laccase conjugate for binding with immobilized antibodies. Kinetic and concentration dependencies for these reactions were studied, and the ELISAs were optimized in accordance with the data obtained. The elaborated systems permit the detection of 2,4-D in concentrations down to 10-20 ng/mL; time of the assays was 1.5-2 h. The main advantage of the laccase label, in comparison with the widely used peroxidase one, lies in the lack of hydrogen peroxide from substrate mixture, because dissolved oxygen plays the role of oxidizer.

A new analytic method for the determination of phenoxy acid herbicides and 2,4-D esters in soil samples by GC-FID was described by Santos-Delgado et.al (29). The esterification reaction with MeOH and H₂SO₄ as catalyst has been used, optimizing experimental variables by the 'Simplex method'. The recoveries in soil samples were found to be between 76 and 97% with RSD between 4 and 7% ($n=4$) at concentration level of 5 and 10 $\mu\text{g ml}^{-1}$ for phenoxy acids and 2,4-D esters, respectively.

Ding et.al (30) described a modified method to analyze chlorophenoxy acid herbicides in water samples. The herbicides 2,4-D, Silvex (2,4,5-trichlorophenoxypropionic acid) and 2,4,5-T were used to evaluate the method. The method involves extraction of samples by a graphitized carbon black cartridge, and on-line derivatization in the GC injection port using a large-volume (10-20 μl) direct sample introduction (DSI) device with tetra alkylammonium salts. The analytes were then identified and quantified by ion-trap GC-MS. The large-volume DSI injection-port derivatization technique provides sensitivity, fast and reproducible results for chlorophenoxy acid herbicides residues, for quantitation at 0.1 to 0.2 $\mu\text{g/l}$ in 500-ml water samples. Recovery of the herbicide residues in spiked various water samples ranged from 70 to 99% while RSDs ranged from 1 to 13%.

Hostetler and Thurman (31) developed analytical methods using high-performance liquid chromatography-diode array detection (HPLC-DAD) and HPLC/MS for the analysis of the following chloroacetanilide herbicide metabolites in water: alachlor ethanesulfonic acid (ESA); alachlor OA, acetochlor ESA; acetochlor OA, metolachlor ESA; and metolachlor OA. Good precision and accuracy were demonstrated for both the HPLC-DAD and HPLC/MS methods in reagent water, surface water, and ground water samples. The average HPLC-DAD recoveries of the chloroacetanilide herbicide metabolites from water samples spiked at 0.25, 0.5 and 2.0 $\mu\text{g/l}$ ranged from 84 to 112%, with RSD of 18% or less. The average HPLC/MS recoveries of the metabolites from water samples spiked at 0.05, 0.2 and 2.0 $\mu\text{g/L}$ ranged from 81 to 118%, with RSD of 20% or less. The LOQ for all metabolites using the HPLC-DAD method was 0.20 $\mu\text{g/L}$, whereas the LOQ using the HPLC/MS method was at 0.05 $\mu\text{g/L}$. These metabolite-determination methods were valuable for acquiring information about water quality and the fate and transport of the parent chloroacetanilide herbicides in water.

A sensitive method for measuring the urinary metabolites of atrazine, diazinon, malathion, 2,4-D, and certain synthetic pyrethroids in human urine was developed by Baker et.al (32). In this method, stable isotopically labeled analogues of the metabolites were spiked into the urine, which was subsequently extracted at both neutral and acidic pH using organic solvents. The extracts were analyzed by HPLC /MS using atmospheric pressure chemical ionization. The method has LOD ranging from 20 to 500 ng/L and RSD of less than 11%.

A rapid SPE method was developed by Thorstensen et.al (33) for the determination of bentazone and the phenoxy acids herbicide like 2,4-D, dichlorprop, MCPA, and mecoprop in Norwegian environmental water samples. Cartridges with a high-capacity cross-linked polystyrene-based polymer were used for off-line preconcentration. Average recovery of >90% was achieved with 500 mg sorbents using 2 mL of methanol with 5% NH_3 as elution solvent. The recoveries were independent of sample pH in the tested range of pH 1-7. Using a sample volume of 200 mL, the LOD for the phenoxy acids and bentazone are 0.02 $\mu\text{g/L}$. Sample volumes up to 2000 mL at a flow rate of 60 mL/min could be handled without any loss of analytes, which makes it possible to lower the LOD. The SPE method was compared to a routinely used liquid-liquid extraction method.

Three different water matrices spiked at 1.0 and 0.05 $\mu\text{g/L}$ were extracted, and the quantification was performed by GC-MS. Both methods permitted the determination of phenoxy acids and bentazone in distilled water, creek water, and well water down to a level of 0.05 $\mu\text{g/L}$ with recoveries >80% for 200 mL samples. Important advantages of the SPE method compared to the liquid-liquid extraction method were the short extraction times, lack of emulsions, use of disposable equipment, and reduced consumption of organic solvents.

A discussion of SPE method development for acidic herbicides was presented by Wells and Yu et.al. (34) that reviews sample matrix modification, extraction sorbent selection, derivatization procedures for GC analysis, and clean-up procedures for HPLC. Acidic herbicides were families of compounds that include derivatives of phenol (dinoseb, dinoterb and pentachlorophenol), benzoic acid (acifluorfen, chloramben, dicamba, 3,5-dichlorobenzoic acid and dacthal--a dibenzoic acid derivative), acetic acid (2,4-D, MCPA and 2,4,5-T), propanoic acid [dichlorprop, fluazifop, haloxyfop, 2-(4-chloro-2-methylphenoxy)propanoic acid (MCPB) and silvex], butanoic acid [4-(2,4-dichlorophenoxy)butanoic acid (2,4-DB) and 4-(4-chloro-2-methylphenoxy)butanoic acid (MCPB)], and other miscellaneous acids such as pyridinecarboxylic acid (picloram) and thiadiazine dioxide (bentazon).

Li and Lee (35) studied a dynamic ion-exchange solid-phase extraction (DIE-SPE) technique, for sample preparation for GC/MS analysis of sixteen acidic herbicides in environmental waters. C18 bonded silica was the solid-phase material used. The optimal sample pH was weakly acidic to neutral. However, for common tap water and surface water, which run pH 6-9, all the acidic herbicides except for Chloramben could be effectively extracted from a sample of 1,000mL volume without pH adjustment. Despite some minimal retention on the upper RP-SPE cartridge, most of the acidic herbicides could be extracted on the lower DIE-SPE cartridge with recovery over 80% except for Chloramben (50%), fenoprop (73%), MCPB (67%), and 2,4-DB (70%) when a 500-mL aqueous sample of pH 9.5 was percolated through the tandem-cartridge system. The effectiveness of the system in removing the long carbon chain fatty acids as well as the basic and neutral organic interfering substances from the sample was also demonstrated.

Heberle et.al (36) described a procedure for simultaneous enrichment, separation, and quantification of acetanilide herbicides and their major ionic OA and ESA metabolites in groundwater and surface water using Carboxen B as SPE material. The analytes adsorbed on Carboxen B were eluted selectively from the solid phase in three fractions containing the parent compounds (PCs), their OA metabolites, and their ESA metabolites, respectively. The complete separation of the three compound classes allowed the analysis of the neutral PCs (acetochlor, alachlor, and metolachlor) and their methylated OA metabolites by GC-MS. The ESA compounds were analyzed by HPLC with UV detection. The use of Carboxen B resulted in good recoveries of the polar metabolites even from large sample volumes (1 L). Absolute recoveries from spiked surface and groundwater samples ranged between 76 and 100% for the PCs, between 41 and 91% for the OAs, and between 47 and 96% for the ESAs. The maximum standard deviation of the absolute recoveries was 12%. The method LOD were between 1 and 8 ng/L for the PCs, between 1 and 7 ng/L for the OAs, and between 10 and 90 ng/L for the ESAs.

Lyubimov et.al (37) described a method to provide medical practitioners who care for members of the agricultural community with a cost-efficient way to conduct exposure assessment. They used a direct 2,4-D enzyme immunoassay (EIA) and measurement of the ratio between 2,4-D-spiked and non-spiked samples of the same urine to quantify 2,4-D levels. This simplified approach minimizes the effects of non-specific interfering substances in urine and eliminates the need for sample extraction and clean-up. Possible urine co-contaminants (2,4-dichlorophenol and 2,5-dichlorophenol) do not significantly interfere with this immunoassay. Coded urine specimens were examined for levels of 2,4-D by HPLC-MS/MS and compared with immunoassay results from the same specimens. A correlation coefficient of $r = 0.982$ with a P value of .0001 for a plot of HPLC-MS/MS versus immunoassay demonstrated that the results from these methods were comparable over urinary dose levels ranging from not detectable (<19 ppb) to 1700 ppb 2,4-D, as determined by immunoassay.

Gallego et. al (38) determined two phenoxyacid herbicides (2,4-D and MCPA) and their six corresponding phenols in soil by using GC/MS for quantitation. An automatic extraction (leaching), preconcentration, and cleanup (sorption) module was developed to

extract these eight compounds from soil. The average recovery of all species, spiked to soil at $\mu\text{g/kg}$ - mg/kg levels, was 95% (average standard deviation $\pm 5\%$). A plot of agricultural clay soil (approximately 12 m^2) was contaminated with both herbicides (approximately 96 g/m^3 , depth 10 cm, density 1.23 g/cm^3) and irrigated with (17 mm) at variable time intervals.

Kahn et al.(39) presented a peer-verified GC method for the weight percent (wt %) determination of acetochlor herbicide in technical and formulated products. The test samples were dissolved in acetone that contained dipentyl phthalate as an internal standard. They were analyzed by GC on a 15 m capillary column by using split injection and a flame ionization detector (FID). Acetochlor (wt %) was determined by comparing the ratios of peak area of acetochlor/peak area of dipentyl phthalate internal standard obtained for the test sample and calibration solutions. Repeatability of the method, expressed as the within-laboratory (between replicates). RSD was found to be 0.09-0.77% for the 5 matrixes.

Dickow et.al (40) developed a method for the simultaneous determination of 2,4-D and MCPA in canine plasma and urine. This method used derivatization of extracted samples with 9-anthrylmethane (ADAM) for analysis by reversed-phase HPLC with fluorescence detection. Precision and accuracy were within the accepted limits of 15% and 85-115%, respectively, for both analytes in plasma and urine. Calibration curves for 2,4-D and MCPA in plasma were linear ($r^2 > 0.99$) between 0.50 and 5.0 mg/L and 5.0 and 100 mg/L. Calibration curves for 2,4-D and MCPA in urine were linear ($r^2 > 0.99$) between 5.0 and 70.0 mg 2,4-D/L and 10.0 and 70.0 mg MCPA/L. The lower LOD was 62.5 ng/mL for both 2,4-D and MCPA.

Dijkman et.al. (41) investigated the effects of matrix interferences on the analytical performance of a triple quadrupole mass spectrometric (MS-MS) detector coupled to various reversed-phase liquid chromatographic (LC) modes for the on-line determination of various types of acidic herbicides in water using external calibration for quantification of the analytes tested at a level of $0.4\text{ }\mu\text{g/l}$. The LC modes included (i) a single-column configuration (ii) precolumn switching and (iii) coupled-column. For detection, electrospray and atmospheric pressure chemical ionization in both positive and negative ionization modes were examined. Salinity and dissolved organic carbon were selected as

interferences to study matrix effects in this type of analysis. The recovery data were used to compare and evaluate the analytical performance of the various LC approaches.

A screening method was developed by Hogendoom et.al (42) for the determination of acidic pesticides in various types of soils. Methodology was based on the use of microwave assisted solvent extraction (MASE) for fast and efficient extraction of the analytes from the soils and coupled-column reversed-phase liquid chromatography (LC-LC) with UV detection at 228 nm for the instrumental analysis of uncleaned extracts. Four types of soils, including sand, clay and peat, with a range in organic matter content of 0.3-13% and ten acidic pesticides of different chemical families (bentazone, bromoxynil, metsulfuron-methyl, 2,4-D, MCPA, MCPP, 2,4-DP, 2,4,5-T, 2,4-DB and MCPB) were selected as matrices and analytes, respectively. Weighted regression of the recovery data showed for most analyte-matrix combinations, including freshly spiked samples and aged residues, that the method provides overall recoveries between 60 and 90% with RSD of the intra-laboratory reproducibility's between 5 and 25%; LODs were obtained between 5 and 50 $\mu\text{g/kg}$.

Casino et.al (43) developed a reliable and sensitive indirect ELISAs for the quantitative determination of metolachlor, alachlor, and acetochlor. Under best conditions, LOD of 0.06, 0.3, and 0.4 $\mu\text{g/L}$ for metolachlor, alachlor, and acetochlor were reached, respectively. The optimized ELISAs were also highly specific, showing little or no cross-reactivity to other similar compounds. Immunoassays were used as a tool to determine critical chloroacetanilide herbicides in water and soil samples without purification steps. The excellent recoveries obtained (mean value ranging between 90% and 98%) confirm the potential of this approach to control these herbicides in the environment being applied as a "screening" method either for field monitoring or laboratory.

An imaging assay analogous to competitive enzyme immunoassays has been developed by Surugiu et.al (44) using a molecularly imprinted polymer instead of an antibody. The antigen 2,4-D was labeled with tobacco peroxidase, and the chemiluminescence reaction of luminol was used for detection. Microtiter plates (96 or 384 wells) were coated with polymer microspheres imprinted with 2,4-D, which were fixed in place by using poly vinyl alcohol as glue. In a competitive mode, the analyte-peroxidase conjugate was incubated with the free analyte in the microtiter plate, after which the bound fraction of

the conjugate was quantified. After addition of the chemiluminescent substrates, light emission was measured in a high-throughput imaging format with a CCD camera. Calibration curves corresponding to analyte concentrations ranging from 0.01 to 100 $\mu\text{g/mL}$ were obtained.

A flow injection competitive assay analogous to enzyme immunoassays was presented by Surugiu et.al (45) using a molecularly imprinted polymer instead of the antibody. A glass capillary was modified by covalently attaching an imprinted polymer to the inner capillary wall. The herbicide 2,4-D was used as a model analyte. The analyte was labeled with tobacco peroxidase, and chemiluminescence was used for detection in combination with a photomultiplier tube or a CCD camera. In a competitive mode, the analyte-peroxidase conjugate was passed together with the free analyte through the polymer-coated capillary mounted in a flow system. After a washing step, the chemiluminescent substrate was injected and the bound fraction of the conjugate was quantified by measuring the intensity of the emitted light. Calibration curves corresponding to analyte concentrations ranging from 0.5 ng/mL to 50 $\mu\text{g/mL}$ (2.25 nM-225 μM) were obtained. A lowered LOD by 2 orders of magnitude was obtained when detection was done in discontinuous mode and the chemiluminescence light was conducted inside the photomultiplier tube by an optical fiber bundle, thus yielding a dynamic range of 5 pg mL^{-1} -100 ng mL^{-1} (22.5 pM-450 nM).

A SPME method was developed by Henriksen et.al (46) for the analysis of acidic pesticide residues in water. The method utilizes in situ derivatization with butylchloroformate, followed by on-line SPME extraction using a PDMS fibre, and analysis by GC-MS. Derivatives of the phenoxy acids mechlorprop (MCP), dichlorprop (DCP), MCPA and 2,4-D and their phenol degradation products 4-chloro-2-methylphenol and 2,4-dichlorophenol (DCP) were identified. LOD at 0.16-2.3 $\mu\text{g/L}$ were achieved. Standard curves in the range 0.5-10.0 $\mu\text{g/L}$ were fitted to a second-degree polynomial. Standard deviation ($n = 5$) was below 10% for the phenol derivatives, but 20-50% for the phenoxy acids.

Ramesh and Ravi (47) developed a method based on SPME for the determination of residues of eight selected herbicides: trifluralin, butachlor, pretilachlor, metolachlor, atrazine, acetochlor, alachlor, and fluroxypyr-meptyl, in ground water samples.

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Carbowax divinylbenzene was used for SPME of the herbicides. Quantification was done at equilibrium. The method was found suitable for the determination of residues of herbicides at concentration levels from 5 to 20 ng L⁻¹. The RSD of the detection limits were calculated. The humic acid content was found to interfere more significantly in the determination of residues. An ionic chloride strength, in terms of sodium chloride, of up to 10% did not have any significant impact on the determination. Furthermore, there was an enhanced sensitivity of detection up to pH 3.0.

Thorstensen et.al (48) described a method for the extraction of bentazone, dichlorprop, and MCPA in three selected Norwegian soils of different textures. Initially three different extraction methods were tested on one soil type. All methods gave recoveries >80% for the pesticide mixture, but extraction with sodium hydroxide in combination with solid-phase preconcentration was used for further recovery tests with soils of different properties spiked at four herbicide concentration levels (0.001-10 µg/g of wet soil). The method was rapid and easy and required a minimum of organic solvents. The recoveries were in the range of 82-109, 80-123, and 45-91% for the soils containing 1.4 (Hole), 2.5 (Kroer), and 37.8% (Froland) organic carbon, respectively. LOQ using GC-MS were 0.0003 µg/g of wet soil for bentazone and 0.0001 µg/g of wet soil for both dichlorprop and MCPA.

García-Campañas et.al (49) presented a combination of a FIA system with micellar-enhanced photochemically induced fluorescence (MEPIF) detection as a powerful alternative for the rapid and sensitive analysis of chlorophenoxyacid herbicides. These compounds do not show native fluorescence but they can be photolysed into strongly fluorescence photoproducts after direct irradiation with ultraviolet light. The use of a cationic surfactant such as cetyltrimethylammonium chloride provides a considerable enhancement of photochemically induced fluorescence intensity and the nature of the technique allows a possible and easy adaptation to FIA system. Linear calibration graphs, with three replicates for each concentration value were established in the range of 0.2-5.0 µg ml⁻¹ for 2,4-D and 0.1-5.0 µg ml⁻¹ for Mecoprop (MCP). The IUPAC LOD were 73.2 and 33.5 ng ml⁻¹ for 2,4-D and MCP, respectively. Satisfactory recoveries were obtained in the analysis of these herbicides in spiked waters.

Hughes et.al (50) described a method that allowed the determination of 2,4-D in human urine. The accurate determination of 2,4-D in urine was an important factor in monitoring worker and population exposure. These studies successfully validated a method for the detection of 2,4-D in urine at a LOQ of 5.00 ppb using GC/MS detection. The 2,4-D was extracted from human urine using a procedure involving hydrolysis using potassium hydroxide, followed by a liquid-liquid extraction into methylene chloride. The extracted samples were derivatized with diazomethane. The methylated fraction was analyzed by GC/MS. Quantitation was made by comparison to methylated reference standards of 2,4-D. Aliquots fortified at 5-, 50-, and 500-ppb levels were analyzed. The overall mean recovery for all fortified samples was 90.3% with a RSD of 14.31%.

Xiang et al (51) described a method for the determination of acetochlor and oxyfluorfen by capillary gas chromatography with FID and an SE-30 capillary column (60 m x 0.53 mm i. d., 1.5 μ m), using dibutyl phthalate as the internal standard. The standard deviations for acetochlor and oxyfluorfen concentration(mass fraction) were 0.44% and 0.47% respectively. The RSD for acetochlor and oxyfluorfen were 0.79% and 0.88% and the average recoveries for acetochlor and oxyfluorfen were 99.3% and 101.1% respectively. The method was simple, rapid and accurate.

Shoemaker (52) developed a method for determination of acetanilide degradation products in drinking water using LC/negative ion electrospray/MS with SPE. A novel chromatographic separation of the acetochlor/alachlor ESA and OA structural isomers was developed which uses an ammonium acetate-methanol gradient combined with heating the analytical column to 70°C. Twelve acetanilide degradates were extracted by SPE from 100 mL water samples using carbon cartridges with mean recoveries >90% and RSDs < or =16%.

A simple and rapid method based on microwave-assisted extraction (MAE) coupled to GC analysis was developed by Vryzas et.al (53) for the analysis of triazine (atrazine, cyanazine, metribuzine, simazine and deethylatrazine, and deisopropylatrazine) and chloroacetanilide (acetochlor, alachlor, and metolachlor) herbicide residues in soils. Soil samples were processed by MAE for 5 min at 80°C in the presence of acetonitrile (20 mL/sample). Mean recovery values of most solutes are >80% in the 10 to 500 μ g/kg

fortification range with respective RSDs < 20%. The LOQ and LOD were 10 and 1 to 5 $\mu\text{g/kg}$, respectively. Residue levels determined in field weathered soils were higher when soils were processed by MAE than with a comparison method based on flask-shaking of soil suspensions overnight. Extracts were analyzed by a GC system equipped either with a thermionic (GC-NPD) or GC-MS.

A simple method for the simultaneous determination of chlorophenoxy acid herbicides and their esters in soil was presented by Rosales-Conrado et.al (54). Compounds studied are 2,4-D, 4-(2,4-dichlorophenoxy)butanoic acid (2,4-DB), 2,4-dichlorophenoxyacetic-1-butyl ester (2,4-D-1-butyl ester), and 2,4-dichlorophenoxyacetic-1-methyl ester (2,4-D-1-methyl ester). The chromatographic analysis was carried out by HPLC, after ultrasonic extraction, on a C_{18} packed capillary column with temperature gradient, large injection volumes and UV detection at 232 nm. Samples were spiked with amounts between 2.5 and 6.0 $\mu\text{g g}^{-1}$ of each herbicide; recoveries obtained were between 72 and 97% ($n=3$ for each spiked level) and LOD were between 0.3 and 0.5 $\mu\text{g g}^{-1}$.

Thurman et.al (55) analyzed the degradates of acetochlor and alachlor (ethanesulfonic acids, ESAs) in both standards and in a groundwater sample using HPLC-time-of-flight mass spectrometry with electrospray ionization. The negative pseudomolecular ion of the secondary amide of acetochlor ESA and alachlor ESA gave average masses of 256.0750 ± 0.0049 amu and 270.0786 ± 0.0064 amu respectively. Acetochlor and alachlor ESA gave similar masses of 314.1098 ± 0.0061 amu and 314.1153 ± 0.0048 amu; however, they could not be distinguished by accurate mass because they have the same empirical formula. On the other hand, they may be distinguished using positive-ion electrospray because of different fragmentation spectra, which did not occur using negative-ion electrospray.

Marchese et.al (56) made an evaluation of the feasibility of using reversed-phase liquid chromatography/tandem mass spectrometry with an electrospray interface (LC/ESI-MS/MS) to measure traces of phenoxyacid herbicides and their metabolites in surface and drinking water samples. The procedure involved passing 0.5 L of river and drinking water samples through a 0.5 g graphitized carbon black extraction cartridge. Recovery

was higher than 85% irrespective of the aqueous matrix in which the analytes were dissolved. A conventional 4.6-mm i.d. reversed-phase LC C-18 column operating with a mobile phase flow rate of 1 mL/min was used to chromatograph the analytes. A flow of 200 μ L/min of the column effluent was diverted to the ESI source. The LOD (signal-to-noise ratio = 3) of the method for the pesticides considered in drinking and surface water samples are less than 0.1 ng/L for phenoxyacid herbicides, and about 5-10 ng/L for their metabolites (2,4-DCP and 4-chloro-2-methylphenol).

A new analytical method was presented by Dagnac et.al (57) that describes the extraction and quantification of OA and SA metabolites of the herbicide acetochlor in soil samples. Experiments were performed on 50 g of soil using a solvent extraction technique with an acetonitrile-water (60:40) mixture in an acidic medium. Analysis was carried out by reversed-phase liquid chromatography and detection by electrospray ionization mass spectrometry in single ion monitoring and negative modes. Four different soil matrices were spiked in triplicate with standards of each degradation compound at three concentration levels between 2 and 80 μ g/kg. The average recoveries ranged from 90 to 120% for both the metabolites, with RSD lower than 15%. The LOQ were about 1 and 2 μ g/kg for the ESA and the OA metabolites, respectively.

A multiresidue analytical method was presented by Yokley et.al (58) for the determination of the chloroacetanilide herbicides, metolachlor, acetochlor, and alachlor, the chloroacetamide herbicide, dimethenamid, and their respective ESA and OA degradates in ground and surface water. A 50mL water sample was subjected to purification using a C-18 SPE column. The four parent components and their eight ESA and OA degradates were isolated using 80/20 methanol/water (v/v) for elution. The eluate was reduced to < 1.0 mL and reconstituted in 10/90 acetonitrile/water (v/v) to the desired final fraction volume. Final analysis was accomplished using LC/electrospray ionization-mass spectrometry/MS in the + (parent compounds) and (ESA and OA degradates) ion modes by monitoring appropriate precursor/product ion pairs for each of the 12 analytes. The method LOQ was 0.10 ppb and the LOD was 0.125 ng injected for each analyte. Average procedural recovery data range from 95 to 105% for fortification levels of 0.10-100 ppb.

Wells et.al (59) described the comparison of quantitative NMR spectroscopy (QNMR) with chromatographic methods such as GC or HPLC for the determination of the purity of and impurities in technical grade agrochemicals, 2,4-D and Dalapon sodium (sodium 2,2-dichloropropionate). Comparison revealed that QNMR was more precise and accurate than the chromatographic methods. Quantitative impurity profiling of technical grade 1 was rapid and accurate using 600 MHz (1) H NMR. Extra dispersion at the relatively high frequency allowed full assignment of the NMR spectrum of 1 and its related organic impurities in technical samples.

Zayed et.al (60) studied the mineralization of 2,4-D in clay and clay loam Egyptian soils under subtropical conditions over a period of 90 d. The mineralization of 2,4-D in clay soil was higher than that in clay loam soil under anaerobic conditions. Under aerobic conditions, the soil type had no influence on mineralization capacity of 2,4-D during 90 d. The soil binding increased with time whereby the extractable ^{14}C residues simultaneously decreased. Chromatographic analysis of the methanol extractable ^{14}C -residues of soils revealed the presence of 2,4-D as a main product together with 2,4-DCP.

Laboratory studies were conducted by Nemeth-Konda et.al (61) to determine the sorption behaviour of six commonly used pesticides (acetochlor, atrazine, carbendazim, diazinon, imidacloprid and isoproturon) on Hungarian brown forest soil with clay alluviation (Luvisol) using the batch equilibrium technique. The adsorption constant related soil organic carbon content (Koc) calculated from Freundlich equation were 314 for acetochlor, 133 for atrazine, 2805 for carbendazim, 1589 for diazinon, 210 for imidacloprid and 174 for isoproturon.

Sixteen pesticides belonging to nine chemical families were monitored by Baraud et.al (62). After extracting the samples and concentrating the recovered solutions, HPLC analysis with UV detection was performed. Identification of the pesticides was done by applying to the HPLC measurements a novel UV-detection procedure based on the normalized absorbance variation with wavelength (Noravawa procedure). The presence of metsulfuron methyl, isoproturon, linuron, deltamethrin (and/or malathion), and chlorophenoxy acids (2,4-D and MCPP) was found at the urban sampling site at levels

ranging from about 1 to 1130 ng m⁻³ of air, depending on the compound and sampling period. On the rural sampling site residues of isoproturon, deltamethrin (and/or malathion), MCPP, and 2,4-D were generally detected at higher levels (19-5130 ng m⁻³) than on the urban site, as expected.

Amarante et.al (63) developed a method for simultaneous extraction and determination of 2,4-D and its major transformation product, i.e., the 2,4-DCP, in soil samples. Recoveries were obtained from soil samples fortified at 0.1, 1.0, 2.0, 3.0 and 4.0 mg kg⁻¹ levels and the results varied from 85 to 111% (for 2,4-D) and from 95 to 98% (for 2,4-DCP). For both compounds, the LOQs were 0.1 mg kg⁻¹, which were the loss level at which the accuracy and the precision were studied. Nevertheless, the LOD, calculated by considering the blank standard deviation and the minimum concentration level, were 0.03 and 0.02 mg kg⁻¹, for 2,4-D and 2,4-DCP, respectively.

Halimah et.al (64) developed improved methods for extraction and clean up of fluroxypyr residue in water. Two methods of fluroxypyr extraction were used, namely, direct measurement of fluroxypyr and concentration of fluroxypyr onto SPE adsorbent, followed by elution with solvent before determination of fluroxypyr. The recovery for direct measurement of fluroxypyr in water containing 8-100 µg L⁻¹, ranged from 86 to 110% with RSD of 0.7 to 2.15%. The recovery of fluroxypyr from the spiked sample at 1 to 50 µg L⁻¹ after eluting through either C₁₈ or C₁₈ end-capped ranged from 40-64% (with RSD of 0.7 to 2.15) and 41-65% (with standard deviation of 1.52 to 11.9). The use of isolate env+, gave better results than the C₁₈, C₁₈ end-capped or the direct measurement methods. The recovery and standard deviation of fluroxypyr from spiked water using isolate env+ ranged from 91-102% and 2.5 to 5.3, respectively.

An aerosol time-of-flight mass spectrometer (ATOFMS) was used by Whiteaker et.al (65) to analyze the size and composition of individual particles containing pesticides. The common names of the pesticides studied were 2,4-D, atrazine, chlorpyrifos, malathion, permethrin, and propoxur. Analysis of the mass spectra produced by single- and two-step laser desorption/ionization of pesticide-containing particles allows for identification of peaks that could be used for detection of pesticide residues in the ambient aerosol.

Results suggest that this technique may be useful for studying the real-time partitioning and distribution of pesticides in the atmosphere immediately following application in agricultural regions.

Zheng et.al (66) described a method for determination of photoproducts of acetochlor in water. The sunlight photodegradation half-lives of 20 mg/L acetochlor were 151, 154 and 169 days in de-ionized water, river water and paddy water, respectively. When exposed to UV light, acetochlor in aqueous solution was rapidly degraded. The half-lives were 7.1, 10.1, and 11.5 min in de-ionized water, river water and paddy water, respectively. Photoproducts of acetochlor were identified by GC/MS and found at least twelve photoproducts resulted from dechlorination with subsequent hydroxylation and cyclization processes. The chemical structures of ten photoproducts were presumed on the basis of mass spectrum interpretation and literature data. Photoproducts were identified as 2-ethyl-6-methylaniline; N,N-diethylaniline; 4,8-dimethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 2-oxo-N-(2-ethyl-6-methylphenyl)-N-(ethoxymethyl) acetamide; N-(ethoxymethyl)-2'-ethyl-6'-methylformanilide; 1-hydroxyacetyl-2-ethoxyl-7-ethylindole; 8-ethyl-1-ethoxymethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 4, 8-dimethyl-1-ethoxymethyl-2-oxo-1,2,3,4-tetrahydroquinoline; 2-hydroxy-2'-ethyl-6'-methyl-N-(ethoxymethyl) acetanilide and a compound related to acetochlor. The other two photoproducts were detected by GC/MS although their chemical structure were unknown.

Sutherland et.al (67) developed a methodology for analysis of soil and water runoff samples concomitantly containing the herbicides simazine (1-chloro-4,6-bis(ethylamino)-s-triazine) and 2,4-D to investigate the potential for runoff and leaching from ornamental nursery plots. SPE was used prior to analysis by GC and LC. Chromatographic results were compared with determination by ELISA. The significant analytical contributions of this research include (1) the development of a scheme using chromatographic mode sequencing for the fractionation of simazine and 2,4-D, (2) optimization of the homogeneous derivatization of 2,4-D using the methylating agent boron trifluoride in methanol as an alternative to in situ generation of diazomethane, and (3) the practical application of these techniques to field samples.

Jankowska et.al (68) described an HPLC procedure for simultaneous determination of chlorophenoxy acid herbicides and their radiolytic degradation products in waters with the use of octadecylsilica column and spectrophotometric detection at 280 nm. The satisfactory separation was achieved with a mobile phase of pH 2.5 consisting of 43.7 mM acetic acid with 40% (v/v) acetonitrile. LOD values for herbicides and phenol derivatives were in the range of 19-41 $\mu\text{g/L}$ and 10-60 $\mu\text{g/L}$, respectively. The developed method was applied for monitoring the effectiveness of radiolytic degradation of herbicides. Studies of products of gamma-radiolysis of 2,4-DCP have shown that the efficiency of this process was affected by the presence of naturally occurring scavengers of gamma-radiation such as carbonates or nitrates.

Nakazawa et.al (69) developed a simple method for the rapid and simultaneous analysis of dichlorvos (DDVP), malathion, carbaryl, and 2,4-D in citrus fruit, which uses flow-injection ion spray ionization tandem mass spectrometry. The method involved the combined use of stable isotopically labeled internal standards (DDVP- d_6 , malathion- d_{10} , carbaryl- d_7 , and 2,4-D- d_5) and a multiple reaction monitoring technique. The average recoveries for the pesticides at the same concentrations as their tolerance levels (DDVP: 0.1–0.2 $\mu\text{g g}^{-1}$; malathion: 0.5–4.0 $\mu\text{g g}^{-1}$; carbaryl: 1.0 $\mu\text{g g}^{-1}$; 2,4-D: 1.0–2.0 $\mu\text{g g}^{-1}$) ranged from 90 to 119% with the RSD ranging from 1.0 to 13.1% ($n = 5$). Analysis time, including sample preparation and determination, was only 15 min.

Electrochemical impedance spectroscopy (EIS) was described by Navratilova et.al (70) for the direct determination of herbicide 2,4-D. Specific antibody against 2,4-D was immobilised onto different gold electrodes. Several methods of antibody immobilisation by covalent linkage to modified surface were studied. Self-assembled monolayers formed using thiocompounds as cystamine, 4-aminothiophenol, 3,3'-dithiopropionic acid di-(N-succinimidyl ester) and 11-mercaptoundecanoic acid were chosen for the sensing surface activation. Three different sensor types were tested: screen-printed disc and finger-like structures and interdigitated array electrodes produced by lithography. The measurements were carried out in a stationary arrangement, and the reaction between hapten and the immobilised antibody was observed online. Changes of impedance parameters were evaluated, and the best immobilisation technique (using 4-

aminothiophenol) was chosen for further measurements. Impedance changes due to immunocomplex formation were evaluated, and the possibility of direct monitoring of 2,4-D binding to the antibody was demonstrated at a fixed frequency. For the strip sensor, the calibration curves were constructed in concentration range from 45 nmol l⁻¹ to 0.45 mmol l⁻¹ of 2,4-D.

Four methods were developed by Halimah et.al. (71) for the analysis of fluroxypyr in soil samples from oil palm plantations. The first method involved the extraction of the herbicide with 0.05 M NaOH in methanol followed by purification using acid base partition. The concentrated material was subjected to derivatization and then cleaning process using a florisil column and finally analyzed by GC-ECD. By this method, the recovery of fluroxypyr from the spiked soil ranged from 70 to 104% with the minimum LOD of 5 µg/kg. The second method involved solid liquid extraction of fluroxypyr using a horizontal shaker followed by quantification using HPLC equipped with UV detector. The recovery of fluroxypyr using this method, ranged from 80 to 120% when the soil was spiked with fluroxypyr at 0.1-0.2 µg/g soil. In the third method, the recovery of fluroxypyr was determined by solid liquid extraction using an ultrasonic bath. The recovery of fluroxypyr at spiking levels of 4-50 µg/L ranged from 88 to 98% with RSD of 3.0-5.8% with a minimum LOD of 4 µg/kg. In the fourth method, fluroxypyr was extracted using the solid liquid extraction method followed by the cleaning up step with OASIS HLB (polyvinyl dibenzene). The recovery of fluroxypyr was between 91 and 95% with RSD of 4.2-6.2%, respectively. The LOD in method 4 was further improved to 1 pg/kg. When the weight of soil used was increased 4 fold, the recovery of fluroxypyr at spiking level of 1-50 µg/kg ranged from 82-107% with RSD of 0.5-4.7%.

Ayranci and Hoda (72) studied the removal of pesticides such as metribuzin, bromacil, 2,4-D and atrazine from aqueous solutions by adsorption on high area carbon cloth. The adsorption process was followed by in situ UV-spectrophotometric technique in a specially designed adsorption cell. Spectroscopic data of the pesticides were determined in separate experiments. The extent of adsorption was quantified by calculating the amount of adsorbate adsorbed per unit area of the carbon cloth and the percentage coverage at the carbon-cloth surface. The order of extent of adsorption of the pesticides

studied was found as metribuzin < 2,4-D < bromacil < atrazine. The adsorption process was found to follow a first-order kinetics and the rate constants were determined. The competitive adsorption of pesticides was also examined by carrying out the adsorption process from a solution of equimolar mixture of bromacil and metribuzin.

Liubavina et.al. (73) developed competitive immunochromatographic assay for the pesticides 2,4-D and simazine (2-chloro-4,6-bis(N-ethylamino)-1,2,5-triazine) in aqueous samples. Monoclonal antibodies to these pesticides labeled with colloidal gold were used to visualize the results. The sensitivity of the 2,4-D and simazine assay was 12 ng/ml, and the time of analysis was 3-7 min. The method does not differ in sensitivity from the competitive ELA using conjugates of monoclonal antibodies to the pesticides with horseradish peroxidase; however, the time of the ELA was 1.5 h.

A multiresidue method using GC coupled to ion-trap tandem mass spectrometry (MS/MS) was developed by Scheyer et.al (74) for the analysis of twenty seven pesticides, commonly used in Alsace, in atmospheric samples (particle and gas phases). To develop this method, analysis of electron impact in single MS was first conducted to choose the parent ions of the pesticides studied. Among the twenty seven pesticides analysed, seven of them required a derivatisation step. This was the case of some ureas (chlorotoluron, diuron and isoproturon), phenoxy acids (2,4-D, MCPA, mecoprop) and of bromoxynil. The derivatization was performed with pentafluorobenzylbromide. With this analytical procedure, the LOD varied between 2.5 and 1,250 pg m⁻³ depending on the compounds studied.

Lekic et.al (75) described an analytical method that could be used for rapid identification and determination of chlorophenoxy herbicides in modestly equipped laboratories for the clinical toxicology. Thin layer chromatography on cellulose layer using neutral red as ion pairing reagent gave the best results in separation of different herbicides, making possible visualisation at the daylight without further reagents or equipment. HPLC methods for separation on C₈ and C₁₈ phases with and without ion-pairing reagent were compared. It was found that HPLC on C₁₈ phase utilizing ion-suppression mode has the best reproducibility, linearity and mass LOD suitable for quantification of chlorophenoxy herbicides after acute poisoning.

Wu et.al (76) investigated an automated dynamic liquid-liquid-liquid microextraction (D-LLLME) controlled by a programmable syringe pump and combined with HPLC-UV for the extraction and determination of five phenoxy acid herbicides in aqueous samples. Good linearity of analytes was achieved in the range of 0.5-500 ng/ml with coefficients of determination, $r_2 > 0.9994$. Good repeatabilities of extraction performance were obtained with RSD lower than 7.5%. The method provided up-to 490-fold enrichment within 13 min. In addition, the LODs ranged from 0.1 to 0.4 ng/mL ($S/N = 3$). D-LLLME was successfully applied for the analysis of phenoxy acid herbicides from real environmental water samples.

Moret et.al (77) developed a procedure using alkaline extraction SPE and HPLC to analyze the polar herbicides 2,4-D and MCPA together with their main metabolites in soils. An ion-pairing HPLC method was used for the determination as it permits the baseline separation of these highly polar herbicides and their main metabolites. The use of a highly cross-linked polystyrene-divinylbenzene sorbent (PS-DVB) gives the best results for the analysis of these compounds. This sorbent allows the direct preconcentration of the analytes at the high pH values obtained after quantitative alkaline extraction of the herbicides from soil samples. Different parameters were evaluated for the SPE preconcentration step. The high polarity of the main analytes of interest (2,4-D and MCPA) makes it necessary to work at low flow rates ($\leq 0.5 \text{ mL min}^{-1}$) in order for these compounds to be retained by the PS-DVB sorbent. A two stage desorption from the SPE sorbent is required to obtain the analytes in solvents that are appropriate for HPLC determination. A first desorption with a 50:50 methanol:water mixture elutes the most polar analytes (2,4-D, MCPA and 2CP). The second elution step with methanol permits the analysis of the other phenol derivatives. The humic and fulvic substances present in the soil are not efficiently retained by PS-DVB sorbents at alkaline pH's and so do not interfere in the analysis.

The relationship between excess thermodynamic function and binding to urease of four chloroacetanilide herbicides was studied by Liu et.al (78) using HPLC and fluorescence spectrum. The linear relationship between the composition of mobile phase of RP-HPLC and the capacity factor of chloroacetanilide herbicides has been obtained. The excess

thermodynamic enthalpy (ΔH^*) of acetochlor, pretilachlor, butachlor and metolachlor was determined, and the binding constant K and the number of binding sites with urease were calculated. The relationship between excess thermodynamic function and the binding constant K was suggested.

Kashyap et.al (79) developed a fast and efficient method by selection of modified extraction apparatus and HPLC-UV conditions for the determination of 2,4-D in soil samples. The method was applied to the study of soil samples collected from the agricultural field. The herbicide was extracted from soil samples by acetonitrile in a modified Soxhlet apparatus. Recoveries obtained varied from 85% to 100% for spiked soil samples. The LOQ and the LOD were 0.010 and 0.005 ppm, respectively, for spiked soil samples. The LOQ and LOD were 0.006 and 0.003 ppm for unspiked soil samples. The measured concentrations of 2,4-D in spiked soil samples were between 0.010 and 0.020 ppm with an average of 0.016 ± 0.003 ppm. For unspiked soil samples it was between 0.006 ppm and 0.012 ppm with an average of 0.009 ± 0.002 ppm. The measured concentrations of 2,4-D in soil samples were generally low and do not exceed the regulatory agencies guidelines.

Three types of solid-phase chemical exposure sampling media: cellulose, polyurethane foam, and XAD-2, were analyzed by Gardner et.al. (80) for 2,4-D and the amine salts of 2,4-D. Individual samples were extracted into acidified methanol, and the extracts were analyzed via liquid chromatography-tandem mass spectrometry using electrospray ionization and a C_8 reversed-phase LC column. ^{13}C 2,4-D and a labeled benzoic acid ^{13}C or D_5 were employed as the surrogate and internal standard, respectively. Over a three-year study of pesticide exposure among agricultural workers involving a high volume of analyses, values of average % recovery for ^{13}C 2,4-D on personal exposure samples ranged from 87 to 98 with a % RSD ranging from 7 to 12.

Cao et.al. (81) developed an indirect cELISA for the nitrile herbicide bromoxynil, by raising the polyclonal antibodies against 2,6-dibromo-4-cyano-phenoxyacetic acid (hapten) conjugated to bovine serum albumin by the N-hydroxysuccinimide-activated ester method. Antiserum with a sufficiently high titer to provide the determinations of

targeted compounds was obtained only 77 days after the primary immunization. Antiserum A2 was applied to the residual analysis of some water samples, under optimized ELISA condition. The quantitative working range was from 10 to 500 ppb with a LOD of 5 ppb. Cross-reactivity to structurally similar agrochemicals and related chemicals was determined. The antiserum showed little cross-reactivity with 2,6-dibromophenol and bromoxynil octanoate ester which have a dibromophenol group as common structure, but showed no cross-reactivity with other herbicides. Each water sample (river water, tap water, purified water, and bottled water) had a matrix effect and was investigated by adding Tween20 in the assay buffer. These four kinds of water samples were fortified with bromoxynil at several concentration levels and were directly analyzed with only dilution with an equal volume of antiserum solution, the mean recovery was 102.3%, and the mean coefficient of variation was 5.96%.

The Hwang et.al (82) applied SPME technique using a 75 mm film of carboxen/polydimethylsiloxane for the analysis of chloroacetanilide herbicides (acetochlor, alachlor, butachlor, metolachlor, and propachlor) residues. The feasibility of SPME with GC-ECD analysis has been evaluated. The method provided a simple and rapid analytical procedure for chloroacetanilide herbicides in water with LOD 0.002-0.065 $\mu\text{g/L}$ for deionized water, and 0.005-0.22 $\mu\text{g/L}$ for farm water. The RSD ($n = 5$) for analyses of farm water were 7-20% for 0.5 $\mu\text{g/L}$ chloroacetanilide herbicides.

Toba and Hay (83) studied the development of a simple solid-phase bioassay for the direct detection of the herbicide 2,4-D in soil using *Ralstonia eutropha* JMP 134-32, a luxCDABE-based 2,4-D whole cell bio reporter. The bio reporter was spotted onto glass microfibre filter discs that allowed its retrieval and analysis after exposure to 2,4-D amended soils. These disc-fixed cells responded in a concentration dependent manner to 2,4-D in solution (0-25 mg/L) and in spiked soil (0-50 mg/kg). The influence of environmental factors on bioavailability was demonstrated in soil with a low moisture content which prevented 2,4-D-induced bioluminescence but which did not affect bioluminescence from already induced cells. This rapid and low cost bioassay provides a proof of concept demonstrating that retrievable disk-fixed cells can be induced in soil, thus providing a measure of solid-phase bioavailability.

An amperometric immunosensor to detect 2,4-D was described by Cai et.al (84). Firstly, 2,4-D-protein conjugate was prepared using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The rabbit antiserum was prepared with substitution degree of 16. Secondly, the immunosensor was fabricated using screen printing technique. The 2,4-D/PLL conjugate was immobilized on the surface of screen printed carbon electrode via cross linking method. The free and immobilized 2,4-D competed the binding sites of anti-2,4-D antiserum. After HRP-labeled goat-anti-rabbit IgG binding with anti-2,4 antiserum, the substrate was added and current response was recorded. The dependency of concentration of antiserum and HRP-labeled anti-IgG on current response was also studied. The result shows that LOD for 2,4-D was 1.69 ng/mL with linear range 1.69 - 30000 ng/mL.

Santacruz et.al (85) studied the degradation of two chlorinated pesticides (2,4-D and DDT) by using a 54-mL glass column packed with tezontle (a low-cost basaltic scoria). Bacteria were cultured in YPG (yeast, peptone, and glucose) liquid medium at 32 °C. The rich medium was pumped during 24 h through the column to inoculate it. Later, the wasted medium was discharged and the pesticide added. Optical densities, TOC, and pesticide concentration were determined. Pesticide removals for 2,4-D (with initial concentration between 100 and 500 mg/L) were about 99%. DDT removal (at initial concentration of up to 150 mg/L) was as high as 55-99%. TOC removals for 2,4-D was in the 36-87% interval, whereas for DDT they were as high as 36-78%.

An indirect electrochemical determination of 2,4-D, has been described by Maleki et.al (86). The method was based on the adsorption and desorption of 2,4-D on mercury electrode. Under the optimum conditions the LOD was $50 \mu\text{g L}^{-1}$. The proposed method was applied to the determination of 2,4-D in real samples such as soil and water.

Shoemaker et.al (87) described a method of SPE with a nonporous graphitized carbon sorbent to extract the ESA and OA acid degradates of propachlor, flufenacet, dimethenamid, alachlor, acetochlor, and metolachlor from finished drinking water matrixes. Separation and quantitation of the target analytes are achieved with liquid chromatography/tandem mass spectrometry. Dimethachlor ESA and butachlor ESA were chosen during the method development as the surrogate and internal standard. Drinking

water samples were dechlorinated with ammonium chloride without adversely affecting the analyte recoveries. Typical mean recoveries of 92-116% in deionized water and 89-116% in ground water were observed with RSD of <5%.

Zhao et.al (88) described a method using single-drop microextraction (SDME) and GC- μ ECD for the determination of chloroacetanilide herbicides (alachlor, acetochlor, metolachlor, pretilachlor and butachlor) residues. The method was proved to be a simple and rapid for chloroacetanilide herbicides in water with LOD 0.0002–0.114 μ g/L. The relative recoveries range from 80% to 102% for all the target analytes, with the RSD varying from 3.9% to 11.7%.

Wang et.al (89) performed successful enantiomeric separation of 10 chiral pesticides by HPLC using cellulose-tris(3,5-dimethylphenylcarbamate) (CDMPC) chiral stationary phase. The mobile phase was n-hexane modified by ethanol, propanol, 2-propanol (IPA), butanol, or isobutanol. The effects of mobile phase composition and column temperature on the separation were investigated. Baseline separation was obtained with ethofumesate, fluroxypyr-meptyl, malathion, benalaxyl, diclofop-methyl, methamidophos, vinclozolin, and lactofen, whereas near baseline separation was obtained with profenofos and acetochlor. Butanol was the best modifier for benalaxyl; isobutanol was the best modifier for lactofen, malathion, diclofop-methyl, and ethofumesate; and IPA was the best modifier for the other five. Better separations were not always at low temperature. The elution orders of the eluting enantiomers were determined by a circular dichroism detector. The quantitative analysis methods for the enantiomers of ethofumesate, benalaxyl, and diclofop-methyl were established. Validation parameters include linearity, precision, and LOD. The enantiomeric residual analysis procedures in soil and water samples were also developed using acetone extraction and C₁₈ SPE. The methods were reliable for residual analysis of the enantiomers.

Norrgran et.al (90) developed a sensitive, selective and precise method for measuring herbicide metabolites in human urine. The method uses automated liquid delivery of internal standards and acetate buffer and a mixed polarity polymeric phase SPE of a 2 ml urine sample. The concentrated eluate was analyzed using HPLC/MS. Isotope dilution

calibration is used for quantification of all analytes. The LOD of method range from 0.036 to 0.075 ng/mL. The within- and between-day variation in pooled quality control samples range from 2.5 to 9.0% and from 3.2 to 16%, respectively, for all analytes at concentrations ranging from 0.6 to 12 ng/mL. Precision was similar with samples fortified with 0.1 and 0.25 ng/mL that were analyzed in each run.

A new, robust and simple method was described by Pawlicova et.al (91) for the chemiluminescent determination of the pesticide bromoxynil. The empirical procedure was performed with the aid of a flow-injection manifold provided with an on-line photo-reactor to obtain chemiluminescent photofragments. After a period of 12 s of irradiation with an 8 W low-pressure mercury lamp, a chemiluminescent oxidation was performed with the system potassium permanganate in a polyphosphoric acid medium. The photolysis required a basic medium (KOH 0.014 mol L⁻¹) with ethanol (1%) as a sensitizer. The method allowed the determination of 134 samples h⁻¹ of bromoxynil in a wide interval of concentrations, over the range 5 x 10⁻³ to 1 mg l⁻¹; the LOD was 5 x 10⁻³ mg l⁻¹. The RSD (n=24) at 0.25 mg l⁻¹ of the pesticide level was 2.3%. The method was applied to a water sample and to a formulation.

A practical method for residue analysis of 2,4-D, dicamba and 2,4,5-T in baked tobacco leaves has been developed by Liu et.al (92) using nonaqueous capillary electrophoresis. The herbicide residues of 2,4-D, dicamba and 2,4,5-T in tobaccos were extracted by ultrasonication with ethyl acetate, followed by a cleanup procedure with gel permeation chromatography. The LOD were ca. 0.4-0.6 µg/mL for the three herbicides, whereas the overall recovery ranged from 80.8 to 84.1%. The proposed method has been successfully applied to measure 300 real tobacco samples, and the residue profiles of the three herbicides in tobacco samples were obtained and evaluated.

kong and Lemely (93) studied, the 2,4-D degradation kinetics of Anodic Fenton treatment (AFT) in a silt loam soil slurry and the effects of various experimental conditions including initial 2,4-D concentration, Fenton reagent delivery rate, amount of humic acid addition, and pH were examined. The 2,4-D degradation in soil slurry by AFT was found to follow a two-stage kinetic model. The optimal pH for 2,4-D degradation in soil slurry by AFT was observed to be in the range of pH 2-3.

Rodriguez et.al (94) studied the effect of one organic amendment consisting of an urban waste compost on the sorption properties of the herbicide 2,4-D on four soils of different physicochemical characteristics. The soils chosen were a Typic Haphorthod (ST), a Typic Endoaquept , an Entic Pelloxerert , and a Typic Eutrochrept . 2,4-D adsorption was the highest on ST soil, whereas the lowest adsorption was for SR soil. The adsorbed amounts of the herbicide on aged organic fertilized soils diminished in three of the amended soils, but was still greater than on unammended soils. In contrast, the ST soil showed the largest adsorption for unammended soil.

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Experimental
Results and Discussion .

3.1: INVESTIGATION OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF FLUROXYPYR (STARANE).

The chemical structure of fluroxypyre is given below.

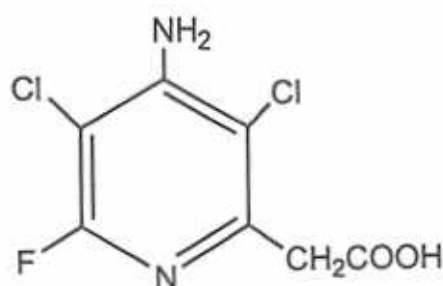
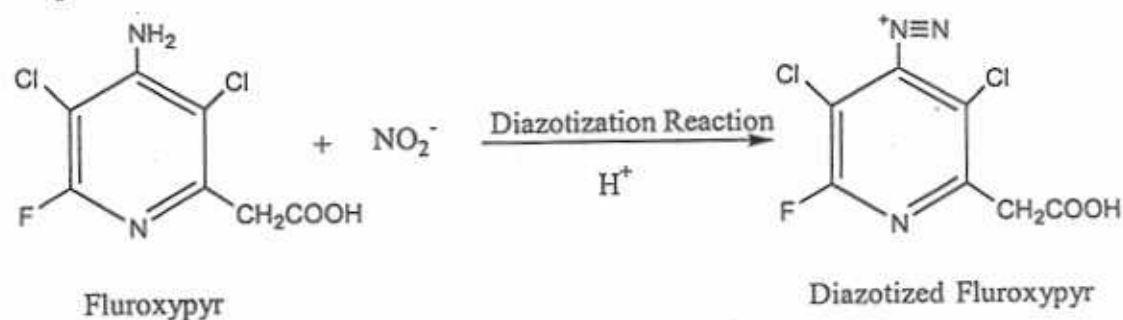


Figure 3.1.1 Chemical structure of fluroxypyr.

3.1.1: Method development strategy for the spectrophotometric determination of fluroxypyr:

Fluroxypyr have primary aromatic amine group in the molecule as shown above in figure 3.1.1. The proposed method for fluroxypyr determination is based on direct exploitation of the primary aromatic amine group for the formation of azo dye. The proposed two step chemical reaction is shown in figure-3.1.2. The first step involves the reaction of fluroxypyr with nitrite solution in acidic medium to form the diazotized product. In the second step the diazotized product is reacted with suitable coupling reagent at room temperature to form a colored azo dye. In this investigation the final orange colored azo dye was used for the determination of fluroxypyr using batch and online spectrophotometric methods.

Step-1: Diazotization Reaction:



Step-2: Coupling Reaction:

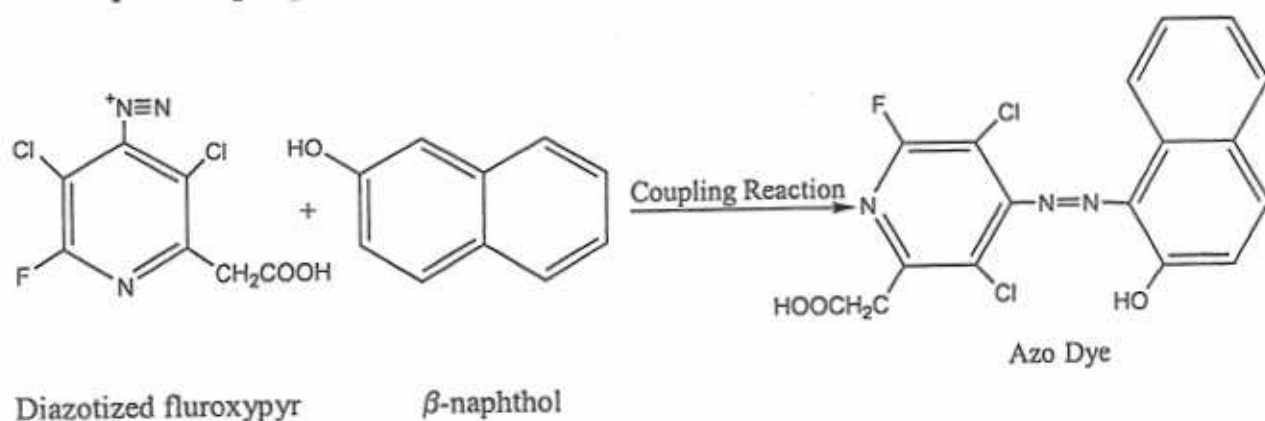


Figure 3.1.2: Proposed chemical reaction for the spectrophotometric determination of fluroxypyr.

3.1.2: Preliminary investigation of the possibility of diazotization and coupling for spectrophotometric determination of fluroxypyr.

Preliminary studies were carried out to investigate the possibility of the formation of expected azo dye. Initially high concentration of fluroxypyr (1000 ppm), relatively large volume of concentrated hydrochloric acid, sodium nitrite and various coupling reagents like aniline, β -naphthol and phenol were tried to form the expected azo dye at room temperature. The formation of the colored product (azo dye) indicated the possibility of proposed reaction and subsequent spectrophotometric determination of fluroxypyr by this method. Further studies were focused on optimization of various parameters and are given below.

3.1.3: OPTIMIZATION STUDIES FOR SPECTROPHOTOMETRIC DETERMINATION OF FLUROXYPYR.

3.1.3.1. Investigation of suitable wavelength for the spectrophotometric determination of fluroxypyr.

Instruments:

UV/Vis spectrophotometer (UNICO UV-2100 United Products and Instruments Inc., Dayton, N.J U.S.A), Digital Analytical Balance (Sartorius handy H51, Germany), Peristaltic pump, V-450 6 port injection valve (Upchurch Scientific, Inc, USA) , PTFE tubing (Becton Dickson,USA) 1.19mm i.d, 3-way connector.

Reagents and chemicals:

All chemicals used were of analytical reagent grade purity. Sodium nitrite, β -Naphthol, hydrochloric acid, ethanol, and sodium hydroxide were used during this work. Standard reference material of fluroxypyr was obtained from ACROS ORGANIC Belgium. Commercial sample containing fluroxypyr were purchased commercially from the local market.

Solution preparation:

- i) **Nitrite solution:** Nitrite solution (1000 ppm) was prepared by dissolving 0.15g of sodium nitrite in 0.36 M HCl solution in a 100ml volumetric flask.
- ii) **β -naphthol solution:** β -naphthol solution (1000ppm) was prepared by dissolving 0.1g of β -naphthol in 1.0% sodium hydroxide solution in a 100ml volumetric flask.
- iii) **Standard fluroxypyr solution:** Fluroxypyr solution (1000 ppm) was prepared by dissolving 0.1 g of authentic standard of fluroxypyr in 50 mL of ethanol and diluted to 100 mL with distilled water. Through stepwise dilution using distilled water 100 and 10 ppm fluroxypyr solution were prepared from 1000ppm solution.

Procedure:

Fluroxypyr standard solution 5ml from 100ppm stock solution was transferred to 50 ml volumetric flask. To this 5ml of 1000ppm nitrite solution was added. The resulting solution was equilibrated for sometime for the formation of diazotization product. Then 3ml of β -naphthol (1000ppm) was added as coupling reagent and diluted to 50ml with

distilled water. Blank solution was prepared through the same procedure without the addition of fluroxypyr. The absorbance of the resulting azo dye was measured from 340 to 580nm using UNICO UV-2100 spectrophotometer for finding out the optimum wavelength. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are given in table 3.1.1 and are shown in figure 3.1.3.

Table 3.1.1: Investigation of suitable wavelength for the spectrophotometric determination of fluroxypyr:

Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
340	0.847	440	0.914
350	0.935	450	0.780
360	0.974	460	0.686
370	1.033	470	0.646
380	1.179	480	0.612
385	1.234	490	0.574
390	1.292	500	0.485
395	1.316	510	0.390
400	1.286	530	0.226
410	1.206	550	0.103
420	1.067	580	0.058
430	1.006		

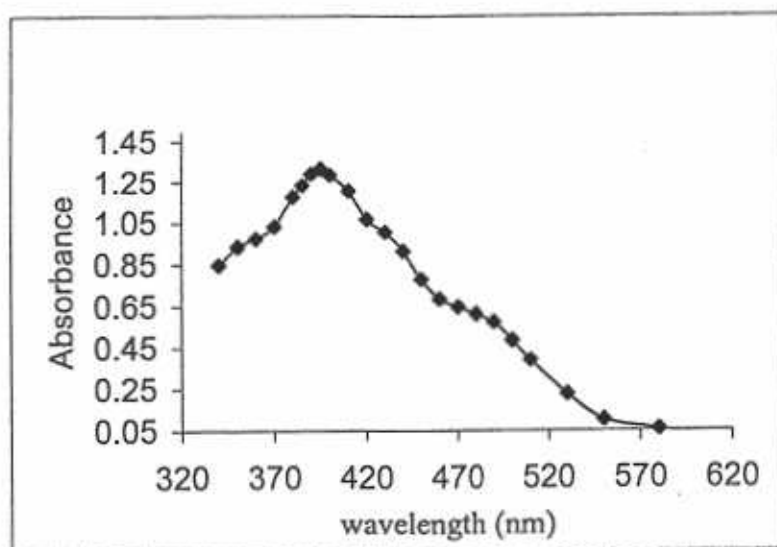


Figure 3.1.3: Wavelength optimization for the spectrophotometric determination of fluroxypyr.

Conditions:	λ_{max}	Varied
	Fluroxypyr solution	10ppm
	Nitrite solution (1000ppm)	5ml
	β -naphthol solution (1000ppm)	3ml.

3.1.3.2: Investigation of the effect of nitrite concentration on the diazotization of fluroxypyr.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to five separate 50ml volumetric flasks. To each of these flasks 5ml of different concentration of nitrite in the range of 500 to 2500ppm was added. To the resulting diazotized product 3ml of β -naphthol (1000ppm) was added. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. Separate blank solutions were prepared using the same procedure without the addition of fluroxypyr. The absorbance of the azo dye was measured at 395nm using UNICO UV 2100 spectrophotometer for

finding out the optimum concentration of nitrite. The results are given in table 3.1.2 and are shown in figure 3.1.4.

Table 3.1.2. Investigation of the effect of nitrite concentration on the diazotization of fluroxypyr.

S.NO	Nitrite concentration (ppm)	Absorbance
1	500	0.633
2	1000	1.143
3	1500	1.417
4	2000	1.400
5	2500	1.399

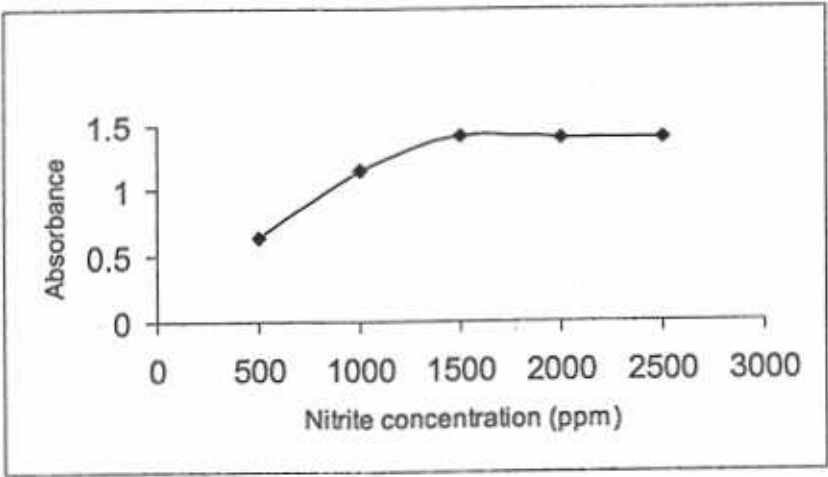


Figure 3.1.4. Investigation of the effect of nitrite concentration on the diazotization of fluroxypre.

Conditions:

λ_{max}	395nm
Fluroxypyr solution	10ppm
Nitrite solution (varied conc.)	5ml
β -naphthol (1000ppm)	3ml

3.1.3.3: Investigation of the effect of volume of nitrite solution on the diazotization of fluroxypyr.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to six separate 50ml volumetric flasks. To these flasks varied volumes of nitrite (1500ppm) solution in the range of 1 to 6 ml was added. To the resulting diazotized product 3ml of β -naphthol (1000ppm) was added. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. Separate blank solutions were prepared through the same procedure without the addition of fluroxypyr. The absorbance of the azo dye was measured at 395nm. The results are given in table 3.1.3 and are shown in figure 3.1.5.

Table 3.1.3 Investigation of the effect of volume of nitrite solution on the diazotization of fluroxypyr.

S.NO	Nitrite volume (ml)	Absorbance
1	1	0.995
2	2	1.400
3	3	1.440
4	4	1.490
5	5	1.540
6	6	1.540

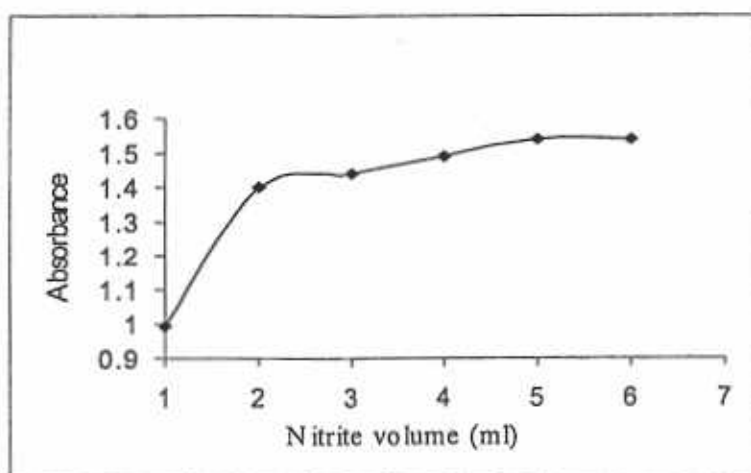


Figure 3.1.5. Investigation of the effect of volume of nitrite solution on the diazotization of fluroxypyr.

Conditions:

λ_{max}	395nm
Fluroxypyr solution	10ppm
Nitrite solution (1500ppm)	varied
β -naphthol (1000ppm)	3ml

3.1.3.4: Investigation of the effect of acidity on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to six separate 50ml volumetric flasks. To each of these flasks 5ml of nitrite solution (1500ppm) prepared in different concentration of hydrochloric acid ranging from 0.1-0.6M was added for finding out the optimum molarity of hydrochloric acid required for the formation of diazotized product. To the resulting diazotized product, 3ml of β -naphthol (1000ppm) was added. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. Separate blank solutions were

prepared through the same procedure without the addition of fluroxypyr. The absorbance of the azo dye was measured at 395nm. The results are given in table 3.1.4 and are shown in figure 3.1.6.

Table 3.1.4 : Invstigation of the effect of acidity on the absorbance behaviour of the azo dye.

S.NO	Concentration of HCl (M)	Absorbance
1	0.12	0.058
2	0.24	0.173
3	0.36	0.372
4	0.48	0.304
5	0.60	0.214

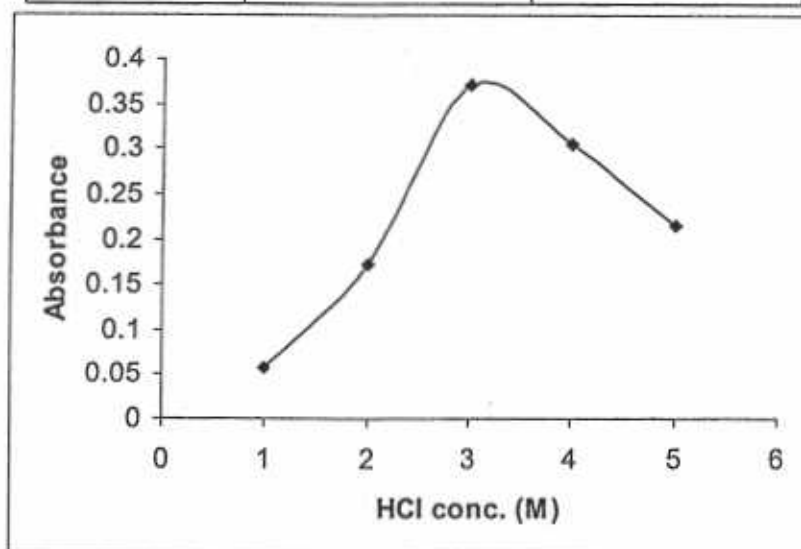


Figure 3.1.6: Investigation of the effect of acidity on the absorbance behaviour of the azo dye

Conditions:

λ_{max}	395nm
Fluroxypyr solution	10ppm
Nitrite solution (1500ppm)	5ml
Hydrochloric acid (M)	varied
β -naphthol (1000ppm)	3ml

3.1.3.5: Investigation of the effect of sodium hydroxide concentration on the absorbance behaviour of the azo dye.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to six separate 50ml volumetric flasks. To each flask 5ml of nitrite (1500ppm) solution was added. To the resulting diazotized product 3ml of β -naphthol (1000ppm) solution prepared in different percent concentration of sodium hydroxide ranging from 0.1 to 1% was added for finding out the optimum amount of sodium hydroxide. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. Separate blank solutions were prepared through the same procedure without the addition of fluroxypyr. The absorbance of the azo dye was measured at 395nm. The results are given in table 3.1.5 and are shown in figure 3.1.7.

Table 3.1.5: Optimization of sodium hydroxide concentration for the maximum formation of azo dye.

S.NO	Sodium hydroxide (%)	Absorbance
1	0.1	1.250
2	0.2	1.850
3	0.4	1.660
4	0.6	1.045
5	0.8	0.984
6	1.0	0.199

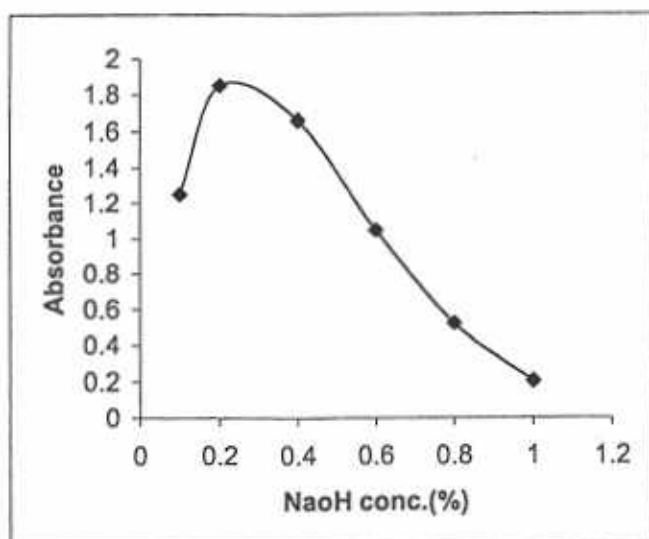


Figure 3.1.7: Optimization of sodium hydroxide concentration for the maximum formation of azo dye.

Conditions:	$\lambda_{\text{max.}}$	395nm
	Fluroxypyr solution	10ppm
	Nitrite solution (1500ppm)	5ml
	β -naphthol (1000ppm)	3ml
	NaOH (%)	varied

3.1.3.6: Investigation of the effect of β -naphthol concentration on the absorbance behaviour of the azo dye

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to seven separate 50ml volumetric flasks. To each of these flasks 5ml of nitrite (1500ppm) solution was added. To the resulting diazotized product 3ml of β -naphthol from the range of 500 to 6000ppm was added for finding out the optimum concentration of β -naphthol. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. The absorbance of the azo dye was measured at 395nm. The results are given in table 3.1.6 and are shown in figure 3.1.8.

Table 3.1.6. Optimization of β -naphthol concentration for the maximum formation of azo dye.

S.NO	β -naphthol concentration (ppm)	Absorbance
1	500	0.117
2	1000	0.245
3	2000	1.060
4	3000	1.580
5	4000	1.750
6	5000	1.999
7	6000	1.999

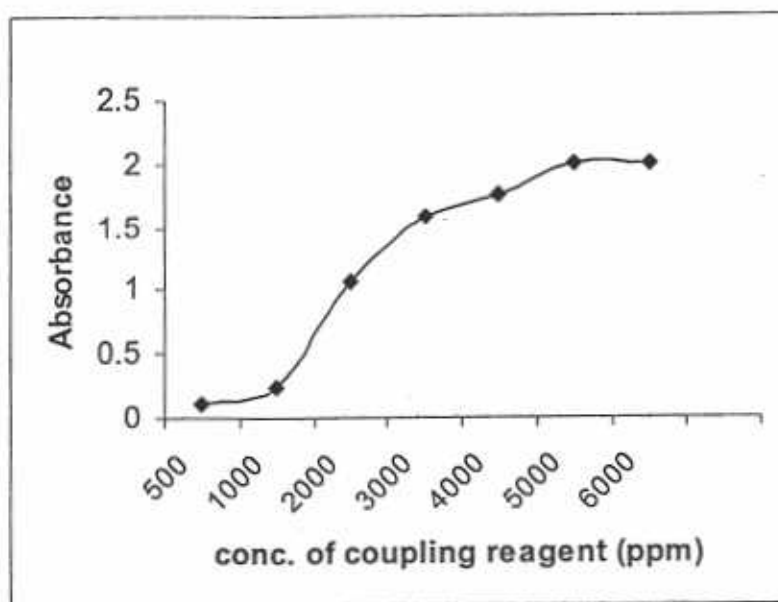


Figure 3.1.8. Optimization of β -naphthol concentration for the maximum formation of azo dye.

Conditions:	$\lambda_{\max}$	395nm
	Fluroxypyr solution	10ppm
	Nitrite solution (1500ppm)	5ml
	β -naphthol (varied)	3ml

3.1.3.7. Investigation of the effect of β -naphthol volume on the absorbance behavior of the azo dye

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Fluroxypyr standard solution 5ml from 100 ppm stock solution was transferred to six separate 50ml volumetric flasks. To each of these flasks 5ml of nitrite (1500ppm) solution was added for finding out the optimum volume of β -naphthol. To the resulting diazotized product varied volume of β -naphthol (5000ppm) solution in the range of 1 to 5ml was added. An orange yellow colored azo dye was formed in each flask and diluted with distilled water up to the mark. The absorbance of the azo dye was measured at 395nm. The results are given in table 3.1.7 and are shown in figure 3.1.9.

Table 3.1.7. Optimization of the β -naphthol volume for maximum formation of azo dye.

S.NO	β -naphthol volume (ml)	Absorbance
1	1	0.448
2	2	0.594
3	3	1.240
4	4	1.420
5	5	1.270

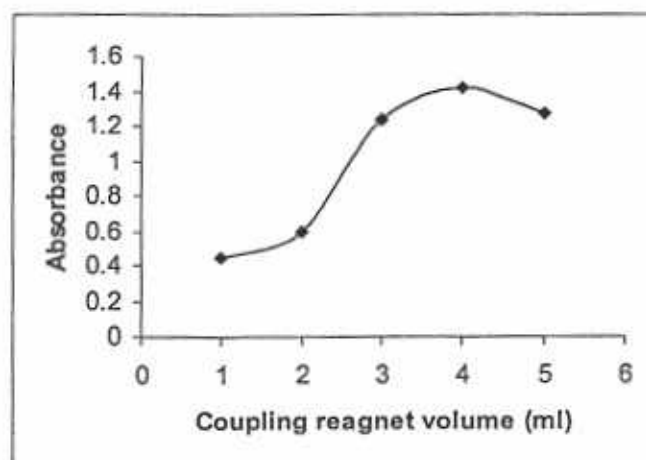


Figure 3.1.9. Optimization of the β -naphthol volume for maximum formation of azo dye.

Conditions:	λ_{max}	395nm
	Fluroxypyr solution	10ppm
	Nitrite solution (1500ppm)	5ml
	β -naphthol (5000ppm)	varied

3.1.3.8: Determination of the limit of detection and quantification of fluroxypyr by batch analysis.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

From standard fluroxypyr solution (100ppm) different volume for 10 to 0.5 ppm were taken in 50 ml volumetric flask. To each flask optimum volume of 1500ppm nitrite solution (5ml) was added and mixed well. To the resulting diazotized product, optimum volume (4ml) of β -naphthol solution (5000ppm) was added. The resulting azo dye was diluted up to the mark with distilled water. Blank solution was prepared in the same way without the addition of fluroxypyr. The absorbance of the resulting azo dye was measured at 395 nm. The results are given in table 3.1.8 and are shown in figure 3.1.10. Molar absorptivity was calculated by using the following formula and found to be $2.19 \times 10^4 \text{ L.mol}^{-1}.\text{cm}^{-1}$.

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

Where A = absorbance, C stands for concentration (mole/liter) and L stands for path length in centimeter.

LOD and LOQ were calculated for six replicates of 0.5ppm solution by using the following formula.

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

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

Where S = Standard Deviation

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

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

Table 3.1.8: Investigation of the effect of concentration on absorbance behavior of fluroxypyr.

Fluroxypyr conc. (ppm)	Absorbance	Fluroxypyr conc. (ppm)	Absorbance
0.5	0.007	4.0	0.481
0.6	0.03	5.0	0.534
0.7	0.067	6.0	0.587
0.8	0.098	7.0	0.640
0.9	0.148	8.0	0.690
1.0	0.285	9.0	0.721
2.0	0.375	10	0.760
3.0	0.428		

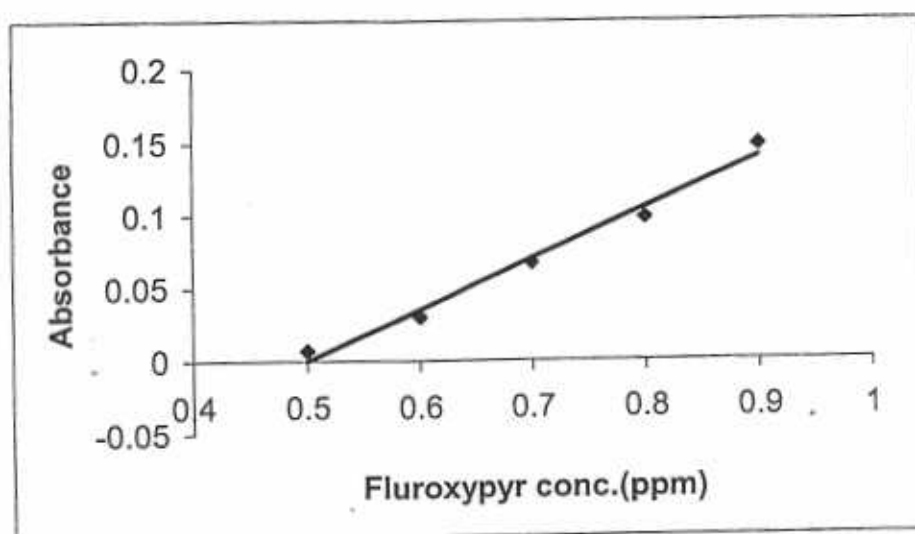


Figure 3.1.10: Investigation of the effect of concentration on absorbance behavior of fluroxypyr at lower concentration.

Conditions:	$\lambda_{\text{max.}}$	395nm
	Fluroxypyr solution (1000ppm)	10ml
	Nitrite solution (1500ppm)	5ml
	β -naphthol (5000ppm)	4ml

3.1.4: INVESTIGATION OF PHYSICAL PARAMETERS FOR ONLINE SPECTROPHOTOMETRIC DETERMINATION OF FLUROXYPYR.

All physical parameters such as the reactor length (R1 and R2), flow rate and injection volume were optimized for the flow injection spectrophotometric determination of fluroxypyr, while all the chemical parameters optimized for the batch analysis of fluroxypyr were kept the same. Since the optimum temperature for diazotization was room temperature, all FI manifolds were set at room temperature. A flow injection assembly is shown in figure 3.1.11.

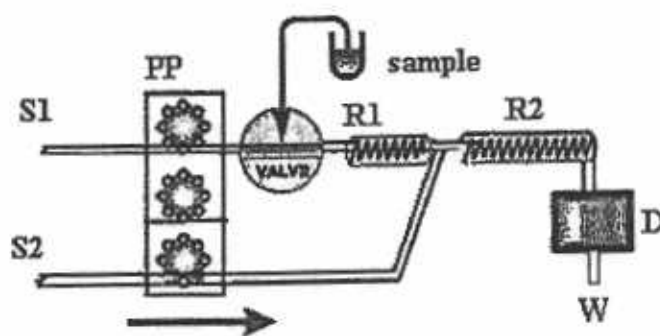


Figure 3.1.11: Schematic diagram of the FI manifold. (S1) nitrite stream, (S2) β -naphthol stream, (R1) diazotization coil, (R2) coupling coil, (pp) peristaltic pump, (W) waste, (D) detector.

3.1.4.1: Investigation of flow rate of system for online spectrophotometric determination of fluroxypyr.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

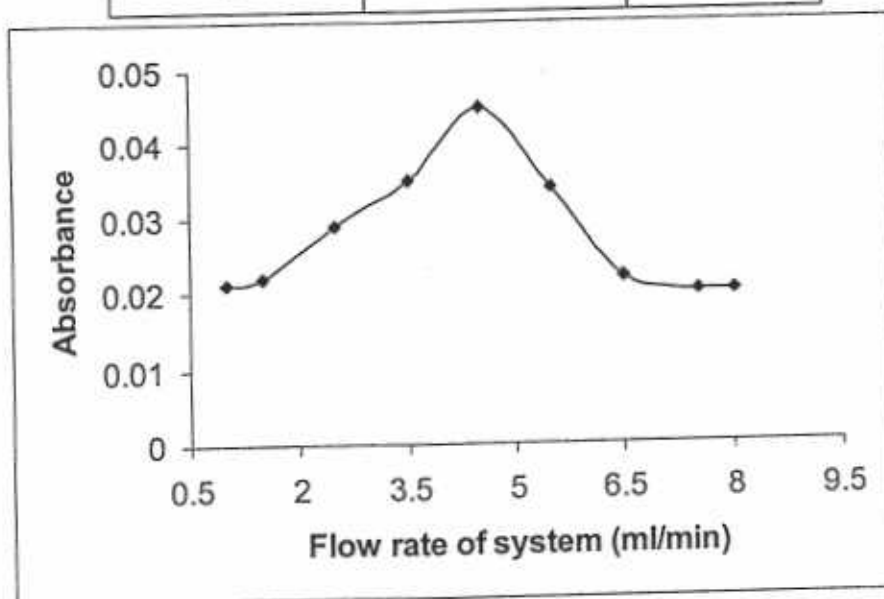
Procedure :

In a flow through assembly nitrite solution (1500ppm) was allowed to flow in tube S1 while the coupling reagent β -naphthol (5000ppm) was allowed to flow in tube S2. Flow rate of the system was studied in the range of 1-8ml/min. The coil length of reactor R1 and R2 was 50cm and 30cm respectively. From 10ppm fluroxypyr solution, 1ml was injected in the nitrite stream through a 6 port injector valve to form diazotized

reagent. This diazotized reagent coupled with β -naphthol in reactor R2. The absorbance of the azo dye formed was measured at 395nm by using UNICO UV 2100 spectrophotometer and displayed through computer programme. Blank was adjusted by flowing continuous stream of nitrite and β -naphthol solution through the system. The results are given in table 3.1.9 and shown in figure 3.1.12.

Table 3.1.9: Investigation of flow rate of system for online spectrophotometric determination of fluroxypyr.

S.NO	Flow Rate (ml/min)	Absorbance
1	1.0	0.021
2	1.5	0.022
3	2.5	0.029
4	3.5	0.035
5	4.5	0.045
6	5.5	0.034
7	6.5	0.022
8	7.5	0.020
9	8.0	0.020



3.1.12: Investigation of flow rate of system for online spectrophotometric determination of fluroxypyr.

Conditions:	$\lambda_{\max}$	395nm
	injection volume (10ppm)	1ml
	Flow rate (ml/min)	varied
	R1	50cm
	R2	30cm

3.1.4.2: Investigation of injection volume for online spectrophotometric determination of fluroxypyr.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

In a flow through assembly nitrite solution(1500ppm) was allowed to flow in tube S1 while the coupling reagent β -naphthol(5000ppm) was allowed to flow in tube S2. Flow rate of the system was 4.5ml/min. The coil length of reactor R1 and R2 was 50cm and 30cm respectively. From 10ppm fluroxypyr solution varied volumes (1-4ml) was injected in the nitrite stream. The absorbance of the azo dye formed was measured at 395nm. The results are given in table 3.1.10 and shown in figure 3.1.13

Table 3.1.10: Investigation of injection volume for online spectrophotometric determination of fluroxypyr.

S.NO	Injection volume (ml)	Absorbance
1	1.0	0.047
2	2.0	0.040
3	3.0	0.040
4	4.0	0.040

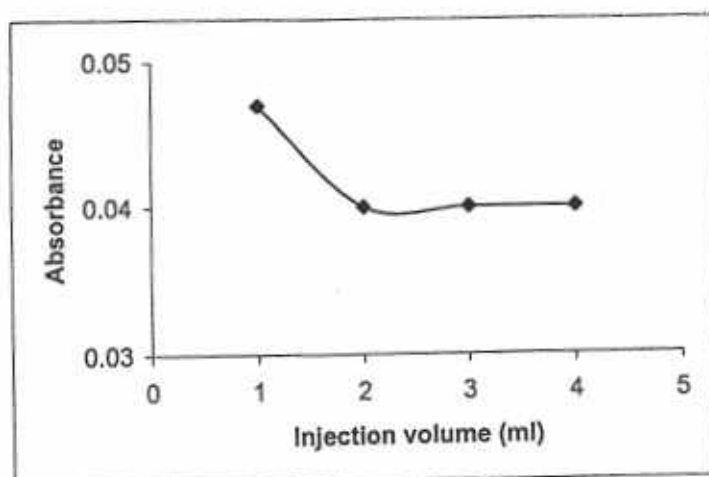


Figure 3.1.13: Investigation of injection volume for online spectrophotometric determination of fluroxypyr.

Conditions:	$\lambda_{\max}$	395nm
	Injection volume	Varied
	Flow rate (ml/min)	4.5
	R1	50cm
	R2	30cm

3.1.4.3: Investigation of diazotizing coil length (R1) for online spectrophotometric determination of fluroxypyr.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

For optimization of diazotization coil length (R1), the same procedure was used as mentioned before only the length of diazotizing coil was varied from 10-80cm. The absorbance of the azo dye formed was measured at 395nm. The results are given in table 3.1.11 and shown in figure 3.1.14.

Table 3.1.11: Optimization of diazotization coil length (R1) for online spectrophotometric determination of fluroxypyr.

S.NO	R1 (cm)	Absorbance	R1 (cm)	Absorbance
1	10	0.105	50	0.210
2	20	0.149	60	0.244
3	30	0.165	70	0.237
4	40	0.190	80	0.238

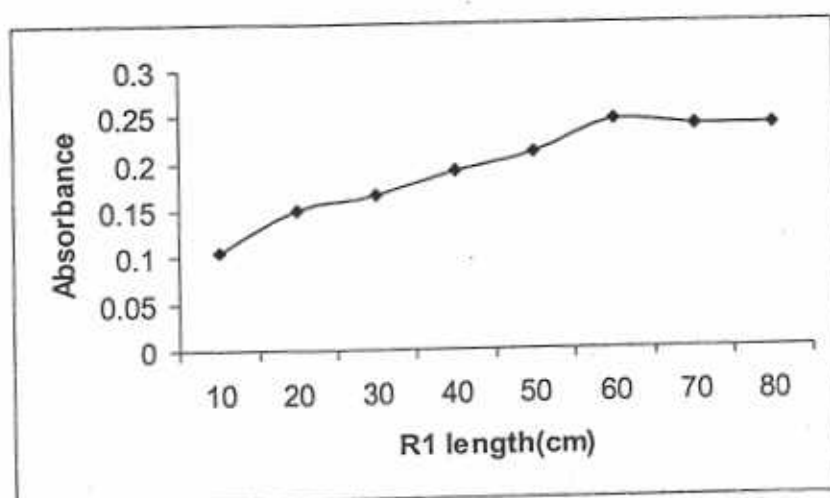


Figure 3.1.14: Optimization of diazotizing coil length (R1) for online spectrophotometric determination of fluroxypyr.

Conditions:	$\lambda_{\max}$	395nm
	Injection volume	1ml
	Flow rate (ml/min)	4.5
	R1 (cm)	varied
	R2	30cm

3.1.4.4: Optimization of coupling coil length (R2) for online spectrophotometric determination of fluroxypyr.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

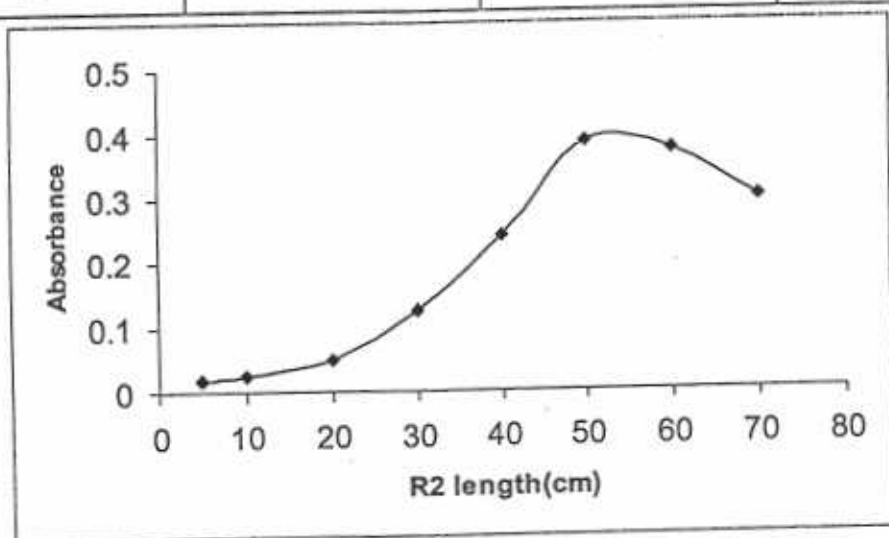
Solutions: The same as mentioned before

Procedure:

For optimization of coupling coil length (R2), the same procedure was used as mentioned before only the length of coupling coil was varied from 5-70cm. Color was developed in the same way as mentioned before. The absorbance of the azo dye formed was measured at 395nm. The results are given in table 3.1.12 and shown in figure 3.1.15.

Table 3.1.12: Optimization of coupling coil length (R2) for online spectrophotometric determination of fluroxypyr.

R2 length (cm)	Absorbance	R2 length (cm)	Absorbance
5.0	0.020	40	0.240
10	0.026	50	0.388
20	0.050	60	0.375
30	0.126	70	0.300



Figur 3.1.15 Optimization of coupling coil length (R2) for online spectrophotometric determination of fluroxypyr.

Conditions:

$\lambda_{\max}$	395nm
Injection volume	1ml
Flow rate (ml/min)	4.5
R1	60cm
R2	varied

3.1.4.5: Investigation of limit of detection and limit of quantification for online spectrophotometric determination of fluroxypyr.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Standard fluroxypyr solution of 0.6ppm –10ppm was prepared in separate 10 ml volumetric flasks by taking the required volume of standard from 100ppm and 10 ppm stock solution and diluting with distilled water. In a flow through assembly nitrite solution (1500ppm) was allowed to flow in tube S1 while the coupling reagent β -naphthol (5000ppm) was allowed to flow in tube S2. The flow rate of the system was maintained at 4.5ml/min. The coil length of reactor R1 and R2 was 60cm and 50cm respectively. From standard fluroxypyr solution 1-ml was injected in the nitrite stream through a 6 port injector valve to form diazotized reagent. This diazotized reagent couple with β -naphthol in reactor R2. The absorbance of the azo dye formed was measured at 395nm by using UNICO UV 2100 spectrophotometer and displayed through computer programme. Blank was adjusted by allowing to flow a continuous stream of nitrite and β -naphthol solution through the system. After each running, system was washed with blank solution and sample coil length was washed with distilled water and dried by passing air through it. This give a maximum sampling throughput of 60 samples per hour. The results are given in table 3.1.13 and are shown in figure 3.1.16. Molar absorptivity was calculated by using the formula given before and found to be $1.5 \times 10^4 \text{ L.mol}^{-1}.\text{cm}^{-1}$

Table 3.1.13: Investigation of the effect of concentration on absorbance behavior of fluroxypyr at lower concentration.

S.NO	Fluroxypyr conc.(ppm)	Absorbance
1	0.6	0.010
2	0.7	0.056
3	0.8	0.103
4	0.9	0.152
5	1.0	0.200
6	2.0	0.340
7	4.0	0.456
8	5.0	0.514
9	6.0	0.572
10	7.0	0.622
11	8.0	0.680
12	9.0	0.745
13	10.0	0.833

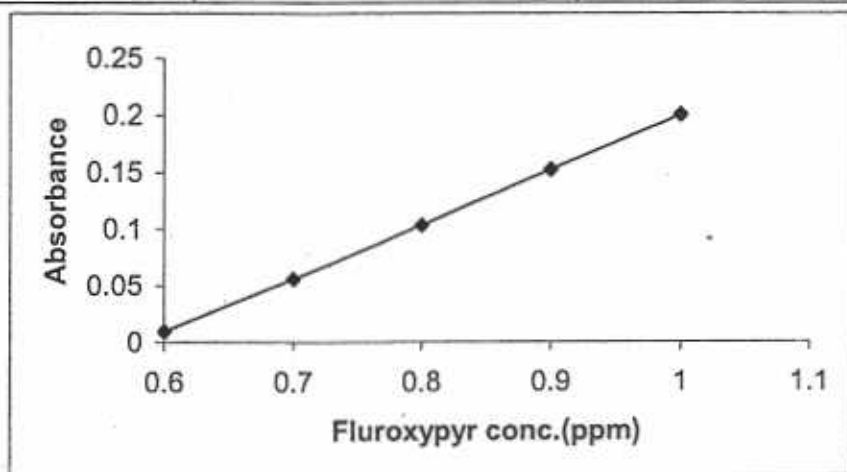


Figure 3.1.16: Investigation of the effect of concentration on absorbance behavior of fluroxypyr at lower concentration.

Conditions: λ_{max} 395nm
Injection volume 1ml
Flow rate (ml/min) 4.5

3.1.5: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF FLUROXYPYR IN REAL SAMPLES.

Instrument: The same as mentioned before

Samples: Maize and wheat samples purchased from market.

Sample extraction procedure:

Standard procedure for the extraction of herbicides from the real sample was used ⁽¹⁾. According to this method, 15 g of homogenized food samples (maize and wheat grain & flour) were taken in a beaker and 50 ml of solvents (petroleum ether and acetone in 1:1) was added to it for extraction of herbicide. The mixture was shaken for 2hr. After equilibrations, the sample was filtered and the filtrate was concentrated on rotary evaporator up to the volume of 2ml.

Procedure

From the extracted sample 2ml were taken in a 50ml volumetric flasks. To each flask 5ml of nitrite solution was added and color was developed in the same way as mentioned before. The absorbance of the colored solution was measured at 395nm.

In cases of online analysis 1ml of the extracted food sample was injected in the nitrite stream through six port injection valve and the color was developed in the same way as mentioned before. The absorbance of the colored solution was measured at 395nm. Three replicate readings were taken for each sample. A comparison of both batch and FI analysis are given in table 3.1.14.

Table-3.1.14: Comparison of quantitative analysis of fluroxypyr in real samples both by batch analysis and online analysis.

Serial NO.	Sample	Batch analysis ($\mu\text{g/g}$)	online analysis ($\mu\text{g/g}$)
1	Wheat (grain)	5.66 ± 0.007	4.00 ± 0.047
2	Wheat (Flour)	3.30 ± 0.016	2.73 ± 0.010
3	Maiz (grain)	3.30 ± 0.001	3.30 ± 0.015
4	Maiz (flour)	2.63 ± 0.013	2.70 ± 0.016

3.1.6: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF FLUROXYPYR IN COMMERCIAL FORMULATION.

Instrument: The same as mentioned before

Sample: Commercial sample (starane) was purchased from local market.

Procedure:

1-3ml of furoxypre formulation was taken in three separate 50ml volumetric flasks and dissolved with 25ml ethanol and diluted up to mark with distilled water. From each solution 5ml were taken in three separate 50ml volumetric flasks and color was developed in the same way as mentioned before for batch analysis. The absorbance of the azo dye formed was measured at 395nm.

For online analysis from sample stock solution dilute solutions was prepared in separate 10ml volumetric flask and 1ml from these dilute solutions was injected in nitrite stream. The absorbance of the azo dye formed was measured at 395nm. Three replicate readings were taken for each sample. The results of both batch and online analysis are given in table 3.1.15.

Table-3.1.15 Active ingredient of herbicide in commercial formulation.

Herbicides Label	Label value active ingredient g/ml	Found value of active ingredient (batch analysis) g/ml	Found value of active ingredient (FIA) g/ml
Starane	0.4	0.32±0.01	0.30±0.03

3.1.7: PERCENT RECOVERY OF FLUROXYPYR FROM REAL SAMPLES AND FORMULATIONS BY USING THE INVESTIGATED METHOD.

Instrument: The same as mentioned before

Procedure:

A recovery test was performed on control samples, fortified with known concentration of fluroxypyr solution. The solvent was evaporated at room temperature for 1hr. The extraction procedure was exactly the same way as for the sample. Six replicates of the fortified samples were analyzed by batch and online method using the same procedure as mentioned before. Percent recovery was calculated by using formula given below. The

results of percent recovery of fluroxypyr in real samples and formulation by batch and online method are given in table 3.1.16.

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

Table 3.1.16: Percent Recovery of fluroxypre in real samples and formulations by using the investigated method.

Samples	FIA	Batch analysis
	% Recovery	%Recovery
Starane	96.00 ±0.016	97.50± 0.013
Maize (grains)	95.50±0.01	95.10± 0.014
Maize (flour)	93.00±0.009	92.00±0.01
Wheat (grain)	96.02±0.03	94.30±0.016
Wheat (flour)	93.00±0.01	90.80±0.012

Results and discussion

The chemical reaction involved in this procedure is shown in figure 3.1.2. Fluroxypyr has a primary aromatic amine group in the molecule. In this procedure the primary aromatic amine group was directly exploited for the formation of azo dye. In the first step fluroxypyr was reacted with nitrite in acidic medium to form the diazotized product, which was subsequently coupled with optimized coupling reagent β -naphthol at room temperature. An orange colored azo dye was the final product, which was measured spectrophotometrically for determination of fluroxypyr.

Various parameters like wavelength, nitrite concentration hydrochloric acid concentration, coupling reagent concentration and physical parameters for flow injection analysis were optimized for the maximum formation of azo dye. Calibration curve and limit of detection was also investigated for the proposed method using both batch and flow injection analysis.

After preliminary experiments the azo dye formed was investigated for the optimum wavelength. The results are given in table-3.1.1 and are shown in figure 3.1.3. As can be

seen from figure 3.1.3 the resulting azo dye has a maximum absorbance at 395nm which was subsequently used as optimum wavelength for further investigation of fluroxypyr. Sodium nitrite concentration was optimized between 500-2500ppm(table 3.1.2) and it can be seen from figure 3.1.4 that 1500ppm was the optimum concentration of nitrite for maximum diazotization. Various volumes of 1500ppm nitrite solution in the range of 1-6ml were used for the maximum formation of azo dye. The results are given in table 3.1.3 and shown in figure 3.1.5. As can be seen from figure 3.1.5 that 5ml of nitrite solution was sufficient for the maximum diazotization. This optimum volume of nitrite solution was used for further investigation of fluroxypyr determination.

The diazotization reaction take place in the acidic media therefore the molarity of hydrochloric acid was also optimized for the maximum formation of azo dye. The results are given in table 3.1.4 and are shown in figure 3.1.6. As can be seen from figure that 0.36M of hydrochloric acid was the optimum concentration for maximum reaction.

Similarly the percentages concentration of sodium hydroxide was studied in the range of 0.1-1% (table 3.1.5). Figure 3.1.7 shows that 0.2% of sodium hydroxide is the optimum concentration.

For coupling reaction, the concentration and volume of coupling reagent β -naphthol was optimized for the formation of maximum azo dye. The results are given in table 3.1.6, 3.1.7 and are shown in figure 3.1.8 & 3.1.9. As can be seen from the figures that 4ml of 1000ppm β -naphthol is the optimum volume for maximum formation of azo dye.

Optimization of parameters for online analysis

For online studies all the physical parameters were optimized for FI analysis of fluroxypyr. The FI assembly was set at room temperature and shown in figure 3.1.11.

The flow rate was optimized in the range from 1-8ml/min for the online determination of fluroxypyr (table 3.1.9). As can be seen from figure 3.1.12 that when the flow rate was increased, the signal increased up to a flow rate of 4.5ml/min, and decreased at higher flow rates. A flow rate of 4.5ml/min was selected as the optimum flow rate to obtain a good sampling rate, peak width and sensitivity. With this rate a sampling through put of 60 samples/h was achieved

The injection volume of sample was also investigated in range of 1-4ml as given in table 3.1.10 and can be seen from figure 3.1.13. A sample loop of 1ml was selected to maintain a good sampling rate.

The coil length of reactor R1 and R2 was investigated in the range 10-80cm and 5-70cm respectively as given in table 3.1.11 and 3.1.12 and shown in figure 3.1.14 & 3.1.15 respectively. As can be seen from figure 3.1.14 that 60cm is the optimum length for reactor R1. Above 60cm there was a slight decrease in the signal of the diazotized product. Similarly figure 3.1.15 show that coupling reaction is fast as compared to diazotization reaction. The signal was maximum at 50cm length of the reactor coil R2, but decreased at greater lengths, because of the decomposition of azo dye.

At optimum conditions, the effect of lower concentration of fluroxypyr (below 1 ppm) on absorbance behavior was investigated to calculate the LOD and LOQ for the investigated method both for batch and online analysis. The results are given in table 3.1.8, 3.1.13 and are shown in figure 3.1.10 & 3.1.16 respectively. The LOD and LOQ were calculated for the investigated method using formula given before and were found to be 0.05ppm and 0.17ppm for FI analysis and 0.073ppm and 0.25ppm for batch analysis respectively.

The RSD was also calculated both for batch analysis and online analysis and was found to be 2.4% and 1.67% respectively. Molar absorptivity was calculated and found to be $1.5 \times 10^4 \text{ L.mol}^{-1}.\text{cm}^{-1}$ and $2.19 \times 10^4 \text{ L.mol}^{-1}.\text{cm}^{-1}$ for online and batch analysis respectively. These parameters indicate the suitability of the method to be adopted for analysis.

Application of the investigated method for the analysis of fluroxypyr in commercial formulation and in real sample.

The investigated method was applied for the determination of fluroxypyr in commercial formulation and also in real samples of wheat, maize grains and in flour (table 3.1.14 & table 3.1.15). Calibration curve of standard fluroxypyr was used for the determination of fluroxypyr in formulation and in real samples. The following formula was used for calculating the active ingredient in formulation and for residue determination in real samples.

$$\text{ppm of fluroxypyr} = \frac{\text{G.R}(\mu\text{g/ml}) \times \text{volume made(ml)} \times \text{D.F}}{\text{Weight of sample taken (g)}}$$

Where G.R represents graphical reading and D.F stands for dilution factor.

For online analysis the residue level in the analyzed wheat grain and flour samples were found to be $4.00 \pm 0.047 \mu\text{g/g}$ and $2.73 \pm 0.01 \mu\text{g/g}$ respectively. In maize grain and flour samples, $3.30 \pm 0.015 \mu\text{g/g}$ and $2.70 \pm 0.016 \mu\text{g/g}$ of fluroxypyr were found respectively. The residue level found were much higher than the NZ food safety authority 2005 MRLs, which is $0.1 \mu\text{g/g}$ for cereal grains. The results of online and batch method were also compared. The methods were successfully applied for the determination of the active ingredient concentration in commercial formulation of fluroxypyr herbicides. The lower value of the active ingredients obtained in the commercial formulations showed the adulteration of herbicides by local dealers.

A recovery test was performed on the control samples for the determination of fluroxypyr (table 3.1.16). The recoveries of fluroxypyr from the fortified wheat and maize samples varied between 93% and 96% for online analysis and 90.8% to 97.5% for batch analysis

Reference:

- 1) J.Shah, M.R.Jan, M.Nafees, S.Noureen and Noureen Bhatti, "Monitoring Pesticide Residues in Fresh Fruits Marketed in Peshawar, Pakistan", American Laboratory , application note ,22, (2005).

3.2: INVESTIGATION OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF ACETOCHLOR.

The chemical structure of acetochlor is given below.

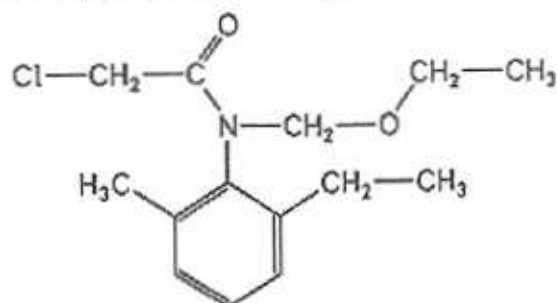


Figure 3.2.1: Chemical structure of acetochlor

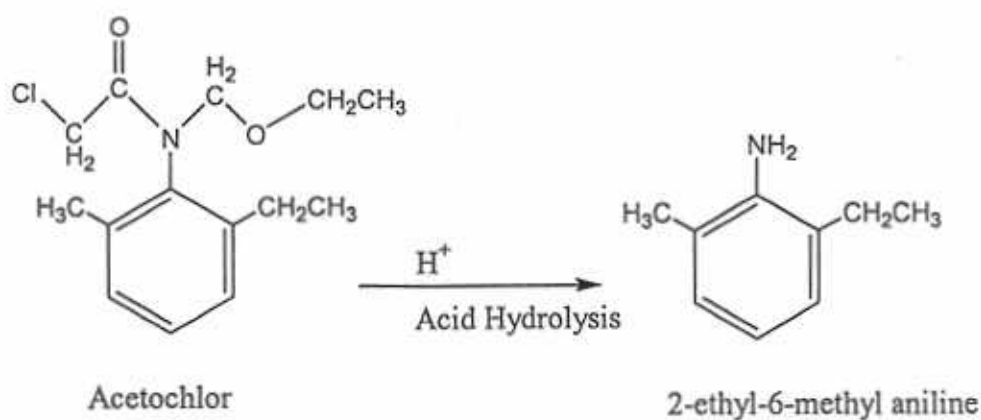
3.2.1 Method development strategy for the spectrophotometric determination of acetochlor.

Acetochlor is chemically 2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide. Looking in to the structure it is clear that on hydrolysis it would lead to degradation product 2-ethyl-6-methylaniline. Acetochlor give the same product when exposed to light ⁽¹⁾. The proposed chemical method is based on using this product as coupling reagent with already diaotized specie. The proposed reaction involves 3 steps as shown in figure 3.2.2.

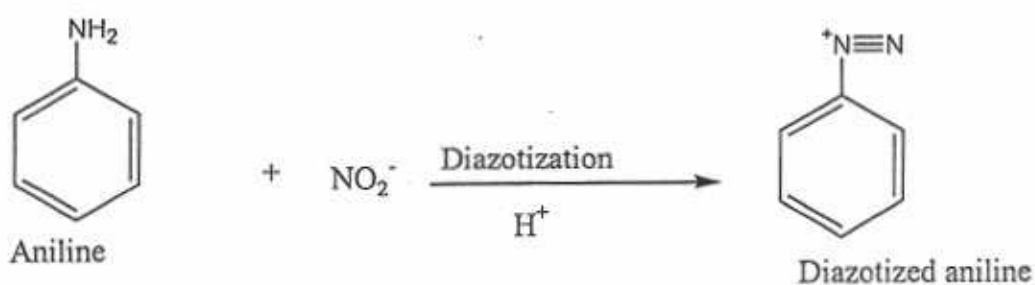
3.2.2. Preliminary investigation of the possibility of hydrolysis and coupling for spectrophotometric determination of acetochlor.

Preliminary studies were conducted to investigate the possible hydrolysis of acetochlor and the subsequent use of the hydrolyzed product as coupling reagent. Initially high concentration of acetochlor (100 ppm), relatively large volume of hydrochloric, sodium nitrite and various diazotizing reagent like sulfanilamide, aniline, p-nitroaniline and sulfanilic acid were tried to form the expected azo dye at room temperature. Both acid and base hydrolysis were tried by heating on waterbath for a time period of 30 minutes. The formation of the colored azo dye with acid hydrolysis indicated the possibility of reaction and subsequent spectrophotometric determination of acetochlor by this method. Further studies were focused on optimization of various parameters and are given below.

Step 1: Hydrolysis



Step 2 :Diazotization



Step 3: Coupling

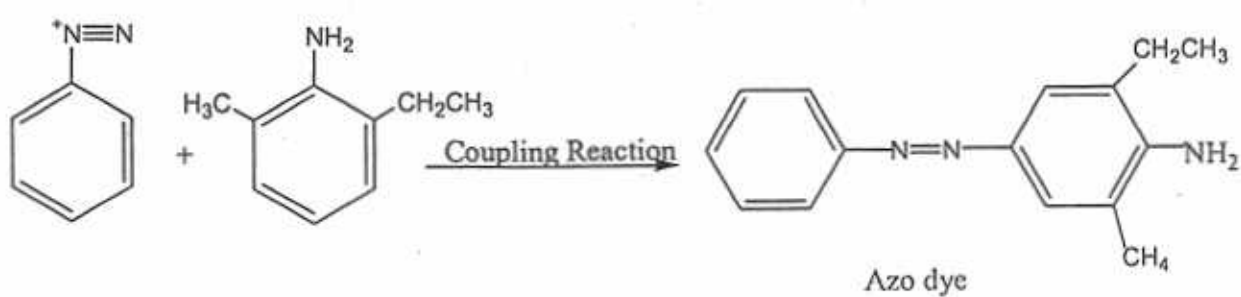


Figure 3.2.2: Proposed reaction mechanism for spectrophotometric determination of acetochlor

3.2.3: OPTIMIZATION STUDIES FOR SPECTROPHOTOMETRIC DETERMINATION OF ACETOCHLOR.

3.2.3.1. Investigation of suitable wavelength for the determination of acetochlor

Instruments:

The FI system comprised a peristaltic pump with PET (i.d. 1.19mm, Becton Dickson, USA) and silicon (i.d.1.71mm) flow tubes, a V-450 6 port injection valve (Upchurch Scientific Inc, USA) and a UV/Vis spectrophotometer (UNICO UV-2100 United Products and Instruments Inc., Dayton, N.J, U.S.A) as a detector was used.

Reagents and chemicals.

All chemicals used were of analytical reagent grade purity. Sodium nitrite, Aniline (Merck), concentrated hydrochloric acid (Merck) and ethanol (Merck), were used during this work. Standard reference material was purchased from Dr.Ehrenstorfer GmbH Germany. Commercial sample containing acetachlor was purchased commercially from the local market.

Solution preparations:

Aniline solution (1%): 1% aniline solution was prepared by adding 1ml of aniline in 50 ml of ethanol and diluted with distilled water up to 100ml.

Nitrite solution (0.15%): Nitrite solution was prepared by dissolving 0.225g sodium nitrite in distilled water and diluted up to 100 ml.

Diazotized aniline solution: Diazotized aniline solution was prepared by mixing nitrite solution (0.15%) and aniline solution (1%) in a 3: 1 ratio.

Standard hydrolyzed acetochlor solution: Acetochlor solution, 1000ppm was prepared by dissolving 0.1g of acetochlor in little ethanol and diluted upto mark with distilled water in a 100ml volumetric flask. A 10ml portion was taken in a flask from 1000ppm stock solution and 2ml of concentrated hydrochloric acid was added to it followed by a few ml of ethanol and distilled water. The resulting solutions was heated on a boiling water bath for 30 minutes, cooled, dissolved in 20ml of ethanol and diluted with distilled water in a 100ml volumetric flask. Working standard solutions were prepared from hydrolyzed stock solution by dilution with distilled water.

Procedure:

From stock solution of diazotized aniline 5ml was taken in a 50ml volumetric flask and 5ml of acetochlor from 100ppm hydrolyzed stock solution was added to it. A red colored azo dye was formed confirming the coupling ability of the hydrolyzed product. Blank solution was prepared in the same way without the addition of acetochlor. The absorbance of the resulting azo dye was measured from 380 to 600nm using UNICO UV-2100 spectrophotometer for finding out the optimum wavelength. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are given in table 3.2.1 and are shown in figure 3.2.3.

Table 3.2.1: Investigation of suitable wavelength for the determination of acetochlor.

Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
360	0.532	470	0.911
370	0.985	480	0.746
380	1.375	490	0.619
390	1.493	500	0.487
400	1.53	510	0.421
410	1.492	520	0.372
420	1.427	530	0.33
430	1.369	540	0.289
440	1.297	550	0.26
450	1.173	590	0.206
460	1.068	600	0.192

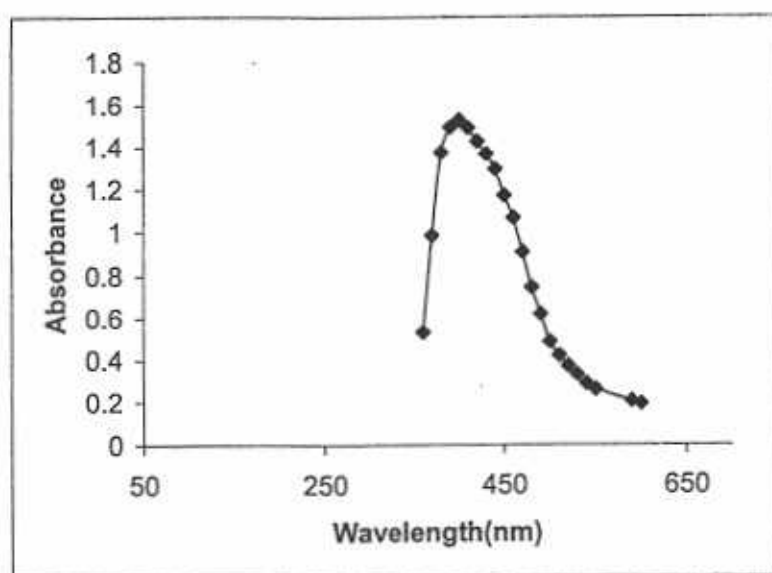


Figure 3.2.3: Investigation of suitable wavelength for the determination of acetochlor

Conditions:	λ_{max}	Varied
	Acetochlor solution	10ppm
	Diazotized reagent	5ml

3.2.3.2: Investigation of suitable diazotizing reagent for the spectrophotometric determination of acetochlor.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: Analytical reagent grade purity sulfanilic acid, sulfanilamide, aniline, and p-nitroaniline were used as diazotizing reagent. Stock solution of 1% of each diazotizing reagents was prepared in four separate volumetric flasks and diazotized with nitrite solution in 1:5 ratios respectively. These stock solutions were used for further investigations

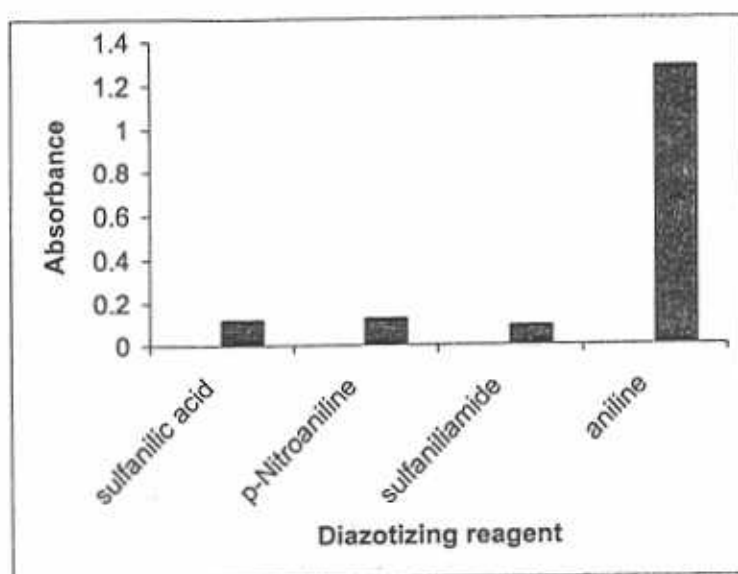
Procedure:

From stock solution of each diazotized reagents, 5ml was taken in a 50ml volumetric flask and 5ml of acetochlor from 100ppm hydrolyzed stock solution was added to it. A red colored azo dye was formed. Separate blank solution was prepared in the same way without the addition of acetochlor. The absorbance of the resulting azo dye was measured at optimum wavelength (400nm) using UNICO UV-2100 spectrophotometer for finding

out the maximum absorption. The results are given in table 3.2.2 and are shown in figure 3.2.4.

Table 3.2.2: Investigation of suitable diazotizing reagent for the spectrophotometric determination of acetochlor.

S.NO	Diazotizing Reagents	Absorbance
1	Sulfanilic acid	0.119
2	Sulfanilamide	0.089
3	P-nitroaniline	0.126
4	Aniline	1.282



3.2.4: Investigation of suitable diazotizing reagent for the spectrophotometric determination of acetochlor.

Conditions:	$\lambda_{\max}$	400nm
	Acetochlor solution	10ppm
	Diazotized reagent	5ml

3.2.3.3: Investigation of acid concentration for hydrolysis of acetochlor.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

10ml of standard acetochlor from 1000 ppm stock solution was taken in five separate titration flasks and varied volume of concentrated hydrochloric acid was added to it to form 0.1-0.5M HCl solution on dilution. This was followed by addition of 1ml of ethanol and 10 ml of distilled water. It was heated on boiling water bath for 30 minutes. After cooling 20ml of ethanol was added to it and diluted up to mark with distilled water in a 100ml volumetric flask. From stock solution of diazotized reagent, 5ml was taken in a 50ml volumetric flask and 5ml of acetochlor from 10ppm hydrolyzed stock solution was added to it. A red colored azo dye was formed. Blank solution was prepared in the same way without the addition of acetochlor. The absorbance of the resulting azo dye was measured at 400nm. The results are given in table 3.2.3 and are shown in figure 3.2.5.

Table 3.2.3: Investigation of acid concentration for hydrolysis of acetochlor.

S.NO	HCl conc. (M)	Absorbance
1	0.1	1.474
2	0.2	1.475
3	0.3	1.475
4	0.4	1.475
5	0.5	1.475

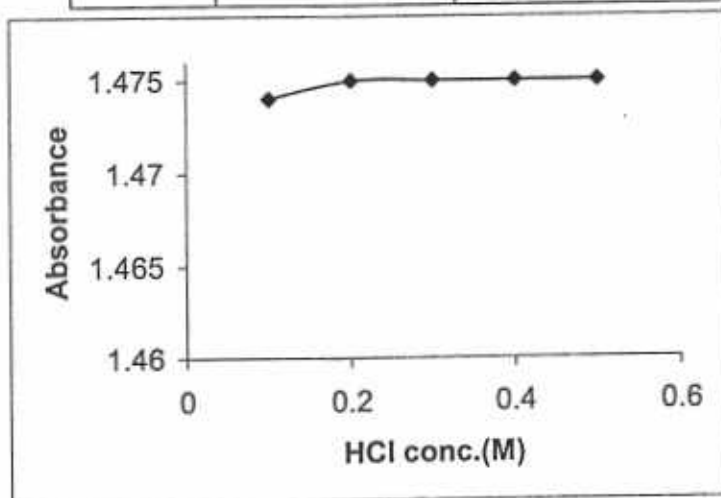


Figure 3.2.5: Investigation of acid concentration for hydrolysis of acetochlor.

Conditions:	$\lambda_{\max}$	400nm
	Acetochlor solution	10ppm
	Diazotized reagent	5ml

3.2.3.4: Investigation of hydrolysis time for spectrophotometric determination of acetochlor.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

10ml of standard acetochlor from 1000ppm stock solution was taken in seven separate titration flasks and hydrolyzed in the same way as mentioned before. The hydrolyzed solution were heated on boiling water bath. The heating time was varied from 5-50 minutes. After cooling 20ml of ethanol was added to it and diluted up to mark with distilled water in a 100ml volumetric flask. Color was developed in the same way as mentioned before. The absorbance of the resulting azo dye was measured at 400nm. The results are given in table 3.2.4 and are shown in figure 3.2.6.

Table 3.2.4: Investigation of hydrolysis time for spectrophotometric determination of acetochlor.

S.NO	Heating Time (min)	Absorbance
1	5	1.538
2	10	1.545
3	20	1.545
4	30	1.538
5	40	1.535
6	50	1.535

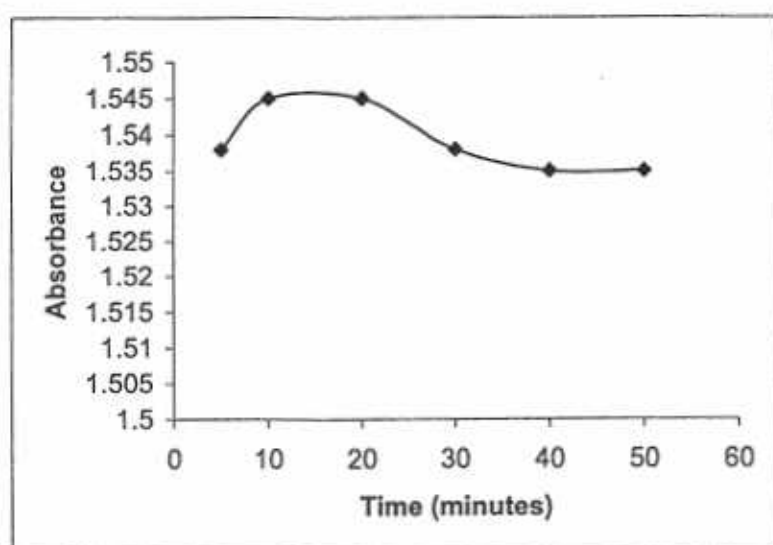


Figure 3.2.6: Investigation of hydrolysis time for spectrophotometric determination of acetochlor.

Conditions:	$\lambda_{\max}$	400nm
	Acetochlor solution	10ppm
	Diazotized reagent	5ml

3.2.3.5: Investigation of the effect of Nitrite concentration on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Hydrolyzed acetochlor solution was prepared in the same way as mentioned above by heating on boiling water bath for 10 minutes. Stock solution of diazotized reagent were prepared by mixing various concentration of nitrite solution i.e. from 0.05-0.3% with aniline solution in 5:1 ratio. From each of these solution 5ml was taken in a 50ml volumetric flask and color was developed in the same way as mentioned before. The results are given in table 3.2.5 and are shown in figure 3.2.7.

Table 3.2.5: Investigation of the effect of Nitrite concentration on the absorbance behavior of the azo dye.

Nitrite conc. (%)	Absorbance	Nitrite conc. (%)	Absorbance
0.05	1.514	0.20	1.542
0.10	1.529	0.25	1.542
0.15	1.542	0.30	1.536

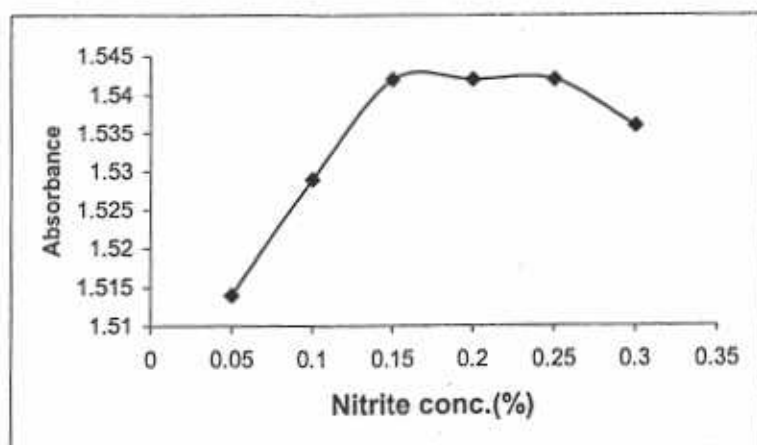


Figure 3.2.7: Investigation of the effect of Nitrite concentration on the absorbance behavior of the azo dye.

Conditions: $\lambda_{\max}$ 400nm
 Acetochlor solution 10ppm
 Diazotized reagent 5ml

3.2.3.6: Investigation of the effect of Nitrite volume on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Stock solution of diazotized reagent were prepared by mixing nitrite (0.15%) solution with aniline solution. The volume of aniline was kept constant i.e. 1ml and that of nitrite was varied from 5-18 ml. From each of these diazotized reagent solution 5ml was taken

in a 50ml volumetric flask and color was developed in the same way as mentioned before. The results are given in table 3.2.6 and are shown in figure 3.2.8.

Table 3.2.6: Investigation of the effect of Nitrite volume on the absorbance behavior of the azo dye.

S.NO	Nitrite volume (ml)	Absorbance
1	5	1.282
2	8	1.523
3	10	1.538
4	12	1.578
5	15	1.573
6	18	1.539

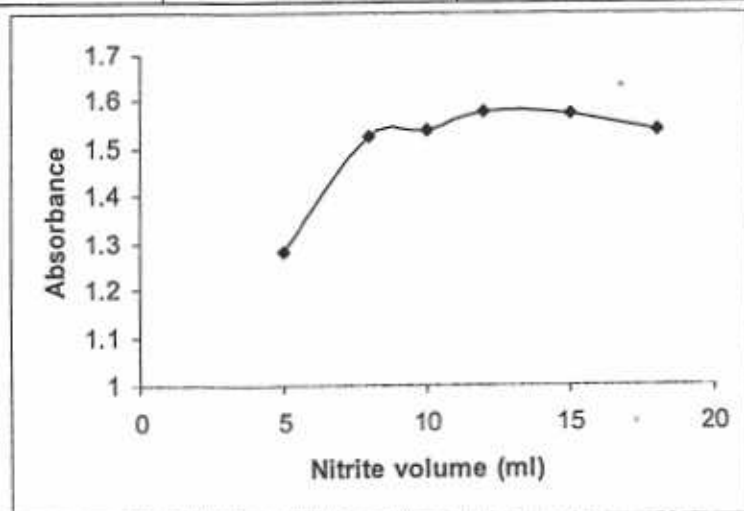


Figure 3.2.8: Investigation of the effect of Nitrite volume on the absorbance behavior of the azo dye.

Conditions: $\lambda_{\max}$ 400nm
 Acetochlor solution 10ppm
 Diazotized reagent 5ml

3.2.3.7: Investigation of the effect of aniline concentration on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Stock solutions of diazotized reagents were prepared by mixing various concentration of aniline solution i.e. from 0.5-2.5% with 0.15% nitrite solution in 1:12 ratio. From each of these diazotized reagent solutions 5ml was taken in a 50ml volumetric flask and color was developed in the same way as mentioned before. The results are given in table 3.2.7 and are shown in figure 3.2.9.

Table 3.2.7: Investigation of the effect of aniline concentration on the absorbance behavior of the azo dye.

S.NO	Aniline conc. (%)	Absorbance
1	0.5	1.090
2	1.0	1.541
3	1.5	1.527
4	2.0	1.452
5	2.5	1.411

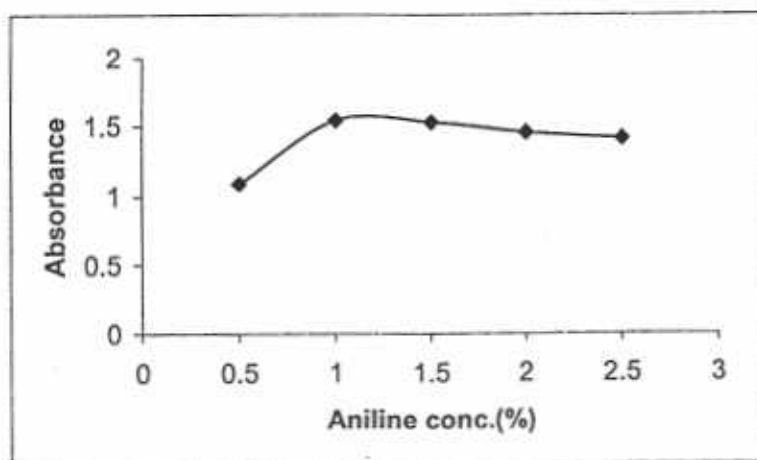


Figure 3.2.9: Investigation of the effect of aniline concentration on the absorbance behavior of the azo dye.

Conditions: λ_{max} 400nm
Acetochlor solution 10ppm
Diazotized reagent 5ml

3.2.3.8: Investigation of the effect of aniline volume on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Stock solution of diazotized reagent was prepared by mixing of 1% aniline solution with 0.15% nitrite solution. The volume of nitrite solution was kept constant (12ml) and that of aniline was varied from 1-8ml. From each of these diazotized reagent solution, 5ml was taken in a 50ml volumetric flask and color was developed in the same way as mentioned before. The results are given in table 3.2.8 and are shown in figure 3.2.10.

Table 3.2.8: Investigation of the effect of aniline volume on the absorbance behavior of the azo dye

Aniline volume (ml)	Absorbance	Aniline volume (ml)	Absorbance
1	0.218	5	1.243
2	1.029	6	0.727
3	1.263	7	0.324
4	1.362	8	0.157

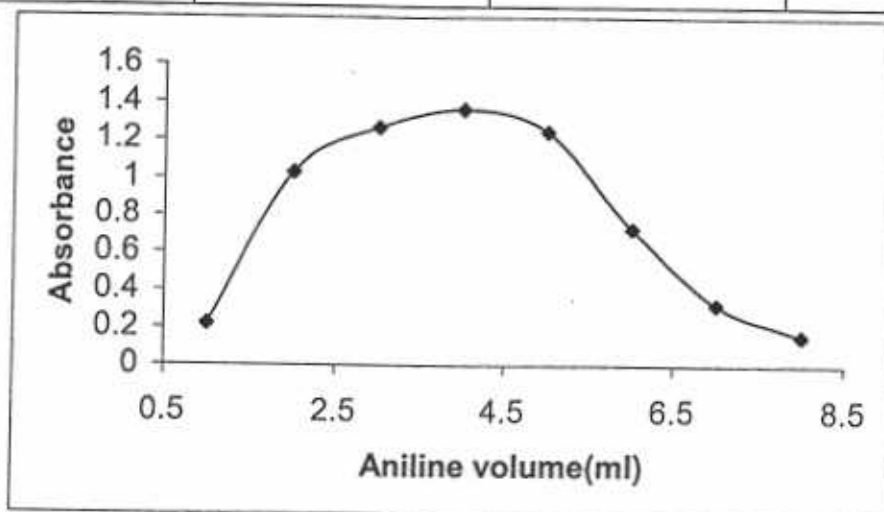


Figure 3.2.10: Investigation of the effect of aniline volume on the absorbance behavior of the azo dye

Conditions:	$\lambda_{\max}$	400nm
	Acetochlor solution	10ppm
	Diazotized reagent	5ml

3.2.3.9: Investigation of the effect of volume of diazotized reagent on the absorbance behavior of the azo dye

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Stock solution of diazotized reagent was prepared by mixing of 1%aniline solution with 0.15% nitrite solution in 1:3 ratio. Various volume of diazotized reagent ranging from 2-14ml were taken in separate 50ml volumetric flasks and 5ml of acetochlor from 100ppm hydrolyzed stock solution was added to each flask. A red colored azo dye was formed. Separate blank solution was prepared in the same way without the addition of acetochlor. The absorbance of the resulting azo dye was measured at 400nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.2.9 and are shown in figure 3.2.11.

Table 3.2.9:Investigation of the effect of volume of diazotized reagent on the absorbance behavior of the azo dye

S.NO	Diazotized reagent volume (ml)	Absorbance
1	2	0.420
2	4	1.523
3	6	1.529
4	8	1.535
5	10	1.541
6	12	1.541
7	14	1.541

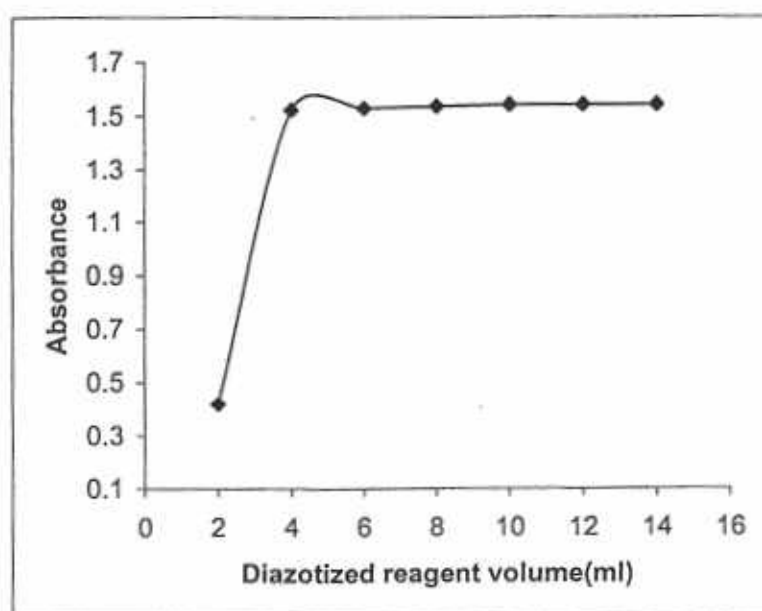


Figure 3.2.11: Investigation of the effect of volume of diazotized reagent on the absorbance behavior of the azo dye

Conditions: $\lambda_{\max}$ 400nm
 Acetochlor solution 10ppm
 Diazotized reagent varied

3.2.3.10: Investigation of the effect of equilibration time on the absorbance behavior of the azo dye

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure:

Stock solution of diazotized reagent was prepared by mixing of 1% aniline solution with 0.15% nitrite solution in 1:3 ratio. Diazotized reagent (12ml) was taken in separate 50ml volumetric flask and 5ml of acetochlor from 100ppm hydrolyzed stock solution was added to each flask. A red colored azo dye was formed. The solutions were equilibrated for different time intervals ranging from 2-60 minute and absorbance was measured at 400nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.2.10 and are shown in figure 3.2.12.

Table 3.2.10: Investigation of the effect of equilibration time on the absorbance behavior of the azo dye.

Time (minutes)	Absorbance	Time (minutes)	Absorbance
2	1.023	30	1.789
5	1.456	35	1.789
10	1.789	40	1.789
15	1.789	45	1.789
20	1.789	50	1.789
25	1.789	60	1.789

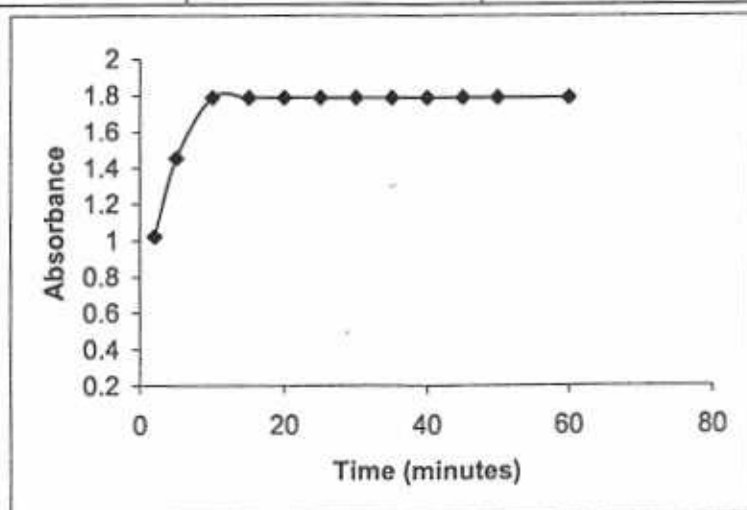


Figure 3.2.12: Investigation of the effect of equilibration time on the absorbance behavior of the azo dye

Conditions: $\lambda_{\max}$ 400nm
 Acetochlor solution 10ppm
 Diazotized reagent 10ml

3.2.3.11: Investigation of the limit of detection and limit of quantification for the spectrophotometric determination of acetochlor.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

For determination of detection limit, different volumes of standard hydrolyzed acetochlor (100ppm) solution for making 10ppm to 9ppb were taken in 50 ml volumetric flask. To each flask optimum volume of diazotized aniline solution (10ml) was added and mixed well and equilibrated for 10 minutes. The resulting azo dye was diluted up to the mark with distilled water. Blank solution was prepared in the same way without the addition of acetochlor. The absorbance of the resulting azo dye was measured at 400 nm using UNICO UV 2100 spectrophotometer. The results are given in table 3.2.11 and are shown in figure 3.2.13. Taking the 9ppb solution seven replicate analysis were done. LOD and LOQ were calculated by using the formula given below.

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

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

Where S = Standard Deviation

3.2.11: Investigation of the effect of concentration on absorbance behavior of acetochlor at lower concentration.

Conc. (ppm)	Absorbance	Conc. (ppb)	Absorbance
10	1.990	80	0.262
8.0	1.853	60	0.188
6.0	1.620	40	0.125
4.0	1.453	30	0.085
2.0	1.200	20	0.049
1.0	1.160	10	0.024
0.5	0.990	9	0.009
0.1	0.720		

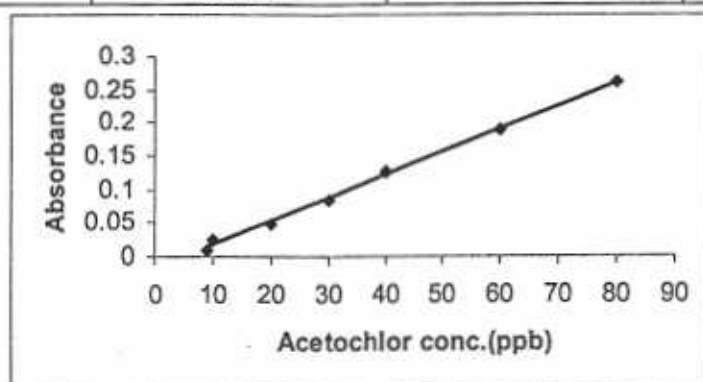


Figure 3.2.13: Investigation of the effect of concentration on absorbance behavior of acetochlor at lower concentration.

Conditions:	$\lambda_{\max}$	400nm
	Acetochlor solution	varied
	Diazotized reagent	10ml

3.2.4 : INVESTIGATION OF PHYSICAL PARAMETERS FOR ONLINE SPECTROPHOTOMETRIC DETERMINATION OF ACETOCHLOR.

All the physical parameters such as the reactor length (R), flow rate and injection volume were optimized for the flow injection spectrophotometric determination of acetochlor, while all the chemical parameters optimized for the batch analysis of acetochlor were kept same. Since the optimum temperature for coupling was room temperature, all of the FI manifolds were set at room temperature. A flow injection assembly is shown in figure 3.2.14.

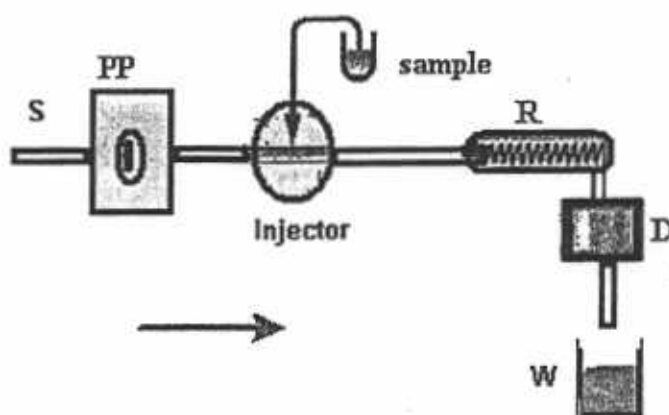


Figure 3.2.14: Schematic diagram of the FI manifold. (S1) diazotizing reagent stream , (R) coupling coil, (pp) persistaltic pump, (W) waste, (D) detector.

3.2.4.1: Investigation of flow rate of system for FI spectrohotometric determination of acetochlor

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure :

In a single channel flow through assembly, diazotized reagent solution was flowing in reagent stream. Flow rate of the system was studied in the range of 0.5-4ml/min. The coil

length of reactor R was 120cm. From 10ppm hydrolyzed acetochlor solution 1ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The coupling reaction take place in reactor R and the absorbance of the azo dye formed was measured at 400nm. Blank was adjusted by flowing diazotized reagent in the system. The optimum flow rate of 1ml/min was found sufficient for complete coupling reaction. The results are given in table 3.2.12 and are shown in figure 3.2.15.

Table 3.2.12:Investigation of flow rate of system for FI spectrohotometric determination of acetochlor.

S.NO	Flow rate (ml/min)	Absorbance
1	0.5	0.686
2	1.0	0.686
3	2.0	0.450
4	3.0	0.342
5	4.0	0.259

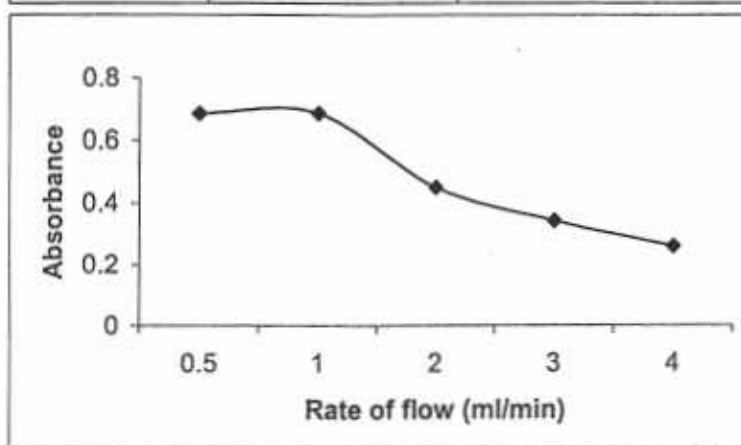


Figure 3.2.15: Investigation of flow rate of system for FI spectrohotometric determination of acetochlor

Conditions: $\lambda_{\max}$ 400nm
 Acetochlor solution 10ppm
 Length of Reactor 120cm

3.2.4.2: Investigation of reactor coil length (R) for FI Spectrophotometric determination of acetochlor

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above.

Procedure :

In a single channel flow through assembly diazotized reagent solution was flowing in reagent stream. Flow rate of the system was 1ml/min. The coil length of reactor R1(coupling reactor)was varied between 40-200cm. From 10ppm hydrolyzed acetochlor solution 1ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The absorbance of the azo dye formed was measured at 400nm by using UNICO UV 2100 spectrophotometer. Blank was adjusted by continuously flowing diazotized reagent in the system. The results are given in table 3.2.13 and are shown in figure 3.2.16

Table 3.2.13: Investigation of reactor coil length (R) for FI Spectrophotometric determination of acetochlor

Coil length (cm)	Absorbance
10	0.006
40	0.161
80	0.301
165	0.754
200	0.858

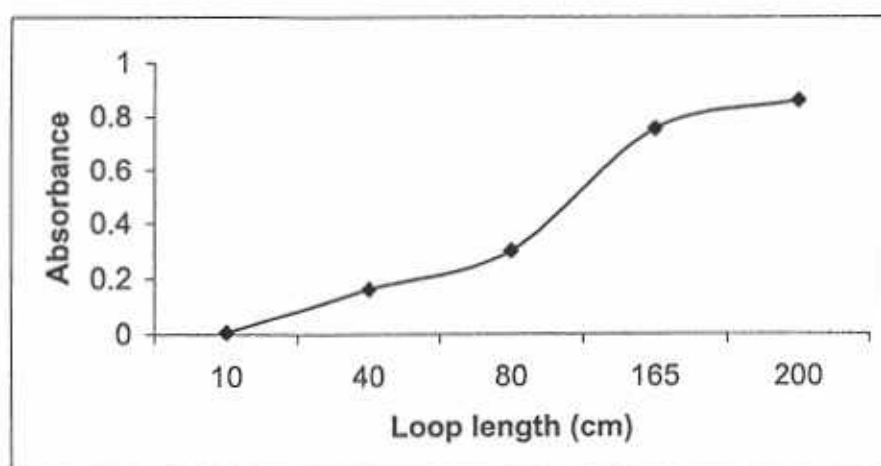


Figure 3.2.16: Investigation of reactor coil length (R) for FI Spectrophotometric determination of acetochlor

Conditions: $\lambda_{\max}$ 400nm
Acetochlor solution 10ppm

3.2.4.3: Investigation of detection limit for online spectrophotometric determination of acetochlor herbicide.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: same as mentioned above

Procedure :

For determination of detection limit, standard solutions of hydrolyzed acetochlor (9ppb to 10ppm) were prepared in a series of 10ml volumetric flasks by dilution from 100ppm hydrolyzed stock solution. In a single channel flow through assembly diazotized reagent solution was flowing in reagent stream. The flow rate of the system was 1ml/min. The length of coupling coil was kept 200cm. From standard solutions 1ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The coupling reaction take place in reactor R and the absorbance of the azo dye formed was measured at 400nm. The results are given in table 3.2.14 and are shown in figure 3.2.17 for lower concentration.

Table 3.2.14: Investigation of the effect of concentration on absorbance behaviour of acetochlor at lower concentration.

Conc. (ppm)	Absorbance	Conc. (ppb)	Absorbance
10	1.801	80	0.208
8.0	1.699	60	0.073
6.0	1.539	50	0.056
4.0	1.400	40	0.048
2.0	1.230	30	0.035
1.0	1.100	20	0.022
0.5	0.856	10	0.010
0.1	0.543	9	0.005

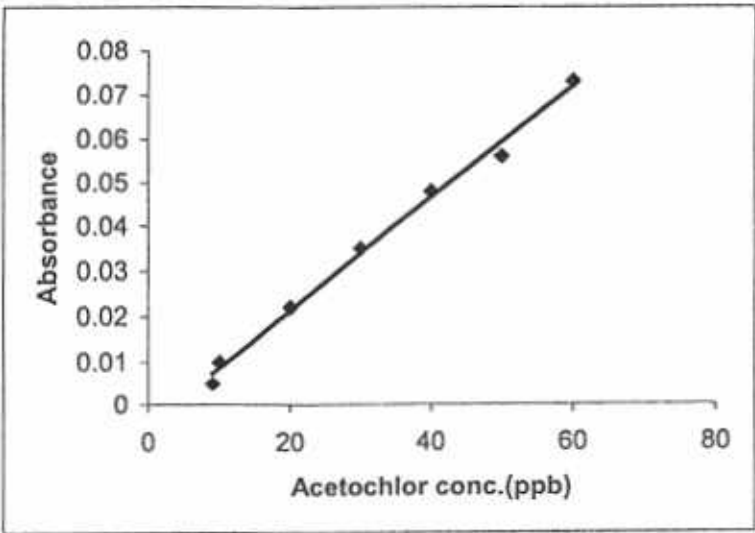


Figure 3.2.17: Investigation of the effect of concentration on absorbance behaviour of acetochlor at lower concentration.

Conditions: λ_{max} 400nm
Acetochlor solution varied
Reactor coil length 1cm
Flow rate 1ml/minute

3.2.5 : APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF ACETOCHLOR IN COMMERCIAL FORMULATION.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Commercial samples: commercial samples of acetochlor was purchased from local market.

Solutions: The same as mentioned before

Procedure:

1-3ml of acetochlor formulation was taken in titration flasks and hydrolyzed in the same way as standards. From these hydrolyzed solutions 2ml were taken in 50ml volumetric flasks and color was developed in the same way as mentioned before for batch analysis. The absorbance was measured against blank at 400nm using Unico 2100 UV spectrophotometer.

For FI analysis 1ml of hydrolyzed herbicide sample was injected in reagent stream and absorbance was measured using the same procedure as for standard.

The active ingredient of acetochlor in commercial formulation was calculated both for batch and FI method using their respective calibration curves. The results are given in table 3.2.15

Table 3.2. 15: Active ingredient of herbicide in commercial formulation.

Herbicide commercial name	Label Amount (g/ml)	Batch Analysis (g/ml)	FIA (g/ml)
Acetore	0.5	0.42±0.01	0.45±0.008

3.2.6: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF ACETOCHLOR IN REAL SAMPLES.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Standard procedure for the extraction of herbicides from the real sample was used, as mentioned before for fluroxypyr. According to this method, 15g of homogenized food sample (maize grain and flour) and 20ml of sugarcane juice were taken in a beaker and 50ml of solvent (petroleum ether and acetone in 1:1) was added to it for extraction of herbicides. The mixture was shaken for 2hr. After equilibration the sample was filtered and passed through 10 g of sodium sulfate. The filtrate was evaporated on rotary evaporator up to a volume of 5ml. The resulting solution was hydrolyzed with 0.2M hydrochloric acid by heating on boiling water bath for 10 minutes. From this hydrolyzed extract 1ml and 2ml was used for online and batch analysis respectively. Color was developed in the same way as mentioned before for batch and FI analysis. The results are given in table 3.2.16.

Table 3.2.16: Residue level of herbicide in real sample.

Sample	FIA method	Batch method
Maiz (grains)	1.37±0.09 µg/g	1.35±0.13 µg/g
Maiz (flour)	1.05±0.20 µg/g	0.99±0.12 µg/g
Sugar cane juice	1.63±0.01 µg/ml	1.6±0.01 µg/ml

**3.2.7: PERCENT RECOVERY OF ACETOCHLOR FROM REAL SAMPLES
USING THE INVESTIGATED METHOD.**

Instrument: The same as mentioned before

Procedure:

A recovery test was performed on control samples, fortified with a known concentration of acetochlor solution. The solvent was evaporated at room temperature for 1hr. the extraction was done exactly the same way as the sample. Six replicates of the fortified samples were analyzed by batch and FI method in the same as mentioned before. The results of percent recovery of acetochlor in real samples and formulation by batch and online method are given in table 3.2.17. Percent recovery was calculated by using the formula mentioned before for fluroxypyr.

Table 3.2.17: Percent recovery of herbicide from real sample.

SAMPLES	FIA	BATCH ANALYSIS
	% Recovery	% Recovery
Maize (grains)	95.5± 0.08	95.1± 0.09
Maize (flour)	93± 0.26	92± 0.45
Sugar cane juice	93.4± 0.43	93±0.36

Results and discussion

After preliminary investigation it was observed that the proposed reaction shown in figure 3.2.2. is feasible for spectrophotometric determination of acetochlor. Then various physical and chemical parameters were optimized and are discussed below.

After preliminary experiments, optimum wavelength for the azo dye formed was investigated. The results are given in table-3.2.1 and are shown in figure 3.2.3. As can be seen from figure 3.2.3 the resulting azo dye has a maximum absorbance at 400nm and was subsequently used as optimum wavelength for further studies of acetochlor.

Different diazotizing reagents (table 3.2.2) were used to form the azo dye to find out the most suitable diazotizing reagent for spectrophotometric determination of acetochlor. Sulfaniliacid, sulfanilamide, p-nitroaniline and aniline were used for this purpose. It was observed that among all the diazotizing reagent investigated aniline was found to be the most suitable (figure 3.2.4) reagent for formation of azo dye therefore, aniline was selected as a diazotizing reagent for further studies.

The concentration of hydrochloric acid required for maximum hydrolysis was optimized in range of 0.1-0.5M (table 3.2.3). An increase in absorbance was observed with increasing final acid concentration up to 0.2 M of hydrochloric acid solution (Figure 3.2.5). Above this concentration the absorbance remained constant, therefore 0.2 M acidity for the final solution was used for further hydrolysis of acetochlor herbicide.

As hydrolysis was done on boiling water bath therefore, the time required for hydrolysis was also optimized in the range 5-50minutes (table 3.2.4). It was observed (figure 3.2.6) that maximum hydrolysis requires 10 minute heating on boiling water bath, beyond this time it remains constant. Therefore, 10 minutes heating on boiling water bath was taken as optimum heating time.

Nitrite concentration was optimized for diazotization in the range of 0.05-0.3% (table 3.2.5) and it can be seen from figure 3.2.7 that 0.15% was the optimum concentration of nitrite for maximum diazotization. Similarly the volume of 0.15% nitrite solution was optimized in the range of 2-18ml (table 3.2.6). It was observed (figure 3.2.8) that 12ml of 0.15% of nitrite solution was sufficient for maximum diazotization and subsequently for maximum formation of azo dye.

Similarly the concentration and volume of aniline solution was optimized (table 3.2.7 and 3.2.8). It was observed (figure 3.2.9) that 1% of aniline is the optimum concentration and above this concentration a decrease in absorbance was observed. Figure 3.2.10 shows that 4ml of 1% aniline is the optimum volume required for maximum formation of azo dye.

The stock solution of diazotized reagent was prepared by adding nitrite and aniline solution in 3:1 ratio respectively. The volume of diazotized reagent required for coupling reaction was studied in the range of 2-12ml (table 3.2.9). It was observed (figure 3.2.11) that 10ml of diazotized reagent is required for maximum formation of azo dye.

The equilibration time for coupling reaction (table 3.2.10 & figure 3.2.12) shows that the reaction is slow and 10 minutes equilibration time is optimum time for completion of reaction. After this time the product is stable and no change in absorbance was observed.

Optimization of parameters for online analysis

The chemical parameters optimized for batch analysis were directly used for online analysis. These optimized parameters were applied for online analysis using a single channel flow injection system. In this system a stream of diazotized reagent was continuously pumped and a volume of 1ml of hydrolyzed sample was injected in reagent stream through a six-port injection valve. The coupling reaction takes place in reactor R and absorbance was measured at 400nm. The physical parameters like flow rate and reactor coil length were optimized. The optimum flow rate of the system (table 3.2.12 & figure 3.2.15) was 1ml/minute. At higher flow rate the absorbance decreased. This is due to the reason that the reaction is slow and it needs some time for completion. The length of reactor R was studied in the range of 30-200 cm (table 3.2.13 & figure 3.2.16) and it was found that with increase of coil length the absorbance of azo dye increases as it finds more dispersion time for completion of reaction. Therefore, a coil length of 200cm was

selected for FI system for keeping good sampling frequency i.e. 60 samples per hour and to avoid the steady state condition.

At optimum conditions, the effect of lower concentration of acetochlor (below 1 ppm) on absorbance behavior was investigated for assessing the LOD and LOQ for the investigated method both for batch and FI analysis. The results are given in tables 3.2.11. & 3.2.14 and are shown in figure 3.2.13 & 3.2.17 respectively. The LOD and LOQ were calculated for the investigated method and were found to be 0.40 ppb and 1.00 ppb for FI analysis and 0.9 ppb and 3.0 ppb for batch analysis respectively.

The RSD was also calculated and was found to be 1.55% and 3.6% for online and batch analysis respectively. Molar absorptivity was calculated and found to be $4.86 \times 10^4 \text{ L.mol}^{-1} \text{ cm}^{-1}$ and $5.37 \times 10^4 \text{ L.mol}^{-1} \text{ cm}^{-1}$ for online and batch method respectively.

Application of the investigated method for the analysis of acetochlore in commercial formulation and in real samples.

The investigated method was applied for the determination of acetochlor in commercial formulation and also in real samples. Calibration curve of standard acetochlor was used for the determination of acetochlore in formulation and in real samples. The following formula was used for calculating the active ingredient in formulation and for residue determination in real samples.

$$\text{ppm of acetochlor} = \frac{\text{G.R (}\mu\text{g/ml)} \times \text{volume made(ml)} \times \text{D.F}}{\text{Weight of sample taken(g)}}$$

Where G.R represents graphical reading and D.F for dilution factor.

The residue level in maize grains was found to be $1.37 \pm 0.09 \mu\text{g/g}$ and $1.35 \pm 0.13 \mu\text{g/g}$ by online and batch method respectively which is much higher than the NZ food and safety authority MRLs, $0.02 \mu\text{g/g}$. In case of flour samples the residue level was found to be $1.05 \pm 0.2 \mu\text{g/g}$ and $0.99 \pm 0.12 \mu\text{g/g}$ and in sugar cane juice the residue level found were $1.63 \pm 0.01 \mu\text{g/ml}$ and $1.6 \pm 0.01 \mu\text{g/ml}$ by online and batch method respectively. The method was successfully applied for the determination of the concentration of active ingredients in commercial formulation of acetochlor herbicides (table 3.2.15). The value of active ingredient determined by using the optimized method was closely related to the label value of active ingredient. The recovery test shows that percent recoveries in

samples varied from 93-95.5% for FI analysis and 92-95.1% for batch analysis (table 3.2.17).

It was further observed that the results of the two methods agreed very closely with each other. Further the sensitivity and reproducibility is almost the same. However keeping in mind the cost and time of analysis FIA would be the method of choice in this case.

Reference:

- 1) H.H.Zheng and C.M.Ye, "Photodegradation of acetochlor in water and UV photoproducts identified by mass spectrometry", J Environ Sci (China).15,6, 783-90 (2003).

3.3: INVESTIGATION OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF 2,4-D.

The chemical structure of 2,4-D is given below.

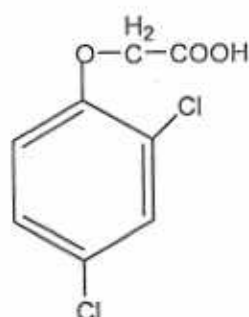
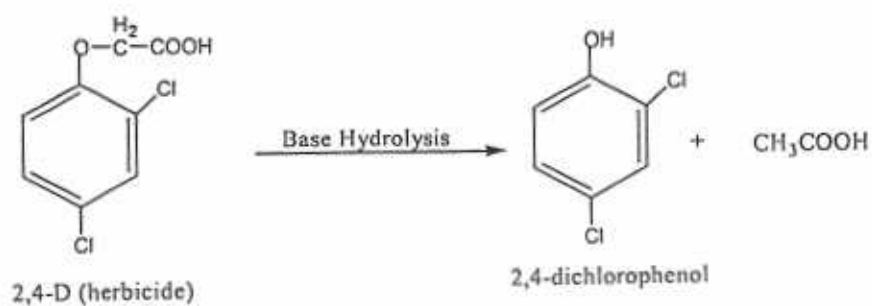


Figure 3.3.1: Chemical Structure of 2,4-D

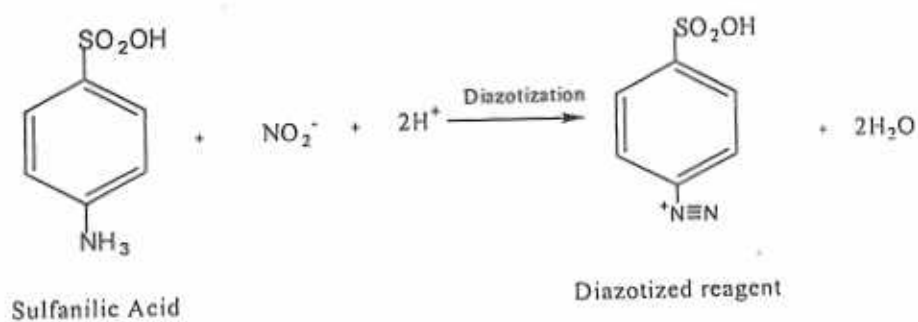
3.3.1: Method development strategy for spectrophotometric determination of 2,4-D.

2,4-D is chemically 2,4-dichlorophenoxy acetic acid. It has 2 chloride group attached, one at position 2 and other at position 4. 2,4-dichlorophenol (2,4-DCP) is the main catabolic metabolite of 2,4-D in aerated soil and it is also used as a precursor in the manufacture of 2,4-D. The proposed method for 2,4-D determination is based on hydrolysis of 2,4-D to form 2,4-dichloro phenol followed by the coupling of this phenol with a suitable diazotized reagent to form an azo dye. The proposed chemical reaction has three steps as shown in figure-3.3.2. The first step involves the base hydrolysis of 2,4-D to 2,4-DCP and acetic acid. The second step involves the formation of diazotized reagent. In the final step the hydrolyzed phenolic product is coupled with already diazotized reagent to form azo dye. In this investigation the expected final product, orange red azo dye formed was used for determination of 2,4-D.

Step 1:Hydrolysis



Step 2: Diazotization



Step 3: Coupling

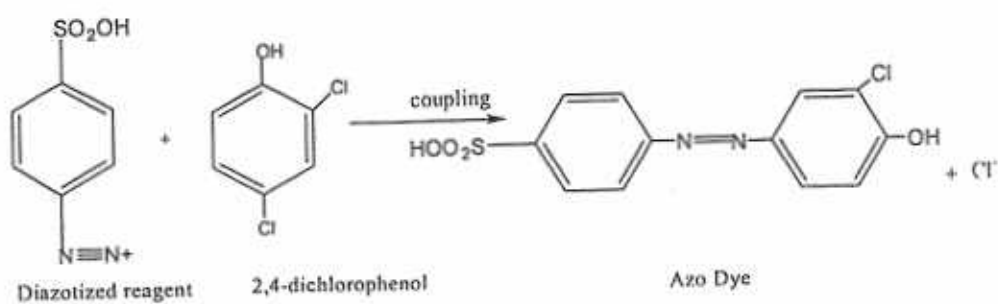


Figure 3.3.2: Proposed chemical reaction for the formation of azo dye from 2,4-D.

3.3.2 : Preliminary investigation of the possibility of hydrolysis and coupling of hydrolyzed product for spectrophotometric determination of 2,4-D.

Preliminary studies were conducted to investigate the possibility of hydrolysis of 2,4-D and the subsequent use of the hydrolyzed product as coupling reagent for the formation of expected azo dye. Initially high concentration of herbicide 2,4-D (1000 ppm), large volume of hydrolyzing reagent sodium hydroxide, sodium carbonate, and diazotizing reagents (sulfanilic acid, sulfanilamide, Aniline, p-amino benzoic acid and nitro aniline) were tried to form the expected azo dye at room temperature. Both acid and base hydrolysis were tried on heating. The formation of the colored azo dye with base hydrolysis indicated the possibility of the proposed reaction and its subsequent application for the spectrophotometric determination of 2,4-D. Further studies were focused on optimization of various parameters and are given below.

3.3.3: OPTIMIZATION STUDIES FOR SPECTROPHOTOMETRIC DETERMINATION OF 2,4-D.

3.3.3.1: Investigation of suitable wavelength for the spectrophotometric determination of 2,4-D.

Instruments:

UV/Vis spectrophotometer (UNICO UV-2100 United Products and Instruments Inc., Dayton, N.J, U.S.A), Digital Analytical Balance (Sartorius handy H51, Germany), Peristaltic pump, V-450 6 port injection valve (Upchurch Scientific, Inc, USA) Polyethylene tubing (i.d. 1.19mm, Becton Dickson, USA), silicon tubing (i.d.1.71mm) , 3-way connector, Thermostatic water bath (SMIC China) and pH meter (WTW pH 422,8120 Wilhelm I, OB, W. Germany) were used during this work.

Reagents and chemicals:

All chemicals used were of analytical reagent grade purity. Sulfanilic acid, Sodium nitrite, sodium carbonate, hydrochloric acid and sodium hydroxide were used during this work. Commercial sample containing 2,4-D was purchased from the local market. Authentic standard of 2,4-D was purchased from dealer.

Solution preparation:

i) **Nitrite solution:** Nitrite solution (1000 ppm) was prepared by dissolving 0.15g sodium nitrite in distilled water and diluted up to mark in a 100 ml volumetric flask.

ii) **Sulfanilic acid solution:** Sulfanilic acid solution (0.6%) was prepared by dissolving 0.6g of sulfanilic acid in 8ml of concentrated HCl and a little distilled water by heating on boiling water bath and then after cooling diluted up to mark in a 100ml volumetric flask.

iii) **Sodium carbonate solution:** Sodium carbonate solution (10%) was prepared by dissolving 10g of sodium carbonate with distilled water in a 100 ml volumetric flask.

iv) **Diazotized sulfanilic acid:** Stock solution of diazotized sulfanilic acid was prepared in a separate flask by mixing sulfanilic acid and sodium nitrite solutions in 1:10 ratio. This stock solution was used for further investigations.

v) **Standard 2,4-D solution:** 0.1 g of authentic standard was dissolved with distilled water in a 100 ml volumetric flask to form 1000ppm solution of 2,4-D.

vi) **Standard Hydrolyzed 2,4-D solution (100ppm):** 10ml of 1000ppm 2,4-D was taken in a separate beaker and 10ml of 1% sodium carbonate was added to it and heated on boiling water bath for 30 minutes. The resulting solution were cooled and diluted up to 100ml with distilled water.

Procedure:

From stock solution of diazotized sulfanilic acid 4ml was taken in a 50ml volumetric flask and 5ml of 2,4-D from 100ppm hydrolyzed solution was added to it. It was followed by the addition of 5ml of sodium carbonate and diluted up to mark with distilled water. A red colored azo dye was formed. Blank solution was prepared in the same way without the addition of hydrolyzed 2,4-D. The absorbance of the resulting azo dye was measured from 320 to 600nm using UNICO UV-2100 spectrophotometer for finding out the optimum wavelength. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are given in table 3.3.1 and are shown in figure 3.3.3.

Table 3.3.1: Investigation of suitable wavelength for the spectrophotometric determination of 2,4-D.

Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
320	0.057	480	0.514
340	0.071	500	0.494
360	0.136	520	0.416
380	0.204	540	0.298
400	0.278	560	0.194
420	0.352	580	0.119
440	0.420	600	0.076
460	0.480		

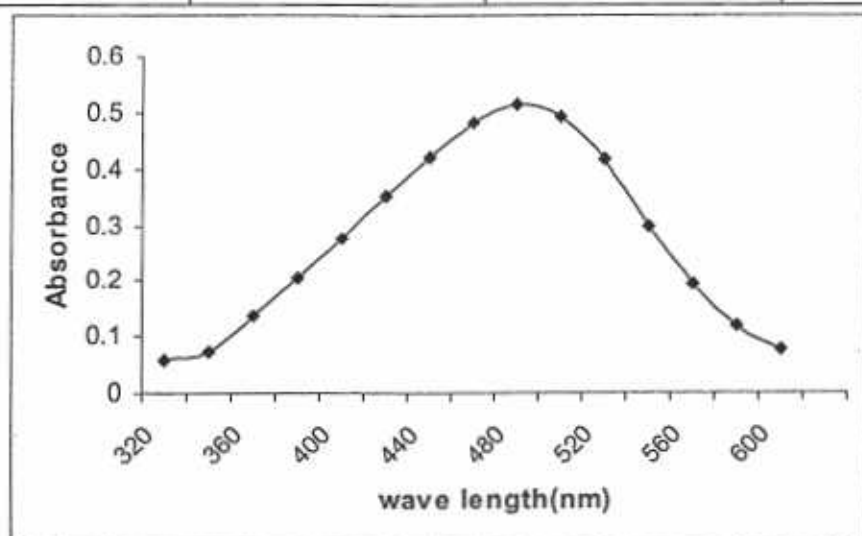


Figure 3.3.3 : Investigation of suitable wavelength for the spectrophotometric determination of 2,4-D.

Conditions:	λ max.	varied
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Sodium carbonate (10 %)	5ml

3.3.3.2: Investigation of suitable diazotizing reagent for spectrophotometric determination of 2,4-D

Instrument: The same as mentioned before

Reagents: The same as mentioned before

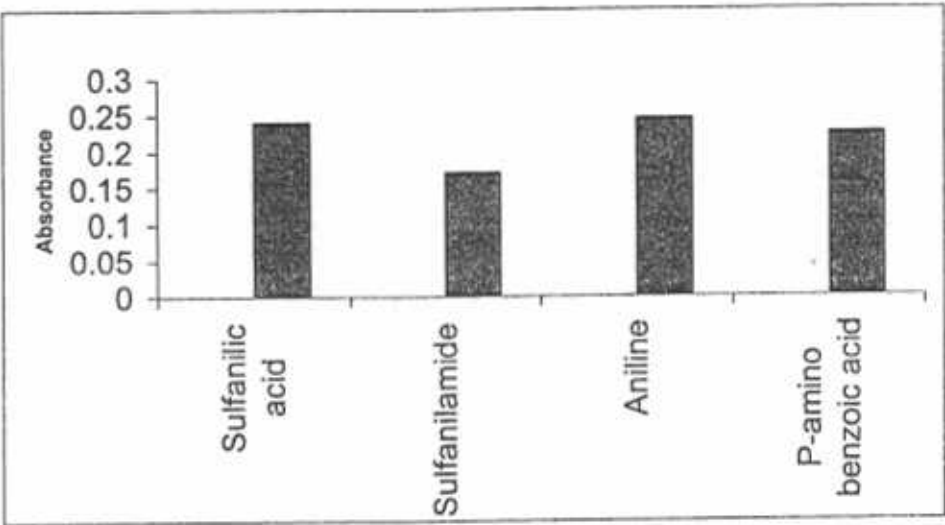
Solutions: Analytical reagent grade purity sulfanilic acid, sulfanilamide, aniline, p-nitro aniline and P-amino benzoic acid were used as diazotizing reagent. Stock solution of 1% of each diazotizing reagents was prepared in five separate volumetric flasks and diazotized with nitrite solution by mixing it in 1:10 ratios with each diazotizing reagent respectively. The resulting stock solutions were used for further investigations

Procedure:

From stock solution of each diazotized reagents 4ml was taken in a 50ml volumetric flask and 5ml of 2,4-D from 100ppm hydrolyzed stock solution was added to it. A red colored azo dye was formed. Separate blank solution was prepared in the same way without the addition of 2,4-D. The absorbance of the resulting azo dye was measured at optimum wavelength (480nm) using UNICO UV-2100 spectrophotometer for finding out the maximum absorption. The results are given in table 3.2.2 and are shown in figure 3.2.4.

3.3.2: Investigation of suitable diazotizing reagent for spectrophotometric determination of 2,4-D.

Diazotizing Reagent	Sulfanilic acid	Sulfanilamide	P-nitro aniline	Aniline	P-amino benzoic acid
Absorbance	0.240	0.171	0.000	0.245	0.225



3.3.4: Investigation of suitable diazotizing reagent for spectrophotometric determination of 2,4-D

Conditions:	$\lambda_{\max}$	varied
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Sodium carbonate (10 %)	5ml

3.3.3.3. Investigation of suitable base for hydrolysis of 2,4-D .

Instrument: The same as mentioned before

Reagents: The same as mentioned before

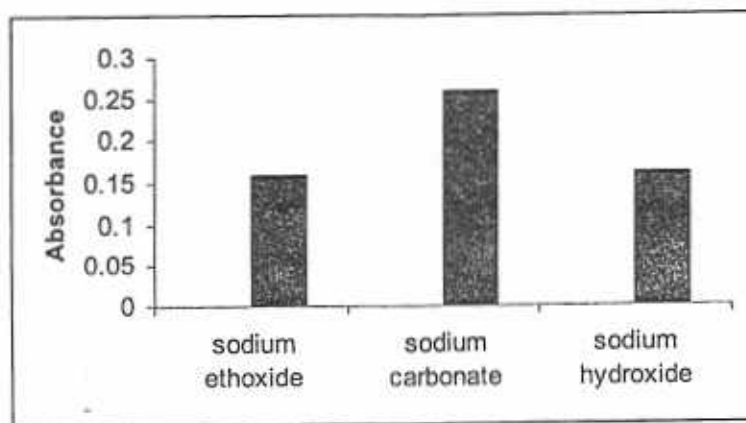
Solutions: The same as mentioned before

Procedure:

10ml of 1% sodium carbonate, 1% sodium ethoxide and 1% of sodium hydroxide were taken in three separate beakers and to each beakers 10ml of 1000ppm 2,4-D was added. All the beakers were covered and heated on boiling water bath for 30 minutes. The resulting hydrolyzed product was cooled and diluted up to mark with distilled water in a 100ml volumetric flask. From these hydrolyzed solution 5ml was taken as a coupling reagent and color was developed in the same way as mentioned before. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.3. and shown in figure 3.3.5 .

3.3.3: Investigation of suitable base for hydrolysis of 2,4-D

Name of Base	Sodium carbonate	Sodium hydroxide	Sodium ethoxide
Absorbance	0.260	0.160	0.165



3.3.5: Investigation of suitable base for hydrolysis of 2,4-D

Conditions:	$\lambda_{\max}$	varied
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Sodium carbonate (10 %)	5ml

3.3.3.4: Investigation of base concentration for hydrolysis of 2,4-D

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ml of 1000ppm 2,4-D was taken in seven separate beakers and 10ml of (0.5%- 6%) sodium carbonate was added to them respectively. All the beakers were covered and heated on boiling water bath for 30 minutes. The resulting hydrolyzed product was cooled and diluted up to mark with distilled water in a 100ml volumetric flask. From these hydrolyzed solution 5ml was taken as a coupling reagent and color was developed in the same way as mentioned before. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.4 and shown in figure 3.3.6.

Table 3.3.4 : Investigation of base concentration for hydrolysis of 2,4-D

Base conc. (%)	Absorbance	Base conc. (%)	Absorbance
0.5	0.098	4	0.201
1	0.173	5	0.198
2	0.224	6	0.190
3	0.201		

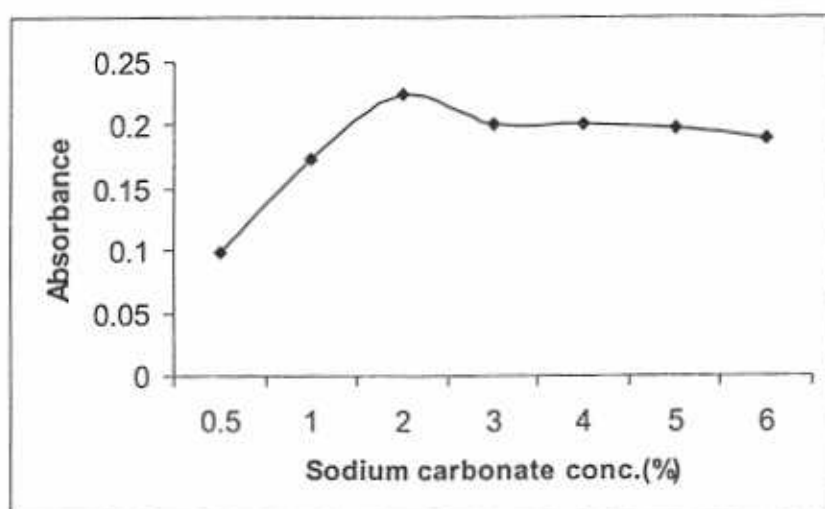


Figure 3.3.6: Investigation of base concentration for hydrolysis of 2,4-D

Conditions:	$\lambda_{\max}$	480nm
	Hydrolysis temperature	100°C
	Hydrolysis time	30 minutes
	Diazotized sulfanilic acid	4ml
	Sodium carbonate(10%)	5ml

3.3.3.5. Investigation of base volume for hydrolysis of 2,4-D

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ml of 1000ppm 2,4-D was taken in seven separate beakers and different volume of sodium carbonate i.e. 5-30 ml was added to them respectively. The solutions were hydrolyzed and color was developed in the same way as mentioned before. The absorbance was measured at 480nm using UV-2100 spectrophotometer. The results are given in table 3.3.5 and shown in figure 3.3.7.

Table 3.3.5. Investigation of base volume for hydrolysis of 2,4-D

Base volume (ml)	Absorbance	Base volume (ml)	Absorbance
5	0.07	20	0.205
10	0.168	25	0.114
15	0.245	30	0.085

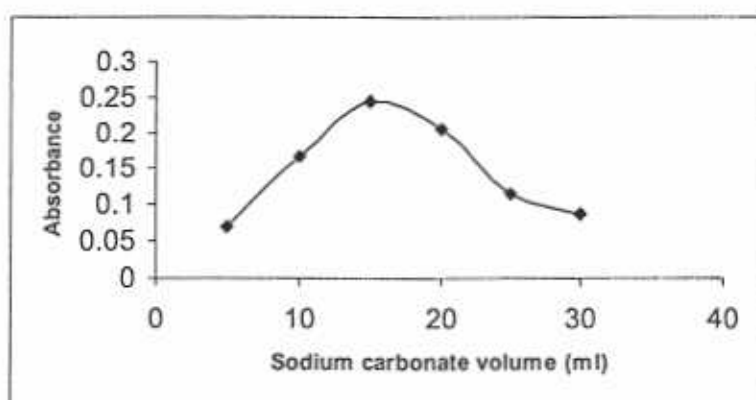


Figure 3.3.7: Investigation of base volume for hydrolysis of 2,4-D

Conditions:	$\lambda_{\max}$	480nm
	Hydrolysis temperature	100°C
	Hydrolysis time	30 minutes
	Diazotized sulfanilic acid	4ml
	Sodium carbonate (10%)	5ml

3.3.3.6: Investigation of optimum temperature for hydrolysis of 2,4-D.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ml of 1000ppm 2,4-D was taken in separate beakers and 15ml of 2% sodium carbonate was added to each beaker. All the beakers were covered and heated for 30 minutes at different temperatures ranging from 20-100°C. In the resulting hydrolyzed solutions color was developed in the same way as mentioned before. The absorbance was measured at 480nm using UV-2100 spectrophotometer. The results are given in table 3.3.6 and shown in figure 3.3.8.

Table 3.3.6: Investigation of optimum temperature for hydrolysis of 2,4-D

Temperature (°C)	Absorbance
20	0.045
40	0.09
60	0.148
80	0.198
100	0.23

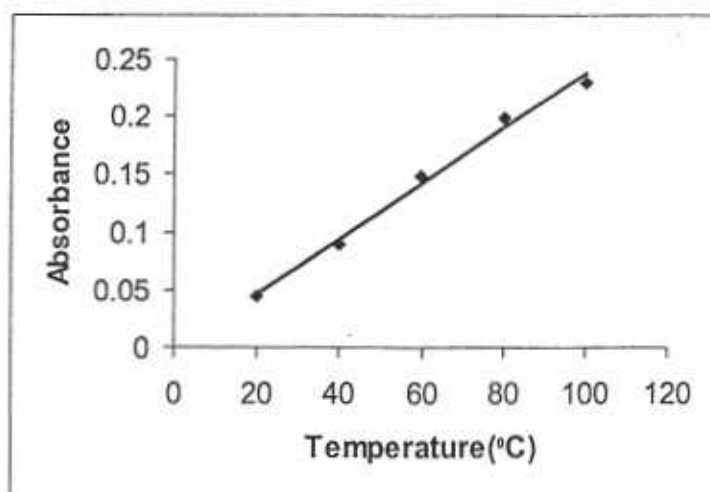


Figure 3.3.8: Investigation of optimum temperature for hydrolysis of 2,4-D.

Conditions:	$\lambda_{\max}$	480nm
	Hydrolysis temperature	varied
	Hydrolysis time	30 minutes
	Sodium carbonate (2%)	15ml
	Diazotized sulfanilic acid	4ml
	Sodium carbonate(10%)	5ml

3.3.3.7: Investigation of heating time for hydrolysis of 2,4-D

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ml of 1000ppm 2,4-D was taken in separate beakers and 15ml of 2% sodium carbonate was added to it respectively. All beakers were covered and heated on boiling water bath for varied time i.e. 5-60 minutes. The resulting solutions were hydrolyzed and color was developed in the same way as mentioned before. The absorbance was measured at 480nm using UV-2100 spectrophotometer. The results are given in table 3.3.7 and shown in figure 3.3.9.

Table 3.3.7 : Investigation of heating time for hydrolysis of 2,4-D

S.NO	Time (min)	Absorbance
1	5	0.181
2	10	0.316
3	20	0.394
4	30	0.519
5	40	0.710
6	50	0.542
7	60	0.423

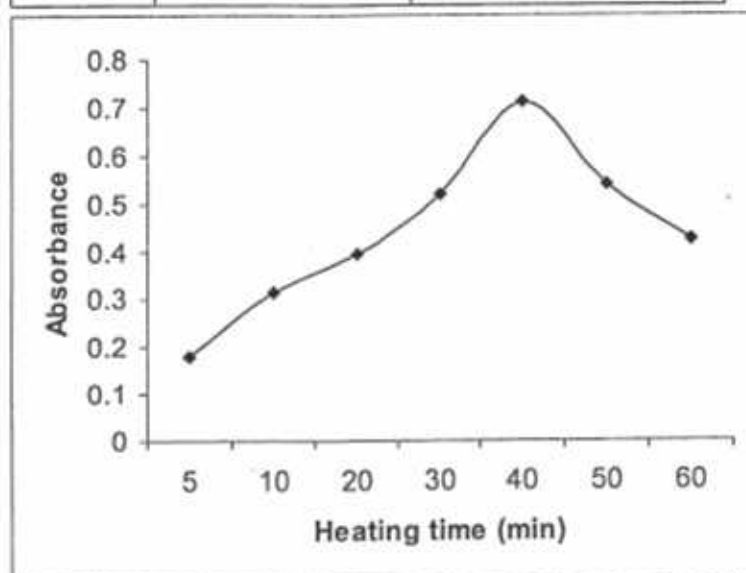


Figure 3.3.9: Investigation of heating time for hydrolysis of 2,4-D

Conditions:	$\lambda_{\max}$	480nm
	Hydrolysis temperature	100°C
	Hydrolysis time	varied
	Sodium carbonate (2%)	15ml
	Diazotized sulfanilic acid	4ml
	Sodium carbonate(10%)	5ml

3.3.3.8: Investigation of the effect of nitrite concentration on the absorbance behavior of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Diazotized sulfanilic acid: Stock solution of diazotized sulfanilic acid was prepared in five separate volumetric flasks by mixing sulfanilic acid (1.0%) and sodium nitrite solution of different concentrations (500-2500ppm) in 1:10 ratio respectively. This stock solution was used for further investigations.

Procedure:

From 100ppm hydrolyzed 2,4-D solution, 5ml was taken in five separate 50ml flasks, to each of these 4ml of varied concentration of diazotized reagent was added. It was followed by the addition of 5ml of sodium carbonate to each flask. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.8 and are shown in figure 3.3.10.

Table 3.3.8: Investigation of the effect of Nitrite concentration on the absorbance behavior of the azo dye.

S.No	Nitrite conc. (ppm)	Absorbance
1	500.0	0.166
2	1000.0	0.243
3	1500.0	0.201
4	2000.0	0.105
5	2500.0	0.083

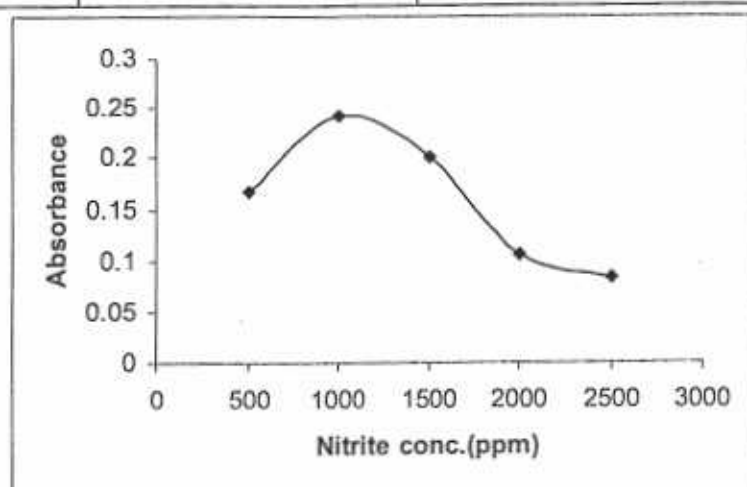


Figure 3.3.10: Investigation of the effect of nitrite concentration on the absorbance behavior of the azo dye.

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Nitrite conc.	Varied
	Sulfanilic acid conc.	1%
	Sodium carbonate (10 %)	5ml

3.3.3.9: Investigation of the effect of sulfanilic acid concentration on the absorbance behavior of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions:

Diazotized sulfanilic acid: Stock solution of diazotized sulfanilic acid was prepared in five separate volumetric flasks by mixing sulfanilic acid solution of various concentration ranging from 0.2%-1% concentration and optimized nitrite solution (1000ppm) in 1:10 ratio .

Procedure:

From 100ppm hydrolyzed 2,4-D solution, 5ml was taken in five separate 50ml flasks. To each of these 4ml of varied concentration of diazotized reagent was added. It was followed by the addition of 5ml of sodium carbonate to each flask. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.9 and are shown in figure 3.3.11.

Table 3.3.9: Investigation of the effect of sulfanilic acid concentration on the absorbance behavior of the azo dye.

S.No	Sulfanilic acid conc. (%)	Absorbance
1	0.2	0.283
2	0.4	0.328
3	0.6	0.457
4	0.8	0.422
5	1.0	0.369

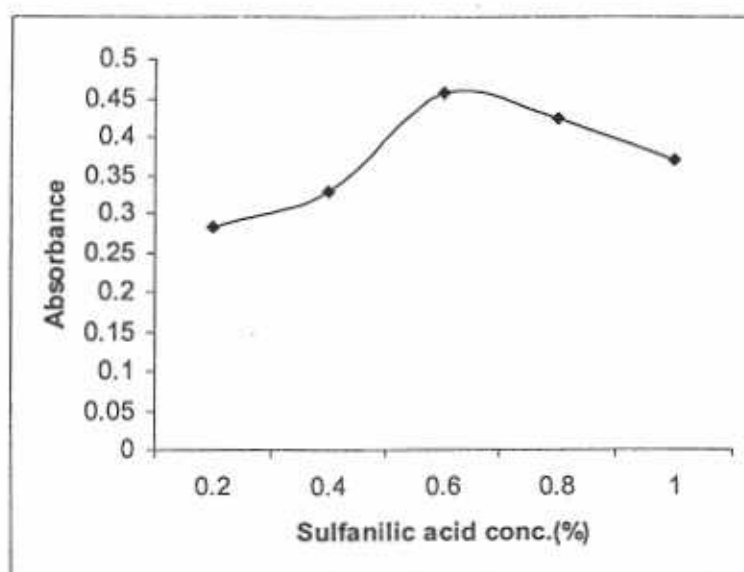


Figure 3.3.11: Investigation of the effect of sulfanilic acid concentration on the absorbance behavior of the azo dye.

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Nitrite conc.	1000ppm
	Sulfanilic acid conc.	varied

3.3.3.10: Investigation of the mixing ratio of nitrite and sulfanilic acid for the formation of diazotized reagent .

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Diazotized sulfanilic acid: Stock solution of diazotized sulfanilic acid was prepared in two series. In series one the volume of nitrite solution (1000ppm) was varied from 2-12ml and sulfanilic acid volume was kept constant. In series two, the sulfanilic acid volume was varied between 1-5ml and nitrite volume was kept constant.

Procedure:

From 100ppm hydrolyzed 2,4-D solution 5ml was taken in two separate series of 50ml volumetric flasks. To each of these 4ml of varied concentration of diazotized reagent was

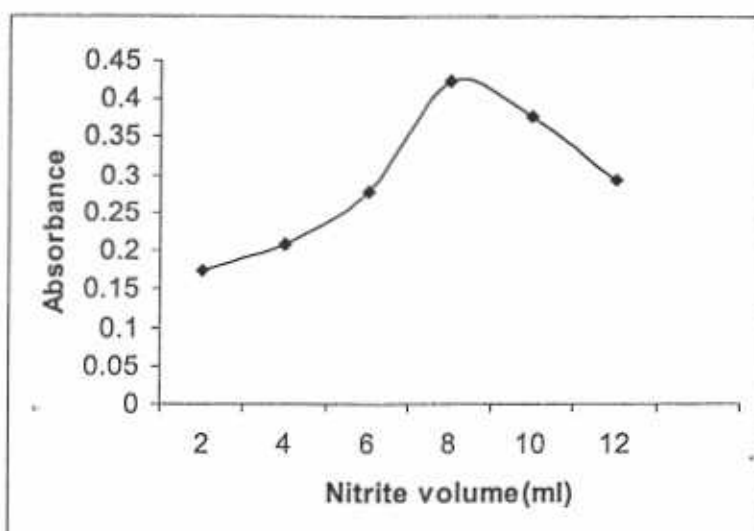
added. It was followed by the addition of 5ml of sodium carbonate to each flask. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer.

The results are given in table 3.3.10 and are shown in figure 3.3.12 (A&B) for the two series respectively.

Table 3.3.10 Investigation of the mixing ratio of nitrite and sulfanilic acid for the formation of diazotized reagent.

Series 1(A)		Series 2(B)	
Nitrite volume (ml)	Absorbance	Sulfanilicacid volume (ml)	Absorbance
2	0.173	1.0	0.262
4	0.209	2.0	0.169
5	0.279	3.0	0.083
8	0.424	4.0	0.015
10	0.377	5.0	0.009
12	0.294		

Series 1(A)



Series 2(B)

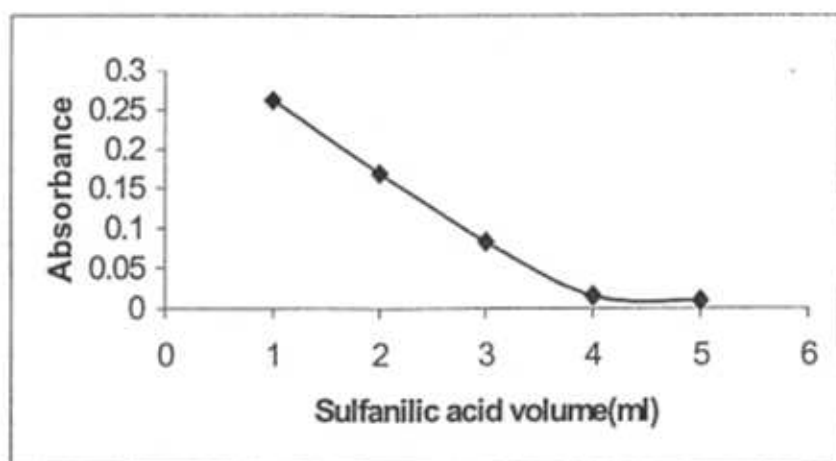


Figure 3.3.12: Investigation of the mixing ratio of nitrite and sulfanilic acid for the formation of diazotized reagent (A) and (B).

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Sodium carbonate (10 %)	5ml

3.3.3.11: Investigation of the effect of acidity on the absorbance behaviour of the azo dye

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Sulfanilic acid solution: sulfanilic acid solution (0.6%) was prepared by dissolving 0.6g of sulfanilic acid in different volumes of hydrochloric acid on boiling water bath. The solutions were cooled and transferred in to 100ml volumetric flasks and diluted with distilled water to make the final acidity of solution ranging from 0. 5M-2.5M.

Procedure:

From 100ppm hydrolyzed 2,4-D solution 5ml was taken in five separate 50ml flasks. To each flask 4ml of diazotized reagent of varied concentration was added. It was followed by the addition of 5ml of sodium carbonate to each flask. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using

UNICO UV-2100 spectrophotometer. The results are given in table 3.3.11 and are shown in figure 3.3.13.

Table 3.3.11: Investigation of the effect of acidity on the absorbance behaviour of azo dye.

S.No	HCl (M)	Absorbance
1	0.5	1.850
2	1.0	1.999
3	1.5	1.999
4	2.0	1.999
5	2.5	1.999

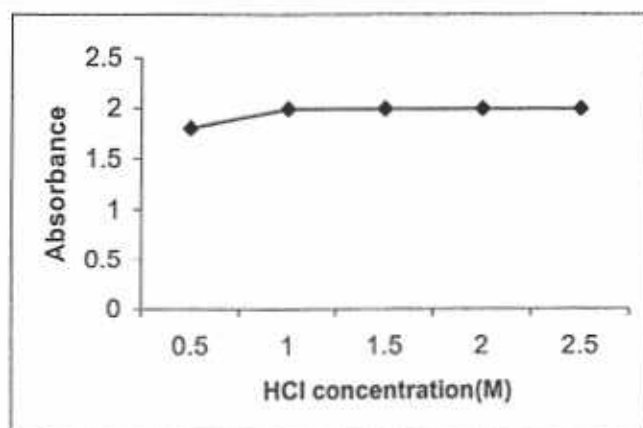


Figure 3.3.13: Investigation of the effect of acidity on the absorbance behaviour of azo dye

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution (hydrolyzed)	10ppm
	Diazotized sulfanilic acid	4ml
	Nitrite conc.	1000ppm
	Sulfanilic acid conc.	0.6%
	Sodium carbonate (10 %)	5ml

3.3.3.12: Investigation of the effect of volume of diazotized reagent on the absorbance behaviour of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Diazotized sulfanilic acid: Stock solution of diazotized sulfanilic acid was prepared by mixing the sulfanilic acid (0.6%) and nitrite solution (1000ppm) in 1:8. This stock solutions were used for further investigations.

Procedure:

From 100ppm hydrolyzed 2,4-D solution 5ml was taken in seven separate 50ml volumetric flasks , to each of these varied volume of diazotized reagent ranging from 2-14ml was added. It was followed by the addition of 5ml of sodium carbonate to each flask. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.12 and shown in figure 3.3.14.

Table 3.3.12 :Investigation of the effect of volume of diazotized reagent on the absorbance behavior of the azo dye.

Diazotized reagent volume (ml)	Absorbance	Diazotized reagent volume (ml)	Absorbance
2	0.558	10	1.300
4	0.628	12	0.900
6	0.716	14	0.854
8	0.935		

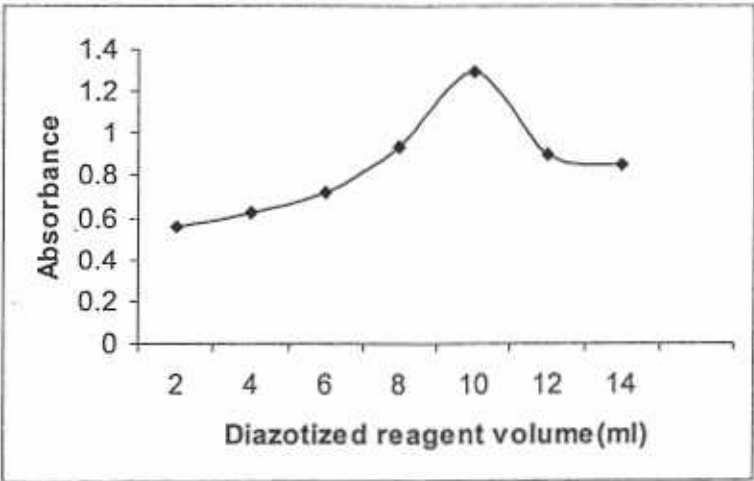


Figure 3.3.14: Investigation of the effect of volume of diazotized reagent on the absorbance behavior of the azo dye.

Conditions:	λ max.	480nm
	2,4-D solution(hydrolyzed)	10ppm
	Diazotized Reagent	varied
	Sodium carbonate (10 %)	5ml

3.3.3.13: Investigation of the effect of pH on the absorbance behaviour of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

From 100ppm hydrolyzed 2,4-D solution 5ml was taken in seven separate 50ml volumetric flasks. To each of these 10ml of diazotized reagent was added followed by the addition of varied volume of 10 % sodium carbonate to maintain the pH between 8-10. The resulting azo dye was diluted up to mark with distilled water. The absorbance was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.13 and are shown in figure 3.3.15.

Table 3.3.13: Investigation of the effect of pH on the absorbance behaviour of azo dye.

S.NO	pH	Absorbance
1	8.0	0.194
2	8.5	0.310
3	9.0	0.332
4	9.5	0.334
5	10	0.338

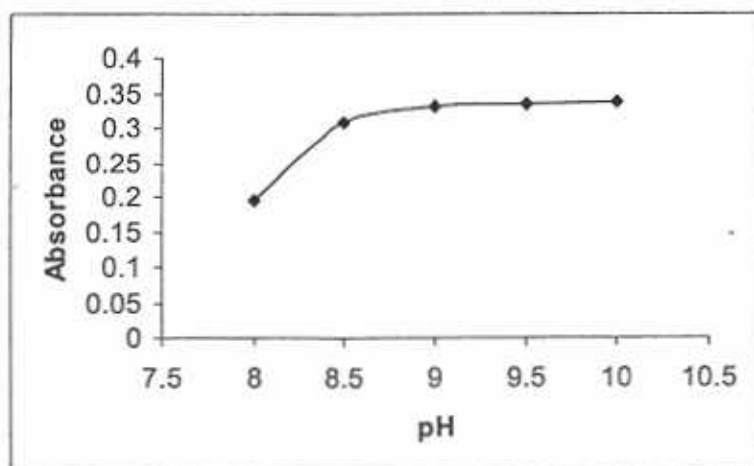


Figure 3.3.15: Investigation of the effect of pH on the absorbance behaviour of the azo dye

conditions :	λ max.	480nm
	2,4-D solution(hydrolyzed)	10ppm
	Diazotized Reagent	10ml
	Sodium carbonate (10 %)	varied

3.3.3.14: Investigation of the effect of concentration on absorbance behaviour of 2,4-D at lower concentration

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

For determination of detection limit, from standard 2,4-D solution (100ppm) different volume for 0.5-20ppm were taken in separate 50 ml volumetric flasks. To each flask optimum volume of diazotized sulfanilic acid solution (10ml) was added followed by addition of 10ml of sodium carbonate to maintain the pH at 10. A red colored azo dye was formed and diluted up to mark with distilled water. The absorbance of the resulting azo dye was measured at 480nm using UNICO UV-2100 spectrophotometer. The results are given in table 3.3.14 and are shown in figure 3.3.16 for lower concentration. LOD and LOQ were calculated using the formula given before for fluroxypyr.

Table 3.3.14: Investigation of the effect of concentration on absorbance behaviour of 2,4-D at lower concentration.

2,4-D conc.(ppm)	Absorbance	2,4-D conc. (ppm)	Absorbance
0.5	0.005	6	0.395
0.6	0.044	8	0.473
0.7	0.076	10	0.588
0.8	0.109	12	0.699
0.9	0.16	14	0.823
1.0	0.184	16	1.014
2.0	0.239	18	1.156
4	0.328	20	1.230

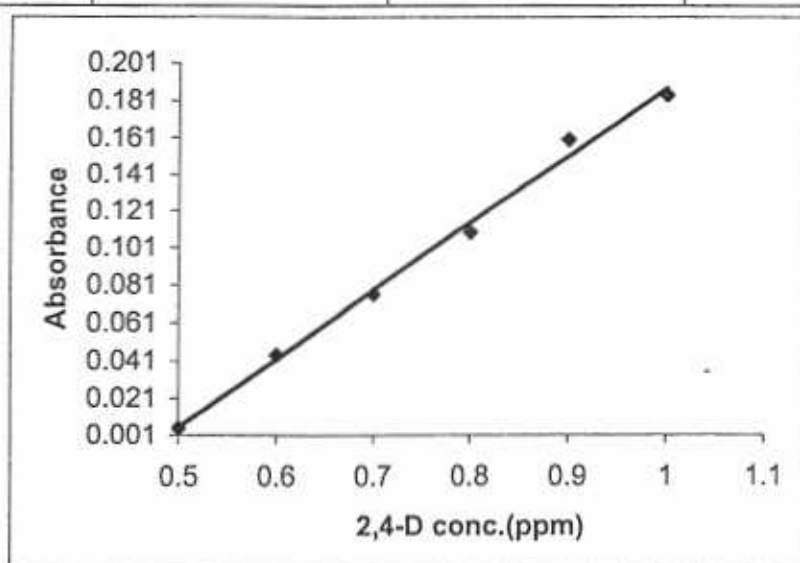


Figure 3.3.16: Investigation of the effect of concentration on absorbance behavior of 2,4-D at lower concentration

Conditions :	$\lambda_{\max}$	480nm
	2,4-D solution	10ppm
	Diazotized Reagent	10ml
	Sodium carbonate (10 %)	10ml

3.3.4: INVESTIGATION OF PHYSICAL PARAMETERS FOR ONLINE SPECTROPHOTOMETRIC DETERMINATION OF 2,4-D.

All physical parameters such as the reactor coil length (R1 and R2), flow rate and injection volume were optimized for the flow injection spectrophotometric determination of 2,4-D, while all the chemical parameters optimized for the batch analysis of 2,4-D were kept the same. Since the optimum temperature for diazotization was room temperature, all of the FI manifolds were set at room temperature. A schematic diagram of flow injection assembly is shown in figure 3.3.17.

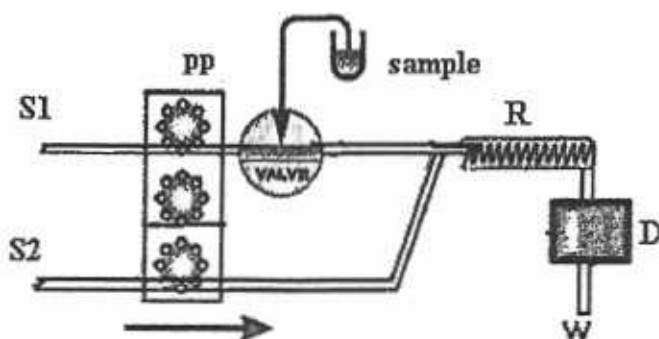


Figure 3.3.17: Schematic diagram of the FI manifold. (S1) Diazotized sulfanilic acid stream, (S2) sodium carbonate stream, (R) coupling coil, (PP) peristaltic pump, (W) waste, (D) detector.

3.3.4.1: Investigation of flow rate of system for FI spectrophotometric determination of 2,4-D.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

In a double channel flow through assembly diazotized sulfanilic acid solution was allowed to flow in tube S1 while sodium carbonate (10%) solution was allowed to flow in tube S2. Flow rate of the system was varied in the range of 1.5-5ml/min. The coil length of reactor R (coupling reactor) was kept 50cm. From 10ppm hydrolyzed 2,4-D

solution 1ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve The coupling reaction take place in reactor R1 where sodium carbonate required to maintaine the pH 10 also mixed with it. The absorbance of the azo dye formed was measured at 480nm by using UNICO UV 2100 spectrophotometer and displayed through computer programme. Blank was adjusted by flowing continouse stream of diazotized reagent and sodium carbonate solution. The results are given in table 3.3.15 and are shown in figure 3.3.18.

Table 3.3.15: Investigation of flow rate of system for FI spectrohotometric determination of 2,4-D .

Flow rate (ml/min)	Absorbance
1.5	0.00
2.5	0.008
3.0	0.349
3.5	0.457
4.0	0.359
4.5	0.274
5.0	0.274

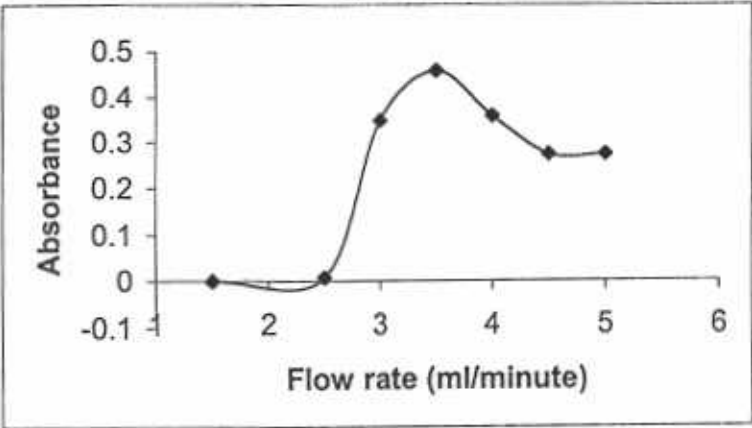


Figure 3.3.18: Investigation of flow rate of system for FI spectrohotometric determination of 2,4-D .

Conditions:	λ max.	480nm
	2,4-D solution	10ppm
	Flow rate (ml/ min)	varied
	Reactor Coil length	50cm

3.3.4.2: Optimization of reaction coil length (R) for FI spectrophotometric determination of 2,4-D .

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

In a double channel flow through assembly diazotized sulfanilic acid solution was allowed to flow in tube S1 while sodium carbonate (10%) solution was allowed to flow in tube S2. Flow rate of the system was maintained at 3.5ml/min. The coil length of reactor R (coupling reactor) was varied between 30-175cm. From 10ppm hydrolyzed 2,4-D solution 1ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The absorbance of the resulting azo dye was measured at 480nm using UNICO UV 2100 spectrophotometer. The results are given in table 3.3.16 and are shown in figure 3.3.19.

Table 3.3.16: Optimization of reaction coil length (R) for FI spectrophotometric determination of 2,4-D .

R length (cm)	Absorbance
5	0.179
15	0.248
25	0.295
35	0.349
45	0.401
55	0.560
65	0.757
75	0.699
85	0.528
95	0.457
105	0.400
125	0.375
145	0.358
175	0.301

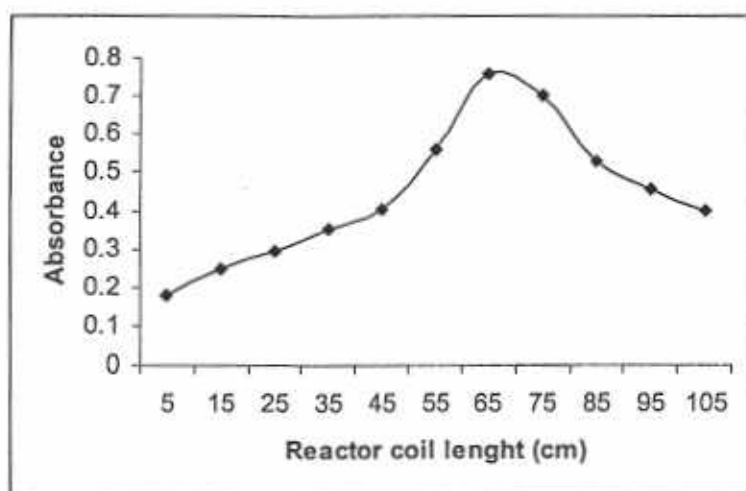


Figure 3.3.19: Optimization of reaction coil length (R) for FI spectrophotometric determination of 2,4-D .

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution (hydrolyzed)	10ppm
	Flow rate (ml/ min)	3.5
	Reactor Coil length	varied

3.3.4.3: Optimization of injection volume for FI spectrophotometric determination of 2,4-D .

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

In a double channel flow through assembly diazotized sulfanilic acid solution was allowed to flow in S1 while sodium carbonate (10%) solution was allowed to flow in tube S2. Flow rate of the system was maintained at 3.5ml/min. The coil length of reactor R (coupling reactor) was 65cm. From 10ppm hydrolyzed 2,4-D solution 1-3 ml was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The absorbance of the resulting azo dye was measured at 480nm using UNICO UV 2100 spectrophotometer. The results are given in table 3.3.17.

Table 3.3.17: Optimization of injection volume for FI spectrophotometric determination of 2,4-D .

Volume injected(ml)	Absorbance
1	0.780
2	0.780
3	0.780

Conditions:	$\lambda_{\max}$	480nm
	2,4-D solution	10ppm
	Flow rate (ml/ min)	3.5
	Reactor Coil length	65cm

3.3.4.4: Investigation of effect of concentration on absorbance behaviour of azo dye at lower concentration.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Standard 2,4-D solution of 0.2ppm –20ppm was prepared in a 10 ml volumetric flask by taking the required volume of standard from 100ppm hydrolyzed solution and diluting with distilled water. In a double channel flow through assembly diazotized sulfanilic acid solution was allowed to flow in tube S1 while the sodium carbonate (10%) solution was allowed to flow in tube S2. Flow rate of the system was maintained at 3.5ml/min. The coil length of coupling reactor was kept 65cm. A 1ml of varied concentration (0.2-20ppm) of 2,4-D solution was injected in the reagent stream of diazotized reagent through a 6 port injector valve. The coupling reaction take place in reactor R where sodium carbonate is also mixed to maintain the pH at 10. The absorbance of the azo dye formed was measured at 480nm by using UNICO UV 2100 spectrophotometer. LOD, LOQ and RSD were calculated using formula given before for fluroxypyr. The results are given in table 3.3.18 and are shown in figure 3.3.20 for lower concentration.

Table 3.3.18: Investigation of effect of concentration on absorbance behaviour of azo dye at lower concentration.

Conc.(ppm)	Absorbance	Conc.(ppm)	Absorbance
0.2	0.017	4.0	0.241
0.3	0.034	6.0	0.305
0.4	0.05	8.0	0.450
0.5	0.071	10.0	0.552
0.6	0.088	12.0	0.654
0.7	0.105	14.0	0.757
0.8	0.122	16.0	0.859
0.9	0.139	18.0	0.962
1.0	0.159	20.0	1.064
2.0	0.199		

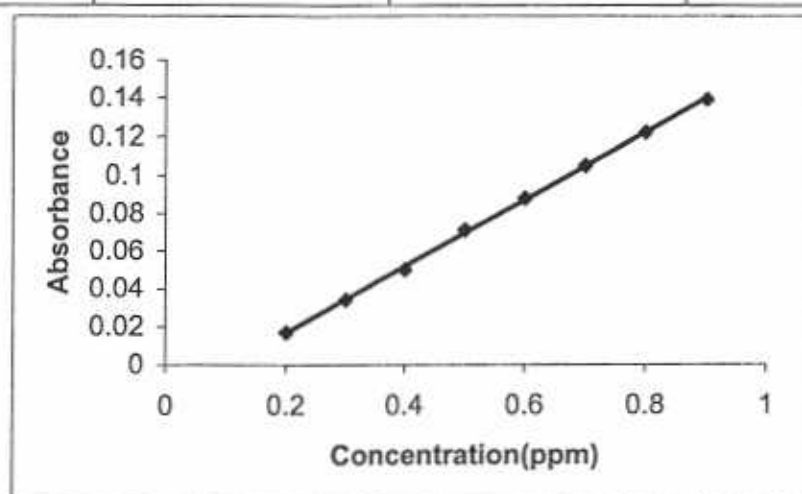


Figure 3.3.20: Investigation of effect of concentration on absorbance behaviour of azo dye at lower concentration.

Conditions: $\lambda_{\max}$ 480nm
 Flow rate 3.5ml/min
 Reactor length 65 cm
 Volume injected 1ml

3.3.5: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF PHENOXY ACETIC ACID HERBICIDES IN COMMERCIAL FORMULATION.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Commercial samples: commercial samples of 2,4-D and MCPA were purchased from local market.

Solutions: The same as mentioned before

Procedure:

A 1-3ml of commercial formulation of 2,4-D and MCPA was taken in separate beakers and 15ml of 2% sodium carbonate was added to each beaker. The beakers were covered and heated on boiling water bath for 40 minutes for hydrolysis. From the resulting hydrolyzed solutions, 5 ml were taken in 50ml volumetric flasks and color was developed in the same way as mentioned before for batch analysis. The absorbance was measured against blank at 480nm using Unico 2100 UV spectrophotometer.

For online analysis 1ml of hydrolyzed herbicide sample was injected in reagent stream and the absorbance was measured using the same procedure as for standard.

The active ingredients of 2,4-D and MCPA in commercial formulation were calculated both for batch analysis and online analysis using their respective standard curves. The results are given in table 3.3.19.

Table 3.3.19: Active ingredient of herbicides

Types of herbicides	Active Ingredient g/ml.		
	Label value	Found (batch analysis)	Found (FIA)
2,4-D	0.35	0.30 ± 0.018	0.299 ± 0.02
MCPA	0.50	0.43 ± 0.09	0.390 ± 0.10

3.3.6:APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS 2,4-D IN REAL SAMPLES.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Sample solution:

30 g of homogenized sample in each case was extracted by using standard procedure mentioned before for fluroxypyr.

Procedure:

From extracted real sample 4ml was taken in separate beakers and hydrolyzed in the same way as mentioned before. From this hydrolyzed extract 1ml and 4ml was used for online and batch analysis respectively. Color was developed in the same way as mentioned before for batch and FI analysis. The results are given in table 3.3.20. The following formula was used for residue determination in real samples.

$$\text{ppm of 2,4-D} = \frac{\text{G.R. } (\mu\text{g/g}) \times \text{volume made} \times \text{D.F.}}{\text{Weight of sample taken (g)}}$$

Where G.R represents graphical reading and D.F for dilution factor.

Table 3.3.20: Residues level of 2,4-D herbicide in food sample

Food samples	Batch Analysis	FIA
	2,4-D ($\mu\text{g/g}$)	2,4-D ($\mu\text{g/g}$)
Wheat (grain)	3.2 ± 0.12	0.87 ± 0.15
Wheat (flour)	6.02 ± 0.42	1.12 ± 0.33
Maize (grain)	3.3 ± 0.14	0.92 ± 0.22
Maize (flour)	5.1 ± 0.28	1.51 ± 0.35

3.3.7: PERCENT RECOVERY OF 2,4-D FROM REAL SAMPLES USING THE INVESTIGATED METHOD.

Procedure:

A recovery test was performed on control samples, fortified with a known concentration of 2,4-D solution. The solvent was evaporated at room temperature for 1hr. Extraction

was done exactly the same way as for the sample. Six replicates of the fortified samples were analyzed by both batch and online method using the same method as mentioned before. Percent recovery was calculated by using the formula given before for fluroxypyr. The results of 2,4-D recovery from real samples and formulation for both batch and online method are given in table 3.3.21

Table 3.3.21: Percent recovery of herbicide from real sample.

Samples	Batch Analysis	FIA
	% Recovery	%Recovery
2,4-D	91.0 $\pm$ 0.16	93.0 $\pm$ 0.22
Maize (grains)	93.6 $\pm$ 0.088	94.5 $\pm$ 0.14
Maize (flour)	90.0 $\pm$ 0.31	93.0 $\pm$ 0.22
Wheat (grains)	94.5 $\pm$ 0.11	95.3 $\pm$ 0.14
Wheat (flour)	90.8 $\pm$ 0.32	92.0 $\pm$ 0.27

Results and discussion

After preliminary investigation it was observed that the proposed reaction shown in figure 3.2.2. is feasible for spectrophotometric determination of 2,4-D. Then various parameters for physical and chemical parameters were optimized and are discussed below.

After preliminary experiments it was observed that azo dye is formed. The wavelength optimization studies were conducted from 320-600nm to find out the most suitable wavelength. As it is clear from the results (table-3.3.1 and figure 3.3.3) the resulting azo dye has a maximum absorbance at 480nm and this wavelength was subsequently used as optimum wavelength for further investigation of 2,4-D.

For finding the most suitable diazotizing reagent, different diazotizing reagents (figure 3.3.4) were investigated for the formation of azo dye. Sulfanilic acid, sulfanilamide, p-

nitro aniline, p-aminobenzoic acid and aniline were used for this purpose. It was observed that among all the diazotizing reagent only p-nitro aniline did not react with hydrolyzed coupling reagent. Aniline and sulfanilic acid gives the same value for formation of azo dye. As aniline is carcinogenic therefore, sulfanilic acid was selected for further studies.

For maximum hydrolysis of 2,4-D various bases like sodium hydroxide, sodium carbonate and sodium ethoxide were optimized. It can be seen from figure 3.3.5 that sodium carbonate show the best result. While sodium hydroxide lead to colored blank solution therefore, sodium carbonate was selected for hydrolysis of 2,4-D.

Similarly the concentration and volume of sodium carbonate required for maximum hydrolysis were also optimized (table 3.3.4 & 3.3.5) respectively. It was found that with increase of concentration from 0.5-2% hydrolysis increases and after this a decrease in hydrolyzed product was observed. It was further observed that at high concentration of sodium carbonate from 5-6% the hydrolyzed product is thick white precipitate. This precipitate was redissolved by using ethanol but it gives lower value of absorbance for azo dye formation (figure 3.3.6) therefore 2% of sodium carbonate was selected for maximum hydrolysis with no precipitation. Similarly the volume of 2% sodium carbonate was studied in the range of 2-14ml. It was observed (figure 3.3.7) that 15ml of 0.2% sodium carbonate is the optimum value for maximum hydrolysis.

As hydrolysis take place on heating, therefore hydrolysis temperature was studied in the range of 10-100°C. Figure 3.3.8 shows that maximum hydrolysis takes place on boiling water bath. Similarly, the time required for hydrolysis was also optimized in the range between 5-60 minutes (table 3.3.7). It was observed (figure 3.3.9) that maximum hydrolysis requires 40 minute heating on boiling water bath and after that a decrease in hydrolysis was observed, probably due to decomposition of hydrolyzed product. Therefore, 40 minutes heating on boiling water bath was selected optimum heating time.

The concentration of nitrite and sulfanilic acid was optimized for the formation of diazotized reagent. Nitrite was used in the range of 500-2500 ppm and Sulfanilic acid in the range of 0.2-1.0% solutions. Maximum diazotization occurred by using solution being 1000ppm in nitrite (Fig. 3.3.10) and 0.6% in sulfanilic acid (Fig. 3.3.11).

The combining ratios of reagents were optimized in the range of 1-5 ml of 0.6% sulfanilic acid and 2-12 ml of 1000ppm nitrite solutions. It was found (figure 3.3.12 A & B) that maximum diazotization occurred at the combining ratio of 1 and 8 ml of sulfanilic acid and nitrite solutions respectively. There was a decrease in absorbance beyond optimum volume of the diazotization reagents.

The effect of acidity on the formation of diazonium ion was also investigated. Constant absorbance was obtained in the range of 1.0- 2.5 M of hydrochloric acid (Fig.3.3.13). Therefore, 1.0M HCl acidity was selected for the formation of diazonium ion.

The effect of volume of diazotized reagent on the formation of azo dye was studied from 2-14ml. It was observed (figure 3.3.14) that 10 ml of diazotized reagent was sufficient for maximum formation of azo dye.

Coupling reaction takes place at basic pH. Therefore, pH of the final solution after addition of 2,4-D was adjusted with 10% sodium carbonate solution in the range of pH 8-10. Maximum absorbance was observed at pH 10 (Fig.3.3.15). It was observed that 10ml of 10% Sodium carbonate is required for the optimum pH. This volume of sodium carbonate was used for further studies.

Optimization of physical parameters for online analysis

The physical variables studied were flow rate, reactor coil length and injection volume.

Flow rate was studied in the range of 1.5-5ml/min (Fig.3.3.18). When the flow rate increased, the signal increased up to the flow rate of 3.5ml/minute and decreased for greater flow rates. A flow rate of 3.5ml/min was chosen as a compromise among adequate sampling rate, peak width, and sensitivity.

The length of reactor R was studied in the range of 30-175 cm and it was found that with increase of coil length the absorbance of azo dye increases up to a length of 65cm (Fig.3.3.19). Therefore a coil length of 65cm was selected for further studies.

The effect of injection volume was investigated with loop in range of 1-3ml volumes. A loop of 1ml was selected to maintain a good sampling rate. Above this volume broadening peak effect was produced without any increase in the peak height. When all the above conditions are coupled a sampling rate of 90/hour was achieved.

At optimum conditions, the effect of lower concentration of 2,4-D (below 1 ppm) on absorbance behavior was investigated for assessing the LOD and LOQ for the

investigated methods. The results are given in table 3.3.14 & 3.3.18 and are shown in 3.3.16 & 3.3.20 respectively. The LOD and LOQ were calculated and were found to be 0.13ppm and 0.42ppm for FI analysis and 0.084ppm and 0.28ppm for batch analysis respectively.

The RSD was also calculated and was found to be 7.2% and 8.2% for the online and batch analysis respectively. Molar absorptivity was calculated and found to be 1.09×10^4 Lmol⁻¹cm⁻¹ and 2.39×10^4 Lmol⁻¹cm⁻¹ for batch and online method respectively.

Application of the investigated method for the analysis of phenoxy acetic acid herbicides in commercial formulation and real samples.

The investigated method is equally suitable for all phenoxy acetic acid herbicides. To demonstrate this, 2,4-D and MCPA were selected. The method was applied for determination of their active ingredients in commercial formulation. The results obtained are given in table 3.3.19. It was observed that the value calculated by this method were very close to the label value. This shows the applicability of method for all phenoxy acetic acid herbicides. On hydrolysis all phenoxy acid herbicide give phenol, which is coupled with suitable agent for the formation of azo dye. Therefore the method cannot be used for speciation but could only be used for total phenoxy acid herbicide determination. In our case the residue determination were done only on those sample on which only 2,4-D was sprayed. Therefore we have reported our results for the same. For FI analysis the residue level in the analyzed wheat grain and flour samples for 2,4-D were 0.87 ± 0.15 µg/g and 1.12 ± 0.33 µg/g while for maize grain and flour samples 0.92 ± 0.22 µg/g and 1.5 ± 0.35 µg/g respectively. The results were also compared with batch analysis (table 3.3.20). The residue level found were much higher than the NZ food and safety authority MRLs 2005 for cereal grains which is 0.05 µg/g.

The recovery test shows that percent recoveries in samples varied from 92-95.3% for FI analysis and 90-94.5% for batch analysis (table 3.3.21).

3.4: INVESTIGATION OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF BROMOXYNIL.

The chemical structure of bromoxynil is given below.

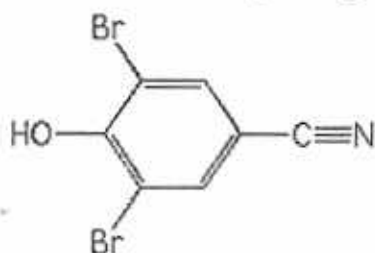


Figure 3.4.1: Chemical structure of bromoxynil

3.4.1: Method development strategy for spectrophotometric determination of bromoxynil.

Bromoxynil is chemically 3,5-dibromo-4-hydroxy benzo nitrile. Looking in to the structure, it was thought that the nitrile group could be reduced to primary amine using acid hydrolysis. The generated amine could be diazotized and coupled with suitable coupling reagent to form azo dye. The proposed chemical reaction is shown in figure 3.4.2.

3.4.2: Preliminary Investigation of the possibility of reduction and coupling for spectrophotometric determination of bromoxynil.

Preliminary studies were conducted to investigate the possibility of the proposed reaction (fig3.4.2) for the formation of expected azo dye. Initially high concentration of bromoxynil (100 ppm), relatively large volume of hydrochloric, sodium nitrite and various coupling reagent like β -naphthol, and aniline were tried for the formation of the expected azo dye at room temperature. Reduction was tried both at room temperature and by heating. The formation of the colored azo dye both at room temperature and upon heating indicated the possibility of the reaction and its subsequent application for spectrophotometric determination of bromoxynil by this method. Further studies were focused on optimization of various parameters and are given below.

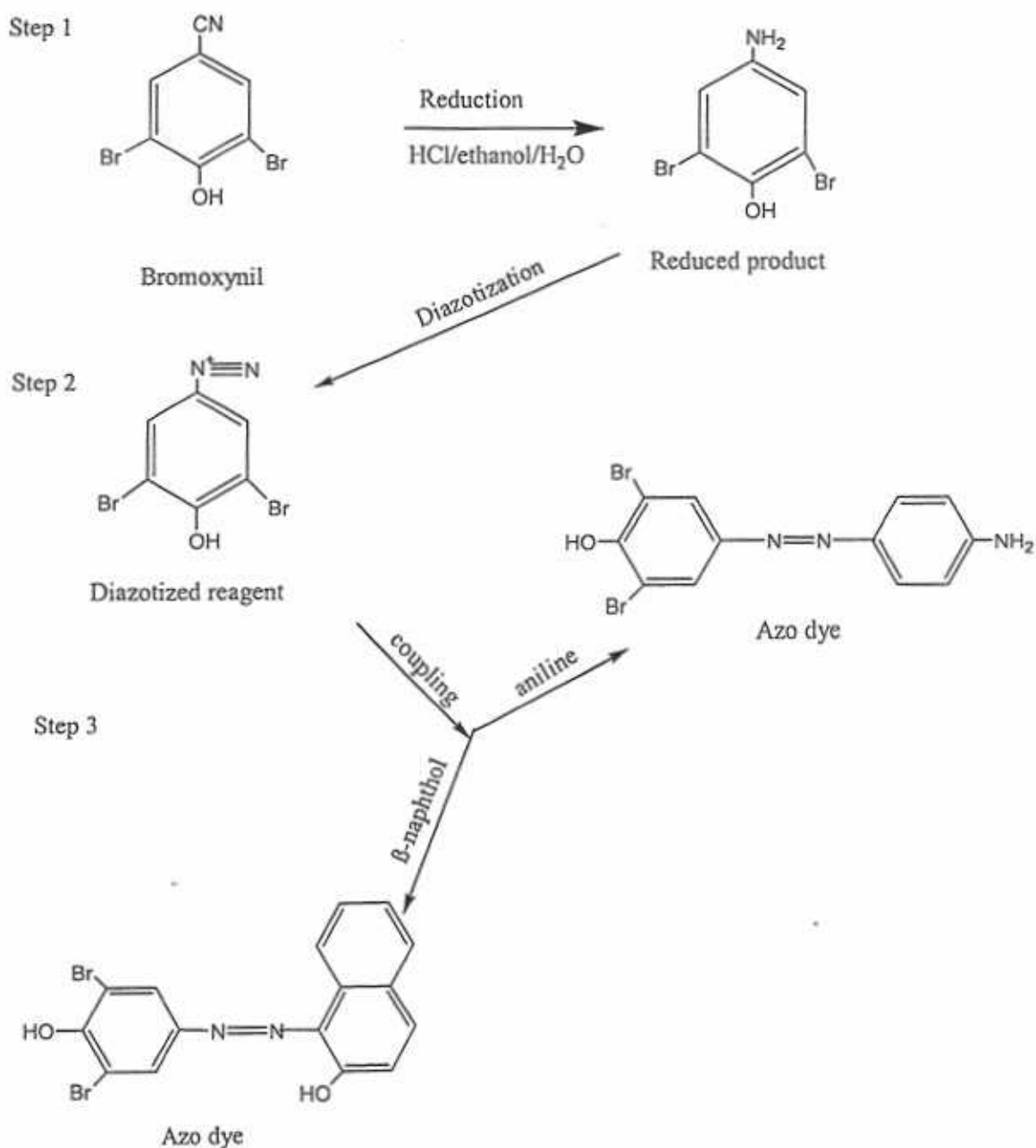


Figure 3.4.2: Proposed reaction mechanism for spectrophotometric determination of bromoxynil

3.4.3: OPTIMIZATION STUDIES FOR SPECTROPHOTOMETRIC DETERMINATION OF BROMOXYNIL

3.4.3.1: Investigation of suitable wavelength for the spectrophotometric determination of bromoxynil

Instruments:

The online system comprised a peristaltic pump with PET (i.d. 1.19mm, Becton Dickson, USA) and silicon (i.d.1.71mm) flow tubes, a V-450 6 port injection valve (Upchurch Scientific Inc, USA) and a UV/Vis spectrophotometer (UNICO UV-2100 United Products and Instruments Inc., Dayton, N.J U.S.A) as a detector was used.

Reagents and chemicals.

All chemicals used were of analytical reagent grade purity. Sodium nitrite, aniline (Merck), β -naphthol, concentrated hydrochloric acid (Merck), and ethanol (Merck), were used during this work. Standard reference material (bromoxynil) was purchased from ACROS ORGANIC, Belgium. Commercial samples containing bromoxynil was purchased from the local market.

Solution preparation

- i) **Nitrite solution:** Nitrite solution (0.15%) was prepared by dissolving 0.225 g sodium nitrite in water and diluted upto 100 mL with distilled water.
- ii) **Aniline solution:** Aniline solution (2%) was prepared by mixing 2 ml of aniline in 70 ml of ethanol and diluting up to 100 ml with water.
- iii) **β -naphthol solution:** β -naphthol (0.2%) was prepared by dissolving 0.2g of β -naphthol in 1.2% sodium hydroxide solution in a 100ml volumetric flask.
- iv) **Standard bromoxynil solution:** Standard bromoxynil solution (1000 ppm) was prepared by dissolving 0.05 g of standard bromoxynil in 50 mL of ethanol in 50 ml volumetric flask. From this stock solution 10 ml was taken and reduced with 0.4M HCl at room temperature. The resulting solution was 100 ppm in reduced product. Through stepwise dilution with distilled water 10 and 1 ppm bromoxynil solution were prepared.

Procedure:

From stock 100 ppm reduced bromoxynil solution 5ml was taken in two separate 50ml volumetric flask. To each flask 10ml of 0.15% nitrite solution was added for diazotization. These solutions were equilibrated for sometime for the completion of

reaction. 10ml β -naphthol (0.1%) was added to one set of solution while 10ml (1%) aniline was added as coupling reagent to the other set. The resulting azo dye was diluted up to mark with distilled water. Separate blank solutions were prepared through the same procedure without the addition of bromoxynil. The absorbance of the azo dye was measured at different wavelength from 390 to 550nm using UNICO UV-2100 spectrophotometer for finding out the optimum wavelength. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are given in table 3.4.1 and are shown in figure 3.4.3.

Table 3.4.1 : Investigation of suitable wavelength for the spectrophotometric determination of bromoxynil

Wavelength (nm)	Absorbance using aniline as coupling reagent	Absorbance using β -naphthol as coupling reagent
390	0.065	0.340
400	0.067	0.345
410	0.069	0.381
420	0.070	0.400
430	0.071	0.403
440	0.073	0.398
450	0.083	0.377
460	0.101	0.371
470	0.116	0.364
480	0.135	0.361
490	0.141	0.359
500	0.142	0.350
510	0.134	0.346
520	0.115	0.333
530	0.080	0.321
540	0.047	0.293
550	0.020	0.270

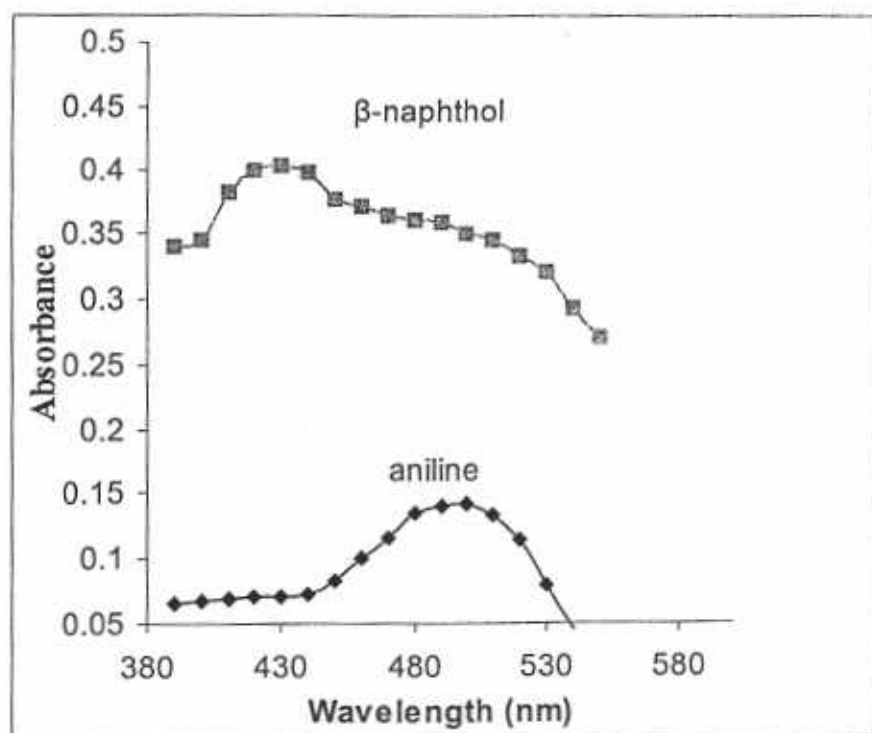


Figure 3.4.3: Investigation of suitable wavelength for the spectrophotometric determination of bromoxynil

Conditions:	$\lambda_{\max}$	varied
	Bromoxynil solution	10ppm
	Nitrite solution (0.15%)	10ml
	β -naphthol solution (0.1%)	10ml
	aniline solution (1%)	10ml

3.4.3.2: Optimization of acid concentration for reduction of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: Same as mentioned above.

Procedure:

10ml of standard bromoxynil from 1000 ppm stock solution was taken in five separate 100ml volumetric flasks and varied volume of concentrated hydrochloric acid was added to it to form 0.1-0.6M HCl solution on dilution. The resulting solutions were 100ppm in bromoxynil.

From each of these 100ppm stock solutions 5ml was taken in two separate series of 50ml volumetric flask. To each flask 10ml of 0.15% nitrite solution was added and were equilibrated for sometime for the formation of diazonium ion. To the resulting diazotized product 10 ml of β -naphthol (0.1%) and (1% aniline) was added as coupling reagent in their respective series and diluted to 50ml with distilled water. The absorbance of the resulting azo dye was measured at 430nm and 500nm for β -nephthol and aniline respectively. The results are given in table 3.4.2 and are shown in figure 3.4.4.

Table 3.4.2: Optimization of acid concentration for reduction of bromoxynil

HCl conc. (M)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
0.1	0.652	0.154
0.2	0.889	0.230
0.3	1.245	0.310
0.4	1.702	0.400
0.5	1.397	0.365
0.6	0.906	0.298

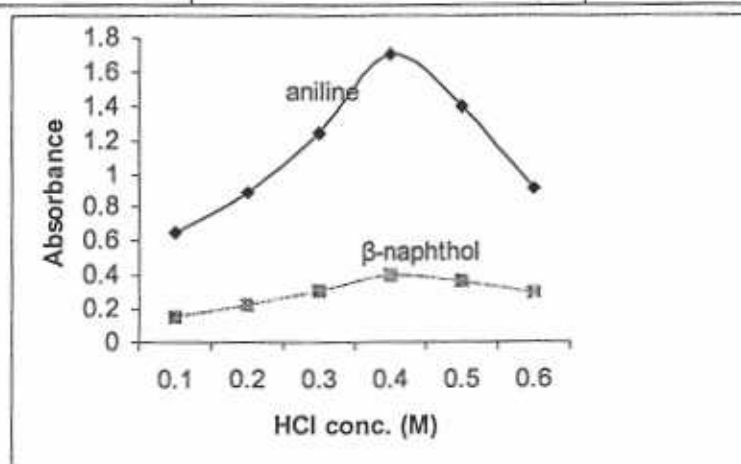


Figure 3.4.4: Optimization of acid concentration for reduction of bromoxynil

Conditions:	$\lambda_{\max}$	430nm & 500nm
Bromoxynil solution		10ppm
Nitrite solution (0.15%)		10ml
β -naphthol solution (0.1%)		10ml
aniline solution (1%)		10ml

3.4.3.3: Optimization of concentration of nitrite solution for diazotization of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10 ppm of hydrolyzed bromoxynil standard solution was taken in two separate series of five volumetric flasks (50 ml). To each flask, 10ml of different concentration of nitrite solution ranging from 0.05%-0.25% were added. Color was developed in the same way as mentioned above. The absorbance of the resulting azo dye was measured at 430nm in case β -naphthol and 500 nm in case of aniline as coupling reagent. The results are given in table 3.4.3 and are shown in figure 3.4.5.

Table 3.4.3: Optimization of concentration of nitrite solution for diazotization of bromoxynil

Nitrite conc. (%)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
0.05	0.275	0.843
0.10	0.350	1.513
0.15	1.203	1.701
0.20	1.146	1.670
0.25	1.096	1.397

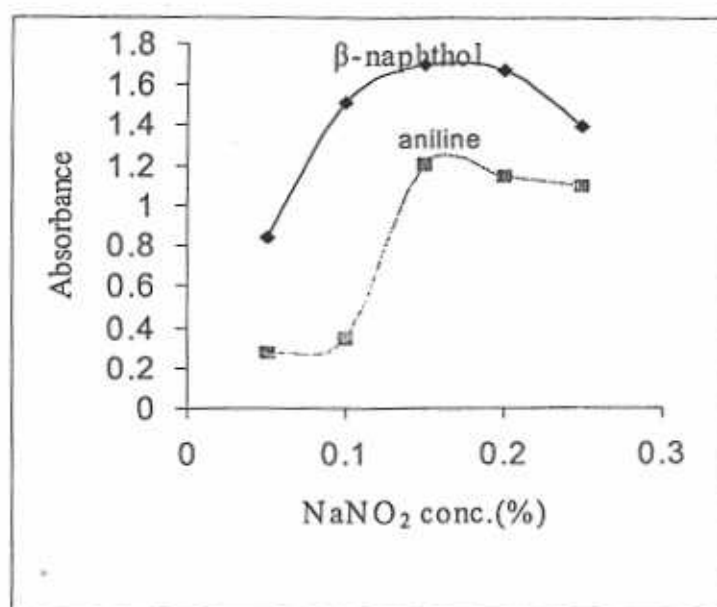


Figure 3.4.5: Optimization of concentration of Nitrite solution for diazotization of bromoxynil

Conditions:	λ_{max}	430nm & 500nm
	Bromoxynil solution	10ppm
	Nitrite solution (varied)	10ml
	β -naphthol solution (0.1%)	10ml
	aniline solution (1%)	10ml

3.4.3.4: Optimization of volume of nitrite solution for diazotization of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

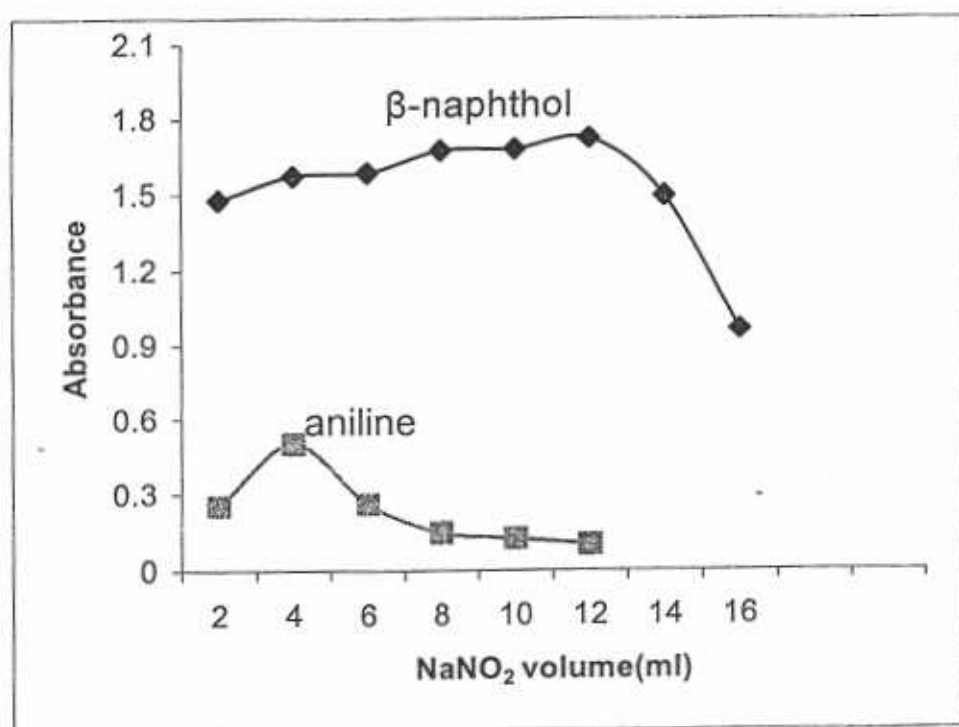
Solutions: The same as mentioned before

Procedure:

The same procedure as mentioned above was used only the volume of 0.15% nitrite solution was varied from 2-14ml. The absorbance of the resulting azo dye was measured at the optimized wavelength. The results are given in table 3.4.4 and are shown in figure 3.4.6.

Table 3.4.4: Investigation of the effect of nitrite volume on the absorbance behavior of the azo dye.

S.NO	Nitrite volume (ml)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
1	2.0	0.255	1.480
2	4.0	0.503	1.572
3	6.0	0.262	1.587
4	8.0	0.147	1.674
5	10	0.126	1.680
6	12	0.102	1.721
7	14	-	1.495
8	16	-	0.963



3.4.6: Optimization of volume of nitrite solution for diazotization of bromoxynil.

Conditions: $\lambda_{\max}$	430nm & 500nm
Bromoxynil solution	10ppm
Nitrite solution (0.15%)	varied
β -naphthol solution (0.1%)	10ml
aniline solution (1%)	10ml

3.4.3.5: Investigation of the effect of coupling reagent concentration on the absorbance behavior of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

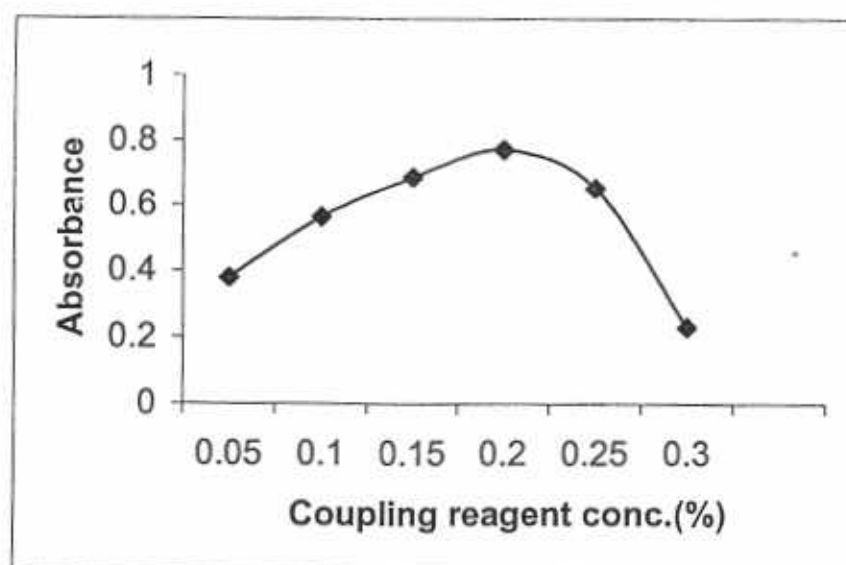
Procedure:

Bromoxynil standard solution 5ml from 100ppm reduced stock solution was transferred to two separate series of six volumetric flasks(50 ml). From 0.15% nitrite solution, 12ml and 4ml was added in series1 and series 2 respectively. In series 1, 10ml of varied concentration of β -naphthol ranging from 0.05%-0.3% were added, while 10ml of varied concentrations of aniline (1-5%) were added as coupling reagent in series 2. The absorbance of resulting azo dye was measured at their respective optimum wavelengths. The results are given in table 3.4.5 and are shown in figure 3.4.7.

Table 3.4.5: Investigation of the effect of coupling reagent concentration on the absorbance behavior of the azo dye.

β -naphthol conc. (%)	Absorbance	aniline conc. (%)	Absorbance
0.05	0.378	1.0	0.186
0.10	0.564	2.0	1.542
0.15	0.686	3.0	1.306
0.20	0.772	4.0	1.249
0.25	0.652	5.0	1.037
0.30	0.230	6.0	0.978

a) Series1



(b) Series 2

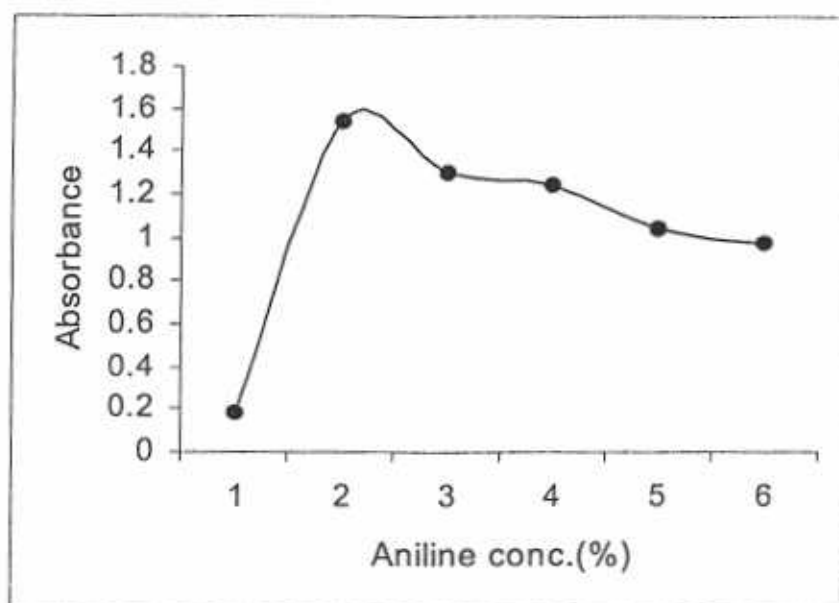


Figure 3.4.7: Investigation of the effect of coupling reagent concentration on the absorbance behavior of the azo dye.

Conditions:

$\lambda_{\max}$	430nm & 500nm
Bromoxynil solution	10ppm
Nitrite solution (0.15%)	12ml & 4ml

β -naphthol solution (varied conc.)	10ml
aniline solution (varied conc.)	10ml

3.4.3.6: Investigation of the effect of volume of coupling reagent on the absorbance behavior of the azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Bromoxynil standard solution 5ml from 100ppm reduced stock solution was transferred to two separate series of 50 ml volumetric flasks. These solutions were diazotized using the optimum volume of 0.15% nitrite solution. To the resulting diazonium ion varied volume ranging from 2-10ml of coupling reagents were added in their respective series. The absorbance of azo dye formed was measured at 430nm and 500nm for series1 and series 2 respectively. The results are given in table 3.4.6 and are shown in figure 3.4.8.

Table 3.4.6: Investigation of the effect of volume of coupling reagent on the absorbance behavior of the azo dye

S.NO	Volume of coupling reagent (ml)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
1	2	0.067	1.084
2	4	1.022	1.440
3	6	1.449	0.700
4	8	1.384	0.696
5	10	1.347	0.612

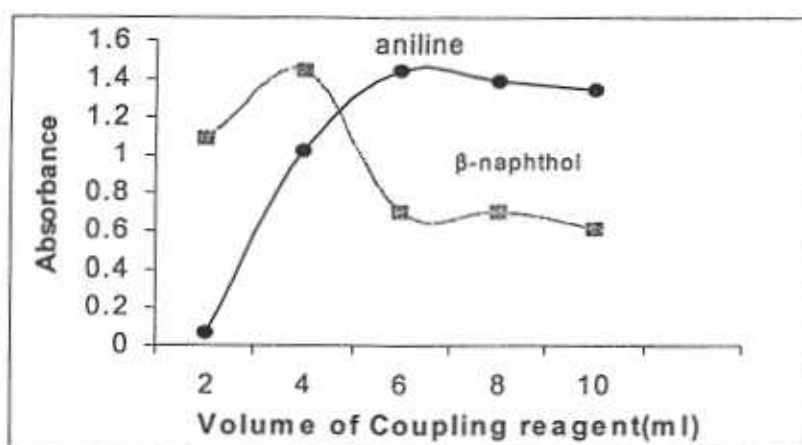


Figure 3.4.8: Investigation of the effect of volume of coupling reagent on the absorbance behavior of the azo dye

Conditions:	$\lambda_{\max}$	430nm & 500nm
	Bromoxynil solution	10ppm
	Nitrite solution (0.15%)	12ml & 4ml
	β -naphthol solution (0.2%)	varied
	aniline solution (2%)	varied

3.4.3.7: Investigation of the effect of sodium hydroxide concentration on the absorbance behavior of the azo dye using β -naphthol as coupling reagent.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Bromoxynil standard solution 5ml from 100ppm reduced bromoxynil stock solution was transferred to seven separate 50 ml volumetric flask. To each flask 12ml of 0.15% nitrite solution was added followed by the addition of 4ml β -naphthol solution prepared in different concentration of sodium hydroxide (0.4-1.8M). The absorbance of azo dye formed was measured at 430nm. The results are given in table 3.4.7 and are shown in figure 3.4.9.

3.4.3.8: Optimization of the effect of time on coupling reaction.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

From 100ppm reduced stock solution of bromoxynil ,5ml was transferred to two separate series of fourteen 50 ml volumetric flasks. Each series was diazotized by the same method as mentioned before. Optimum volume of coupling reagents were added in their respective series. The resulting azo dye was equilibrated for different time intervals ranging from 4-30 minutes. The absorbance was measured at the optimized wavelengths. The results are given in table 3.4.8 and are shown in figure 3.4.10

Table 3.4.8: Optimization of the effect of time on coupling reaction

Time (minutes)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
4	1.644	1.701
6	1.730	1.703
8	1.750	1.703
10	1.754	1.708
12	1.757	1.708
14	1.757	1.708
16	1.757	1.708
18	1.757	1.708
20	1.757	1.708
22	1.757	1.708
24	1.757	1.708
26	1.757	1.708
28	1.757	1.708
30	1.757	1.708

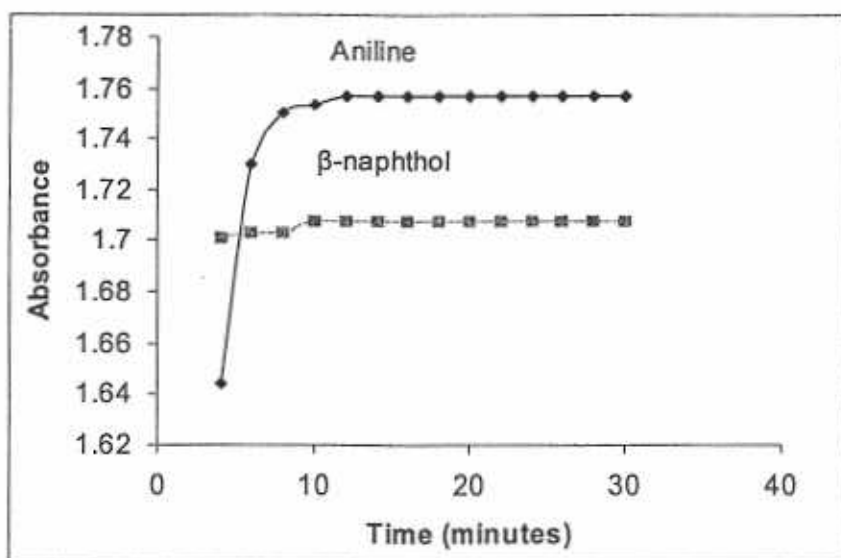


Figure 3.4.10: Optimization of the effect of time on coupling reaction.

Conditions:	λ max.	430nm & 500nm
	Bromoxynil solution	10ppm
	Nitrite solution (0.15%)	12ml & 4ml
	β -naphthol solution (0.2%)	4ml
	aniline solution (2%)	6ml

3.4.3.9: Investigation of the limit of detection and quantification for spectrophotometric determination of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

From standard reduced bromoxynil solution (100ppm) different volume for 0.02-5.0 ppm were taken in two separate series of 50 ml volumetric flask. The optimum volume of nitrite solution and coupling reagents were added in each series and kept for equilibration. The absorbance of azo dye formed was measured at 430nm incase of β -naphthol and at 500nm incase of aniline as coupling reagent. The results are given in

table 3.4.9 and are shown in figure 3.4.11. Molar absorptivity, LOD, LOQ and RSD were calculated by using the formula given before for fluroxypyr.

Table 3.4.9: Investigation of the effect of concentration on absorbance behavior of bromoxynil at lower concentration.

Standard conc.(ppm)	Absorbance		Standard conc. (ppm)	Absorbance	
	Coupling reagent aniline	Coupling reagent β -naphthol		Coupling reagent aniline	Coupling reagent β -naphthol
0.02	0.005	-	0.4	0.070	0.058
0.03	0.008	-	0.5	0.093	0.075
0.04	0.012	-	0.6	0.105	0.097
0.05	0.016	-	0.7	0.133	0.134
0.06	0.018	-	0.8	0.199	0.160
0.07	0.20	-	0.9	0.217	0.290
0.08	0.022	-	1.0	0.308	0.370
0.09	0.024	0.006	2.0	0.821	0.635
0.10	0.027	0.010	3.0	1.335	0.701
0.20	0.036	0.023	4.0	1.495	1.181
0.30	0.050	0.051	5.0	1.662	1.455

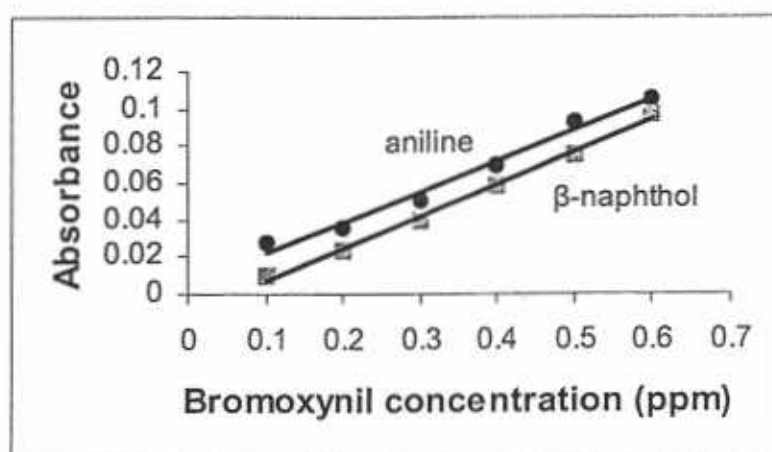


Figure 3.4.11: Investigation of the effect of concentration on absorbance behavior of bromoxynil at lower concentration.

Conditions:	$\lambda_{\max}$	430nm & 500nm
	Bromoxynil solution	10ppm
	Nitrite solution (0.15%)	12ml & 4ml
	β -naphthol solution (0.2%)	4ml
	aniline solution (2%)	6ml

3.4.4: OPTIMIZATION OF PHYSICAL PARAMETERS FOR ONLINE SPECTROPHOTOMETRIC DETERMINATION OF BROMOXYNIL

All physical parameters such as the reactor coil lengths (R1 and R2), flow rate and injection volume were optimized for the flow injection spectrophotometric determination of bromoxynil, while all the chemical parameters optimized for the batch analysis of bromoxynil were kept the same. Since the optimum temperature for diazotization was room temperature, all of the FI manifolds were set at room temperature. A schematic diagram of flow injection assembly used in this work is shown in figure 3.4.12.

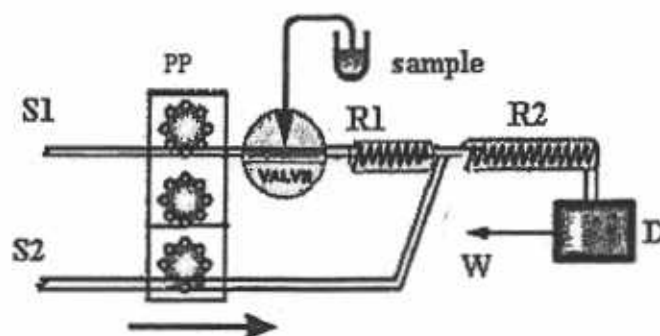


Figure 3.4.12: Schematic diagram of the FI manifold. (S1) nitrite stream, (S2) coupling reagent stream, (R1) diazotization coil, (R2) coupling coil, (PP) peristaltic pump, (W) waste, (D) detector.

3.4.4.1: Investigation of flow rate for online spectrophotometric determination of bromoxynil.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

In a flow trough assembly, nitrite solution (0.15%) was allowed to flow in reagent stream S1 while the coupling reagent (aniline or β -naphthol) was allowed to flow in reagent stream S2. Flow rate of the system was varied in the range of 1.5-6.5ml/min. The coil length of reactor R1 and R2 was kept 60cm and 50cm respectively. From 10ppm reduced bromoxynil solution 1ml was injected in the reagent stream of nitrite through a 6 port injector valve to form diazotized reagent. This diazotized reagent coupled with coupling reagent in reactor coil R2. The absorbance of the azo dye formed was measured at 430nm incase of β -naphthol and at 500nm in case of aniline as coupling reagent. The results are given in table 3.4.10 and are shown in figure 3.4.13.

Table 3.4.10: Investigation of flow rate of system for online spectrophotometric determination of bromoxynil.

Flow rate(ml/min)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β - naphthol)
1.5	0.110	0.092
2.5	0.135	0.102
3.5	0.156	0.102
4.5	0.129	0.102
5.5	0.100	0.100
6.5	0.070	0.099

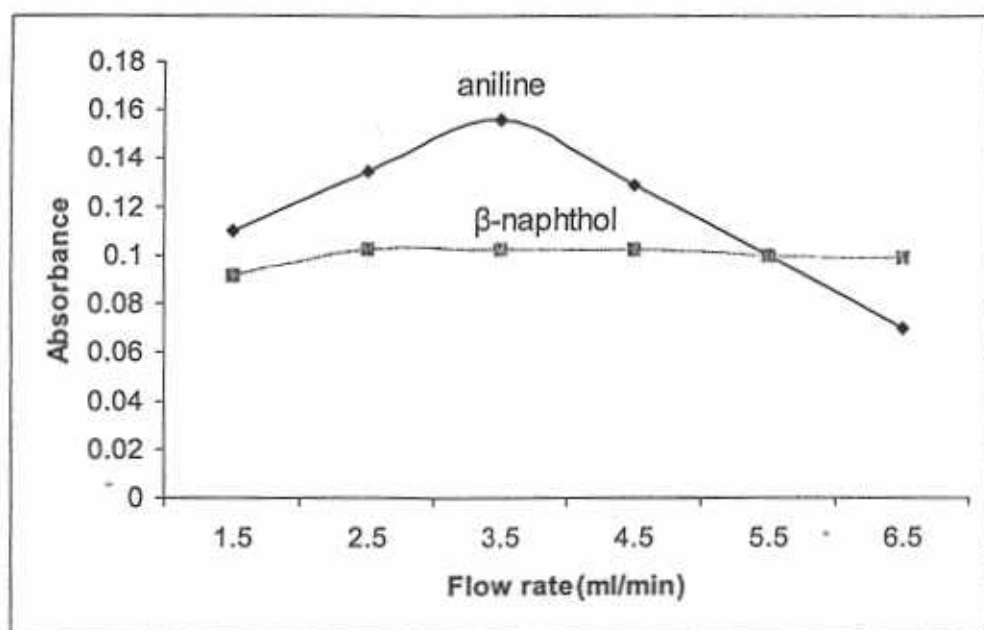


Figure 3.4.13: Investigation of flow rate of system for online spectrophotometric determination of bromoxynil.

Conditions:	$\lambda_{\max}$	430nm & 500nm
	Injection volume (10ppm)	1ml
	Flow rate (ml/min)	varied
	R1	60cm
	R2	50cm

3.4.4.2: Investigation of injection volume for online spectrophotometric determination of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

For volume optimization, varied volume (1-4ml) of 10ppm reduced bromoxynil solution was injected to the nitrite stream and absorbance was measured at optimized wavelengths. The results are given in table 3.4.11 and are shown in figure 3.4.14.

Table 3.4.11: Investigation of injection volume for online spectrophotometric determination of bromoxynil.

Injected volume(ml)	Absorbance (coupling reagent β -naphthol)	Absorbance (Coupling reagent aniline)
1	0.105	0.168
2	0.105	0.160
3	0.096	0.102
4	0.083	0.079

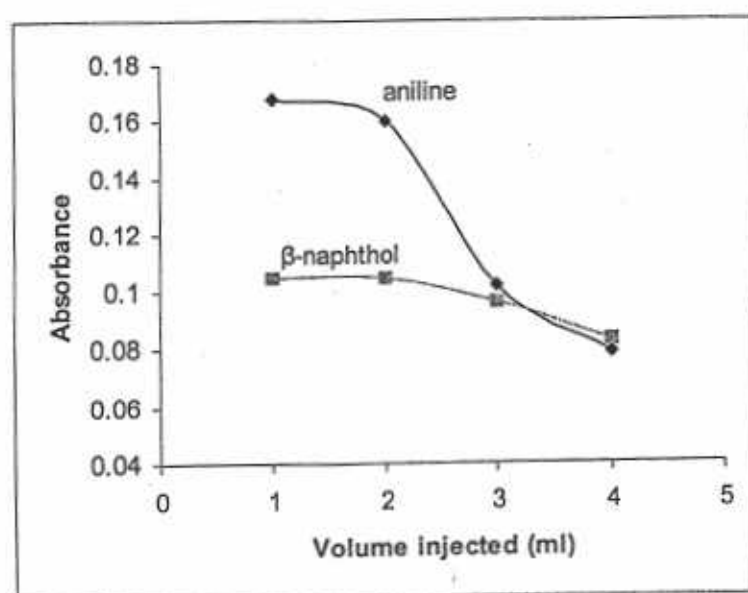


Figure 3.4.14: Investigation of injection volume for online spectrophotometric determination of bromoxynil.

Conditions:	λ max.	430nm & 500nm.
	Injection volume (10ppm)	varied
	Flow rate	3.5(ml/min)
	R1	60cm
	R2	50cm

3.4.4.3: Investigation of diazotizing coil length (R1) for online spectrophotometric determination of bromoxynil

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

For optimization of diazotization coil length (R1), the same procedure was used as mentioned before here only the diazotizing coil length was varied from 30-180cm. The absorbance of the azo dye formed was measured at optimum wavelengths. The results are given in table 3.4.12 and are shown in figure 3.4.15.

Table 3.4.12: Investigation of diazotizing coil length (R1) for online spectrophotometric determination of bromoxynil

R1 length (cm)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
30	0.079	0.053
60	0.156	0.100
90	0.189	0.145
120	0.240	0.180
150	0.120	0.172
180	0.065	0.150

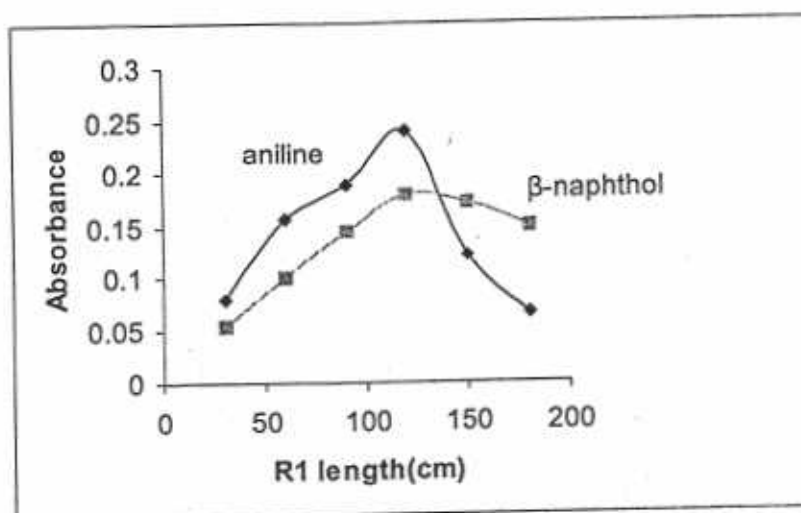


Figure3.4.15:Investigation of diazotizing coil length (R1) for online spectrophotometric determination of bromoxynil

Conditions:	$\lambda_{\max}$	430nm & 500nm
	Injection volume (10ppm)	1ml
	Flow rate	3.5(ml/min)
	R1	varied
	R2	50cm

3.4.4.4: Investigation of coupling coil length (R2) for online spectrophotometric determination of bromoxynil

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure :

For optimization of coupling coil length (R2), the same procedure was used as mentioned before, only the coupling coil length was varied from 50-200cm. The absorbance of the azo dye formed was measured at optimum wavelengths. The results are given in table 3.4.13 and are shown in figure 3.4.16.

Table 3.4.13: Investigation of coupling coil length (R2) for online spectrophotometric determination of bromoxynil

R2 length (cm)	Absorbance (Coupling reagent aniline)	Absorbance (coupling reagent β -naphthol)
50	0.236	0.109
60	0.250	0.145
90	0.298	0.228
130	0.340	0.360
165	0.385	0.360
180	0.380	0.360
200	0.380	0.360

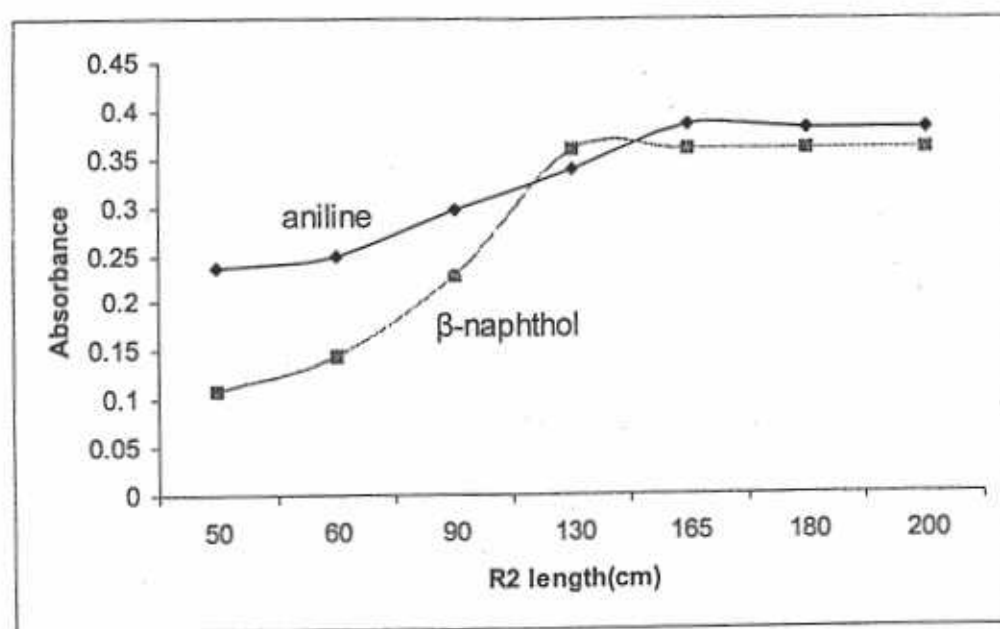


Figure 3.4.16: Investigation of coupling coil length (R2) for online spectrophotometric determination of bromoxynil

Conditions: λ max.	430nm & 500nm
-Injection volume (10ppm)	1ml
Flow rate	3.5(ml/min)
R1	120cm
R2	varied

3.4.4.5: Investigation of limit of detection and quantification for online spectrophotometric determination of bromoxynil.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before.

Procedure:

Standard reduced bromoxynil solution in the range of 0.01-5ppm was prepared in separate, 10ml volumetric flasks by taking the required volume of standard from 100ppm reduced stock solution and diluted with distilled water. In a flow through assembly, nitrite solution (0.15%) was allowed to flow in reagent stream S1 while the coupling reagent (aniline or β -naphthol) was allowed to flow in reagent stream S2. The flow rate

of the system was 3.5ml/min. The coil length of reactor R1 and R2 was kept 120cm and 165cm respectively. From different concentration of reduced bromoxynil solution 1ml was injected in the reagent stream of nitrite through a 6 port injector valve to form diazotized reagent. This diazotized reagent coupled with coupling reagent in reactor R2. The absorbance of the azo dye formed was measured at 430nm in case of β -naphthol and at 500nm in case of aniline as coupling reagent. The results are given in table 3.4.14 and are shown in figure 3.4.17.

Table 3.4.14: Investigation of the effect of concentration on absorbance behavior of bromoxynil at lower concentration.

Standard conc.(ppm)	Absorbance		Standard conc. (ppm)	Absorbance	
	Coupling reagent aniline	Coupling reagent β -naphthol		Coupling reagent aniline	Coupling reagent β -naphthol
0.01	0.004	-	0.7	0.113	0.108
0.04	0.042	-	0.8	0.150	0.133
0.05	0.047	0.006	0.9	0.187	0.153
0.06	0.062	0.016	1.0	0.250	0.254
0.08	0.070	0.025	2.0	0.408	0.301
0.10	0.084	0.043	3.0	0.471	0.390
0.30	0.094	0.052	4.0	0.539	0.425
0.50	0.098	0.073	5.0	0.592	0.479
0.6	0.103	0.090			

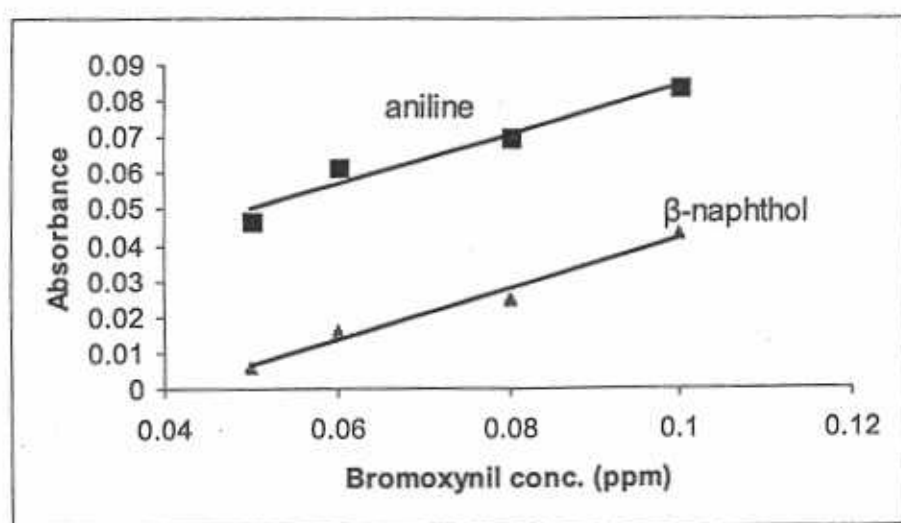


Figure 3.4.17: Investigation of the effect of concentration on absorbance behavior of bromoxynil at lower concentration.

Conditions:	$\lambda_{\max}$	430nm & 500nm
	Injection volume (10ppm)	1ml
	Flow rate	3.5ml/min
	R1	120cm
	R2	165cm

3.4.5: APPLICATION OF THE INVESTIGATED METHOD FOR THE ANALYSIS OF BROMOXYNIL IN COMMERCIAL FORMULATION.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before.

Commercial sample: commercial sample containing bromoxynil was purchased from local market. Commercial sample was a mixture of bromoxynil and MCPA in 1:1 ratio.

Procedure:

For the determination of bromoxynil in commercial formulation, first bromoxynil was separated from MCPA (2-methyl-4-chlorophenoxy acetic acid) which is in 1:1 ratio in commercial products, using cyclohexane. For this, 0.5 ml of the sample was diluted to 20ml with distilled water and extracted twice with 10 ml of cyclohexane using separatory funnel. The extract was then evaporated on boiling water bath and the remaining residue

was dissolved in ethanol, and reduced with 0.4M hydrochloric acid in a 10ml volumetric flask. The resulting Solution was diazotized and color was developed in the same way as mentioned before.. The absorbance of the colored products was measured at 500 nm and 430 nm respectively for aniline and β -naphthol used as coupling reagent. Six replicate readings were taken for each sample both for batch and online method. Standard calibration plot was prepared and the amount of bromoxynil was calculated using the following formula. The results are given in table 3.4.15.

$$\text{ppm of bromoxynil} = \frac{\text{G.R } (\mu\text{g}) \times \text{volume made (ml)} \times \text{D.F}}{\text{volume of sample taken (ml)}}$$

Where G.R represents graphical reading and D.F for dilution factor.

Table 3.4.15: Determination of active ingredient of bromoxynil in commercial formulations.

Types of herbicid	Label amount of active ingredient (g/ml)	Batch method (g/ml)		Online method (g/ml)	
		Coupling reagent aniline	Coupling reagent β -naphthol	Coupling reagent aniline	Coupling reagent β -naphthol
Bromoxynil	0.20	0.2 ± 0.01	0.20 ± 0.09	0.19 ± 0.007	0.15 ± 0.10

3.4.6: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF BROMOXYNIL IN REAL SAMPLES.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Extraction from real samples: Extraction procedure used was the same as mentioned before for fluroxypyr.

Procedure:

The extracted samples were reduced in the same way as standard was reduced with final molarity of 0.4M HCl solution in a 10ml volumetric flask. From this hydrolyzed extract 1ml and 2ml was used for online and batch analysis respectively. Color was developed in the same way as mentioned before for batch and online analysis. Residue level in real sample was calculated by using the formula given before for fluroxypyr. The results are given in table 3.4.16.

Table 3.4.16: Residue level of herbicide in real sample.

Food samples	Batch Method ($\mu\text{g/g}$)		Online Method ($\mu\text{g/g}$)	
	β -naphthol as coupling reagent	aniline as coupling reagent	β -naphthol as coupling reagent	aniline as coupling reagent
Wheat(grain)	96.0 ± 0.01	96.6 ± 0.01	89.9 ± 0.02	90.0 ± 0.007
Wheat(flour)	91.0 ± 0.007	90.0 ± 0.01	87.00 ± 0.13	88.0 ± 0.07
Maize (grain)	70.9 ± 0.05	73.3 ± 0.043	71.6 ± 0.09	71.0 ± 0.009
Maize (flour)	70.0 ± 0.05	70.0 ± 0.004	69.3 ± 0.08	70.0 ± 0.006

3.4.7:PERCENT RECOVERY OF BROMOXYNIL FROM REAL SAMPLES USING THE INVESTIGATED METHOD.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

A recovery test was performed on control samples, fortified with a known concentration of bromoxynil solution. The solvent was evaporated at room temperature for 1hr. The extraction and reduction was done exactly the same way as the sample mentioned before. Six replicates of the fortified samples were analyzed by batch and online method in the same way as mentioned before. Percent recovery was calculated by using formula given before for fluroxypyr. The results of percent recovery of bromoxynil in real samples and formulation by batch and online method are given in table 3.4.17.

Table 3.4.17: Percent recovery of herbicide from real sample and formulations.

SAMPLES	% Recovery (batch method)		% Recovery (online method)	
	β -naphthol as coupling reagent	aniline as coupling reagent	β -naphthol as coupling reagent	aniline as coupling reagent
Bromoxynil formulation	96.0 $\pm$ 0.10	97.0 $\pm$ 0.002	90.7 $\pm$ 0.09	91.0 $\pm$ 0.008
Maize (grain)	88.0 $\pm$ 0.01	88.9 $\pm$ 0.02	90.6 $\pm$ 0.01	90.2 $\pm$ 0.010
Maize (flour)	80.0 $\pm$ 0.05	81.48 $\pm$ 0.05	94.6 $\pm$ 0.09	95.1 $\pm$ 0.013
Wheat (grain)	88.0 $\pm$ 0.10	87.55 $\pm$ 0.10	87.0 $\pm$ 0.01	87.8 $\pm$ 0.029
Wheat (flour)	88.6 $\pm$ 0.08	88.8 $\pm$ 0.10	90.0 $\pm$ 0.05	90.0 $\pm$ 0.009

Results and discussion

After preliminary investigation it was observed that the proposed reaction shown in figure 3.4.2. is feasible for spectrophotometric determination of bromoxynil. Then various physical and chemical parameters were optimized and are discussed below.

The azo dye formed was investigated for the optimum wavelength. The results are given in table-3.4.1 and are shown in figure 3.4.3. As can be seen from figure 3.4.3 the resulting azo dye has a maximum absorbance at 430nm if β -naphthol is used as coupling reagent and at 500nm in case of aniline used as coupling reagent. Therefore these two wavelength were used for further investigation of bromoxynil.

The concentration of hydrochloric acid required for maximum reduction was optimized in range of 0.1-0.6M (table 3.4.2). An increase in absorbance was observed with increasing final acid concentration up to 0.4M of hydrochloric acid solution (figure 3.4.4). Above this a decrease in absorbance was observed. Therefore 0.4 M acidity for the final solution was used for further reduction of bromoxynil herbicide.

Nitrite concentration was optimized for diazotization in the range of 0.05-0.25% (table 3.4.3) and it can be seen from figure 3.4.5 that 0.15% of nitrite was the optimum concentration for maximum diazotization. Similarly the volume of 0.15% nitrite solution was optimized in the range of 2-14ml (table 3.4.4). It was observed (figure 3.4.6) that 12ml of 0.15% of nitrite solution was sufficient for maximum diazotization when β -naphthol was used as coupling reagent and 4ml of 0.15% nitrite was found to be the optimum volume when aniline was used as coupling reagent.

Similarly the concentration of coupling reagents (aniline and β -naphthol) was optimized (table 3.4.5). It was observed (figure 3.4.7) that 2% of aniline and 0.2% of β -naphthol are the optimum concentrations of coupling reagents and above this optimum concentration a decrease in absorbance was observed.

Volume of coupling reagents was also optimized for maximum formation of azo dye. It was observed (figure 3.4.8) that 6ml of 2% aniline and 4ml of 0.2% β -naphthol are the optimum volumes for maximum formation of azo dye.

Coupling reaction take place in basic media and for maximum coupling the concentration of sodium hydroxide was optimized in the range of 0.4-1.8M. It was observed (figure

3.4.9) that 1.2 M sodium hydroxide is the optimum concentration required for maximum formation of azo dye.

Time study was conducted for optimization of equilibration time for coupling reaction by using both coupling reagents. It was found (figure 3.4.10) that when β -naphthol was used as coupling reagent coupling reaction is spontaneous while in case of aniline, coupling reaction is slow and takes 12 minutes for completion. However, it was observed that in both case the resulting azo dye is stable.

Optimization of parameters for online analysis

The chemical parameters optimized for batch analysis were directly used for online analysis. These optimized parameters were applied for online analysis using a double channel flow injection system. In this system a stream of nitrite solution and coupling reagent was continuously pumped and a volume of 1ml of reduced sample was injected in reagent stream through a six-port injection valve. The diazotization and coupling reactions take place in reactor R1 and R2 respectively. The absorbance of azo dye formed was measured at 430nm in case of β -naphthol and at 500nm in case of aniline used as coupling reagent.

The physical parameter like flow rate and reactor coil length were optimized. The flow rate ranging from 1.5-6.5ml/minute was optimized for the online determination of bromoxynil using both coupling reagents (table 3.4.10). It was observed (figure 3.5.13) that in case of β -naphthol signal increased up to a flow rate of 2.5ml/minute and remained constant at higher flow rate. While for aniline, signal increased up to a flow rate of 3.5ml/minute and decreased at higher flow rate. Therefore, a flow rate of 3.5ml/minute was selected as optimum flow rate for both coupling reagents.

The volume of sample injected was optimized in the range of 1-4ml. Figure 3.4.14 shows that with increase of sample volume absorbance decreases possibly because of insufficient time for diazotization reaction and peak broadening was also observed.

The coil length of reactor R1 was investigated in the range of 30-180 cm (table 3.4.12 & figure 3.4.15) and it was found that with increase of coil length the absorbance of azo dye increases up to a coil length of 120cm and decreased when the coil length was further increased. Similarly the coil length of reactor R2 was optimized in range between 50-200cm. Figure 3.4.16 shows that maximum coupling take place at 165cm and 130cm

when aniline and β -naphthol were used as coupling reagent respectively. Above the optimum lengths no change in absorbance was observed. Therefore, the coil length of 165cm was selected for online system for keeping good sampling frequency i.e. 80 samples per hour and to avoid the steady state condition.

At optimum conditions, the effect of lower concentration of bromoxynil (below 1 ppm) on absorbance behavior was investigated for assessing the LOD and LOQ for the investigated batch and online methods. The results are given in table 3.4.9. & 3.4.14 and are shown in figure 3.4.11 & 3.4.17 respectively. The LOD and LOQ were calculated for the investigated methods using formula given before. For bromoxynil- aniline couple LOD and LOQ were found to be 6.0 ppb and 22.0 ppb for batch analysis and 9.0 ppb and 30.0 ppb for online analysis respectively. While for bromoxynil- β -naphthol couple LOD and LOQ were found to be 21.0 ppb and 70.0 ppb for batch analysis and 24.0 ppb and 80.0 ppb for online analysis respectively. Aniline is the best coupling reagent from sensitivity point of view, while reaction is faster in case of β -naphthol.

The RSD was also calculated for the batch and online analysis and was found to be 0.32% and 3.6% for bromoxynil- aniline couple respectively. Similarly for bromoxynil- β -naphthol couple RSD was found to be 12.77% and 10.50% for the batch analysis and online analysis respectively. Molar absorptivity was calculated and was found to be $5.14 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$ & $1.27 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$ for batch and online method respectively when aniline used as coupling reagent. For β -naphthol couple molar absorptivity was found to be $1.4 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$ and $3.285 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$ for online and batch method respectively.

Application of the investigated method for the analysis of bromoxynil in commercial formulation and in real samples.

The investigated methods were applied for the determination of bromoxynil in commercial formulations and in real samples like wheat and maize.

The value of active ingredient determined by using the optimized methods were closely related to the labeled value of active ingredient (table 3.4.15) in case of online methods. While for batch methods experimentally determined values are similar to the label value. The residue levels found in food samples were higher in grains than in flour in both cases (table 3.4.16). This could be due to heat losses during processing. For batch and online

methods the residue levels found were much higher in grains than US EPA MRLs 2004, which are 0.05ppm and 0.3ppm for wheat and maize grains, respectively and NZ food safety authority 2005, which is 0.02ppm for cereal grains. Further, the results of both methods using bromoxynil- aniline couple and bromoxynil- β -naphthol couple are also comparable. Percent recovery test were performed for food samples and formulations. The results are given in table 3.4.17. The recovery was good and the results of batch and online methods were comparable.

3.5: INVESTIGATION OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF CHLORAMBN.

The chemical structure of chloramben is given below.

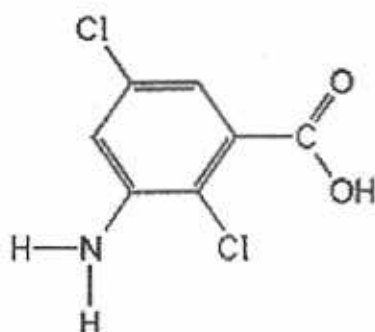


Figure 3.5.1 Chemical structure of chloramben

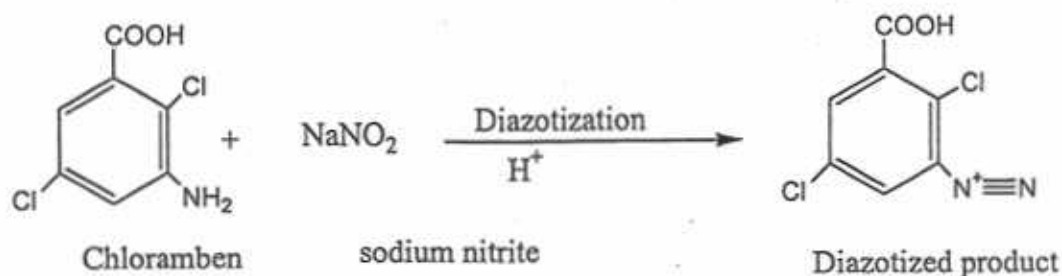
3.5.1: Method Development Strategy for Spectrophotometric Determination of Chloramben:

Looking at the structure of chloramben, it also has an amino group like fluroxypyr. It was thought that it could be used as a diazotizing reagent directly and after coupling with a suitable coupling reagent it will give an azo dye. The resulting azo dye could be determined by spectrophotometric method. The proposed reaction is shown in figure 3.5.2.

3.5.2: Preliminary investigation of the possibility of diazotization and coupling for spectrophotometric determination of chloramben.

Preliminary studies were conducted to investigate the possibility of diazotization, coupling and the formation of expected azo dye. Initially high concentration of chloramben (100 ppm), relatively large volume of concentrated hydrochloric acid, sodium nitrite and various coupling reagents like aniline, β -naphthol and phenol were tried for the formation of expected azo dye at room temperature. The formation of the colored azo dye indicated the feasibility of the reaction and its subsequent application for the spectrophotometric determination of chloramben. Further studies were focused on optimization of various parameters and are given below.

Step 1: Diazotization reaction



Step 2: Coupling reaction

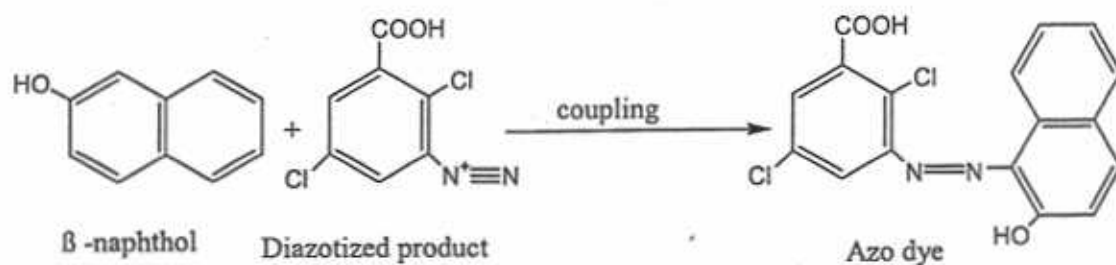


Figure-3.5.2. Proposed chemical reaction for the formation of azo dye for chloramben.

3.5.3: OPTIMIZATION STUDIES FOR SPECTROPHOTOMETRIC DETERMINATION OF CHLORAMBEN.

3.5.3.1. Investigation of suitable wavelength for the determination of chloramben.

Instruments:

The FI system comprised a peristaltic pump with PET (i.d. 1.19mm, Becton Dickson, USA) and silicon (i.d.1.71mm) flow tubes, a V-450, 6 port injection valve (Upchurch Scientific Inc, USA) and a UV/Vis spectrophotometer (UNICO UV-2100 United Products and Instruments Inc., Dayton, N.J, U.S.A) as a detector was used.

Reagents and chemicals:

All chemicals used were of analytical reagent grade purity. Sodium nitrite, β -Naphthol, hydrochloric acid, ethanol and sodium hydroxide were used during this work. Standard reference material was obtained from Acros Organic, Belgium.

Solution preparation:

- i) **Nitrite solution (0.1%):** Nitrite solution was prepared by dissolving 0.15g sodium nitrite in a little distilled water in a 100ml volumetric flask and 3ml of concentrated HCl was added to it. The resulting solution was diluted up to mark with distilled water.
- ii) **β -naphthol solution (0.1%):** β -naphthol solution was prepared by dissolving 0.1g of β -Naphthol in 6% of sodium hydroxide solution in a 100ml volumetric flask.
- iii) **Standard chloramben solution:** Chloramben solution (1000 ppm) was prepared by dissolving 0.1 g of authentic standard of 3-amino-2, 5-dichlorobenzoic acid (chloramben) in 50 ml of ethanol and diluted to 100 ml with distilled water. Through step wise dilution 100 and 10-ppm chloramben solution were prepared.

Procedure:

10ppm of chloramben standard solution was diazotized with 10ml of 0.1% nitrite solution in a 50ml volumetric flask. This solution was equilibrated for sometime for the formation of diazonium ion. To the resulting solution, 10ml β -Naphthol (0.1%) was added as coupling reagent for the formation of azo dye and diluted up to mark with distilled water. Blank solution was prepared through the same procedure without the addition of chloramben. The absorbance of the azo dye was measured at different wavelength from 460 to 520nm using UNICO UV-2100 spectrophotometer for finding out the optimum wavelength. The absorbance was noted with increasing wavelength and each wavelength was calibrated with blank solution. The results are given in table 3.5.1 and are shown in figure 3.5.3.

Table 3.5.1: Wavelength optimization for the spectrophotometric determination of chloramben.

Wavelength (nm)	Absorbance
450	0.810
455	0.869
460	0.925
465	0.995
470	1.085
475	1.120
480	1.110
485	1.065
490	0.935
500	0.778
510	0.420
520	0.166

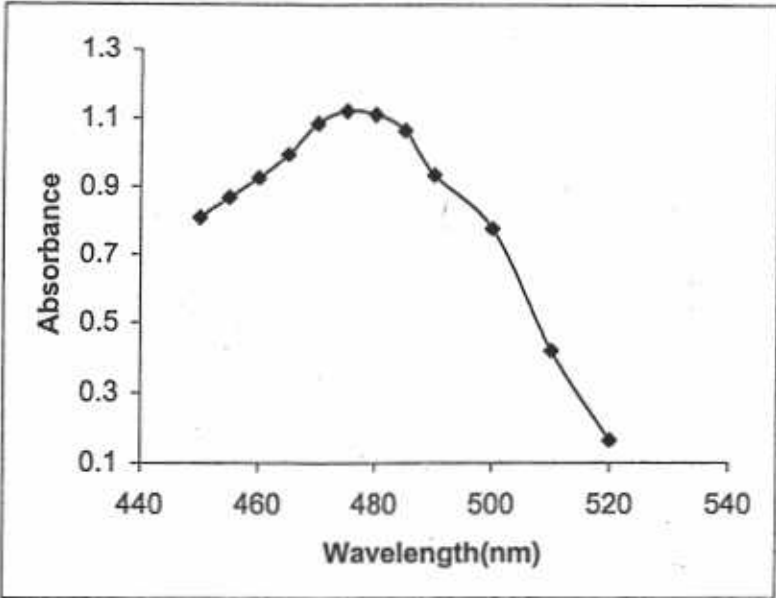


Figure 3.5.3: Wavelength optimization for spectrophotometric determination of chloramben.

Conditions:	λ max.	Varied
	Chloramben solution	10ppm
	Nitrite solution (0.1%)	10ml
	β -naphthol solution (0.1%)	10ml

3.5.3.2: Optimization of nitrite concentration for diazotization of chloramben.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10 ppm of chloramben standard solution was transferred to six separate 50ml volumetric flasks. To each flask 10ml of different concentration of nitrite ranging from 0.05%-0.3% was added. Color was developed in the same way as mentioned before. The absorbance of the resulting azo dye was measured at 475nm. The results are given in table 3.5.2 and are shown in figure 3.5.4.

Table 3.5.2: Optimization of nitrite concentration for diazotization of chloramben.

Nitrite concentration (%)	Absorbance
0.05	0.019
0.10	0.205
0.15	0.382
0.20	0.482
0.25	0.350
0.30	0.240

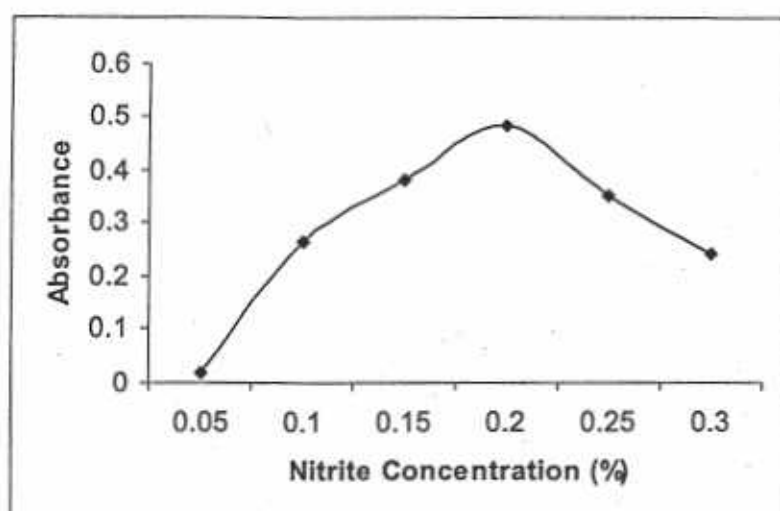


Figure 3.5.4: Optimization of nitrite concentration for diazotization of chloramben.

Conditions: λ_{max} .	475nm
Chloramben solution	10ppm
Nitrite solution (varied conc.)	10ml
β -naphthol (0.1%)	10ml

3.5.3.3: Volume optimization of nitrite solution for diazotization of chloramben.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ppm chloramben standard solution was taken in separate 50ml volumetric flasks. To each flask 0.2% nitrite solution ranging from 1-8ml was added for diazotization. After diazotization the color was developed in the same way as mentioned before and absorbance of the azo dye was measured at 475nm. The results are given in table 3.5.3 and are shown in figure 3.5.5.

Table 3.5.3: Volume optimization of nitrite solution for diazotization of chloramben.

S.NO	Nitrite volume (ml)	Absorbance
1	1	0.419
2	2	0.433
3	3	0.495
4	4	0.555
5	5	0.520
6	6	0.146
7	7	0.140
8	8	0.138

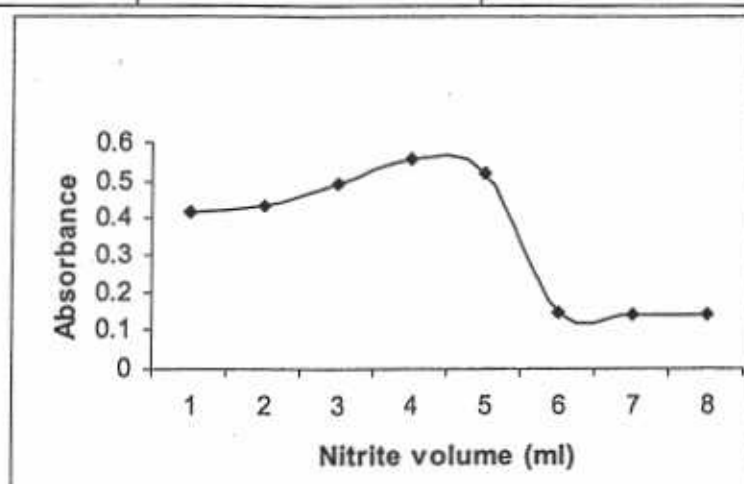


Figure 3.5.5: volume optimization of nitrite solution for diazotization of chloramben.

Conditions:	λ_{max}	475nm
	Chloramben solution	10ppm
	Nitrite solution (0.2 %)	varied
	β -naphthol (0.1%)	10ml

3.5.3.4: Optimization of solution acidity for the formation of diazotized product.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Chloramben standard solution 5ml from 100 ppm stock solution was transferred to six separate 50ml volumetric flasks. To each of these flasks 4ml of nitrite solution (0.2%) prepared in different concentration of hydrochloric acid ranges from 0.1-0.6M was added for finding out the optimum molarity of hydrochloric acid, that is required for the formation of diazotized product. Color was developed in the same way as mentioned before and absorbance of the azo dye was measured at 475nm. The results are given in table 3.5.4 and are shown in figure 3.5.6.

Table 3.5.4: Optimization of solution acidity for the formation of diazotized product.

S.NO	Molarity of HCl (M)	Absorbance
1	0.12	0.514
2	0.24	0.528
3	0.40	0.632
4	0.50	0.602
5	0.60	0.580

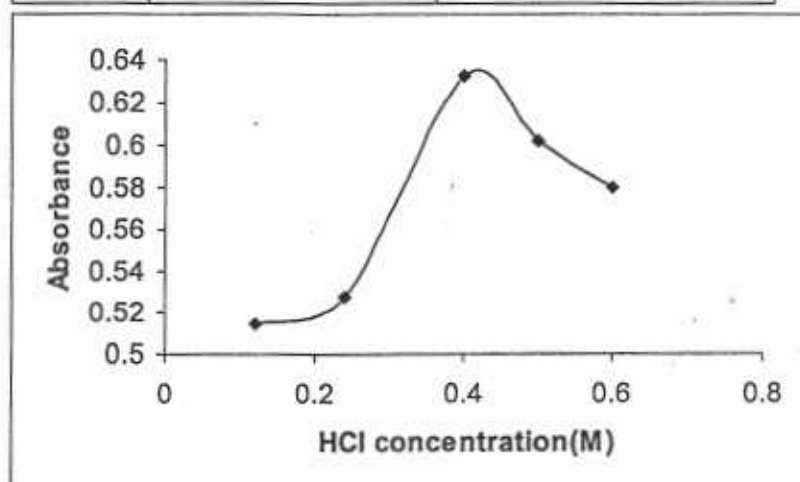


Figure 3.5.6: Optimization of solution acidity for the formation of diazotized Product.

Conditions:

λ_{max} .	475nm
Chloramben solution	10ppm
Nitrite solution (0.2%)	4ml
Hydrochloric acid (ml)	varied

β -naphthol (0.1%)

10ml

3.5.3.5: Optimization of the effect of sodium hydroxide for coupling reaction.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10 ppm Chloramben solution was diazotized with 4ml of 0.2% nitrite solution (prepared in 0.4M HCl solution) in six separate 50ml volumetric flask. To each flask 10ml of β -naphthol (0.1%) prepared in different concentration of sodium hydroxide from 2-12% were added. The absorbance of resulting azo dye was measured at 475nm. The results are given in table 3.5.5 and are shown in figure 3.5.7.

Table 3.5.5: Optimization of the effect of sodium hydroxide for coupling reaction.

S.NO	NaOH (%)	Absorbance
1	2	0.203
2	4	0.293
3	6	0.717
4	8	0.615
5	10	0.417
6	12	0.400

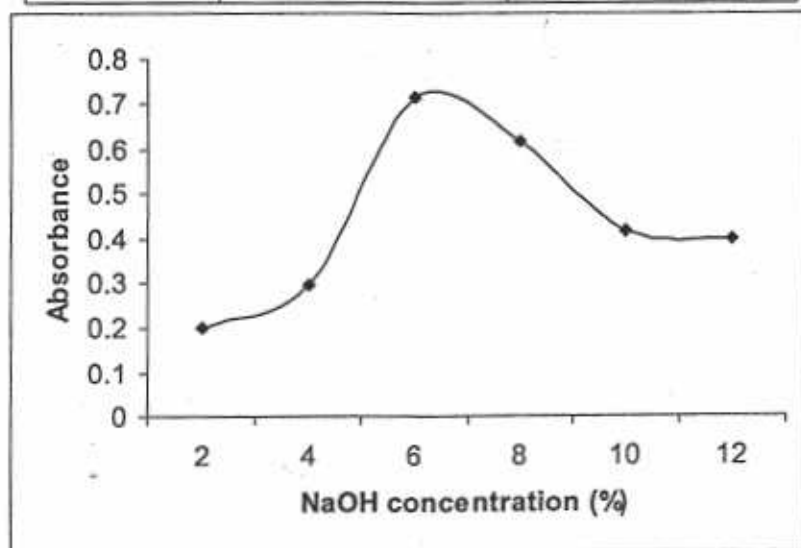


Figure 3.5.7: Optimization of the effect of sodium hydroxide for coupling reaction.

Conditions : λ_{max} .	475nm
Chloramben solution	10ppm
Nitrite solution (0.2%)	4ml
Sodium hydroxide (%)	varied
β -naphthol (0.1%)	10ml

3.5.3.6: Optimization of β -naphthol concentration for maximum formation of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ppm Chloramben solution was diazotized in five separate 50ml volumetric flasks using the same procedure as mentioned before. The resulting solution was coupled with 10 ml of varied concentration of β -naphthol ranging from 0.05-0.25%. The absorbance of the azo dye was measured at 475nm. The results are given in table 3.5.6 and are shown in figure 3.5.8.

Table 3.5.6: Optimization of the effect of β -naphthol concentration for maximum formation of azo dye

β -naphthol conc. (%)	Absorbance
0.05	0.510
0.10	0.651
0.15	0.529
0.20	0.450
0.25	0.414

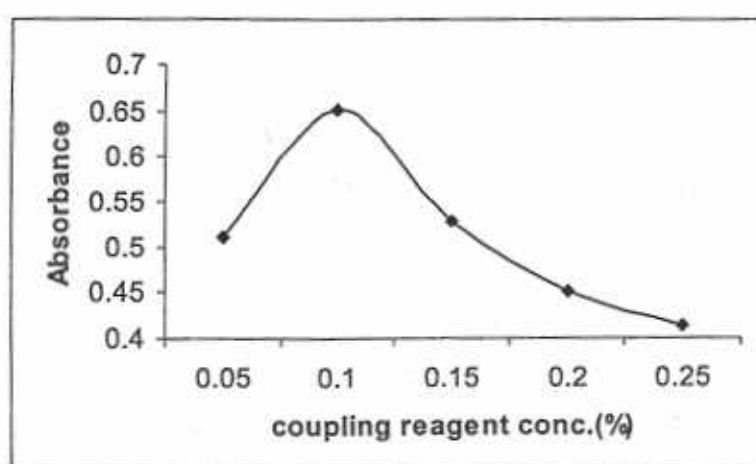


Figure 3.5.8: Optimization of β -naphthol concentration for maximum formation of azo dye.

Conditions:	$\lambda_{\text{max.}}$	475nm
	Chloramben solution	10ppm
	Nitrite solution (0.2%)	4ml
	β -naphthol (varied)	10ml

3.5.3.7: Optimization of β -naphthol volume for maximum formation of azo dye.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ppm Chloramben solution was diazotized in five separate 50ml volumetric flasks by the same way as mentioned before and coupled with varied volume of 0.1% β -naphthol solution ranging from 2-10ml. The absorbance of the azo dye was measured at 475nm. The results are given in table 3.5.7 and are shown in figure 3.5.9.

Table 3.5.7: Optimization of β -naphthol volume for maximum formation of azo dye.

S.NO	β -naphthol volume (ml)	Absorbance
1	2	0.345
2	4	0.633
3	6	0.511
4	8	0.402
5	10	0.372

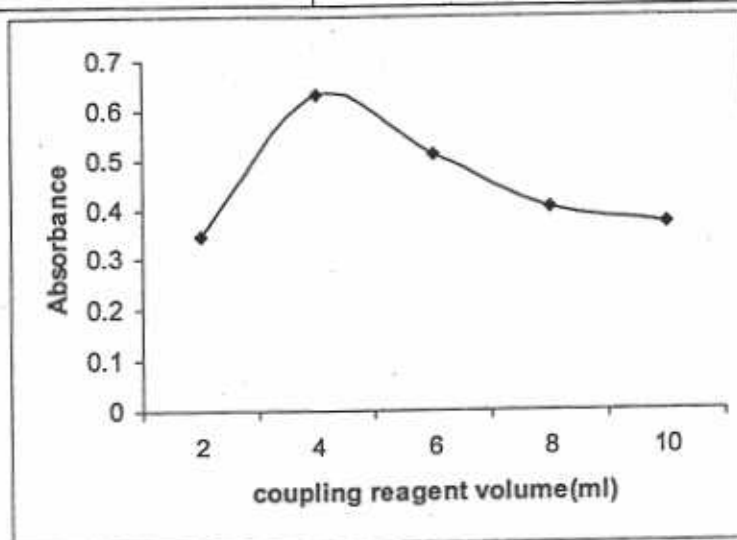


Figure 3.5.9: Optimization of β -naphthol volume for maximum formation of azo dye.

Conditions: λ_{max} 475nm
 Chloramben solution 10ppm
 Nitrite solution (0.2%) 4ml
 β -naphthol (0.1%) 4ml

3.5.3.8: Optimization of the effect of time for diazotization reaction .

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ppm Chloramben solution was diazotized with 4ml of 0.2% nitrite solution in six separate 50ml volumetric flask. Each solution was equilibrated for diazotization using different time intervals ranging from 1-20 minutes. Color was developed in each solution by the same way as mentioned before. The absorbance of the azo dye was measured at 475nm. The results are given in table 3.3.8 and are shown in figure 3.3.10.

Table 3.5.8: Optimization of the effect of time for diazotization reaction.

S.NO	Time (minutes)	Absorbance
1	1	0.875
2	5	0.905
3	10	0.900
4	15	0.895
5	20	0.895

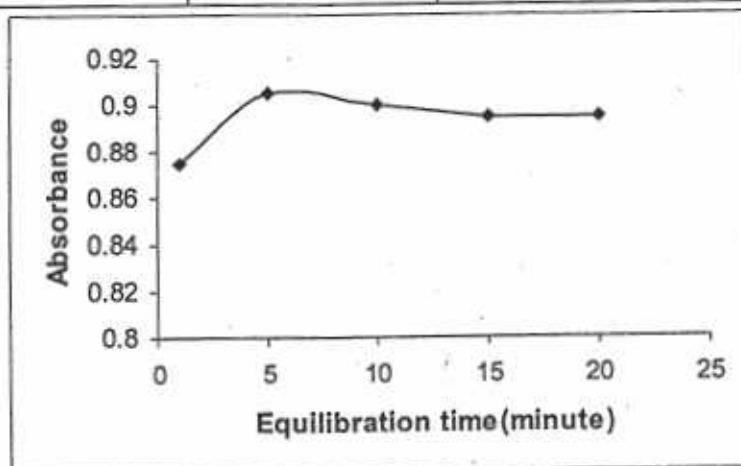


Figure 3.5.10: Optimization of the effect of time for diazotization reaction.

Conditions :	λ_{max} .	475nm
	Chloramben solution	10ppm
	Nitrite solution (0.2%)	4ml
	β -naphthol (0.1%)	4ml

3.5.3.9: Optimization of the effect of time on coupling reaction.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

10ppm Chloramben standard solution was diazotized with 4ml of 0.2% nitrite solution for 5 minutes in nine separate volumetric flasks. To the resulting diazotized product 4ml of 0.1% β -naphthol was added. These resulting solutions were allowed to equilibrate for different time intervals ranging from 1-40 minutes. The absorbance of the azo dye formed was measured at 475nm. The results are given in table 3.5.9 and are shown in figure 3.5.11.

Table 3.5.9: Optimization of the effect of time on coupling reaction.

S.NO	Time (minutes)	Absorbance
1	1	0.615
2	5	0.615
3	10	0.609
4	15	0.567
5	20	0.559
6	25	0.548
7	30	0.540
8	35	0.536
9	40	0.528

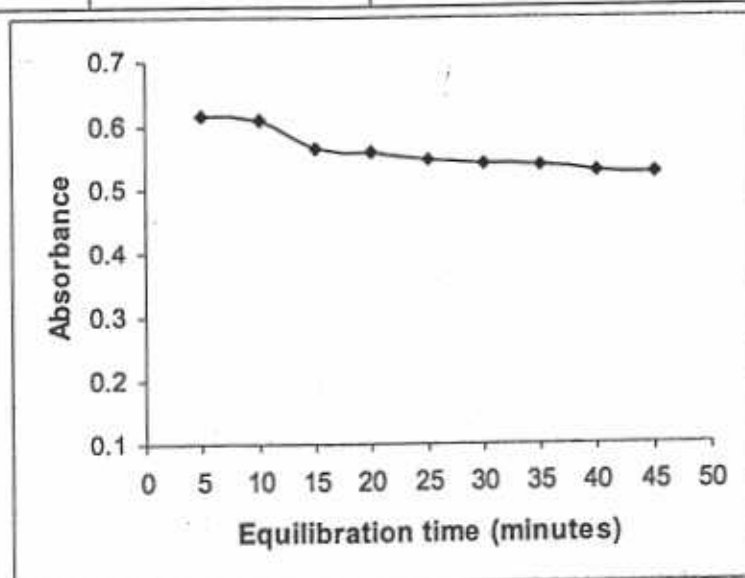


Figure 3.5.11: Optimization of the effect of time on coupling reaction

Conditions:	$\lambda_{\text{max.}}$	475nm
	Chloramben solution	10ppm
	Nitrite solution (0.2%)	4ml
	β -naphthol (0.1%)	4ml
	Diazotization time	5minutes

3.5.3.10: Investigation of the effect of concentration on absorbance behaviour of chloramben at lower concentration.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

From standard chloramben solution (100ppm) different volume for 0.4-10 ppm were taken in separate 50 ml volumetric flasks. To each of flask 4ml of nitrite (0.2%) solution was added and equilibrated for 5 minutes. To the resulting diazotized product 4ml of 0.1% β -naphthol was added. An orange red colored azo dye was formed. The absorbance of the azo dye was measured at 475nm. The results are given in table 3.5.10 and are shown in figure 3.5.12 for lower level concentration. Molar absorptivity, LOD, LOQ and RSD were calculated by using the formula given before fluoxypyr.

Table 3.5.10: Investigation of the effect of concentration on absorbance behavior of chloramben at lower concentration.

Concentration (ppm)	Absorbance	Concentration (ppm)	Absorbance
0.4	0.005	1.0	0.029
0.5	0.009	2.0	0.098
0.6	0.012	4.0	0.254
0.7	0.015	6.0	0.444
0.8	0.018	8.0	0.630
0.9	0.020	10	0.914

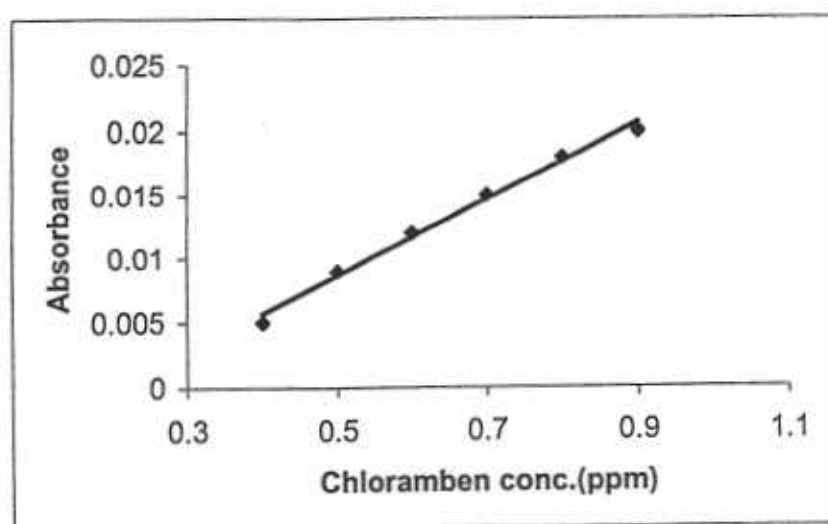


Figure 3.5.12: Investigation of the effect of concentration on absorbance behavior of chloramben at lower concentration.

Condition:	λ_{max}	475nm
	Chloramben solution	10ppm
	Nitrite solution (0.2%)	4ml
	β -naphthol (0.1%)	4ml
	Diazotization time	5minutes

3.5.4: INVESTIGATION OF PHYSICAL PARAMETERS FOR ONLINE SPECTROPHOTOMETRIC DETERMINATION OF CHLORAMBEN.

All physical parameters such as the reactor coil lengths (R1 and R2), flow rate and injection volume were optimized for the flow injection spectrophotometric determination of chloramben, while all the chemical parameters optimized for the batch analysis of chloramben were kept the same. Since the optimum temperature for diazotization was room temperature, all FI manifolds were set at room temperature. A schematic diagram of flow injection assembly used in this work is shown in figure 3.5.13.

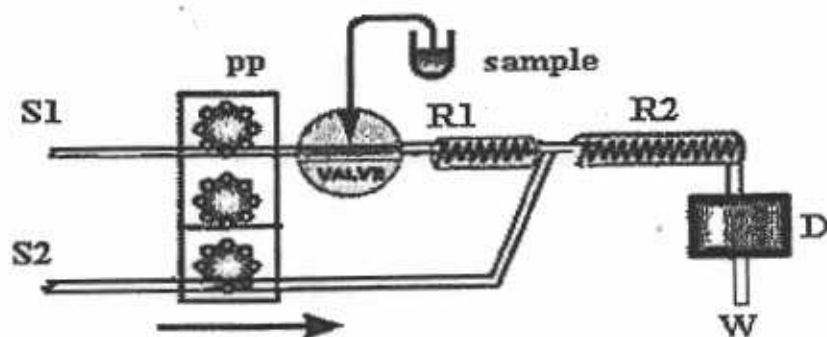


Fig. 3.5.13. Schematic diagram of the FI-manifold. (S1) nitrite stream, (S2) β -naphthol stream, (R1) diazotization coil, (R2) coupling coil, (PP) Peristaltic pump, (S) sample injected, (V) valve, (W) Waste, (C) cell, (D) detector, (RD) readout device.

3.5.4.1 : Investigation of flow rate for FI spectrophotometric determination of chloramben.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

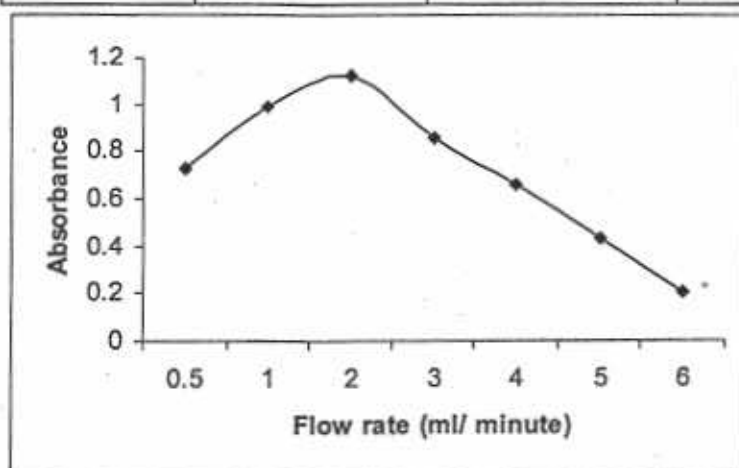
Solutions: The same as mentioned before

Procedure :

In a flow through assembly nitrite solution (0.2%) was allowed to flow in reagent stream S1 while the coupling reagent β -naphthol (0.1%) was allowed to flow in reagent stream S2. Flow rate of the system was varied in the range of 0.5-6ml/min. The coil length of reactor R1 and R2 was kept 50 cm. From 10ppm chloramben solution 1ml was injected in the reagent stream of nitrite through a 6 port injector valve to form diazotized reagent. This diazotized reagent couple with β -naphthol in reactor R2. The absorbance of the azo dye formed was measured at 475nm by using UNICO UV 2100 spectrophotometer. Blank was adjusted by flowing continuous stream of nitrite and β -naphthol solution through the system. The results are given in table 3.5.11 and shown in figure 3.5.14.

Table3.5.11: Investigation of flow rate for FI spectrophotometric determination of chloramben.

Flow rate (ml/minute)	Absorbance	Flow rate (ml/minute)	Absorbance
0.5	0.725	4.0	0.660
1.0	0.990	5.0	0.432
2.0	1.125	6.0	0.201
3.0	0.850		



Figur 3.5.14: Investigation of flow rate for FI spectrophotometric determination of chloramben.

Conditions:	λ_{max}	475nm
	Injection volume	1ml
	Flow rate (ml/min)	varied
	R1	50cm
	R2	50cm

3.5.4.2: Investigation of Injection volume for FI spectrophotometric determination of chloramben.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

For volume optimization varied volume (1-4ml) of 10ppm chloramben solution was injected in the nitrite stream and the absorbance was measured at 475nm. The results are given in table 3.5.12 and shown in figure 3.5.15.

Table 3.5.12: Investigation of Injection volume for FI spectrophotometric determination of chloramben.

S.NO	Injection volume (ml)	Absorbance
1	1	0.753
2	2	0.742
3	3	0.720
4	4	0.700

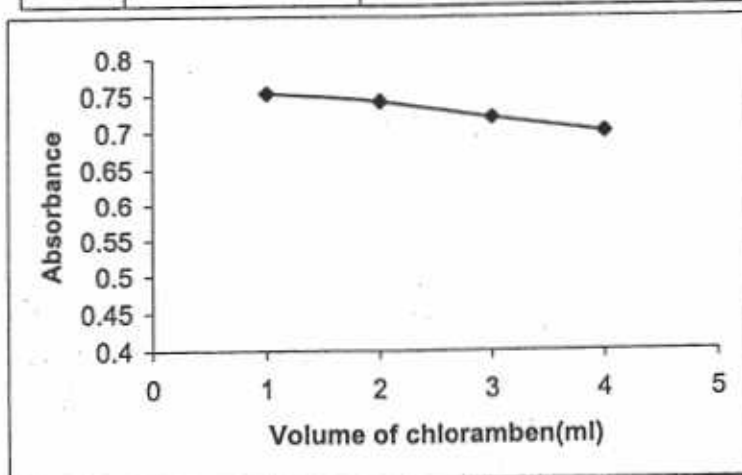


Figure 3.5.15: Investigation of Injection volume for FI spectrophotometric determination of chloramben.

Conditions:	λ_{max}	475nm
	Injection volume	varied
	Flow rate	2ml/min
	R1	50cm
	R2	50cm

3.5.4.3: Investigation of diazotizing coil length (R1) for FI spectrophotometric determination of chloramben.

Instruments: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

For optimization of diazotization coil length (R1), the same procedure was used as mentioned before only the length of diazotizing coil was varied from 20-100cm. The absorbance of the azo dye formed was measured at 475nm. The results are given in table 3.5.13 and shown in figure 3.5.16.

Table 3.5.13: Investigation of diazotizing coil length (R1) for FI spectrophotometric determination of chloramben.

S.NO	R1 legth (cm)	Absorbance
1	20	0.024
2	30	0.078
3	40	0.120
4	50	0.198
5	60	0.270
6	70	0.333
7	80	0.333
8	90	0.333
9	100	0.333

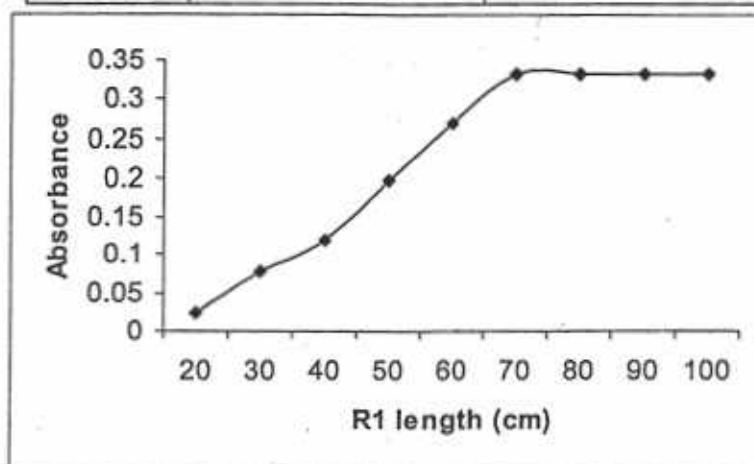


Figure 3.5.16: Investigation of diazotizing coil length (R1) for FI spectrophotometric determination of chloramben.

Conditions:	λ_{max}	475nm
	Injection volume	1ml
	Flow rate	2ml/min
	R1	varied
	R2	50cm

3.5.4.4: Investigation of coupling coil length (R2) for FI spectrophotometric determination of chloramben.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

For optimization of coupling coil length (R2), the same procedure was used as mentioned before only the length of coupling coil was varied from 10-80cm. Color was developed in the same way as mentioned before. The absorbance of the azo dye formed was measured at 475nm. The results are given in table 3.5.14 and are shown in figure 3.5.17.

Table 3.5.14: Investigation of coupling coil length (R2) for FI spectrophotometric determination of chloramben.

S.NO	R2 length (cm)	Absorbance
1	10	0.119
2	20	0.223
3	30	0.348
4	40	0.540
5	50	0.850
6	60	0.850
7	70	0.850
8	80	0.850

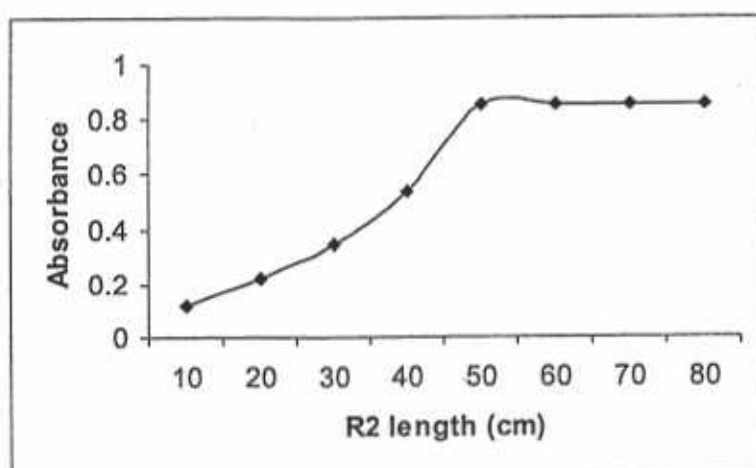


Figure 3.5.17: Investigation of coupling coil length (R2) for FI spectrophotometric determination of chloramben.

Conditions:	λ_{max}	475nm
	Injection volume	1ml
	Flow rate	2ml/min
	R1	70cm
	R2	varied

3.5.4.5: Investigation of the effect of concentration on absorbance behavior of chloramben at lower concentration.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Procedure:

Standard chloramben solution in the range of 0.2ppm –10ppm was prepared in separate 10 ml volumetric flask by taking the required volume of standard from 100ppm and 10 ppm stock solution and diluting with distilled water. In a flow through assembly nitrite solution(0.2%) was allowed to flow in reagent stream S1 while the coupling reagent β -naphthol(0.1%) was allowed to flow in reagent stream S2. The Flow rate of the system was maintained at 2ml/min. The length of reactor R1(diazotizing reactor)was 70cm and for reactor R2 (coupling reactor) was 50cm. From different concentration of chloramben standard solution 1ml was injected in the reagent stream of nitrite through a 6 port injector valve to form diazotized reagent. This diazotized reagent coupled with β -

Naphthol in reactor R2. The absorbance of the azo dye formed was measured at 475nm. Blank was adjusted by allowing to flow a contineouse stream of nitrite and β -naphthol solution through the system. After each running, system was washed with blank solution and sample coil length was washed with distilled water and dried by passing air through it. The results are given in table 3.5.15 and shown in figure 3.5.18.

Table 3.5.15: Investigation of the effect of concentration on absorbance behavior of chloramben at lower concentration.

Chloramben conc.(ppm)	Absorbance	Chloramben conc.(ppm)	Absorbance
0.2	0.006	0.9	0.116
0.3	0.016	1.0	0.168
0.4	0.024	2.0	0.232
0.5	0.044	4.0	0.416
0.6	0.058	6.0	0.605
0.7	0.073	8.0	0.815
0.8	0.095	10	0.980

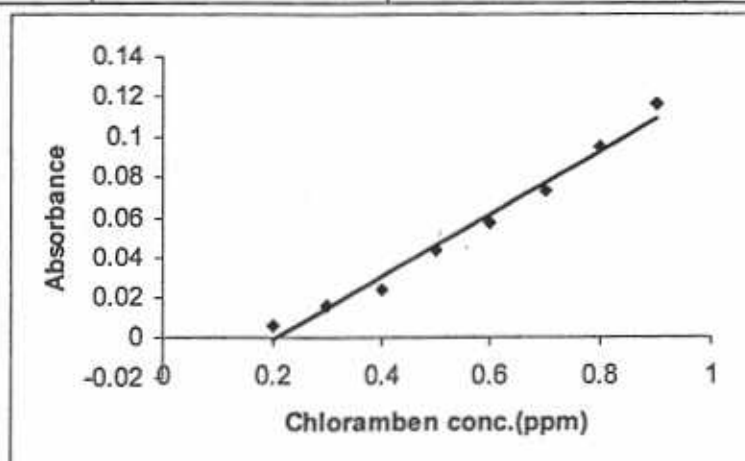


Figure 3.5.18: Investigation of the effect of concentration on absorbance behaviour of chloramben at lower concentration.

Conditions:	$\lambda_{\max}$	475nm
	Injection volume	1ml
	Flow rate	2ml/min
	R1	70cm
	R2	50cm

3.5.5: APPLICATION OF THE INVESTIGATED METHODS FOR THE ANALYSIS OF CHLORAMBEN IN REAL SAMPLES.

Instrument: The same as mentioned before

Reagents: The same as mentioned before

Solutions: The same as mentioned before

Collection of samples: Real samples were purchased from the local market.

Extraction from real samples:

25 g of real sample were taken and extraction was done using the same procedure as mentioned before for fluroxypyr.

Procedure:

2ml of the extracted sample was taken in three separate 50ml volumetric flask. To each flask 4ml of nitrite solution was added and color was developed in the same way as mentioned before for batch analysis. The absorbance of the colored solution was measured at 475nm.

For FI analysis 1ml of the extracted sample was injected in reagent stream and absorbance was measured using the same procedure as for standard.

The residue level of chloramben in real samples was calculated both for batch and FI analysis. The following formula was used for calculating the residue level of chloramben in real samples.

$$\text{ppm of chloramben} = \frac{\text{G.R } (\mu\text{g}) \times \text{volume made} \times \text{D.F}}{\text{Weight of sample taken (g)}}$$

Where G.R represents graphical reading and D.F for dilution factor.

Table 3.5.16: Residue level of chloramben in real samples.

Name	Batch analysis ($\mu\text{g/g}$)	FIA ($\mu\text{g/g}$)
Tomato paste	0.130 $\pm$ 0.130	0.12 $\pm$ 0.110
Corn	0.103 $\pm$ 0.09	0.10 $\pm$ 0.33
Corn flour	0.100 $\pm$ 0.22	0.09 $\pm$ 0.07

3.5.6: PERCENT RECOVERY OF CHLORAMBEN FROM REAL SAMPLES USING THE INVESTIGATED METHOD

Instrument: The same as mentioned before

Procedure:

A recovery test was performed on control samples, fortified with a known concentration of chloramben solution. The solvent was evaporated at room temperature for 1hr. Extraction was done exactly the same way as for the real samples mentioned before. Six replicates of the fortified samples were analyzed by batch and FI method. The results of percent recovery of chloramben in fortified real samples by both methods are given in table 3.5.17. Formula used to calculate percent recovery was same as for fluroxypyr.

Table 3.5.17: Percent recovery of chloramben in real samples.

Name	Batch analysis	FIA
Tomato paste	88.0±0.03%	89.6±0.09
Corn	90.1±0.01%	96.0±0.10
Corn flour	90.0±0.01%	95.0±0.08

Results and discussion

The chemical reaction involved in this procedure is shown in figure 3.5.2. Chloramben have a primary aromatic amine group in the molecule. In this method the primary aromatic amine group was directly exploited for the formation of azo dye. In the first step chloramben was reacted with nitrite in acidic medium to form the diazotized product, which was subsequently coupled with coupling reagent β -naphthol at room temperature. An orange colored azo dye was the final product, which was measured spectrophotometrically for determination of chloramben.

After preliminary investigation it was observed that the proposed reaction is feasible for spectrophotometric determination of chloramben. Wavelength optimization studies were conducted from 450-520nm to find out the most suitable wavelength. It is clear from the results (table-3.5.1 and figure 3.5.3) that the azo dye has a maximum absorbance at 475nm which was subsequently used as optimum wavelength for further investigation of chloramben.

For diazotization reaction, nitrite concentration was optimized in the range 0.05-0.25% and it was observed (figure 3.5.4) that 0.2% was the optimum concentration of nitrite required for maximum diazotization. For volume optimization various volumes of 0.2% nitrite solution in the range of 1-8ml were used for the maximum formation of diazonium ion (table 3.5.3). It was observed (figure3.5.5) that 4ml of 0.2% of nitrite solution was sufficient for maximum diazotization and subsequently formation of azo dye.

The diazotization reaction takes place in acidic media, therefore the effect of acidity was investigated for maximum formation of diazonium ion. Optimum concentration of nitrite solution was prepared in HCl solution of varied acidity ranging from 0.12-0.6M. It can be seen from figure 3.5.6 that 0.4M HCl solution is the optimum molarity of acid required for maximum diazotization reaction.

For coupling reaction concentration of sodium hydroxide was optimized in the range of 2-12%. It was observed (figure3.5.7) that 6% of sodium hydroxide is the optimum concentration required for maximum formation of azo dye.

Concentration and volume of coupling reagent β -naphthol was optimized for the formation of maximum azo dye. It can be seen from figure 3.5.8 & 3.5.9 that 4ml of 0.1% β -naphthol was sufficient for maximum azo dye formation.

Time study was conducted for optimization of equilibration time for diazotization and coupling reactions. It was found that diazotization reaction is slow (figure3.5.10) and need 5 minutes for completion. Diazonium ion formed was stable after 15minutes at room temperature. While coupling reaction is fast (figure 3.5.11). The azo dye formed was stable up to 5 minutes and after that it slowly decomposed.

Optimization of parameters for online analysis

The chemical parameters optimized for batch analysis were directly used for online analysis. These optimized parameters were applied for online analysis using a double channel flow injection system. In this system a stream of nitrite solution and coupling reagent was continuously pumped and a volume of 1ml of chloramben sample was injected in reagent stream through a six-port injection valve. Diazotization reaction takes place in reactor R1 and coupling reaction take place in reactor R2. Absorbance of the resulting azo dye was measured at 475nm. Physical parameter like flow rate and reactor coil length were optimized.

The flow rate ranging from 0.5-6ml/minute was optimized for the online determination of chloramben (table 3.5.11). It was observed (figure 3.5.14) that with the increase in flow rate, the signal increased up to a flow rate of 2ml/min, and decreased at higher flow rates. A flow rate of 2ml/min was selected as the optimum flow rate. At higher flow rate absorbance decreases because the product comes out before the formation of diazonium ion and subsequently azo dye. The volume of sample injected was optimized in the range of 1-4ml. Figure 3.5.15 shows that with increases of sample volume absorbance decreases possibly because of insufficient time for diazotization reaction and peak broadening was also observed..

The coil length of reactor R1 and R2 were optimized in range between 20-100cm and 10-80cm respectively. It can be seen from figure 3.5.16 that 70cm is the optimum coil length for R1 and above this length no change in absorbance was observed. Figure 3.5.17 shows that maximum coupling reaction take place at a coil length (R2) of 50cm. Above this length absorbance was the same. It was further observed that coupling reaction is faster than diazotization reaction.

At optimum conditions, the effect of lower concentration of chloramben on absorbance behavior was investigated to calculate LOD and LOQ for the investigated method both for batch and FI analysis. The results are given in table 3.5.10 and 3.5.15 and are shown in figure 3.1.12 & 3.1.18 respectively. The LOD and LOQ were calculated for the investigated method and were found to be 0.01ppm and 0.04ppm for FI analysis and 0.05 ppm and 0.17 ppm for batch analysis respectively.

The relative standard deviation was also calculated both for online and batch analysis and was found to be 8.3% and 10.6% respectively. Molar absorptivity was calculated and found to be $2.45 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ and $2.64 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ for batch and online method respectively.

Application of the investigated method for the analysis of chloramben in commercial formulation and in real samples.

The investigated method was applied for the determination of chloramben in real samples.

The values for chloramben found in tomato, corn and corn flour were 0.12 ± 0.110 ppm, 0.10 ± 0.33 ppm, 0.09 ± 0.07 ppm for FI analysis and 0.13 ± 0.130 ppm, 0.103 ± 0.09 ppm, 0.1 ± 0.22 ppm for batch analysis (table 3.5.16) respectively.

The percent recovery from samples was also investigated by fortifying the sample with known concentration of standard chloramben. The results are shown in table 3.5.17 both for batch and FI analysis. The results of the % recovery varied from $88.0 \pm 0.03\%$ - $96.0 \pm 0.10\%$.

CONCLUSION

Five different spectrophotometric methods were developed for residue analysis and determination of active ingredients in commercial formulation one each for fluroxypyr, 2,4-D, acetochlor, bromoxynil and chloramben.

The method for fluroxypyr determination is based on direct exploitation of primary aromatic amine group for diazotization followed by formation of azo dye. Various analytical conditions like wavelength and reagent concentrations were optimized for batch analysis and applied directly for online analysis. Physical parameters like flow rate, diazotization coil length and coupling coil length were optimized for online analysis and were found to be 4.5ml/min, 60cm and 50 cm respectively. Using the optimized conditions a sampling frequency of 60 sample/hr was achieved for online analysis for fluroxypyr. The LOD and LOQ for the investigated method were found to be 0.05ppm and 0.17ppm for online and 0.073 ppm & 0.246ppm for batch method respectively. The method is applicable for determination of fluroxypyr in commercial formulation and for residue analysis. The results of both batch and online method are comparable.

Similarly spectrophotometric method has been investigated for the determination of acetochlor in formulation and for residue analysis. Acetochlor was hydrolyzed with 0.2M HCl by heating on a boiling water bath for 10 minutes. The hydrolyzed product (2-ethyl-6-methylaniline) was coupled with 10ml of diazotized aniline solution and the resulting azo dye was measured at 400nm. In case of online analysis, a single channel manifold was set at room temperature with a flow rate of 1ml/minute. By using the optimum conditions, a sample through put of 60sample/hr was achieved. The LOD and LOQ were found to be 0.40 ppb and 1.00ppb for online analysis and 0.9 ppb and 3.0 ppb for batch analysis respectively. The percent recovery ranges from 92%-95.1% for batch method and 93%-95.5% for online method.

Spectrophotometric method for 2,4-D determination is based on base hydrolysis of 2,4-D followed by the coupling of hydrolyzed product with diazotized sulfanilic acid solution. Various analytical conditions like concentration and ratio of nitrite and sulfanilic acid solutions were optimized for formation of diazotized sulfanilic acid and it was found that 1:10 ratio of 0.6% sulfanilic acid and 0.15% nitrite is sufficient for formation of

maximum diazotized sulfanilic acid. Molar absorptivity was calculated and found to be $1.09 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ and $2.39 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ for batch and online method respectively. The method was validated for batch and online analysis of 2,4-D determination in real samples. The method is simple, fast reliable and can be used for all type of phenoxy acetic acid herbicides. Online version has a sample through put of 90 samples / hr .

A new and simple spectrophotometric method was developed for the determination of bromoxynil by using two different coupling reagents like aniline and β -naphthol. The method is based on reduction of bromoxynil with 0.4M HCl at room temperature and diazotization of reduced product with nitrite solution followed by coupling with two coupling reagents. Reagent concentration and physical parameters were optimized for the investigated method by using both coupling reagents. Method is spontaneous when β -naphthol is used as coupling reagent and takes 12 minutes for complete formation of azo dye incase of aniline as coupling reagent. However, the sensitivity of method is higher incase of aniline. The method has been successfully applied for residue determination in real samples and active ingredient determination in formulations. Percent recovery was calculated and the result of batch and online method were compared . For online analysis sampling frequency was found to be 80 samples/hr.

The last method for chloramben determination is based on direct exploitation of primary aromatic amine to form a diazotized product with nitrite solution followed by coupling with a suitable coupling reagent β -naphthol. Chemical and physical parameters were optimized for the batch and online method. FI manifold was set at room temperature with a flow rate of 2ml/minute and diazotization coil length of 70cm and coupling coil length of 50cm. At optimum conditions a sampling frequency of 90 sample/hr was achieved. Molar absorptivity was calculated and found to be $2.45 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ and $2.64 \times 10^4 \text{ L.mol}^{-1}\text{cm}^{-1}$ for batch and method respectively. Percent recovery ranges from $88.0 \pm 0.03\%$ - 96.0 ± 0.10 . RSD was found to be 7.6% and 8.3% for batch and online method respectively.