AFRICA CENTER OF EXCELLENCE FOR WATER MANAGEMENT

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DETERMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN WATER, SEDIMENT, AND SOIL USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

By

STELLA JAMES

A Thesis Submitted to the African Centre of Excellence for Water Management (ACEWM) in Partial Fulfilment of the Requirement for Master's Degree in Water Management with Specialization in Water Quality Management

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STELLA JAMES

Advisor: Dr. Estifanos Ele Yaya

Co-Advisors: Dr. Feleke Zewge, Prof. Bhagwan Singh Chandravanshi

APPROVED BY BOARD OF EXAMINERS

This is to certify that we undersigned have examined this MSc thesis titled "**DETERMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN WATER, SEDIMENT, AND SOIL USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY** "and that in our opinion; it is fully adequate, in scope and quality, as an MSc thesis for the Degree of Master of Science in Water Management (Water Quality Management).

Advisor: Dr. Estifanos Ele Yaya

Signature _____ Date 08/09/21

Co-Advisor: Dr. Feleke Zewge

Signature _____ Date 08/09/21

Co-Advisor: Prof. Bhagwan Singh Chandravanshi

Signature _____ Date 08/09/21

Eternal Examiner: Dr. Eyobel Mulugeta

Signature _____ Date 08/09/2021

Internal Examiner: Dr. Andualem Mekonnen

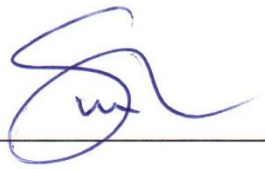
Signature _____ Date 08/09/21

Chairperson: Dr. Getachew Dagnew

Signature _____ Date 08/09/21

Statement of the author

I, Stella James, declare that this research report is my original effort and work and to the best of my knowledge, the findings have never been previously presented to the Addis Ababa University or elsewhere for the award of any academic qualification. Where assistance was sought, it has been accordingly acknowledged. The findings, interpretations, and conclusions expressed in this study neither reflect the views of Addis Ababa, African Centre of Excellence for Water Management (ACEWM), nor those of the individual members of the MSc Examination Committee.

Signature 

Date 02/09/2021

Dedicated

to

My grandmother Alice Imani.

For believing in me more than I believed in myself, for your support and love, and for all the times, you went out of your way to ensure that I received an education, even though you never had that opportunity yourself.

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ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide throughout the world to control broad-leaved herbs in various crops. Despite widespread use in a number of countries, studies have revealed that 2,4-D is a possible carcinogen and has a number of neurotoxic effects. The purpose of this study was to develop a method for extracting and determining 2,4-D acid from the soil, sediment, and water, as well as to investigate the compound's stability in water. The analysis was performed using high-performance liquid chromatography with UV detection at 230 nm. The performance of the developed analytical method was evaluated by extracting the analyte from spiked soil and water samples; recoveries ranged from 88 to 100%. A calibration curve for the method using known concentrations of 1, 10, 20, 40, 60, 80, and 100 mg/L of the analyte showed good linearity ($R^2 \geq 0.9996$). The LOD of the developed method was determined to be 0.45 µg/mL while the LOQ was 2 µg/mL. From the analysis of the samples, no 2,4-D was detected in sediment or soil samples from the Wafiko or Kontola sites, respectively. 2,4-D concentrations in soil samples from Bochessa and water samples from Wafiko and Sher site were generally high and exceeded USEPA regulatory agency standards. On the nature of the compound in water, a 45-day experiment on spiked water samples from Lake Koka demonstrated that the acidic form of 2,4-D is stable in water. An average recovery 73.46 ± 2.00% was achieved in this study. Thus, this study suggests that the developed method can be used to quantitatively extract 2,4-D residues and other chemical pollutants with similar physicochemical properties from contaminated samples originating from various sources.

KEYWORDS: 2,4-Dichlorophenoxyacetic acid, Lake Koka, Water, Soil, Sediment, High performance liquid chromatography

Table of Contents

List of Figures

List of Tables

ABBREVIATIONS AND ACRONYMS

2,4-D	2,4-Diphenoxyacetic Amin/Acid
ASD	Autism Spectrum Disorder
ATPPSC	Adami Tulu Pesticide Processing Share Company
CRV	Central Rift Valley
DAD	Diode Array Detector
DI-SPME	Direct-Immersion Extraction
DLLME	Dispersive Liquid-liquid Microextraction
EC	Electric Conductivity
ECD	Electron Capture Detection
EPA	Environmental Protection Agency
FRDME	Fiber Rotating Disk Microextraction
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
GPC	Gel Partition Chromatography
HF-LPME	Hollow-Fiber Liquid-Phase Microextraction
HPLC	High Performance Liquid Chromatography
HS-SPME	Headspace Extraction
LC	Liquid Chromatography
LLE	Liquid-liquid Extraction
MCLGs	Maximum Contaminant Level Goals
MCLs	Maximum Contaminant Level
MS	Mass Spectrometry
OCPs	organochlorines
ONRs	Oromia National Regional State
OPPs	Organophosphates
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RDSE	Rotating Disk Sorptive Extraction
SDME	Single-Drop Microextraction
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
UA-LLME	Ultrasound-Assisted Liquid-liquid Microextraction

US EPA	United States Environmental Protection Agency
WHO	World Health Organisation

1. INTRODUCTION

1.1 Background of the study

The environment is continually affected by agricultural practices such as extensive use of pesticides. In particular, water bodies located in agricultural catchment face more serious challenges due to drainage systems, runoff and rapid increase in the application of pesticides (Chau et al., 2015). The World Health Organization (henceforth, WHO) defines 'pesticides' as toxic substances released purposely into the environment to prevent, destroy or control pests. Substances in this category are designed to kill weeds (herbicides), insects (insecticides), fungus (fungicides), rodents (rodenticides), and other (WHO, 2020; Đurović-Pejčev & Orđević, 2012). In fact, Chen et al. (2018) reported that over two million tons of pesticides are applied annually across the globe as farming input.

In the environment, pesticides residues may undergo physicochemical and biotransformation processes. However, due to their persistence and solubility, they may remain in the environment for a long time (Gul et al., 2020). These pesticides and/or their residues can be transported into water bodies through surface runoff, agricultural drainage systems and spray drifts, among other means (Jergentz, 2005; Papadakis et al., 2018). A study that was conducted by Chau et al. (2015) in Mekong Delta, Vietnam, revealed heavy traces of more than a dozen different pesticides in harvested rainwater and bottled water above the internationally recommended levels (0.1 and 0.5 μg L^{-1}, respectively)**.**

Boström and Fogelfors (2002) have noted that of the agrochemicals utilized, herbicides are the most frequently used pesticides in many countries. One of the most widely used herbicides in cultivation today for broadleaf weed control is 2, 4-dichlorophenoxyacetic acid (2, 4-D). It is classified as a *phenoxyalkane* acid (Kashyap et al., 2005). The. The herbicide is used in cereals, sugar cane, fruit trees, farms, and forests to suppress weeds (Barba et al., 2019). Due to their high toxicity to dicotyledonous plants and low toxicity to monocotyledonous plants, 2, 4-D herbicides are often used as selective herbicides (Dehghani et al., 2014). Apart from their usage as herbicides, these compounds are often used in high-input farming as seedless fruit germination promoters, defoliating agents, and typically as hormone growth regulators (Kashyap et al., 2005). Kashyap et al. (2005) reported that 2, 4-D is usually used due to its limited persistence period, low cost, and well-understood aquatic dynamics.

Decades ago, herbicides were believed to be harmless to wildlife. However, 2, 4-D has been related to polychlorinated dibenzodioxins; commonly 2,3,7,8-tetrachloro-p-dibenzodioxins. This substance is a well-documented toxin, teratogen, and carcinogen (Dehghani et al., 2014). Dehghani and co-workers explained that the growing body of toxicological data shows that the herbicide itself can have cytotoxic effects on animals. Generally, 2, 4-D is both persistent and toxic, and also classified as an environmental endocrine disruptor and a carcinogenic substance. Additionally, the researchers stated that 2, 4-D has been frequently detected in surface or groundwater worldwide because of its poor biodegradability and volatility.

For several decades, the 2,4-D has been used and monitored. Thus, various chromatographic methods for the determination of 2,4-D in soil, water, cereals, fruits, and vegetables are available in the literature. Both Cheng et al. (2011) and Mnif et al. (2011) observe that due to their occurrence at trace levels and the complexity of environmental and food samples, the analysis of these pesticides requires selective and efficient sample preparation methods that can extract and pre-concentrate them simultaneously, prior to their instrumental determination (Pena-pereira et al., 2020). Due to its high sensitivity, most of these methods employ gas chromatography (GC) with an electron capture detector (ECD). In addition, mass spectrometric detection (MS), which possesses a high degree of selectivity, or even a combination of ECD and MS detection, can be used. However, because 2,4-D is not a volatile compound, it must be derivatized before GC analysis.

Alternatively, the detection limitation in 2,4-D acid analysis can be overcome by using high-performance liquid chromatography (HPLC) methods, which are more straightforward for determining acid herbicides and employing UV detection at 230 or 280 nm. Thus, this study aimed to optimize a method for determining 2,4-dichlorophenoxyacetic acid in water, sediments and soil samples, using HPLC and also monitor the levels of 2,4-D in Lake Ziway and the surrounding agricultural fields. By manipulating several parameters, this study suggested the most cost-effective, rapid, and environmentally friendly approach for detecting 2,4-D at trace level.

1.2 Problem statement

It is well known that a significant portion of pesticides applied in fields enters environmental components such as lakes, rivers, and oceans, and they may ultimately accumulate in plants and animals. Srivastava et al. (2010) stated that the accumulation of these pesticides brings possible harm to the environment. Therefore, assessing the different types of herbicides that runoff into rivers and reservoirs is essential. Chromatographic techniques such as liquid chromatography (LC), gas chromatography (GC), electrochemical sensors, and capillary electrophoresis have been used to determine 2,4-D (Luo et al., 2014; Merkle et al., 2015). However, the 2,4-D concentration in environmental samples is extremely low, and the matrix of some of these samples is complex, posing difficulties for quantitative analysis (Amani et al., 2011).

Currently, gas chromatography (GC) with electron capture detection (ECD) is the most frequently utilized and, in general, the most sensitive tool for measuring 2,4-D residues. However, the study of 2,4-D acid with GC has historically been challenging due to the herbicides' low volatility; in many cases requiring a derivatization step before analysis. Martín-Pozo et al. (2019) and Rezaee et al. (2006) claim that although HPLC is less sensitive than GC–ECD (nanogram vs. picogram levels); it is of advantageous over GC as it does not require derivatization of less volatile compounds during analysis.

Given the significant increase in 2,4-D usage in Ethiopia (Agmas & Adugna, 2020; Tamru et al., 2017), it is necessary to develop rapid, environmentally friendly, low-cost, and sensitive methods for monitoring the presence of 2,4-D in environmental samples. In addition, there is lack of data on the levels of 2,4-D acid in Ethiopia, making it impossible to take necessary measures. Thus, the purpose of this study was to develop a rapid, sensitive, and selective method for detecting 2,4-D acid at trace levels in the water, sediments and soil samples using HPLC. By altering several variables during pretreatment, this study proposes the most cost-effective methodology that is rapid, accurate, and examines materials at trace levels. Also, this new methodology is better than the GC approach in that it eliminates the need for a derivatization step before analysis.

1.3 Study objectives

1.3.1 General objective

The overall objective of this study was to develop a method for determining levels of 2,4-D in water, sediments and soil samples using high performance liquid chromatography and to determine the stability of 2,4-D in water.

i. To optimize HPLC and extraction conditions for quantitative determination of 2,4-D at trace level using HPLC.

ii. To determine the stability of 2,4-D in water.

iii. To determine the levels of 2,4-D in water, sediment and soil samples.

1.4 Research questions

i. What is the optimal condition for the quantitative determination of 2,4-D at trace levels?

ii. What is the stability of 2,4-D acid in water?

iii. What are the levels of 2,4-D in water, sediments and soil at locations where it is widely used?

1.5 Significance of the study

Pesticide-related health effects prove to be a greater obstacle for developing countries' agricultural practices. There is clear evidence that most pesticides are chronically unsafe and carcinogenic (Saraji et al.., 2014; Boström and Fogelfors, 2002). On the other side, they may be very toxic in the short term and play a major role in suicide attempts (Hammami et al., 2017). Despite convincing evidence that 2, 4-D is a widely used herbicide in Ethiopia, there is no information on the levels of 2, 4-dichlorophenoxyacetic acid in the environment that would allow for effective intervention.

The results of this investigation will therefore contribute to the development of a knowledge base that can help address the current information gap, include baseline evidence that the government will use to implement programs to mitigate the impact of 2, 4-D. Secondly, the findings in this paper will lay the groundwork for future toxicity and pathway research.

1.6. Scope and Limitation of the Study

The purpose of this study was to develop a method for determining the presence of 2,4-D acid in the water, sediment, and soil, as well as to investigate the nature of 2,4-D in water. Water, and sediment samples were collected from Wafiko and Sher site, soil samples were collected from Bochessa and Kontola site analyzed for 2,4-D. Seasonal variability of 2,4-D was not assessed in this study because it was beyond the studies scope. The study omitted the effect of physical parameters, while examining the nature of 2,4-D acid in the water because they were outside the scope. Within the above-mentioned scope, the study was conducted in a way that the findings are credible and of great importance to Ethiopia. The objectivity of the findings from this work provides the potential to have the methods repeatable and adaptable in various regions of the world.

2. LITERATURE REVIEW

2.1 Pesticides

2.1.1 General overview of pesticides

The environment contains complex chemicals and biological systems transmitted via natural processes or human activity from one particular environmental (atmosphere, hydrosphere, and lithosphere) compartment. Battaglin and Fairchild (2002) proposes that an excellent example of this would be water bodies which contain numerous compounds such as organic and inorganic pollutants, minerals, salts, and many others. These pollutants, according to Lin et al. (2011), originate from industrial wastes, agrochemical processes, municipal discharges, and natural processes such as the decay of living things.

Many chemical pesticides are used in various industrial and agricultural contexts including tree logging, railroad maintenance, control of insect infestation that transmits parasitic diseases to humans, and farming of crops infested by weeds and insects. In the WHO terminology, pesticides are toxic substances deliberately introduced into the environment to kill living pests (WHO, 2020). **Figure 1** illustrates a farmer spraying pesticides on crops. These substances are used for dealing with weeds, insects, fungi, rodents, and many more. The pesticides have both synthetic and natural varieties. For example, synthetic pesticides are created and developed in laboratories (Fournier et al., 2018). In contrast, natural pesticides are created by other species for their defense or are derived from a natural source such as a mineral or plant. Weeds are by far the most prevalent pests in agriculture in terms of the share of treatments used to control them (Obeng et al., 2018).

Figure 1: Farmer wearing a protective suit while spraying pesticides (Pesticidewise, 2016)

The world's population is growing, and a large population means that more food and food products will be required. Because new agricultural areas are currently limited, it is critical that the farming areas already in use be significantly improved. Chen et al. (2018) reported that over two million tons of pesticides are being applied annually across the globe as a farming input. While pesticides are still commonly used in most developing countries, such practices pose increasing environmental problems that can cause water contamination, ecosystems' disruption and natural habitats' contamination (Marquis, 2013). Large amounts of pesticides are used on farmlands; these pesticides end up in a surface runoff, which is then carried to nearby water reservoirs, where they either leach into groundwater or evaporate into the atmosphere (Mormeta, 2017). Residues of such compounds may be a significant source of environmental pollution, found in the soil on which the crop was grown, in the atmosphere, and or in runoff water that results from heavy rain or irrigation.

Pesticides, in general, can be hazardous, especially to people who come into contact with them daily. However, due to the challenges that pests pose to crop production, farmers are becoming more interested in using pesticides in agricultural fields. However, most farmers in developing countries have little or no knowledge of pesticide safety and disposal measures. Studies done have revealed low to moderate levels of knowledge about pesticides (Sankoh et al., 2016), non-use of personal protective equipment (PPE) (Mormeta, 2017). To add, there is unsafe pesticide storage at home, improper disposal of empty pesticide containers (Mormeta, 2017; Sankoh et al., 2016), pesticide misuse, and relatively low knowledge about pesticide safety labels.

A study by Agmas & Adugna (2020) on farmers' safety practices in Ethiopia, revealed no PPE is usually used (Agmas & Adugna, 2020; Mormeta, 2017). **Figure 2** illustrates some of the unsafe measures' farmers use during pesticide application. The unsafe application and interaction with these agrochemicals have disastrous health consequences for farmers, chemical applicators on commercial farms, and small-holder farms (Marquis, 2013). These farming activities also harm local populations' health. For example, a heavily pesticide-applied field upstream may cause an aquatic environment downstream to become too polluted to support local organisms' lives.

In addition, pesticides have also been linked to various adverse health effects in humans, ranging from minor symptoms like headaches and nausea to more severe outcomes like cancers, reproductive damage, and endocrine disorders (Kasozi et al., 2006). Neural, ear, and eye damage and headaches, dizziness, nausea, vomiting, and bacterial poisoning can be devastating and sometimes life-threatening. Chronic effects on health may arise many years after exposure to the polluted environment or through food and water contamination with herbicides.

Figure 2: A farmer mixing potentially harmful pesticide using bare hands adapted from (Sankoh et al., 2016)

2.1.2 Classification of pesticides

Pesticides are chemical compounds that exhibit a wide variety of physical, biological, and chemical properties (Kim et al., 2012). These characteristics serve as descriptors for their classifications. The three most commonly used pesticide classifications are based on the chemical composition of the pesticide, the target pest species, and the mode of action. The sections below provide detailed information about pesticides classification based on their chemical composition, mode of action, and target pest.

2.1.2.1 Pesticide classification based on the chemical composition

Pesticides are classified chemically into four groups: organochlorines, organophosphates, organonitrogen, and pyrethroids (Islam et al., 2018).

2.1.2.1.1 Organochlorine pesticides

Pesticides containing organochlorines (OCPs) have been widely used throughout the world. This compound class includes DDT, dieldrin, chlordane, toxaphene, mirex, kepone, lindane, and benzene hexachloride (**Figure 3** shows some of the structures of OCPs). Due to their low volatility, extreme stability, and widespread use, OCPs have high persistence in the environment and organisms following application (Rashid et al., 2010). Due to their lipophilic nature, they accumulate in fatty tissues and bioaccumulate throughout the food chain (Zhou et al., 2009). OCP exposure can also occur through low-level food contamination.

Internationally, considerable attention has been paid to this class of substances in recent decades. This is because it was discovered that they are transported through the environment and that critical concentrations have been reached in some areas, although they were never produced or used. While many countries prohibited the use of OCPs in the 1970s and 1980s, several countries, most notably the United States, continued to do so after the ban was implemented (Obeng et al., 2018; Rashid et al., 2010).

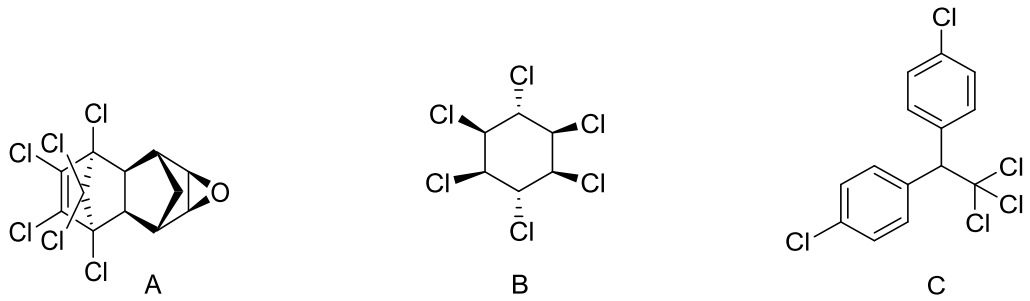
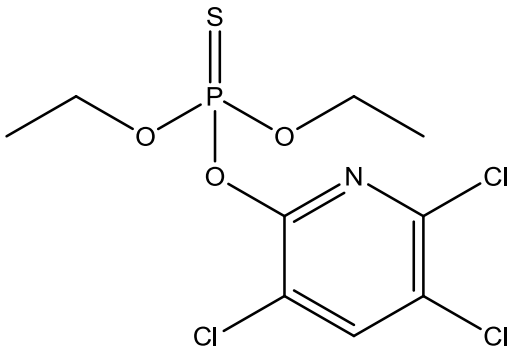


Figure 3:Structures of dieldrin (A), hexachlorocyclohexane (B) and DDT (C), organochlorine pesticides

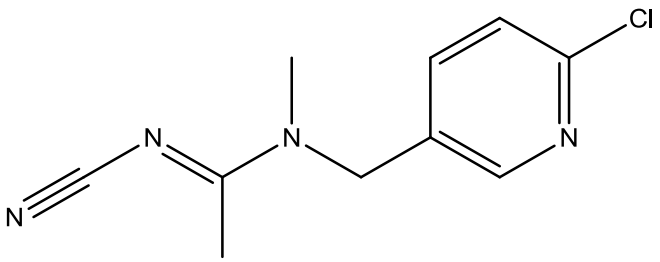
2.1.2.1.2 Organophosphate and pyrethroids

Organophosphates (OPPs), also known as phosphoric acid esters or thiophosphoric acid esters, were discovered in the 1930s and 1940s. Chlorpyrifos, parathion, diazinon, famphur, phorate, terbufos, and malathion are some of their examples (Russo et al., 2002). OPPs are less toxic to mammals than other pesticides, but they are toxic to certain organisms, such as insects. They can result in cancer, have a detrimental effect on the immune system, and disrupt hormonal functions (Garrido et al. 2010). There is a need to monitor these types of compounds in fatty matrices closely (Macirella et al., 2020; Papadakis et al., 2018).



Chlorpyrifos an example of an organophosphate

Pyrethroids are naturally occurring chemicals found in the pyrethrum, but they lose their insecticidal activity over time due to their inherent instability when exposed to light (Meng et al., 2021). Acetamiprid, lamda-cyhalothrin, imidacloprid, and deltamethrin are all examples of pesticides in this class. Even at low concentrations, these compounds are toxic to fish, aquatic arthropods, and honeybees (Jin et al., 2014). Even at low concentrations, repeated exposure increases the risk of anaphylaxis and allergic reactions (Huizhen Li et al., 2014; Papadakis et al., 2018; Wells & Yu, 2000).



Acetamiprid an example of pyrethroids

2.1.2.1.3 Organonitrogen pesticides

Organonitrogen pesticides (ONP) are a diverse class of chemicals. These plant protection agents are referred to by various names, including triazines, carbamates, and their derivatives (Chowdhury et al. 2012; Berg et al. 2002). Carbamates are typically used to control or eliminate insects. They degrade rapidly in the environment and are potentially harmful to humans and other organisms (Chowdhury et al., 2012). Carbamates are used to kill insects by affecting their brains and nervous systems as sprays or appetites. Long-term exposure to carbamates could lead to anorexia, weakness, loss of appetite, and general malaise. Carbamates can inactivate the enzyme Acetylcholinesterase in a reversible manner (Ortiz-

Hernández and Sánchez-Salinas 2010). It is unknown if they are cancer-causing in humans (Chowdhury et al., 2012).

Triazine herbicides (**Figure 4**) are widely used in weed control. They are moderately persistent in water and relatively mobile in soil. They are particularly susceptible to leaching into groundwater and runoff from the application site into surface waters due to their physicochemical properties. Water can become contaminated on the surface or in the ground due to its high solubility in water (Wang et al., 2010). Triazine herbicide has low acute toxicity for most species tested but has a more significant impact on birds than on mammals.

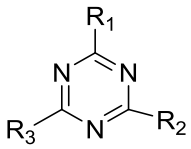


Figure 4: General structure of triazine

2.1.2.2 Pesticide classification based on the mode of action

2.1.2.2.1 Selective and non-selective pesticides

Selective pesticides kill target organisms while causing no harm to other species when applied to a mixed population. Selective herbicides, for example, are used in crop fields, lawns, gardens, and grasslands. Diggle et al. (2003) list selective herbicides examples used on cropland including 2,4-D, atrazine, trifluralin, alachlor, butachlor, fluchloralin, and pendimethalin.

Non-selective pesticides, on the other hand, kill pests indiscriminately. Non-selective herbicides control vegetation on industrial sites, fallow land, aquatics, and tennis courts (Diggle et al., 2003; Monaco et al., 2002).

2.1.2.2.2 Contact or systemic pesticides

A contact herbicide kills only the pest that comes into contact with it. For adequate control, therefore, consistent spray coverage and particle size are essential. Some common contact pesticides are paraquat, diquat, propanil, and petroleum oils (Garabrant & Philbert, 2002)**.** Systemic pesticides translocate widely from the point of absorption to the location of action in a vascular system of a plant. Glyphosate is an example of this pesticide class (Chu et al., 2006).

2.1.2.3 Pesticides classification based on the target pests

2.1.2.3.1 Herbicides

Herbicides are chemicals that are used to kill or stop weeds from growing (Brodowska, 2020). They are the most commonly used pesticides in the world, followed by insecticides and fungicides. Normally, they are used as pre-and post-emergence weed control in crops (Garabrant & Philbert, 2002). They can harm or kill plants, and they can be used to eliminate all vegetation. They contribute to global food security and play a significant economic role in various agricultural, horticultural, turf, and landscape management sectors. Weeds reduce crop yields dramatically by competing for light, water, and nutrients. Depending on the crop and region, yield losses have been reported from 30 percent to over 90 percent. In one extreme case, Venezuela's cassava crops failed 90 percent of the time in 1987 (Agmas & Adugna, 2020; Gul et al., 2020; Thiel et al., 2020). Weeds account for between 8% and 30% of all crop yield losses globally each year, although losses are usually minor. Weeds were also by far the most significant contributor to crop losses between 2001 and 2003, accounting for 34% of crop losses, ahead of animal pests (18%) and pathogens (16%) (Oerke, 2006). According to recent surveys conducted by the Weed Science Society of America, the total annual value of weed-related losses in corn crops in the United States between 2010 and 2015 was $27 billion and $16 billion, respectively (Abate Jote, 2019; S. S. Mohanty & Jena, 2019).

2.1.2.3.2 Insecticides

Insecticides are pesticides that are either chemical or biological (Agmas & Adugna, 2020; Papadakis et al., 2018). Control may entail killing the insect or preventing it from engaging in destructive behaviors. Insecticides can be natural or synthetic, and they are used to control pests in a variety of formulations and delivery systems. Sparks and Nauen (2015) recognize the impact of the science of biotechnology. They stipulate that, in recent years, incorporated bacterial genes coding for insecticidal proteins into various crop plants have compacted death to unsuspecting pests that feed on them. Insecticides are a broad category of pesticides, including Malathion, Mercarbam, DDT, Aldicarb, Carbofuran, Pyrethrum, and Allethrin (Chu et al., 2006; Prado-lu, 2015).

2.1.2.3.3 Fungicides

Fungicides are chemical compounds or biological organisms for killing fungi, such as the mold fungus, which cause severe damage in agriculture, resulting in substantial yield, quality, and profit losses. The fertilizers can be applied directly to soil or sprayed onto agricultural

fields (Agmas & Adugna, 2020). Fungicides have proven successful against a vast variety of pathogenic fungi. Fungicides used to treat a wide range of fungi infections include sulfur, Mancozeb, and Captan.

2.2 2, 4-Dichlorophenoxyacetic acid (2, 4-D)

2.2.1 General overview of 2,4-dichlophenoxyacetic acid

Dicholorophenoxyacetic acid (2, 4-D) is an organic compound with the chemical formula $C_8H_6Cl_2O_3$ that belongs to chlorophenoxyacetic herbicides (**Figure 5)**. 2,4-D is a commonly used herbicide to combat broad-leaved weeds and other plants on rangelands, lawns, golf courses, forests, roadways, and parks (Amani et al., 2011; Wells & Yu, 2000). It is also used as a preservative on turfs, aquatic sites, forestry sites, and many other areas (Amani et al., 2011). An ether bond connects a substituted aromatic ring to a carboxylic acid residue in this compound. Carbon–chlorine (C–Cl) bonds and carbon–methyl group bonds in the aromatic ring are critical structural elements that influence phenoxy acid reactivity and lipophilicity. The C–Cl bond is highly stable due to the coupling of chlorine atom electrons with π-electrons of the aromatic ring (Brodowska, 2020; Jin et al., 2014).

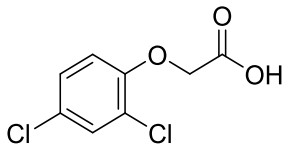


Figure 5: 2,4-Dichlorophenoxyacetic acid

2,4-D was the first commercial herbicide introduced to prevent broadleaf weeds in the 1940s. It appears to be one of the most widely used herbicides globally due to its low cost, selectivity, efficacy, and broad weed control range. Additionally, its high solubility in water and other solvents makes it quickly penetrable into roots or leaves (Islam et al., 2018). Thus, it is considered a more potent herbicide. Furthermore, 2,4-D controls plant growth by acting as an auxin imitation, facilitates cell division and elongation, and extends fruit shelf life at low concentrations (Abate Jote, 2019; Garabrant & Philbert, 2002).

Zhou (2020) suggests that 2,4-D contains esters, acids, and different salts with a wide range of chemical properties, environmental behavior, and toxicity. The parent acid molecule consists of 2,4-D salts and esters (**Figure 6)**. The most widely used forms are dimethylamine salt (DMA) and the 2-ethylhexyl ester (EHE), with global use of approximately 90% to 95% (De Amarante et al., 2003; Wu et al., 2010). About 1500 herbicide drugs use 2,4-D as an

active ingredient, and 2,4-D has been used as a bioweapon in warfare before. An infamous herbicide, Agent Orange, was used by US military forces in Vietnam during the Vietnam War with the double intention to defoliate forest areas that might conceal enemy forces while also destroying crops to starve the enemy forces. Agent Orange was mainly composed of equal amounts of the unpurified butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Islam et al., 2018).

Figure 6: Structures of 2, 4-D methyl ester (A) and 2, 4-D dimethyl amine salt (B)

2,4-D does not break down quickly. It has a half-life ($t_{\frac{1}{2}}$) ranging between 20 and 312 days depending on the environmental conditions (Ordaz-Guillén et al., 2014). To ensure the herbicide's full effect, it is applied directly to the surface or sprayed over fields (Sharma et al., 2019). Due to its low adsorption coefficient (the rate at which surfactant molecules are adsorbed at the surface) and high solubility in water, 2,4-D has frequently been found in surface and groundwater, posing a significant environmental threat and health risk (Gaultier et al., 2008; Kearns et al., 2014; Shareef and Shaw, 2008). Over 90 percent of 2,4-D enters the environment through water bodies (Mountassif et al., 2008).

Zabaloy and Gómez (2014) expressed concern when they explained that exposure to 2,4-D endangers the plants and animals that are subjected to it. Unfortunately, they continued, 2,4-D is toxic to many non-target organisms because it has the potential to slow growth rates, cause reproductive abnormalities, change appearance or behavior patterns, and even decimate non-target species such as plants, animals, and microorganisms. Even at trace levels, it can act as endocrine disruptors to developmental processes (Pattanasupong et al., 2004).

2.2.2 Global pesticide and 2,4-D usage

2,4-D is a high production volume herbicide used as illustrated in **Figure 7** on livestock at a 46-million-pound average; 66 percent of this is used on livestock, 23 percent on pasture, and 11 percent on residential uses (RED, 2005). 2,4-D's overall demand has snowballed by over 40 percent between 2002 and 2011. The United States of America, South America, Europe, and Russia are the main markets for herbicides (USDA, 2014). The product was used in the

US at a rate of over 15,000 tons per annum, becoming the third most widely used pesticide in 2001 (Abate Jote, 2019). In the United Kingdom, it was the seventh most frequently used herbicide on prairie and fodder crop production in 1992, and the eighteenth used herbicide on fresh fruit orchards (Chu et al., 2006; Vashisht et al., 2020). According to Zhang et al. (2010), China utilizes approximately 5,000–8,000 tons of 2,4-D butyl ester to combat weeds in wheat, soybean, corn, and other crops every year.

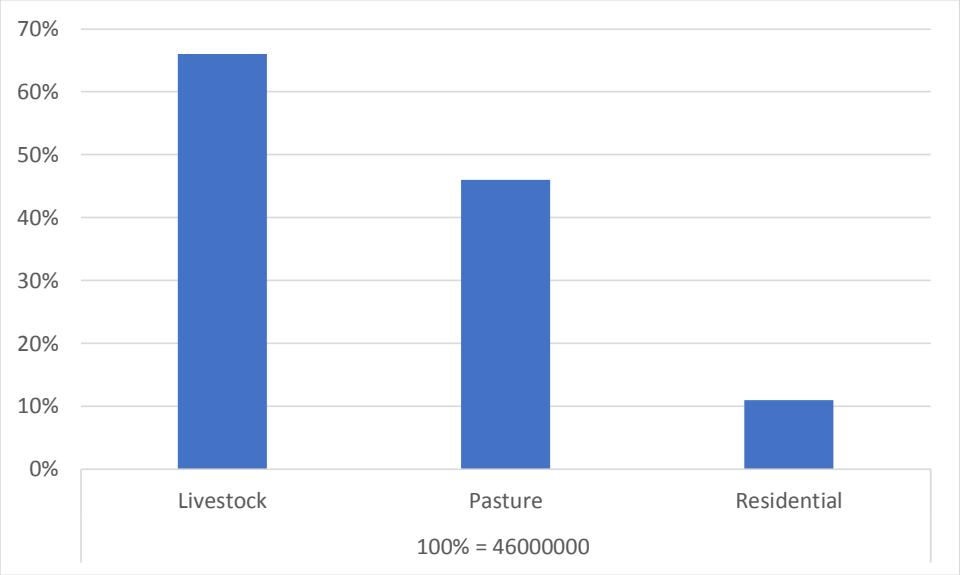


Figure 7: Global 2,4-D usage in a variety of applications (Vashisht et al., 2020)

Furthermore, 2,4-D is widely used in developing countries as shown in **Figure 8** like India, which consumed 1,300 tons of the chemical in 1994 (Thiel et al., 2020). According to (Merini et al., 2007), the approximate number of 2,4-D herbicides applied to crops in Argentina each year is 2,200 tons. This rapid increase in the use of 2,4-D has occurred in tandem with a recent rise in the number of harmful weed infestations. When it is agreed on, using crops that are tolerant to 2,4-D will likely result in 2,4-D getting into the environment and affecting the agroecosystem and the climate (Fournier et al., 2018).

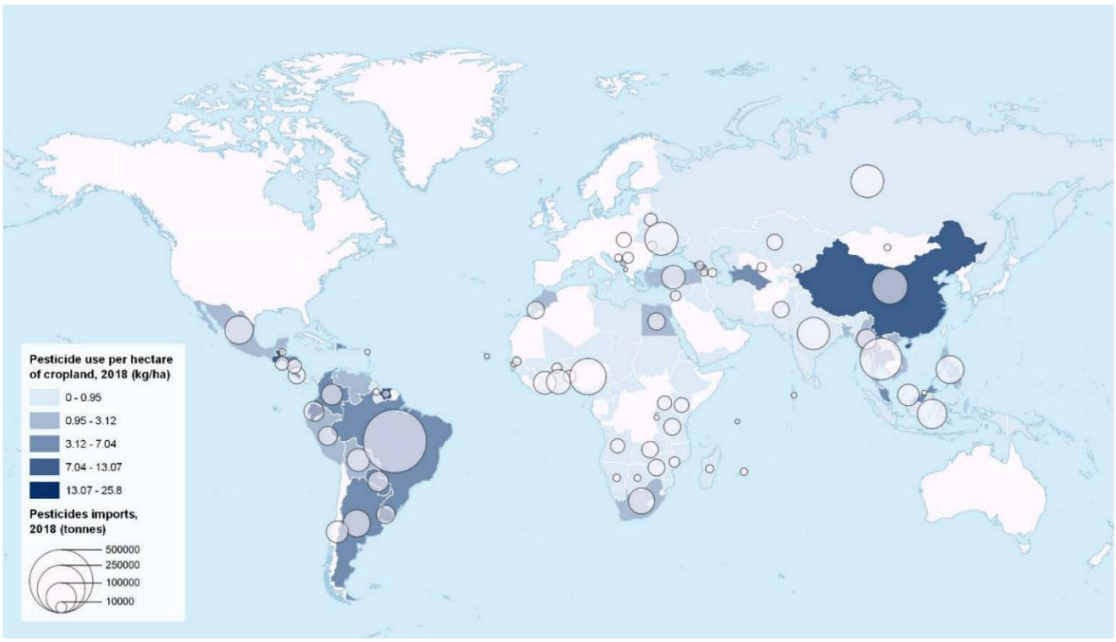


Figure 8: Scale of pesticide use, selected developing countries (FAO, 2018).

2.2.3 Pesticide and 2,4-D usage in Africa

Africa's economy is primarily reliant on agricultural produce, with approximately 59 percent of the population relying on agriculture for consumption and income generation (Abate, van Huis, & Ampofo, 2000). Surprisingly, the African continent contributes about 4% of the global market share of pesticides. This is the lowest percentage of pesticide usage globally (Abate, van Huis, & Ampofo, 2000). The projected growth in the continent's population and demand for food will necessitate increased food production over the coming decades. In turn, the use of pesticides for agricultural practices is likely to increase (Obeng et al., 2018). Snyder and co-workers (2020) study found that pesticide use decreased in the Congo and Mauritius, while it increased in Sudan, Malawi, Togo, and Rwanda between 2010 and 2014.

In Africa, a lack of awareness about pesticides handling has led to the careless application of pesticides that fall under the WHO risk classification system. According to the Pesticide Risk Reduction Program (Tamru et al., 2017), in Ethiopia, 160 out of 302 registered pesticides contain active chemical compounds classified as class II (moderately hazardous) chemicals by WHO (Agmas & Adugna, 2020). According to Obopile et al. (2008), about 50 percent of farmers in Botswana use Cypermethrin and Malathion as pesticides (Class II chemicals). A similar study discovered that in Botswana, Methomyl was used by 7.1 percent of farmers, Demeton-S-methyl was used by 2.7 percent, and Dichlorvos

was used by 1.8 percent. These chemicals are classified as Class Ib (highly hazardous) pesticides.

Oluwole and Cheke (2009) found out that majority of farmers in Nigeria use Monocrotophos (WHO Class Ib chemical). The study specified that Atrazine and Metolachlor are commonly used. These are classified as WHO class III chemicals (slightly hazardous). However, other pesticides in common use in Nigeria fell under WHO Class II. A research study from two sub-Saharan African countries revealed that over 41 percent of farmers in Zambia use Monocrotophos (Class Ib), whereas, in Malawi, Parathion (Class Ia) is used by over a quarter of farmers (Nyirenda et al., 2011). Reporting on West African countries like Benin, Ghana, and Senegal, Williamson, Ball, & Pretty (2008) complained about the frequent use of various Class II and Class III pesticides.

Harmful levels of pesticide ingredient toxicity are classified according to the WHO recommended classifications. These classifications are based on an investigation into the amount of each ingredient that causes toxicity in various organs and other areas of the body (Sharma et al., 2019). In addition to the health hazards pesticides can pose to humans, chronic toxicity has also been taken into consideration. **Table 1** provides a summary of WHO classifications based on Acute Toxicity Hazard Categories from the Globally Harmonized System (GHS). In order to manage risks associated with hazardous chemicals, Hazard (2019) notes, GHS has both classified the hazards of those chemicals and communicated information about the potential health and safety issues associated with their labels and safety data sheets.

Table 1: The WHO Recommended Classification of Pesticides by Hazard, 2019

		LD_{50} for the rat (mg/kg body weight)	
Class		**Oral**	**Dermal**
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–2000	200–2000
III	Slightly hazardous	Over 2000	Over 2000
U	Unlikely to present acute hazard	5000 or higher	

There is a risk that the pest population will become resistant to pesticides resulting in crop failure, regardless of how carefully the pesticides are applied (Kung et al., 2019). Some farmers in the western part of Africa claim that pyrethroid pesticides have caused a rise in tomato bollworm and diamond black moth resistance (Ryu et al., 2020). In addition, aphids exhibit resistance to pyrethroid and organophosphate pesticides; on top of resistance to these two pesticides, whiteflies have developed resistance to neonicotinoid pesticides (Houndete et al., 2010).

2.2.4 Pesticides and 2,4-D usage in Ethiopia

Scholars argue that despite the compelling personal health consequences pesticides may pose, pesticides' benefits outweigh their risks, hence, the increase in their use (Sharma et al., 2019). In Ethiopia, the second most populous nation in Africa, with 85 percent of its population (currently estimated to be 114 million individuals) living in rural areas, the agricultural sector is necessary for both personal needs and as a source of employment. Currently, the agricultural sector accounts for 47 percent of the country's Gross National Product (Ethiopian Economy, 2015). Agriculture production has seen a significant increase over the last decade.

Specifically, there have been emerging farming systems in Ethiopia, whose goal is to increase crop production. The purpose is, according to Tamru et al. (2017), to rectify Ethiopia's persistent food security challenge and increase national income through the export of agricultural products such as cut flowers and vegetables. As a result of Ethiopia's agricultural development policies, the use of inorganic fertilizers and chemical pesticides has increased as a means of increasing agricultural output (Mormeta, 2017). For instance, there is still an upward trend in pesticide use attributed to farming. Such an increase has also been observed in the areas surrounding Lake Tana and Lake Ziway, which are densely populated and have a variety of agricultural activities. Areas around these Lakes cultivate crops three times a year.

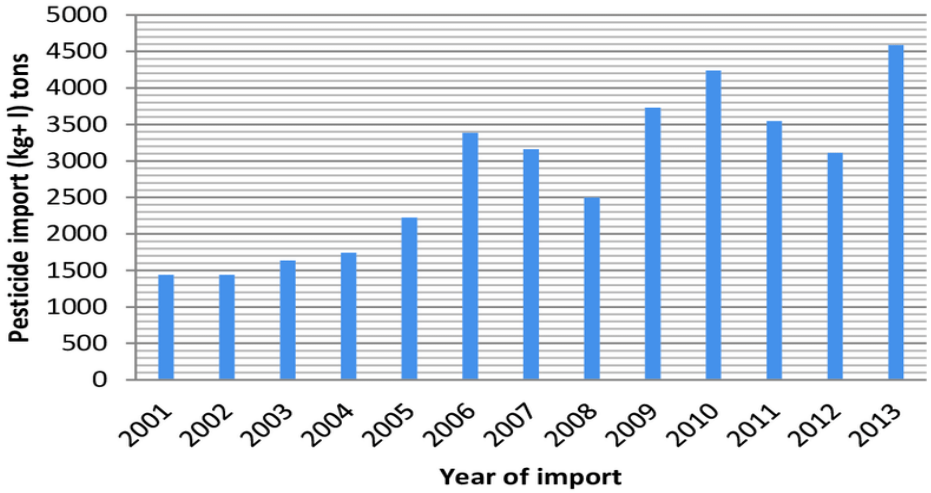


Figure 9: Trends in annual amount of pesticide import in Ethiopia from 2001 to 2013 (Adapted from Mormeta, 2017).

Accordingly, high levels of pesticides have been discovered in the environment and fish foods. According to Birhan Agmas and Marsht Adugna (2020) and Mengistie et al. (2017), all farmers (approximately 100 percent) in Ethiopia use insecticides to kill insects, along with herbicides (96 percent) and fungicide chemicals (66 percent).

Pesticides' use is generally declining in developed countries in response to their adverse effects on the environment. However, the opposite is true to developing countries (Sadasivaiah et al., 2007; Thompson et al., 2017; WHO, 2007). Pesticide contamination of the environment (soil and fish) has been discovered in Ethiopia along the Rift Valley region (Deribe et al., 2011; Yohannes et al., 2013). Studies have also found vast quantities of pesticides applied into nearby natural ecosystems, such as ponds, lakes, and rivers. Here, as reported, they accumulated by the different organisms living there (Srivastava et al., 2010).

2,4-D is a chronic and toxic compound frequently referred to as an endocrine disruptor and carcinogen. However, as a result of its low cost, it has been designated one of Ethiopia's most commonly used herbicides (Agmas & Adugna, 2020). Between 2010 and 2014, the Adami Tulu Pesticide Processing Share Company (ATPPSC), a state-owned entity, imported approximately 65 percent of all pesticide imports. Around 70% of its herbicide imports were 2-4-D, while nearly 100% of its herbicide imports in 2015 were 2-4-D (Tamru et al., 2017).

2.2.5 2,4-D in the environment

Technological advances have led to improved modern agricultural practices, which have increased the global food supply (Zhang et al., 2008). However, these advances have

also degraded the environment; for example, improper pesticide use leads to pollution (Amiri et al., 2018). It is noted that certain herbicides persist in the environment and harm species that are not actual targets. Unfortunately, minimizing the use of these agrochemicals without reducing crop yields is unfeasible. FAO and WHO project that global agricultural demand will increase by more than 70 percent by the year 2050 (McLaughlin and Kinzelbach, 2015; FAO, 2006). According to Soloneski et al. (2016), an estimate of less than 0.1 percent of pesticides used on crops worldwide achieve their intended goals, resulting in an influx of chemical residues that are free to enter the atmosphere. Consequently, agrochemical contamination of food, water, and air has emerged as a severe threat to human and ecosystem health. Pesticides residues in natural water during and after field application constitute a significant source of concern for the environment (Bilal et al., 2019; Rasheed et al., 2019).

Almost all of the 2,4-D pollution in the environment can be attributed to two things: emissions from the factories that produce and ship the herbicide and runoff from the areas where the herbicide is used (Brito et al., 2020; Chu et al., 2006) (**Figure 10**). Another possible source of 2,4-D in the surface water is pollution from point sources such as faulty spray equipment, sewage treatment plants, leaking waste tanks, and washing. 2,4-D's esters and amines are acidic carboxyl groups with pK_a of 2.8 and have low soil adsorption. This enables them to move quickly in aquatic environments, which also accounts for the ubiquitous presence of 2,4-D in these ecosystems (Atamaniuk et al., 2013; Borges et al., 2004). The maximum concentration of 2,4-D allowed in drinking water is 0.1 μg L^{-1} for individual pesticides (Nalcaci et al., 2006; Salman and Hameed, 2010).

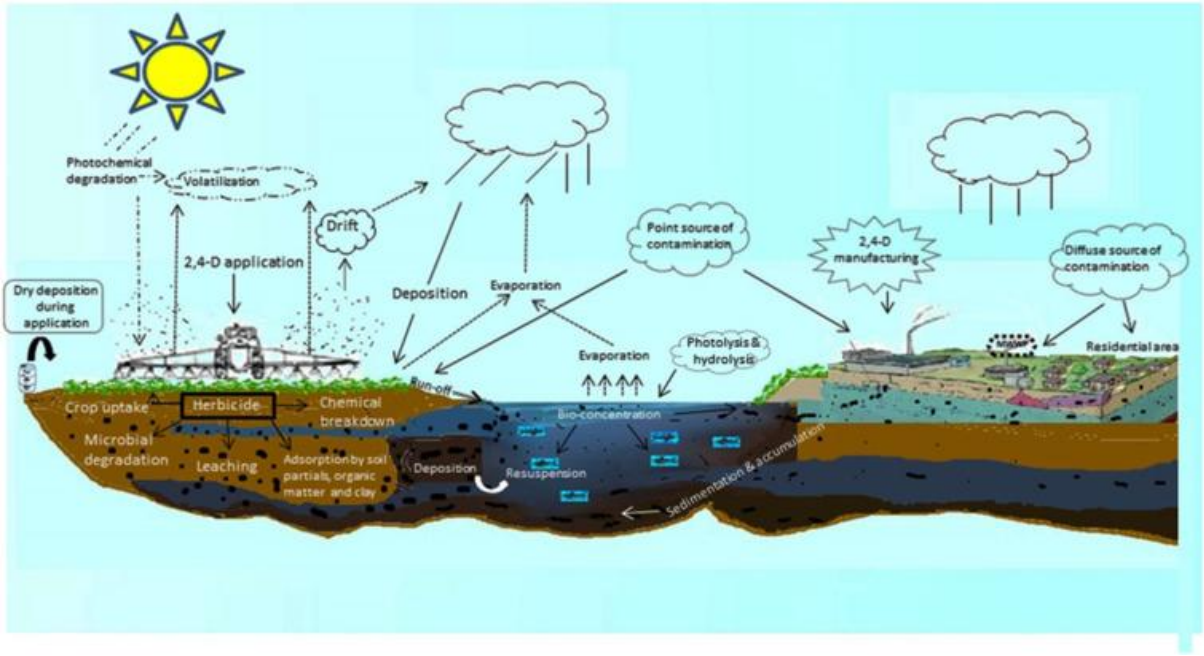


Figure 10: Fate of 2,4-D in the environment after agricultural and domestic application. (Adapted from Islam et al., 2018).

Various policies have been enacted in various countries around the world to protect the environment from toxins and toxic chemicals. For example, in 1974, the United States of America (USA) Congress approved the Safe Drinking Water Act. This legislation requires the Environmental Protection Agency (EPA) to establish appropriate levels for contaminants in drinking water that may or may not cause health problems. These non-enforceable limits are known as "maximum contaminant level goals" (MCLGs). MCLGs are solely concerned with expected health risks and exposure (Honghong Li et al., 2018). The MCLG for 2,4-D set by EPA is 70 µg/L. This is considered a safety standard that will not have any negative health effects.

The EPA defined an enforceable requirement known as a "maximum contaminant level" (MCL) based on MCLG. Chau et al. (2015) report that MCLs are set as close to the MCLGs as possible, taking into account public water systems' ability to identify and eliminate pollutants using proper treatment techniques. Further, the MCL is also set at 70 µg/L, which EPA upholds is the lowest level at which water supplies can plausibly be expected to minimize this pollutant if it exists in drinking water.

2.2.6 Accumulation and distribution of 2,4-D after application

Ding et al. (2000) reported that the highest 2,4-D pollution of fields and water bodies is expected to occur during each planting season since farmers use herbicides heavily during

this time to control weeds on their fields. Flooding fields after pesticide application can carry 2,4-D trace elements to surface waters via runoff. If farmers are unaware of the dangers of environmental pollution, residues from the application may be thrown away carelessly onto fields or surroundings, which, too, pose a substantial risk of environmental pollution. Thus, argue Ismail et al. (2015), water sources near cultivation areas are more susceptible to producing high levels of contaminants. The pre-treatment of 2,4-D for crop spray and subsequent cleaning of spray tanks are usually done in the same area, acting as an entry point of 2,4-D input into the environment.

2.2.7 Factors that permit long term persistence of 2,4-D in the environment

Fenner et al. (2013) warned that it is essential to have a comprehensive understanding of herbicide persistence, bioavailability, and degradation patterns to examine and measure the potential environmental risks posed by herbicides. These factors play an essential part in herbicide persistence, bioavailability, and degradation rates in the environment (Mercurio et al., 2016). It is possible that the long-term persistence of 2,4-D in the environment may be due to the limited capacity of the ecosystem microorganisms to metabolize the herbicide. In natural environments, microorganisms have more sources of carbon from which they can readily assimilate than organic compounds (Mercurio et al., 2016).

Seawater-grown bacterial communities are dependent on adaptation to a wide range of carbon sources. The level of concentration of a complex herbicide needs to be higher than the "utilization threshold" (1 to 100 μg L^{-1}) to facilitate specific enzymatic pathways to break down the complex herbicide. When other carbon sources are present, herbicide's bacterial metabolism is inefficient, so the concentration of most herbicides in the environment is relatively low (Mercurio et al., 2016).

As organic matter levels rise, report Zhang et al. (2010), the 2,4-D toxicity increases. Soil organic matter increases the adsorption of 2,4-D, resulting in less impact on soil biota. They further posit that a higher organic matter in soil microbial populations protects them from herbicide pollution.

Concentration of 2,4-D in effluents of wastewater treatment plants ranges from 83 to 110 mg L^{-1} (Chau et al., 2015; Geissen et al., 2015; Stefanakis & Becker, 2015). The approximate half-life of 2,4-D increases up to 5-fold times under light and sediment conditions compared to dark or without sediment. Light influences bacterial decomposition as it prevents them

from metabolizing the herbicides or changes the nature of other organic carbon in the system, making them more metabolically available (Mercurio et al., 2016). Mercurio and friends proposed that considerable data collected on herbicide persistence in sensitive environments can help explain the year-round and widespread 2,4-D presence in the environment.

2.2.8 General harmful effects of pesticides and 2,4-D

Many pesticide-related health issues range from temporary impacts like headaches and nausea to more persistent conditions such as cancer, reproductive damage, and endocrine disorders. Severe dangers include nerve damage, skin damage, and eye damage, headaches, dizziness, nausea, fatigue, and systemic poisoning. This may change dramatically, take a drastic turn for the worse, and may even lead to death (Garabrant & Philbert, 2002; Thiel et al., 2020). Even years after limited exposure to pesticides in the environment or through food and water contamination, serious health effects can arise. A study found that organochlorine pesticides increased the risk of developing autism spectrum disorders (ASD) by six-fold in children born to mothers who were exposed to them (Kasozi et al., 2006; California Public Health Institute, 2007).

For a long time, herbicides were considered to be innocuous to animals. However, 2,4-D has been associated with polychlorinated dibenzodioxins, particularly 2,3,7,8-tetrachloro-*p*-dibenzodioxins. This compound is a well-known toxicant, teratogen, and carcinogenic agent (Dehghani et al., 2014). Mounting toxicological evidence points to the fact that the herbicide itself may be associated with cytotoxic effects on animals.

2.2.9 Toxicity of 2,4-D in aquatic organisms

Pesticides may enter the aquatic system via the surface or subsurface hydrological pathways. This is usually triggered by spray drift, runoff water, and drainage water (Çipa & Kauffmann, 2018). Improper operations, such as the filling sprayers, cleaning measuring utilities, disposal of used pesticide containers and cleaning of spraying equipment, may also lead to polluting the environment. In Ethiopia, the pesticide sprayers mix or fill pesticides near to water sources, contaminating the water. Conversely, such practices impact the health of the surrounding ecosystems. Furthermore, chemicals applied to home lawns can seep into the sewers and find their way into streams, lakes, and rivers (Gerecke et al., 2002; Mekonen et al., 2016).

Fish are crucial to both aquatic ecosystems and various food webs because of their significant influences on several trophic levels and nutrient cycling. Fish have the ability to metabolize and accumulate agrochemicals efficiently. The *Rhamdia quelen* can absorb up to 30 percent of the 2,4-D that has been applied within 96 hours (Fonseca et al., 2008). 2,4-D exposure altered the metabolic rate of primary hepatic cells of *Metynnis roosevelti*, with results showing a significant change in cellular oxygen consumption and ammonium excretion (Salvo et al., 2015; Barbieri, 2009).

To add, in another species, significant histopathological modifications in the hepatopancreas, such as deformations in the tubule lumen and melanization of the gill lamella, were found with the label dose of 2,4-D in the copepod, *Astacus leptodacty*lus (Benli et al., 2016). Under certain conditions, a very low concentration of 2,4-D in the culture media may be a powerful decoupler of oxidative phosphorylation (Salvo et al., 2015). These alterations can occur regardless of the time of exposure to 2,4-D. deArcaute et al. (2016) presented an example that 2,4-D exposure to *Cnesterodon decemmaculatus* tends to cause nuclear abnormalities. The *Rhamdia quelen* has shown consistent abnormalities in its final weight, total body length, and rate of growth following long-term exposure to low levels of 2, 4-D, according to Menezes et al. (2015).

Again, the mucus layer is essential because it protects fish from parasites in the environment and protects fish from injury caused by water exposure. 2,4-D also affects the structure and morphology of the mucus layer by decreasing glucose levels and increasing protein content (Menezes et al., 2015). Moreover, Fonseca et al. (2008) specified that after being exposed to 2,4-D polluted water, fish exhibit glycogen depletion in the liver, muscles, kidney, and an increase in lactate level (Cattaneo et al., 2008; Menezes et al., 2015). So, the fish's immune system becomes weakened and is more vulnerable to disease (Menezes et al., 2015).

Lipid peroxidation in fish indicates oxidative damage. Phenoxyacetic acid herbicides are implicated in the production of lipid peroxidation in various fish tissues (Pretto et al., 2011; Fonseca et al., 2008). Lipid peroxidation causes genotoxicity at both the chromosomal and DNA levels in fish like *C. decemmaculatus* (de Arcaute et al., 2016). It is noted that 2,4-D damages DNA in fish cells. 2,4-D treated fish DNA breaks were observed in *C. batrachus* and *Oncorhynchus mikiss* using a comet assay (Ateeq et al., 2005; Martnez-Tabche et al., 2004). DNA break was also observed in fish cells derived from *Cyprinus carpio* (0.05–0.4 g

mL^{-1}) and *Metynnis roosevelti* (0.3–0.6 g mL^{-1}) when exposed to 2,4-D (2.0–10.0 g mL^{-1}) (Salvo et al., 2015).

Chemoreception is used by fish for various purposes, such as food searching, seeking a mate, swimming with a school, and avoiding predators (Fedotov, 2009). 2,4-D may result in significant behavioral and physiological changes in fish, with the most notable being changes in reduced responsiveness to mating cues and predator signals. This, in turn, may result in lower body weights and fitness (Browne Moore, 2014).

2.3 Analytical methods for the determination of 2, 4-D in the environment

Petrie et al. (2015) suggested that 2,4-D is easily dissolved in water and transported through the drainage systems, posing a high risk to aquatic organisms and humans. The presence of 2,4-D in wastewater, surface and groundwater, and drinking water has increased substantially, resulting in an unknown amount of parent compounds and transformation products. The determination of these compounds in water or wastewater has become a major scientific task that necessitates highly sophisticated analytical methods and techniques for detecting in nanograms per liter (ng L^{-1}) (Pena-pereira et al., 2020). Consequently, there is a clear need for in-depth information on 2,4-D from an analytical chemistry standpoint. The systemic analysis can provide information on the application of broad-ranging monitoring methods as well as the development of rapid and efficient evaluation methods used to determine these chemicals.

Ledoux (2011) acknowledge that 2,4-D residues are the subject of significant progress recently. Reliable methods to detect and quantify trace levels and amounts have been developed. A variety of reviews have highlighted the various 2,4-D residue analyzers in complex matrices of water, soil, and food with new methodology and application (Sherma, 2001; Beyer and Biziuk, 2008; Chung and Chen, 2011; Llorent-Martinez et al., 2011).

Currently, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are preferred over techniques for determination of 2,4-D, especially when utilizing mass spectrometry (MS) and tandem mass spectrometry (MS/MS) (Vashisht et al., 2020; Wu et al., 2010). The main advantages of GC and HPLC 2,4-D residue analysis are high sensitivity and high selectivity. However, sample pre-treatment steps have to be followed since the concentrations of analytes and samples are very low and have complex matrices (Dimpe & Nomngongo, 2016; Hu et al., 2015).

2.3.1 Sample preparations methods for the analysis of 2,4-D residues in environmental and food matrices

As part of preparations, the compound must be enriched before analysis. This is done to separate pesticide pollutants from the matrix (Koning et al. 2009). As stated previously, direct analysis of emerging pollutants in complex matrices is complicated because their concentrations are generally low and are connected with the sample matrix. Due to this, it is difficult to predict their environmental transport and fate. To overcome this situation, researchers must carefully examine the preparation step for samples. To prepare samples, pre-concentration, clean-up, filtration, extraction, and pH adjustment processes may be used (Rezaee et al., 2006). Recently, sample preparation methods are moving towards environmental friendliness, low cost, miniaturization, automation, and simplicity. Various pre-treatment techniques for systemic purification or concentration of 2,4-D have been developed and tested (Martín-Pozo et al., 2019; Wu et al., 2010).

Sample preparation is a necessary step in the analysis and is frequently a bottleneck in obtaining sensitive and accurate results for trace pollutant detection (Shegefti et al. 2009). For accurate pesticide measurements, the most effective extraction and pre-concentration procedure must be chosen. Dabiri et al. (2005) advises that sample preparation must be done. They explain that refers to the process of isolating components of interest from a sample matrix and concentrating them into forms suitable for the analytical procedure. This reduces other sample components that may interfere with the analysis. A dilution step improves the analysis's selectivity, sensitivity, reliability, accuracy, and reproducibility (Koning et al., 2009; Somenatb, 2009). The following are some reasons why sample preparation is an important step according to Shegefti et al. (2009):

a) The state of matter the sample is in is incompatible with the analytical method.
b) Other compounds present in the sample may interfere with the measurement results and produce incorrect or negative results for the target analyte.
c) The composition of the analyte in the sample is too low to be detected by conventional methods.

In order to meet these requirements, analytical methodologies should be capable of detecting residue concentrations at shallow levels while also presenting unequivocal evidence to prove the individuality and concentration of any detected residue. A lot of the work in separation science and related fields has been dedicated to the development of new sample preparation

techniques, which are faster, more effective, and which use fewer organic solvents (Gilar et al. 2001; Shegefti et al. 2009; Xiao et al. 2009).

2.3.2 Conventional sample preparation techniques

A wide range of conventional analytical techniques, including Soxhlet, liquid-liquid extraction (LLE), and (solid-phase extraction) SPE, have been used to selectively and quantitatively extract 2,4-D residues from environmental, biological, food, pharmaceutical, and other samples.

2.3.2.1 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a flexible traditional sample preparation technique. LLE is a separation method based on the difference in solubility of a compound in two immiscible solvents at an appropriate pH level (Amani et al., 2011) . LLE has been widely used to extract non-polar pesticides such as organochlorine and organophosphorous pesticides from water samples using organic solvents such as hexane and cyclohexane. Its application also tends to medium polarity organic compounds; carbamates, triazine. To add, urea pesticides have been successfully extracted using dichloromethane or chloroform (Matias et al., 2019). LLE has been used for other matrices including the analysis of multi-residue pesticides from fruits and vegetables (Kim et al., 2012). Farre (2013) give an example crayfish samples with methylene chloride as a multi-residue pesticides extraction.

LLE has significant advantages in trace analysis, such as toxic substance pre-concentration, simplicity, low cost, and compatibility with analytical systems. However, LLE has drawbacks of its own. For instance, Merkle et al. (2015) proves that the use of toxic and costly organic solvents, which may endanger the health of analytical laboratory staff as well as environmental safety, and the occurrence of emulsions, which may necessitate time-consuming extraction steps, are some. Moreover, Hu et al. (2015) clearly shows that the method itself takes time and frequently necessitates pre-concentration prior to analysis. Recently, however, researchers have focused on developing simplified, miniaturized, and improved sample pre-treatment and clean-up procedures that can modify or substitute this method.

LLE technique is one of the oldest well-explored sample preparation techniques that uses the immiscibility properties of organic solvents to partition target analytes from raw extract to the extractant (Hu et al., 2015; Merkle et al., 2015; Wu et al., 2010). It is a non-selective clean-up

method, and widely used solvents are ethyl acetate, hexane, isooctane, toluene, chloroform, and methylcyclohexane.

The use of large volumes of organic solvents, which are environmentally unfriendly, is the main drawback of LLE (Vashisht et al., 2020). It has been used for the extraction of pharmaceuticals and hormones in aquatic samples because of its simplicity and rapidity, but SPE is preferred over LLE simply because it can overcome LLE drawbacks. Despite its drawbacks, LLE has been recently reported for the extraction of emerging pollutants (Martín-Pozo et al., 2019; S. Mohanty, 2000; Vashisht et al., 2020).

2.3.2.2 Solid-phase extraction

Solid-phase extraction (SPE), developed between 1980 and 1990, is an alternative to LLE. The SPE process uses solid sorbent packed in a cartridge and a liquid sample flowing through the sorbent. Inorganic particles, polymeric compounds, and ion-exchange resins are commonly found in the solid phase. Different solid-phase extraction mechanisms rely on adsorption, partitioning, or ion exchange (Ye et al., 2007). The column sorbent chiefly influences the applicability of SPE. Nowadays, numerous sorbents are readily available, including chemically modified silica gel, polymer sorbents, and graphitized or porous carbon (wir-Ferenc and Biziuk 2006).

Ye et al. (2007) outlines two fundamental approaches to SPE. In the first approach, the analyte of interest is retained, and the matrix interferences are washed away. In the second approach, the analyte of interest is washed through, and the matrix interferences are retained. Many different types of SPE cartridges are available in various chemistries, adsorbents, and gauges. The key to effective sample selection is to know which products are appropriate for each application and sample.

The SPE procedure has a number of features that are quite appealing when compared to classical solvent extraction methods. SPE is simple to use, flexible, highly selective, fast, have higher enrichment factors and does not have emulsification and different sorbents. However, it has some limitations. Some of the most critical limitations of SPE are listed below. They represent criticism from Buszewski and Ligor (2002), Ye et al. (2007) and Żwir-Ferenc and Biziuk 2006).

a) As a result, materials such as heavy oils, animal oils, and vegetable oils, as well as solid materials of any kind in the sample, clogging of the pores may occur.

b) Despite the decrease in solvent consumption in SPE compared with LLE, SPE needs at least 100 µL of solvent.
c) SPE is a time-consuming method due to several operation steps, including; conditioning, sample loading, and elution.

The main disadvantages include using a significant number of organic solvents (still lower than LLE), disposable cartridges, and discs with a special manifold. However, researchers tend to overlook these limitations, such that other researchers such as Mogolodi and Nomngongo (2016) have reported on the application of SPE for pre-concentrating of different pollutants in environmental matrices. Also, new SPE materials have been developed as in fabric phase sorptive extraction (FPSE), a new device of very high sorbent loading in an ultra-thin coating (Kabir et al., 2017). These innovations represent new possibilities in the analysis of ECs in complex environmental samples including those of surface waters.

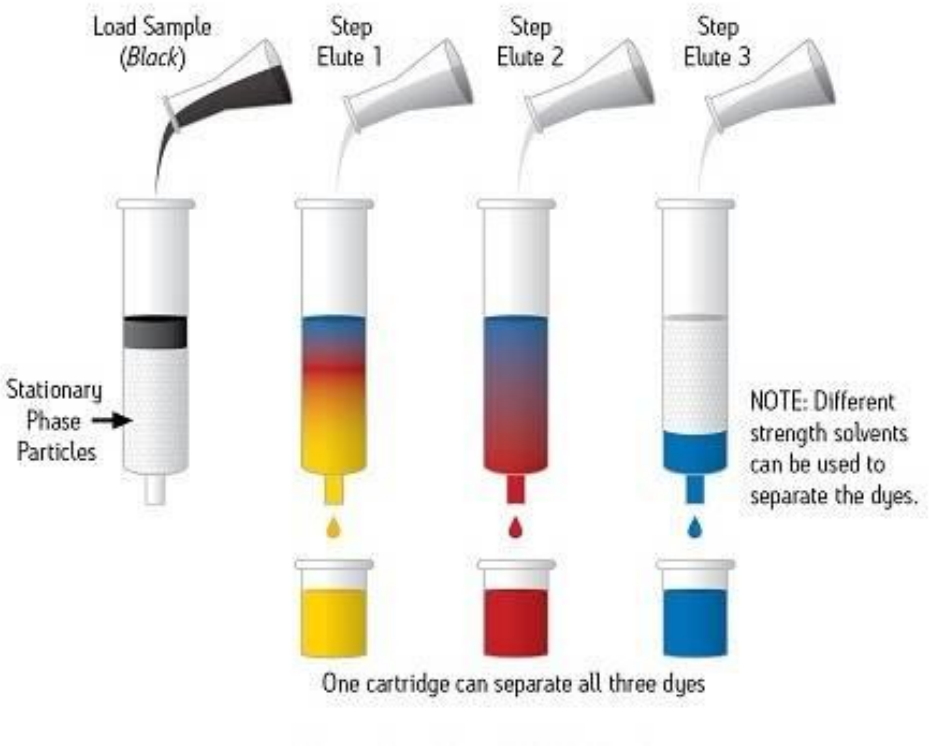


Figure 11: Solid-phase extraction steps adapted from https://www.waters.com/waters/de_DE/Solid-Phase-Extraction-SPE

2.3.2.3 Soxhlet extraction

In a conventional implementation of Soxhlet extraction (SE), a thimble is filled with condensed fresh extractant from a distillation flask (Martín-Pozo et al., 2019). As shown in **Figure 12**, once the liquid reaches the overflow level, a siphon is used to transfer the solution into a distillation flask, carrying the extracted analytes into the bulk liquid. The extraction is

repeated until complete. This performance makes Soxhlet a hybrid continuous-discontinuous technique. Extractant can be considered a batch system. However, extractant recirculation also leads to a continuous characteristic (López-Bascón and de Castro, 2019).

The advantages of conventional SE include the following:

a) Improved displacement of the mass transfer equilibrium, the sample is repeatedly placed in contact with fresh portions of the extractant. This creates a novel mass transfer equilibrium.
b) The system's temperature remains relatively high because some of the heat applied to the distillation flask achieves the extraction cavity.
c) Once the leaching step is complete, no filtration is required.
d) As the basic equipment is inexpensive, increased sample throughput can be obtained by running the process simultaneously while also extracting.

The following are the main drawbacks of SE as compared to other conventional techniques for solid sample preparation:

a) The lengthy extraction process and a large amount of extractant waste are not only expensive to dispose of but can also cause additional environmental issues.
b) Samples are typically extracted at the extractant's boiling point for an extended period of time, and the possibility of thermal decomposition of thermolabile compounds cannot be overlooked.
c) A Soxhlet extractor cannot provide agitation, which would increase the amount of time required for the extraction step.
d) After the extraction, it is necessary to perform an evaporation /or concentration step due to a large amount of solvent used.
e) The technique is only applicable to solvent selectivity and is therefore not easy to automate

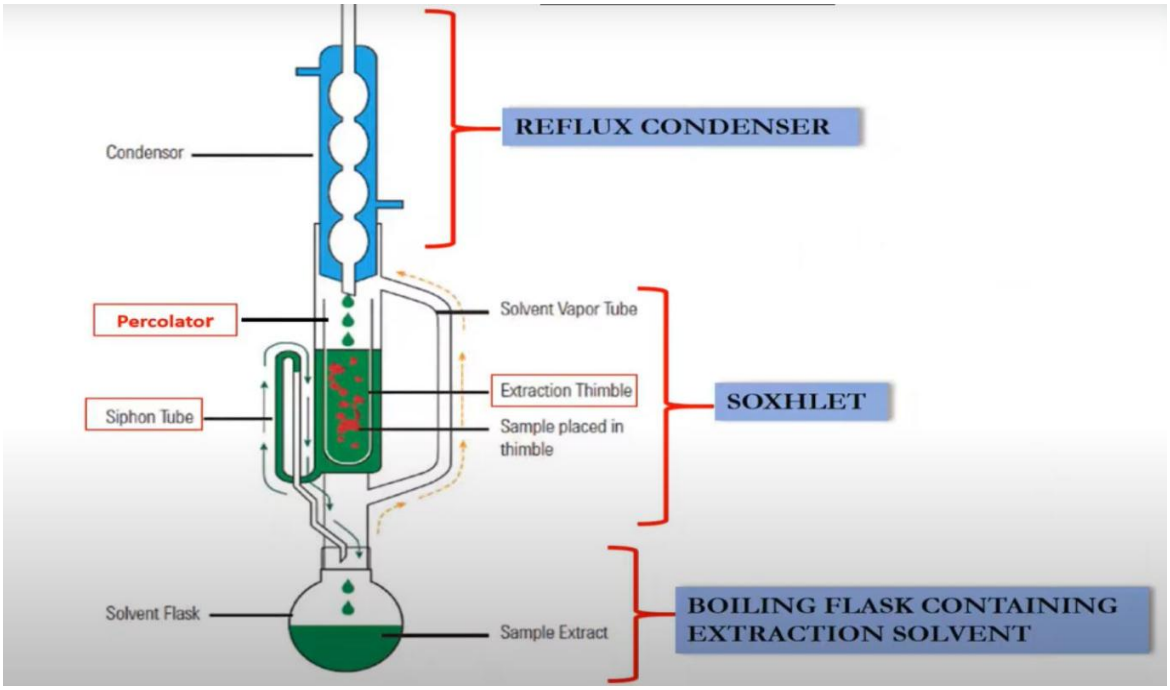


Figure 12: Soxhlet set up (Kashyap et al., 2005)

2.3.2.4 Modern sample preparation techniques for pesticides analysis

Studies in the last few years have placed increased emphasis on developing less expensive, yet effective, miniaturized and automated extraction techniques that could reduce the amount of toxic organic solvents consumed (Saraji et al. 2014; Tobiszewski et al. 2009). So, in order to reach this goal, dispersive liquid-liquid microextraction (DLLME), solid-phase microextraction (SPME), single-drop microextraction (SDME), and hollow-fiber liquid-phase microextraction (HF-LPME) techniques have been developed and are currently widely used as techniques of choice for effectively minimizing or avoiding the use of organic solvents in sample preparation procedures (Merdassa et al. 2015; Chen et al. 2010).

However, SPME is expensive, its fiber is fragile, and has a limited lifetime, which typically causes its effects to be carried over and to require sorbent conditioning for a long time (Peruga et al., 2013). Consequently, this method process can fail to meet expectations in SDME (Merdassa et al. 2015), and reproducibility can be adversely affected by the manual cutting of the membranes in HF-LPME (Bradney et al. 2017, Peruga et al. 2013).

2.3.2.5 Solid-phase microextraction

A microextraction technique is a sample preparation technique using a small amount of extraction phase (μL) compared to the sample volume (mL) (Martín-Pozo et al., 2019). Compared to SPE, it is a much more reliable and effective alternative extraction method. A system setup can be divided into dynamic and static techniques. A stirred sample mode

enables the static techniques to be performed. A thin film fiber microextraction, rotating disk sorptive extraction (RDSE), and fiber rotating disk microextraction (FRDME) are employed. Dynamic techniques, however, are referred to as tube, tip, and needle microextraction and capillary microextraction (Merkle et al., 2015). In SPME, we do not use solvents, so there is no danger of any damaging vapors. Also, Martín-Pozo et al., (2019) noted that SPME process does not require toxic or carcinogenic vapors since the SPME process does not utilize toxic or carcinogenic coating (Hinshaw, 2003) .

SPME is designed to adsorb analytes on a fiber that is coated with a stationary phase that was previously delivered using a syringe (Merkle et al., 2015). A partition coefficient between the fiber stationary phase and the sample has a major impact on the sensitivity of SPME. These techniques can be grouped into three distinct groups: headspace extraction (HS-SPME), direct-immersion extraction (DI-SPME), and membrane-protected SPME (Martín-Pozo et al. 2019).

According to Hinshaw (2003), the SPME is advantageous over other extraction methods in that it requires less manipulation and is not laborious. One of the primary advantages of SPME over other extraction methods is its efficient use of small sample volumes, saving valuable resources in the process. Other advantages of SPME are that it is able to obtain analyte concentration from liquid, gaseous, and solid samples. SPME is a solvent-free extraction technique that is very simple to automate for large-scale use. However, the principal drawback of this technique is that the polymeric extractant phase and the desorption process are major weaknesses.

This challenge can be resolved by using a hollow fiber membrane, which is made from a metal oxide-based membrane. In this method, a membrane is used as the adsorption material that integrates sampling, extraction, and pre-concentration into a single step (Martín-Pozo et al., 2019). A small sample preparation is supported by using solvent and instrumentation extraction techniques that have been developed to limit extraction and promote quick and simple sample preparation (Martín-Pozo et al., 2019). There is a new feature of SPME that is process automation, for example, automatic SPME-GC/MS for surface and groundwater pesticide determination.

2.3.2.6 Dispersive liquid-liquid microextraction (DLLME)

An extension to the existing work was introduced in 2003, including the implementation of a dispersive solid phase (micro) extraction (DSPE/DSPME) (Vashisht et al., 2020). It is an

approach in which a single sample extraction is carried out with a solid adsorbent, and after that, a clean-up process is performed. The solvent system has a ternary structure in which the actions of a second solvent have the effect of dispersing a small amount of extracting solvent. It is a promising and precise sample preparation technique that is better for the environment than other techniques, and it can be used to process a variety of environmental samples, such as food, water, and soil (Meng et al., 2021).

The overall liquid ratio in this process is a modified version of a standard solvent extraction method, where the acceptor to donor phase ratio is much lower than in other methods (Chen et al., 2010; Farajzadeh et al., 2009). To establish the ternary component solvent system, in which extraction and disperser solvents are rapidly introduced into the aqueous sample, DLLME utilizes a trace enrichment technique based on a ternary component solvent system in which the solvent extraction and dispersion solvents are utilized to create a cloudy solution. Zacharis et al. (2010) observed that extraction equilibrium is quickly reached because there are many surface contacts between the droplets of the extraction solvent and the aqueous sample solution. The stability of the tiny extraction droplets in the dispersed system depends on various characteristics of the emulsion interface, including surface electrical charge and Van der Waals forces.

It is important to remember that factors such as agitation speed, temperature, viscosity, and the presence of impurities have a major impact on the effectiveness of demulsification (Chen et al. 2010; Farajzadeh et al. 2009). After centrifugation, the extraction solvent is either sedimented at the bottom of the tube if the density of the extractant is higher than that of water or is floated to the top of the tube. This happens if the density of the extractant is lower than that of water. Then, it is taken with a microsyringe for chromatographic analysis. DLLME has various advantages, including its simplicity of use, rapidity, low cost, high recovery, and the usage of inexpensive and commonly available laboratory equipment and environmentally safe practices (Ma et al., 2012).

Vashisht et al. (2020) explained that dispersive solid-phase extraction (DSPE) is a highly developed form of SPE. However, DSPE forms the fraction of the so-called quick, easy, cheap, effective, rugged, and safe (QuEChERS). However, the extraction performance of DSPE chiefly dependent on the choice of the adsorbent materials. In DSPE, sorbents such as bonded silica, activated carbon, and primary, secondary amine are directly dispersed into the sample solution instead of being packed in SPE columns. Subsequent to the extraction process, the adsorbent-containing adsorbed analytes are separated by filtration or

centrifugation. This method allows the complete interaction of the sorbent and the sorbent particles, accomplishing a great capacity per amount of sorbent and avoiding the blockage of cartridges in traditional SPE. However, the main drawback of using DSPE/DSPME is the inability to change solvent between the extraction and pre-concentration steps.

The complete exposure of the sorbent to the sorbent particles can be achieved. This means there is a tremendous capacity for sorbent in relation to the amount of sorbent. Furthermore, the traditional SPE method does not involve this interaction. So, cartridges can never become blocked. Despite these advantages, DSPE/DSPME tends not to be a popular choice due to the fact that it is challenging to make a change in the solvent in the middle of the extraction and pre-concentration steps.

2.3.2.7 Hollow fiber liquid-phase microextraction (HF-LPME)

Liquid phase microextraction methods such as dispersive liquid-liquid microextraction (DLLME), ultrasound-assisted liquid-liquid microextraction (UA-LLME), and, among others, due to the advantages they offer compared with LLE techniques, have gained attention (Martín-Pozo et al., 2019). Liquid-phase microextraction (DLLME) is the low cost, significant reduction in acceptor volumetric and the specificities, clean-up samples, and high enhancement factor of hollow fiber liquid-phase microextraction are preferred (Ganjali et al., 2015).

The HF-LPME principles are of the same caliber as SPMEs. HF-LPME only requires the solvent to be inside the channel of the hollow fiber. Then, the solvent's pores are inserted into the channel by the aqueous solution (acceptor) (Ganjali et al., 2015). The analytes are usually extracted into an acceptor solution within hollow fiber pores by using the organic phase in the pore. While the HF-LPME process is relatively slow, there are no commercially available equipment pieces to utilize in this process (Merkle et al., 2015). Furthermore, HF bubbles are left on the surface. They have an effect of reducing the number of times the extract can be taken from the compound and the amount be transported per rate (Martín-Pozo et al., 2019).

2.3.3 Sample clean-up methods

Matrix constituents can be co-extracted and later co-eluted with the analytes they contain, and this can interfere with the identification and quantification of the analytes. In addition, compounds that have been co-extracted, particularly lipids, tend to adsorb in GC systems such as injection ports and columns, which reduces chromatographic performance (Amani, et al., 2011). By thoroughly cleaning up after any data acquisition, Amani and co-workers

observed that all matrix issues are avoided, sensitivity is improved, repeatability is increased, and the lifetime of the capillary column is increased. Several different methods have been sought to eliminate the compounds that have co-extracted in the extracts, including freezing centrifugation, liquid-liquid partitioning, gel permeation chromatography (GPC), and SPE.

A majority of the literature mentions the use of anhydrous sodium sulfate as part of a water removal process in the extraction solvent system, which generally occurs during clean-up procedures such as solvent partitioning or purification columns (as one of the layers of the column). However, a few authors used sodium sulfate from the grinding step during the extraction procedure to help break down the sample further (Kashyap et al., 2005).

2.3.4 Selection of extraction solvent

The selection of appropriate extraction solvent is the primary step in the optimization procedure. Generally, in liquid-liquid techniques, extraction solvent should meet the following requirements. It should have a different density with water, be insoluble in water, have high extraction capability for the target analytes and have good chromatographic behavior (Matsadiq et al. 2011; Wang 2016; Yang et al. 2012). However, the solvent should dissolve the analyte on Soxhlet extraction, not too volatile, have high extraction capability, not toxic, and have good chromatographic behavior.

Abuin et al. (2006) explained that volatile solvents such as hexane, benzene, ether, ethyl acetate, and dichloromethane are commonly used in liquid-liquid extraction semi-volatile compounds from water. Hexane is excellent for extracting nonpolar compounds such as aliphatic hydrocarbons, benzene is best for extracting aromatic compounds, and ether and ethyl acetate are perfect for extracting polar compounds (Kim et al., 2012). De Amarante et al. (2003) stated that dichloromethane extracts a wide variety of nonpolar to polar compounds effectively as it is easy to separate from the water and is non-flammable due to its higher specific gravity. Additionally, due to its low boiling point and ease of re-concentration following extraction, it is suitable for simultaneous analysis. However, like benzene, dichloromethane is carcinogenic (Dekant et al., 2021), and recent trends indicate that these solvents should be avoided when performing liquid-liquid extractions.

According to Maštovská & Lehotay, (2004), acetonitrile (MeCN), acetone, and ethyl acetate (EtOAc) are the three most commonly used extraction solvents for the isolation of multiple pesticide residues from produce, and each has been shown to provide acceptable recoveries

for a wide variety of pesticides. Compared to MeCN and acetone, EtOAc is practically insoluble in water (at 20 °C, only 7.94%, w/w, of water is soluble in EtOAc), which can be easily removed from EtOAc extracts using a drying agent (usually anhydrous Na_2SO_4) (Gilbert-López et al., 2009). However, acetone requires the addition of a non-polar solvent to achieve a distinct separation from the water phase, which results in dilution and possibly lower recoveries for more polar analytes. MeCN is approximately 1.4 and 1.7 times more expensive than comparable grades of EtOAc and acetone, respectively (Anastassiades et al., 2003; Maštovská & Lehotay, 2004; Truong et al., 2019).

Soxhlet extraction is carried out using nonpolar solvents such as benzene or dichloromethane, polar solvents such as methanol, or mixtures of polar and nonpolar solvents with boiling points close to ethanol/benzene or acetone/hexane (Martín-Pozo et al., 2019). Benzene is an excellent solvent for extracting polycyclic aromatic hydrocarbons (PAHs), whereas acetone effectively extracts sulfur-containing compounds. The results of a study conducted by Truong et al. (2019) indicated that different solvents resulted in a significant difference in extraction yield. Methanol had the highest extraction yield (33.2%), followed by distilled water (27.0%), ethanol (12.2%), acetone (8.6%), chloroform (7.2%), and dichloromethane (4.9%), indicating that highly polar solvents have a higher extraction efficiency.

2.3.5 Separation and determination

The majority of pesticides are highly toxic, and they contribute to thermal instability and low volatility. It is the reason why HPLC and GC employ derivatization reactions, lengthy treatment processes, and large quantities of organic solvents. GC has established itself as a reliable alternative method for pesticide residue analysis (Nannou, Boti, & Albanis, 2018). Water is used as a solvent for pesticides that can dissolve in water and require fewer organic solvents.

The drawback of this method is that it can only be used on pesticides that dissolve in water. HPLC is widely used due to its high sensitivity and selectivity, and it requires adequate sample preparation techniques to identify pesticide residues at trace levels. It is less eco-friendly than standard HPLC, which uses approximately 50 ml of organic solvent per sample. Processing samples can also take longer and be more precise than traditional HPLC due to the use of a diode array detector (DAD) (Wen, et al., 2015).

Before the advent of high-performance liquid chromatography (HPLC), gas chromatography (GC) analysis of 2,4-dichlorophenoxyacetic acid has been quite a challenge because GC analysis of the herbicides is complicated due to their low volatility. Currently, gas-liquid chromatography (GLC) with electron capture detection (ECD) is the most commonly used and, typically, the most sensitive method (a picogram level) for measuring 2,4-D residues. When conducting an investigation, a combination of analytical methods is used to achieve increased sensitivity. For increased sensitivity, the 2,4-D has to be transformed (derivatized), often to a methyl ester by reacting with BF3-methanol, diazomethane, or concentrated sulfuric acid-methanol (De Amarante et al., 2003; Kashyap et al., 2005).

3. EXPERIMENTAL

3.1 Study area

The study was conducted at Lake Ziway and Koka Dam in the Ethiopian Rift Valley's main rift valley. The Lake Ziway watershed, which is primarily comprised of smallholder agricultural lands, covers an area of 7032 km^2, ranging from latitudes 7° 22' 36"N to 8° 18' 21"N and longitudes 37° 53' 40"E to 39° 28' 9" E. (**Figure 13**). It stretches from the western border of the Gurage Mountains to the eastern border of the Arsi Mountains. It rises above 3500 m above sea level in two Ethiopian administrative regions, with 73.6 percent located in Oromia National Regional State (ONRS and the remainder in Southern Nation Nationalities and Citizens Regions (Mekonen, et al., 2016)

The lake is located 160 km south of Addis Ababa, just east of the ONRS town of Ziway. It covers approximately 434 km^2 and has a maximum depth of 9 m, a mean depth of 2.5 m, and a shoreline length of 137 km (Hengsdijk and Jansen, 2006). It is the highest upstream lake in Ethiopia's Central Rift Valley (CRV). The watershed's runoff enters the lake via two feeder channels, Katar and Meki. The retention time in the lake is approximately 1.5-2 years (Spliethoff et al., 2009).

The watershed's climate patterns are not uniform. It receives an average annual precipitation of 729.8 mm and 1227.7 mm, respectively, with an average annual temperature of 18.5 °C. Between June and September, the rainy season accounts for approximately 55% of annual precipitation, while the dry season accounts for 45% (Billi and Caparrini, 2006).

The lake is of freshwater quality and is a vital component of Ethiopia's Central Rift Valley region, serving as a source of water for closed and open field irrigation and the town of Batu's

source of drinking water. Additionally, it benefits the fishing community's livelihood. It provides shelter for wildlife such as fish, birds and mammals such as hippopotamuses. Numerous bird species are also protected by the marshes that surround it, which provide roosting areas for hundreds of cranes, herons, ducks, and geese, among others (Spliethoff et al., 2009). The Bulbula River is an outflow from Lake Ziway to the south, feeding the terminal lake, Lake Abijata. The north-south gradient of groundwater rises from Lake Ziway to feed Lakes Langano, Abijata, and Shala (Tenalem, 2001), all of which fall at lower altitudes, with Lake Shala as the final receiver.

Lake Koka is located in southern Ethiopia, at an elevation of 1590 m above sea level. It is a man-made lake formed when a concrete dam was built across the Awash River to generate hydroelectricity. There are two inflowing rivers into the lake: Awash (major) and Modjo (minor), which enter from the west, and one outlet river (Awash River) that exits from the east. The lake has a surface area of 220 km^2 and a maximum and mean depth of 14 and 9 m, respectively (Agmas & Adugna, 2020). Between mid-June and mid-September, the region's climate is characterized by a dramatic increase in precipitation and relative humidity, although mean monthly temperatures are less variable. The lake water conductivity was 251 S/cm at 25 °C and had a pH of 7.4 during the study period.

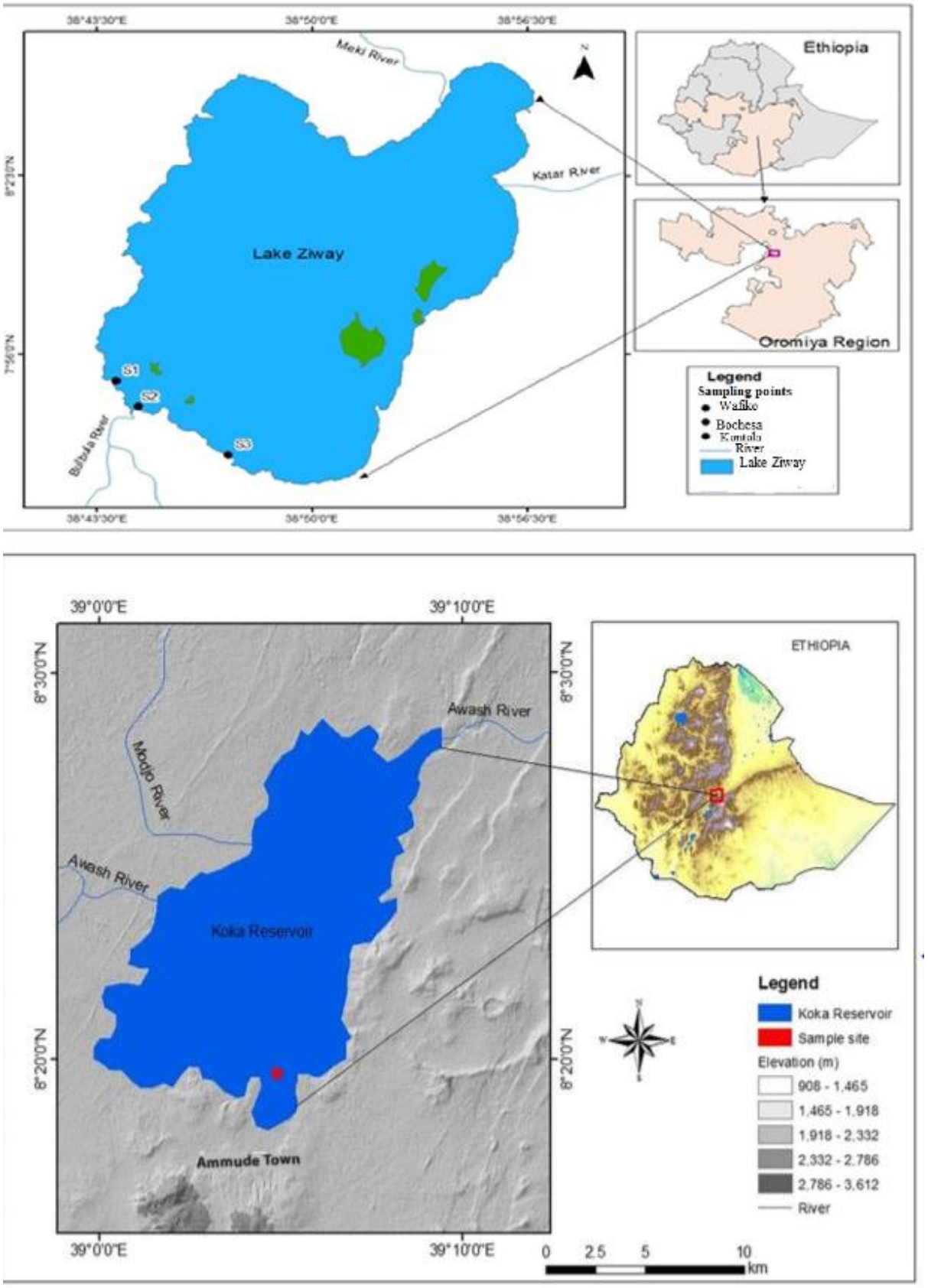


Figure 13: Location of Lake Ziway and Lake Koka (Sampling sites)

3.2 Sampling and sample preparation

3.2.1 Sampling

Sampling points were purposively chosen for the sampling of water, soil and sediment samples by taking in to consideration the areas where farmers apply the chemical. US EPA Method 8151A sampling procedure for water were followed. Water samples were collected from Koka Dam and Ziway Lake (Wafiko and Sher site) for method validation and 2,4-D monitoring, respectively. For 2-4, D monitoring, samples were collected in May at the following coordinates: 7° 22' 36"N and 8° 18' 21"N, and longitudes of 37° 53' 40"E and 39° 28' 9" E **(Figure 13)**. Three different points were sampled using a bottom water sampler, as illustrated in **Figure 14**. Six samples were collected in total, with one taken at the surface and another at a depth of 1m at each point. All water samples collected for this study were stored in 2 L amber bottles and transported to the organic chemistry laboratory at Addis Ababa University. Water samples not exposed to 2,4-D, for method validation were collected from Koka Dam.

Figure 13: Collection of water samples using a bottom water sampler

US EPA method 5053 for soil sampling was followed Three replicates (100 g) Soil samples were collected for method validation (spiking) at three different sampling locations in an agriculture field not exposed to 2,4-D herbicide at Addis Ababa University's College of Natural Sciences (Ethiopia). The samples were taken using an auger from the land surface to a depth of 15 cm. They were then placed in polyethylene soil sample bags and brought to the University of Addis Ababa's organic chemistry laboratory. Soil samples for 2,4-D monitoring were collected from Bochessa and kontola site at six different sampling locations in polyethylene bags from suspected 2,4-D-exposed agricultural fields near Lake Ziway. The samples were collected using an auger from the land surface to a depth of 10 and 15 cm at each point. Twelve 300-gram soil samples were collected and transported to the laboratory.

Sediment sampling was conducted following US EPA method 5035. Six sediment samples were collected using a sediment grab sampler from three different sampling points at Wafiko and Sher site. The samples were taken at the precise water sampling locations. They were then placed in polyethylene sample bags and transported to the organic chemistry laboratory at the University of Addis Ababa.

3.2.3 Sample preparation

Water samples were filtered through 0.45 cellulose acetate filter papers (0.45 μm, Micro Science and 110 mm Smith F1/KA4, Germany) for further analysis. After filtering the samples, the pH was immediately determined. Soil samples were air-dried for 24 hours, homogenized, sieved (150 mesh), and stored at room temperature until the analysis. Sediment samples were suspended in water and then water was then shaken vigorously for half an hour on a shaker. Suction filtration was used to filter the sample. The filtrate was kept for extraction.

3.2.3 Chemical, reagents and instruments

2,4-D Amin-salt solution (dimethyl amine salt) was purchased from Addis Ababa pesticide vendors. Analytical-grade dichloromethane, HPLC grade acetone, methanol, acetonitrile, hexane, sodium sulfate, methanol, ethylacetate, hydrochloric acid and chloroform were obtained from Sigma Aldrich (Seelze, Germany). Whatman filter paper number 1 of medium porosity was obtained from Schleicher and Schuell. All other ingredients were of analytical grade, unless stated otherwise. The chemicals were used for calibration of instrumentation, estimation of analytes, and validation of analytical methodology.

Chromatographic analyses were performed using Agilent Technologies, High-performance liquid chromatograph (HPLC) (Agilent 1260 Infinity, Germany) coupled to a diode array detector (DAD), and 7820A gas chromatography (GC) equipped with Agilent Technologies 5977E inert mass spectrometry (MS) detector for method recovery rates comparison. An electronic balance (Adam Equipment Company, UK) was used for weighing during various experiments. To measure pH values Adwa pH meter, model 1020 (Romania) was used. Ultrasonic heater (Decon F5100b, England) was used during recrystallization. Mortar and pestle were used in grinding soil sample and suction filtration apparatus with vacuum pump was used to filter sediment samples. Rotary evaporator (Heidolph Instruments GmbH & Co. KG), Soxhlet extractor (Supeorior, Germany) and liquid-liquid extractor (Witeg NS29, 2/32, Germany) were also used in the sample preparation stages.

3.3 Optimization of analytical methods for quantitative determination of 2,4-D using HPLC-DAD

During the optimization process, a new method which lasts for 8 min was developed. The optimized method included selecting an extraction apparatus, extraction and elution solvents, an optimized extraction time, and optimized HPLC conditions. For the HPLC analysis Agilent 1260 infinity equipped with photodiode array detector was used. The flow rates (mL/min) and detector wavelength were varied to optimize the HPLC parameters. Samples were Soxhlet-extracted at 2, 4, 5, and 7 hours to determine the optimal extraction time. Methanol was found to be the most appropriate solvent for extracting 2,4-D amine from soil. On the other hand, various solvent volumes were investigated to determine the one with highest recovery rate.

3.3.1 Method development

3.3.1.1 Extraction of 2,4-D amine salt from 2,4-D amine solution

The 2,4-D amine salt was obtained from the amine solution (86% w/v) by freeze-drying using a Modylo-D Thermos Avant freeze dryer for 24 hours at a temperature of -15 °C. The crude 2,4-D amine salt was subjected to recrystallization for further purification.

3.3.1.2 Purification of the 2,4-D amine salt

10 g of 2,4-D amine salt was weighed using an electronic balance. Next, 40 mL of acetone was added into the beaker containing 2,4-D and placed on a heater. The solution was stirred until all the contents were dissolved. Then it was removed from the heater and cooled at room temperature. The crystallization process from acetone was initiated by scratching bottom of

the beaker using a glass rode. Finally, crystals were separated by gravity filtration. Purity of the compound was checked using nuclear magnetic resonances (1D-NMR).

3.3.1.3 Preparation of 2,4-D acid (acidification)

1 g of purified 2,4-D (amine salt) was measured. Then 30 mL of aqueous hydrochloric acid of pH 1 was added into the beaker containing 2,4-D amine salt crystals. The sample was stirred for 15 min and then extracted three times with 20 mL of ethyl acetate (LLE). Then the organic layer was separated, combined and was dried over anhydrous sodium sulfate. After that, the solution was filtered and concentrated using rotary evaporator. Next, the average percentage yield was calculated. Purity of the product was determined using TLC (80% hexane and 20% ethyl acetate), NMR, HPLC and GC-MS. The acidification was done in triplicate.

3.3.1.4 Preparation of 2,4-D methyl ester (esterification)

Methyl ester was prepared by Fischer esterification (Ganeshpure et al., 2007). 2 g of 2,4-D acid standard was dissolved in 2 mL of methanol. 2 mL of concentrated sulfuric acid was added to the mixture in a 50 mL round bottom flask. A reflux setup was arranged and the mixture was heated to 70 ℃ for 1 hour. The mixture was cooled at room temperature. Then it was diluted in 30 mL of chloroform and the mixture was transferred to a separatory funnel. 30 mL of deionized water was added and shaken for 3 min and kept aside to late layers formation and the organic layer was separated. The organic layer was washed with 30 mL of sodium hydrogen carbonate saturated solution, separated and the organic layer was dried over sodium sulfate, filtered and then concentrated. TLC, GC-MS and NMR were used to check for purity of the ester. The average percentage yield was calculated. Concentrations of 100, 50, 10 and 1 mg/L were prepared for GC-MS analysis (calibration curve construction).

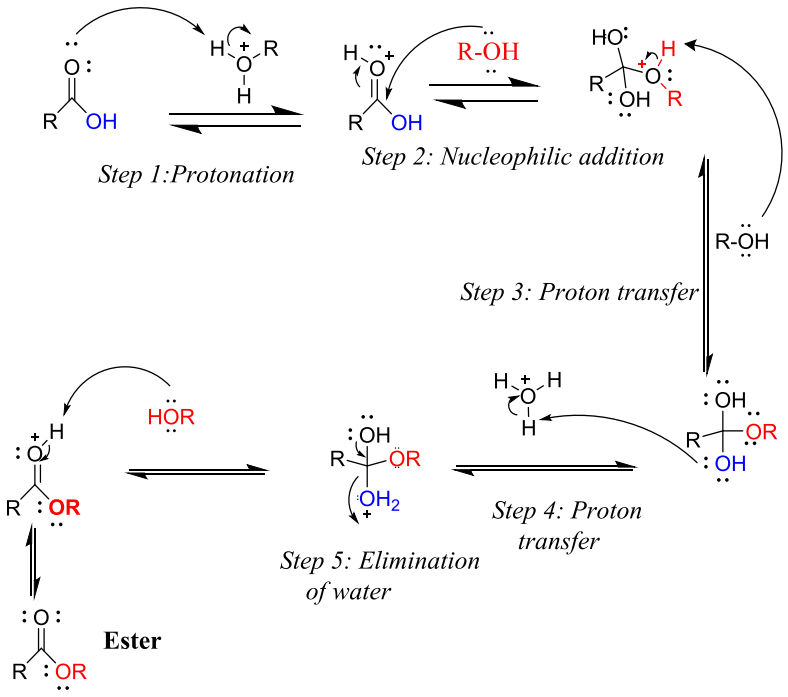


*Figure 14**: Esterification mechanism***

3.3.1. 5 Preparation of standards and working solutions for quantitative analysis

Two sets of stock solutions of 2,4-D were prepared for GC-MS and HPLC analysis. The first set was prepared via the reconstitution of 2 mg of 2,4-D ester into 20 mL of dichloromethane to a final concentration of 100 mg/L. The working solutions were then prepared via the serial dilution of stock solutions with the same solvent dichloromethane, reaching the following concentrations: 0.25, 1, 10, 20, 40 and 60 mg/L. All standard solutions were maintained in amber bottles and stored at -20 °C pending analysis.

A 100 mg/L stock standard solution of 2-4-D acid was prepared by dissolving 0.0005 g of 2,4-D acid in 5 mL of methanol. To determine the method's linearity, a series of calibration solutions containing 1, 10, 20, 40, 60, and 80 mg/L of the working solutions were prepared in methanol. Until analysis, all standard solutions were stored at -20 °C in amber bottles. To optimize the parameters, uncontaminated soil and water samples were spiked with diluted standard solutions of the target analytes (0.25 mL and 1 mL). Similarly, 0.25 mL and 1 mL pesticide containing solutions were prepared and extracted in the same manner as described previously to determine the percent recovery.

3.3.1.6 Recovery rates of the standards and compound

Recovery rates of the standards and also spiked compounds were calculated using the following formula;

$$Percentage\ yield = \frac{Actual\ yield}{Theoretical\ yied} \times 100\%$$

Actual yield: Mass of sample after extraction. Theoretical yield: Mass of sample before extraction.

The theoretical yield was measured using a balance. The actual yield was the mass of the vial minus the total mass after extraction and concentration (weight of the sample-vial).

3.3.1.7 Spiking of the soil samples and water samples for method validation

Pesticide free soil was spiked following the principle described by Tor et al. (2006). A sieved soil sample (10 g) was weighed in a beaker and spiked at the required concentration level by adding different volumes of the standard (0.25mL and 1 mL of 40 and 10 g/mL solution) respectively.

Water samples were spiked following the principle by (Rezaee et al., 2006). 15 beakers of 100 mL water samples were measured. Every 100 mL of the water was then spiked with 100 mg of 2,4-D amine salt. The accuracy of the analytical method was evaluated through recovery studies for both the soil and the water samples.

3.3.2 Extraction of spiked 2,4-D from water and soil samples

To validate the method, 100 mL of spiked water was measured, acidified with aqueous HCl to pH 1, transferred to a separatory funnel (1 L volume), and then partitioned with ethylacetate (50 mL) as shown in **Figure 17** after it was vigorously shaken for 3 min. The organic phases were separated mixed together and washed with sodium bicarbonate saturated solution and the organic extract was dried over anhydrous sodium sulfate and filtered. The extract was then evaporated to dryness using a rotary evaporator in a water bath set to 40 °C, as shown in **Figure 18**. The same procedure was followed to extract samples from Lake Ziway.

Figure 15: Liquid-Liquid extraction of the 2,4-D acid

Figure 16: Concentration of samples extract in a rotary evaporator

Soil samples were extracted using Soxhlet apparatus **(Figure 19).** The spiked soil 100 g (different concentrations) was transferred into a thimble; a round-bottom flask was placed in a cork ring, anti-bumping granules and 90 mL methanol were added, the flask was clamped and transferred to a heating mantle. The thimble was placed in the Soxhlet and 10 mL of solvent (methanol) was added from top. Soxhlet apparatus was attached to the water condenser. The methanol in the round bottomed flask was heated to boiling. The heating process was kept for various extractions times to evaluate complete extraction of the analyte. The heating process was stopped; the methanol extract was then filtered and concentrated to dryness. The residue was dissolved in acidified water and liquid-liquid extraction was carried out using ethyl acetate. The organic phases were separated, mixed dried over sodium sulfate, filtered and concentrated. Percentage yields were determined for various extraction times. The extraction took place in replicates of every variable (time of extraction and amount of concentration). The same procedure was followed in monitoring 2,4-D acid from the soil samples obtained from Bochessa and Kontola agricultural fields in Ziway.

Figure 17: Soxhlet extraction of soil samples

3.3.3 High-performance liquid chromatography analysis

For the analysis, different chromatographic conditions were tested to obtain optimal peak shape and resolution. It includes different combinations and ratios of the mobile phases, column temperature optimization, flow rate and maximum UV-absorbance (wavelength) selections. The HPLC-DAD used was Agilent made 1260 infinity equipped with G1311B quaternary pump, G1329B auto-liquid sampler, G1315C diode array detector (DAD) and G1316A thermal column compartment (TCC). The mobile phase was acetonitrile: water (0.2% formic acid) in 75 to 25 ratio for 8 min in an isocratic mode. The injection was made via the auto sampler and the injection volume ranges from 5-10 µL in a standard mode. Reversed phase C-18 column with particle size 5µm, internal diameter of 4 mm and length of 250 mm was used for separation and identification of components. The DAD wavelength was set at 230 and 280 nm for 2,4-D determination. **Table 2** shows a summary of HPLC–UV conditions.

Table 2. Chromatographic conditions (HPLC–UV)

Used for detection of 2,4-D	***Parameters details***
Mobile phase	ACN-0.2% FA in H_20-(75:25)
Isocratic mode	_
Flow rate	1 mL/min
Temperature	25 °C
Injection volume	5-10 μL
Detection	230 and 280 nm

* ACN = acetonitrile, *FA = formic acid, *H_2O = water

3.3.4 Calibration curve construction

A 100 mL of 100 mg/L standard was prepared and serially diluted to 0.25, 1, 10, 20, 40, 60 and 80 mg/L. 1 mL of the sample from each concentration was transferred into HPLC vials and analyzed at 25 °C with the injection volume of 10 μL. Data was recorded at 230 and 280 nm wavelength. All the analysis were done in triplicate from three separate standards, and each of the triplicate samples were also analyzed separately by HPLC.

3.4 Determination of the stability of 2,4-D amine in water

Stability test was conducted by spiking 2 L of the lake water with 2 g recrystallized 2,4-D amine salt. The samples were extracted on time course bases for over a period of 45 days from a zeroth hour to check degradation of the compound. For the analysis, the spiked lake water was placed on a shaker for 5 min to maintain homogeneity of the solution and 100 mL was measured and acidified (as described above), extracted with ethyl acetate (EtOAc) three times, the organic phases were combined and washed with sodium bicarbonate and dried over anhydrous sodium sulfate, filtered and concentrated. This procedure was repeated for all the analysis days (from 0-45th day). The identity and purity of the compound was checked using TLC co-spotting technique. For the first nine days, spiked water samples were extracted every 24 hours. However, after the 10th extraction the water samples were extracted after every 5 days. Recovery rate of the compound was calculated so as to monitor the degradation

of the compound or the stability in the lake water. For the HPLC analysis to check if there were any degradation or side products, the crude extract was dissolved in methanol (20 µg/mL) and 10 µL was injected.

3.5 Determination of the levels of 2,4-D in water, sediment and soil samples

The analysis followed EPA method 1699 for determination of pesticides in soil, water and sediment samples (EPA, 2007). A total of 6 water and 12 soil samples were collected from Lake Ziway. The samples were analyzed in accordance with the aforementioned method on spiked soil and water samples. On water samples the method developed using the standards for extraction and analysis of 2,4-D was exactly adopted. Soil samples were also extracted using the Soxhlet extractor. 100 g of soil sample was measured and extracted in 150 mL methanol. After extraction, the extract was concentrated to dryness. It was then dissolved in 15 mL of water and then acidified to pH 1. After acidification, it was extracted three times in ethyl-acetate (15 ml x 3).

For sediment samples, 100 g of the sediment was weighed in a beaker and transferred to a conical flask. 100 mL of distilled water was then added. It was then shaken vigorously for half an hour on a shaker. Suction filtration was used to filter the sample. After that, it was acidified and set aside for 15 minutes. The acidified compound was extracted with ethyl acetate and concentrated before being dissolved in 1 mL methanol for HPLC analysis. For HPLC analysis of the soil extracts, the crude was dissolved in 2 mL methanol and 5 µL was injected. **Figures 20, 21 and 22** summarize the above processes.

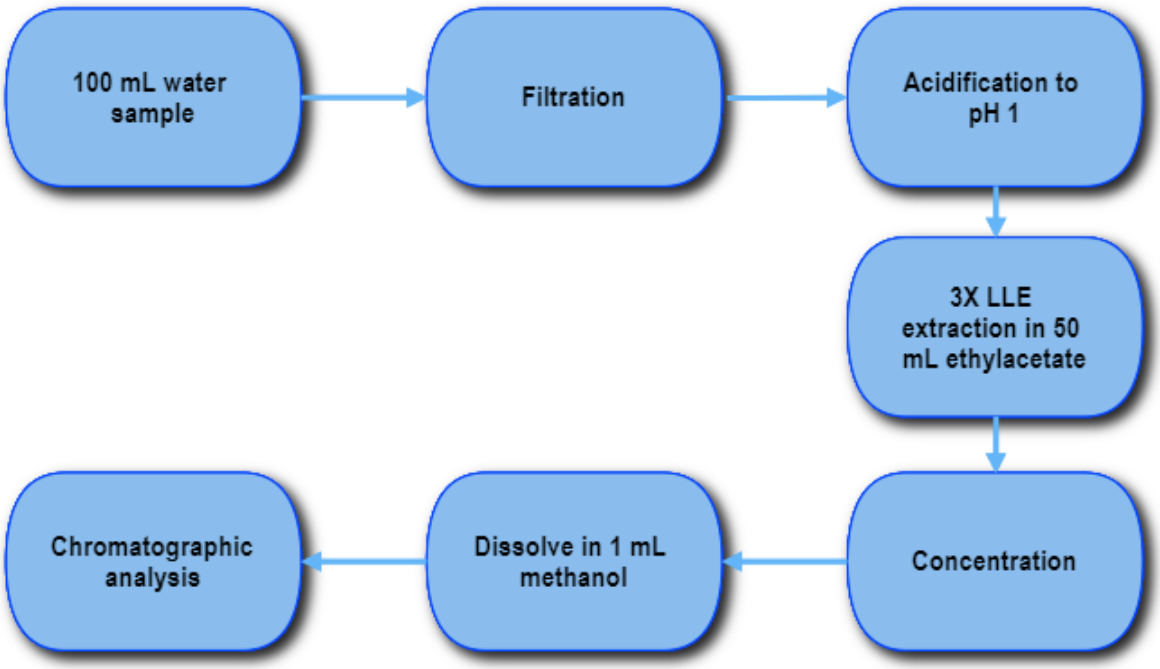


Figure 20: Water sample preparation for chromatographic analysis 2,4-D herbicide by HPLC

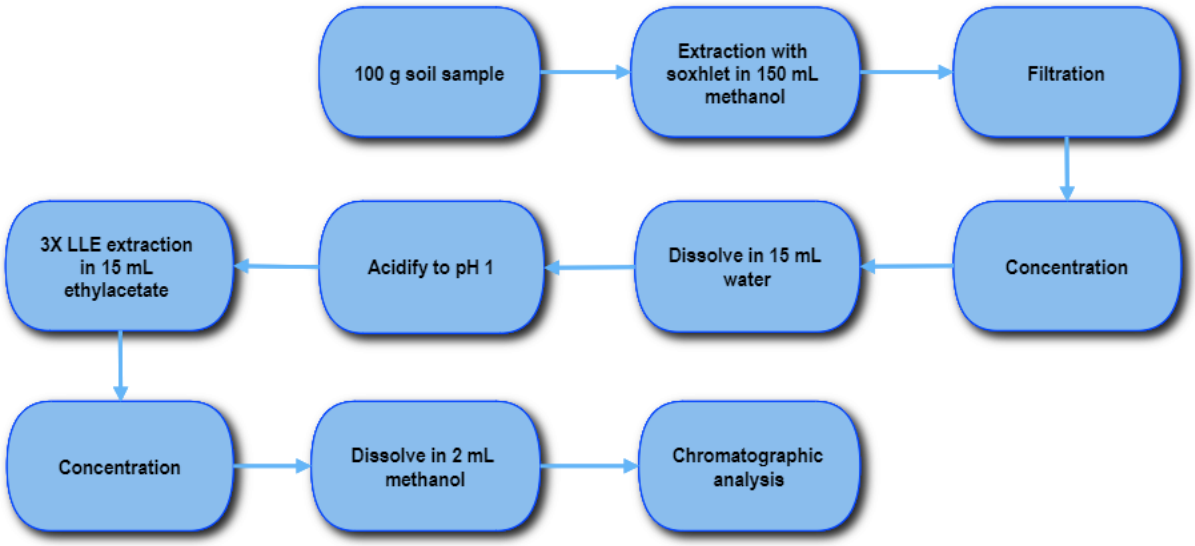


Figure 21: Soil sample preparation for chromatographic analysis 2,4-D herbicide by HPLC

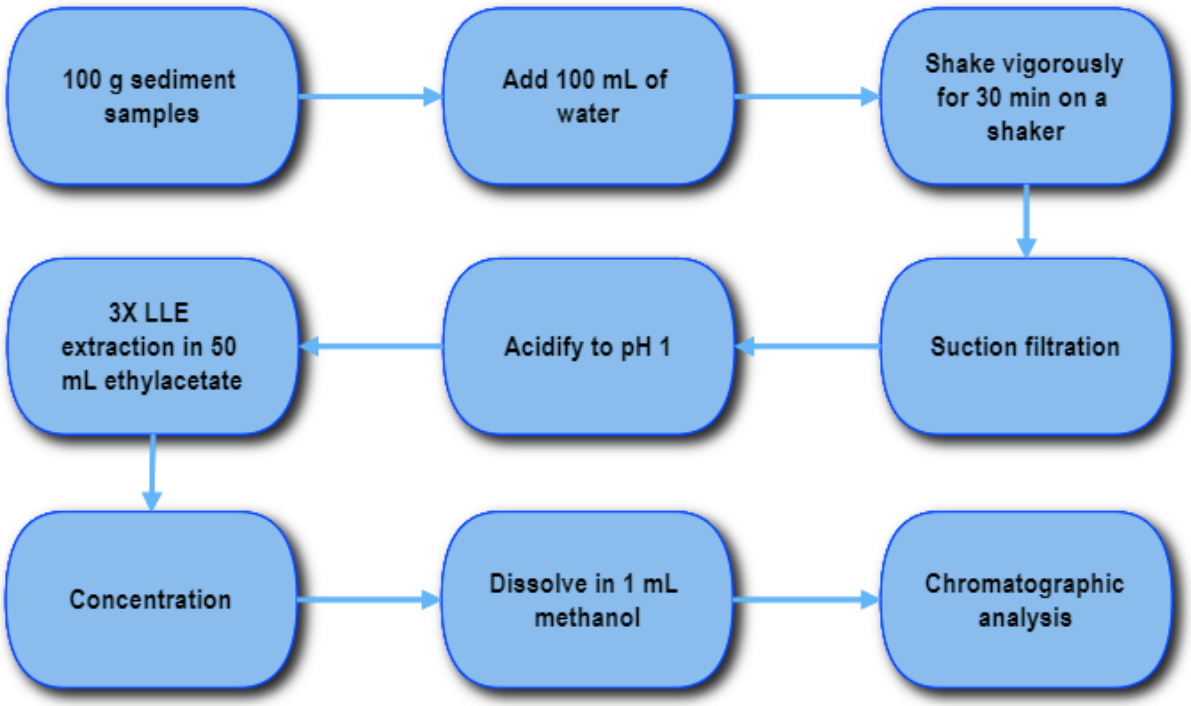


Figure 22: Sediment sample preparation for chromatographic analysis 2,4-D herbicide by HPLC

4. RESULTS AND DISCUSSION

4.1 Optimization of analytical methods for quantitative determination of 2,4-D amine at trace level using HPLC

4.1.1 Method development

4.1.1.1 Extraction, recrystallisation, acidification and esterification of the 2,4-D amine salt

A solution of 2,4-D amine turned into brownish crystals after freeze-drying, which was subsequently purified via recrystallization. Recrystallization step was necessary to remove any impurities before preparing working standards. The recrystallized 2,4-D salt turned from brownish to clear crystals, **Figure 22 C**. The compound was then acidified to obtain 2,4-D acid for HPLC analysis. The acidified compound was finally esterified because 2,4-D acid is not a volatile compound and thus cannot be analyzed by gas chromatography. In addition, the esterification step was performed to compare the recoveries of 2,4-D from the amine salt in the acidic and ester forms. The acidified compound was in the form of a powder, while the ester had a needle-like shape. **Figure 22** shows the 2,4-D solution, freeze-dried, recrystallized, acidified and esterified in that order. The formation of only one spot on a developed **TLC** plate confirmed the purity of every compound at each step which NMR also confirmed. The NMR results included in the appendix confirm the 2,4-D's molecular structure at various steps **(appendix i-vi)**. Purity of the ester was confirmed by performing GC-MS analysis **(appendix vii).**

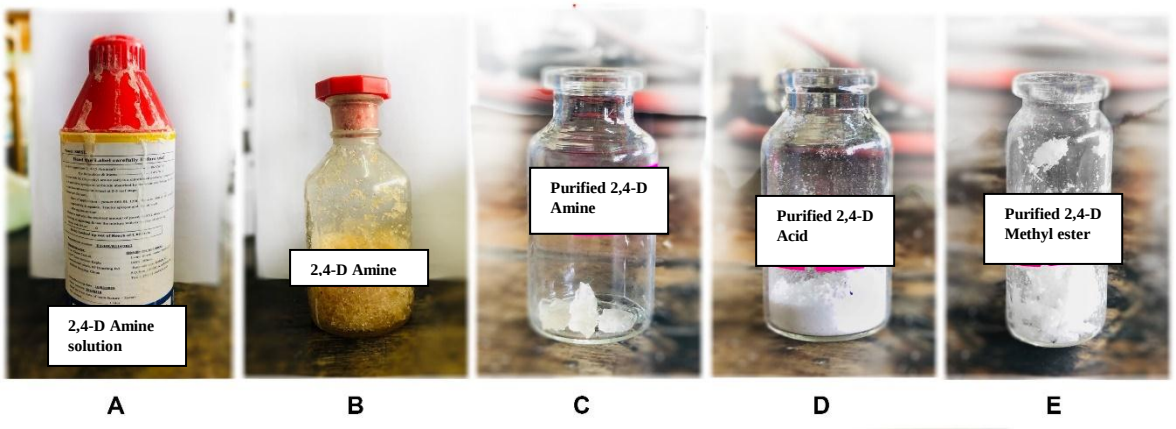


Figure 18: 2,4-D amine salt solution (A), Freeze dried 2,4-D amine salt (B), Purified 2,4-D amine salt (C), Acidified 2,4-D (D), Esterified 2,4-D (E).

4.1.1.2 Preparation of standards for HPLC and GC-MS analysis

This study was attempted to analyze 2,4-D using both HPLC and GC-MS. Standards preparation took less than an hour for HPLC analysis and more than 2 hours for GC-MS analysis. The preparation of GC-MS samples required concentrated sulfuric acid and chloroform during sample preparation and dichloromethane during analysis. The study recommended HPLC based on the recovery rates where the average was 97.9±3.5% for HPLC standards and 86.8±0.22% for GC-MS standards (**Table 3**), the time required to prepare samples for both GC-MS and HPLC analysis was also another factor, the steps needed to prepare standards for HPLC and GC-MS analyses, and the ability to avoid the use of certain hazardous chemicals such as dichloromethane and chloroform. Generally, GC (Alvarez-Rivera et al., 2019; Park et al., 2011), GC/MS (Thorstensen et al., 2000), or capillary electrophoresis is used to detect 2,4-D (Baggiani et al., 2000). It has been demonstrated that GC and some CE analyses exhibit adequate selectivity and sensitivity (Bajoub et al., 2016; Hu et al., 2015; Shin et al., 2011). However, due to the low volatility of acidic herbicides caused by hydrogen bonding between their carboxylic acids, these methods require derivatization. Additionally, they are adsorbed onto the GC stationary phase due to their polar nature, possibly resulting in peak asymmetry (Behbahani & Najafi, 2014; Tran et al., 2007; Wang et al., 2016). Furthermore, many chemicals used in derivatization are toxic to humans (Sajid, 2018).

Utilizing an HPLC system makes it possible to eliminate the need for derivatizations and hazardous reagents while still generating reliable experimental results. Additionally, HPLC is a more attractive methodology than GC because derivatization adds a step in sample preparation (Cairns et al., 2008). Besides the extra time consumption and toxic nature of the common derivatizing reagents, there is also the chance to introduce contaminations to the sample (Pintado-herrera et al., 2012). Finally, there is a higher chance of degrading the compound during esterification as heating is employed. The latter also explains the lower recovery rates on the esters compared to the acids as obtained in this study.

Table 3: Recovery ranges of 2,4-D acid and 2,4-D ester

2,4-D Acid recovery (%)	*2,4-D Methyl ester recovery (%)*
93.84 ± 0.1	87.06 ± 0.6
99.93 ± 0.1	86.63 ± 0.1
99.98± 0.1	

4.1.1.3 Optimization of the analysis procedure

In order to realize a reliable or applicable analysis and extraction procedure, investigation of the effects of various experimental parameters and determining the optimum conditions must always be considered. In this analytical method, some experimental variables affecting the performances of the technique including the effect of volume of extraction solvent, extraction time have been studied and optimized. In addition, the type of suitable extraction solvent was chosen after reviewing a number of literatures on solvent extraction in liquid-liquid and Soxhlet extraction of polar compounds. For analyses of the 2,4-D acid, peak areas were used to evaluate the extraction efficiency and establish the optimum extraction condition.

4.1.1.4 Optimized sample extraction

One point to keep in mind is that for HPLC analysis, pre-concentration and chromatographic separation must be carried out in acidic conditions, preferably at a pH lower than the herbicide's pK_a, to ensure that ionization is suppressed (Cairns et al., 2008; Kodamatani et al., 2016). Considering that 2,4-D amine salt was used in this study to develop the method, the compound was acidified with HCl before liquid-liquid extraction and chromatographic analysis to suppress ionization and extract it into an organic solvent. The suppression of ionization decreases the analyte's hydrophilicity, allowing it to be extracted into an organic phase (Park et al., 2011). In addition, acidification was necessary since pre-conception and liquid chromatography analysis is typically performed under acidic conditions. The same procedure was followed in extracting samples obtained from Ziway because it was impossible to predict whether the compound present in the soil, sediment, and water samples was 2,4-D amine salt or 2,4-D acid. Furthermore, 2,4-D is an acidic pesticide with a pK_a value of between 2.7 and 4.8 (Han et al., 2010; Xi et al., 2010), which is described as a weak acid hence the need to still acidify so that it can be extracted.

4.1.1.4.1 Selection of extraction solvent

A vigorous literature review was done on analytical performances of different solvents for polar organic compounds in this study. Based on the papers reviewed, methanol was chosen for Soxhlet extraction and ethyl acetate was selected for liquid-liquid extraction. Based on the reasons that ethyl acetate is capable of providing acceptable recoveries as the other solvents used in extraction of polar compounds, yet cheaper than acetonitrile, and also does not require an addition of any non-polar solvent during the extraction just like acetone, thus, this

study opted for ethyl acetate as the best solvent for extracting 2,4-D acid from both water and sediment samples. Methanol was selected in reference to the study that was done by Truong and colleagues' study as the solvent of choice for extracting 2,4-D acid from a soil sample in this study.

4.1.1.4.2 Optimal extraction time

Table 4 and F**igure 23** summarizes the soil extracts obtained after varying the number of hours of extraction. The experimental results revealed that for the different levels of spiked amount of the analyte, 4 hours extraction time was optimum. It produced the exact percentage yield as 5 and 7 hours extraction. Where 7 hours is the recommended time for a standard Soxhlet extraction (López-Bascón-Bascon & Luque de Castro, 2019; Shen & Shao, 2005). This may be attributed to the very fast mass transfer taking place initially but before establishment of the equilibrium state, which was achieved later, around 4 hours. Therefore, extraction time of 4 hours was found to be the optimum time and used throughout this study.

Table 4: Recovery of 2,4-D herbicide in spiked soil

Amount of standard spiked	***Duration of extraction (h)***	***% Recovery***
250 µL		
	2	45%± 0.3
	4	98%± 0.2
	5	100%± 0.2
	7	100%± 0.1
2 mL		
	2	60%± 0.2
	4	100%± 0.4
	5	100%± 0.2
	7	100%± 0.1

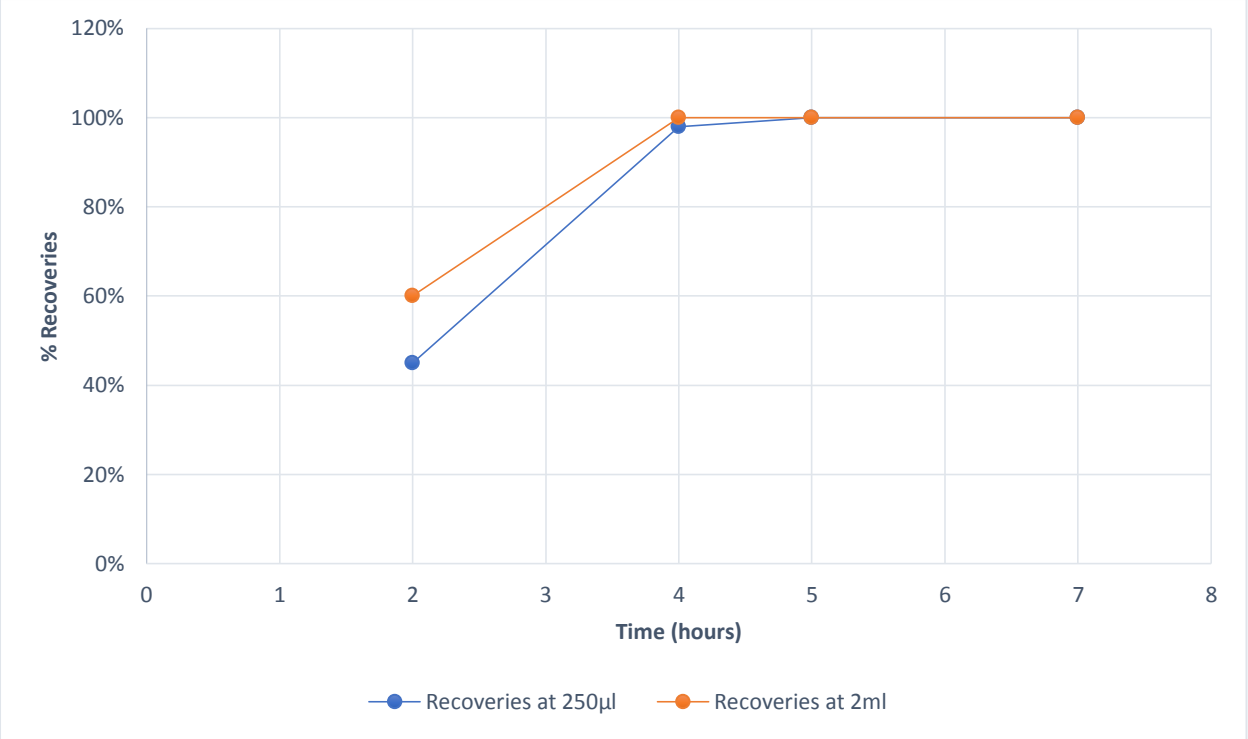


Figure 19: Percentage recoveries spiked 2,4-D at different time intervals and concentration

4.1.1.5 HPLC-DAD optimization

In order to optimize the HPLC parameters, the conditions were monitored by varying the flow rates (mL/min) and the detector wavelength. It was observed that the maximum absorbance of the analyte and best selectivity of the peak was the detector wavelength of 230 nm and 280 nm and flow rate of 1 mL/min. The best chromatography separation was achieved on a RP-C18 Column at 25 ℃ column temperature (25 cm length, 4.0 mm ID, 5 µm particle size) which was purchased from Agilent Technology and the system was operated isocratically. For routine analysis a mobile phase consisting of a mixture of ACN, and 2% HCOOH in H_2O was used and the sample was injected through a fixed sample loop having a volume of the 10 µL. The UV-DAD detector was set at 230 and 280 nm. All samples were analyzed in triplicate from three separate extracts, and each of the triplicate samples was analyzed by HPLC-DAD for detection and determination of 2,4-D. The HPLC method was faster, taking only 8 minutes, compared to the 16 minutes done by Chen et al. (2018).

4.1.1.6 Comparison of the proposed method with similar literature reports

The figures of merit of the Soxhlet method developed in the presented study for determination of 2,4-D amine salt in water, sediment and soil samples have been compared with other reported techniques (Nagaraju and Huang 2007; Rocha et al. 2008). Details of the relevant results of the methods and that of this study are provided in **Table 5** and **6**. Based on

these findings, comparison was made and it was observed that the proposed method involves minimum labor and requires short extraction time. In addition, performances of the developed technique were compared with that of the previously reported techniques in terms of relative recovery and regression coefficient (R^2) and the findings confirmed that the developed technique are found to be comparable or better. Furthermore, it could also be noted that the developed method utilizes simpler and traditional laboratory equipment which could be accessible in most common research laboratories.

In summary, the advantages of the extraction method developed in this study include the use of a small volume of organic solvent (80-150 mL) in comparison to the "traditional" method, a shorter extraction time (4 hours) when compared to the "traditional" method as well, and higher recovery rates (91% ± 2%). **Table 5** compares the standard and optimized methods in a variety of ways. The study discussed here demonstrates that the extraction procedures used improved the ease, reproducibility, and throughput of 2,4-D herbicide analysis. **Table 6** summarizes the comparison of this study and other studies in terms of recovery with percent standard deviation (% SD) in spiked soil samples, the amount of solvent, time for extraction and concentration and also the linearity of the method. According to the results, the optimized method appears to be a highly effective tool for sample extraction. It has a higher extraction efficiency than the widely used standard Soxhlet, particularly when it comes to extracting 2,4-D from soil. The findings indicate that this method facilitated sample extraction, identification, and quantification. A one tailed paired t-test performed in excel on the recovery rates of the optimized method and the standard method with variance unknown gave a p-value of 1.0051×10^{-7}. The obtained p-value was less than 0.05, hence recoveries obtained in this study were significant and did not occur by chance.

Table 5: Recovery of 2,4-D herbicide in spiked soil sample

Soil sample number	Overall percentage recovery ± % using standard method (acetonitrile) (Kashyap et al., 2005b)	Overall percentage recovery ± % using optimized method (methanol)
1	55% ± 5%	90%± 3%
2	60% ± 4%	91 % ± 2%

3	50% ± 4%	94 % ± 5%
4	58%± 6%	93% ± 2 %
5	49% ± 6%	88% ± 2%
6	55% ± 5%	91% ± 5%

Table 6: Comparison of the optimized method with literature data for 2,4-D acid extraction

Methods	***Type of solvent***	***Amount of solvent (mL)***	***Extraction time (h)***	***Concentration time (min)***	***Average recovery***	***LOD (µg/L)***	***R^2***	***References***
Soxhlet-HPLC (UV)	Methanol	80-150	4	15	89.9% ± 5%	0.45	0.9996	This study
Soxhlet-HPLC-(UV)	Acetonitrile	250-500	6	20	55% ± 4%	-	0.991	(Kashyap et al., 2005a)
Soxhlet-HPLC	Methanol	200	48	-	97%	-	-	(Atalay & Hwang, 1999)

4.1.2 Method validation

4.1.2.1 Spiking the soil samples and water samples for method validation

To validate the method, 2,4-D amine salt was spiked to soil and water samples. The calibration curve **(Figure 25)** and linear ranges of the detector response were determined using standard solutions of 2,4-D acid with concentrations ranging from 1 to 100 mg/L. Precision was determined using repeatability in recoveries and evaluated using replicates when extracting and analyzing soil samples at various spiking concentrations (0.25 and 1 g/L) (86 % w/v). The accuracy of the analytical method was established through recovery studies on soil and water samples (**Table 7)**. The presence of the target analyte in spiked water and soil samples were established using thin-layer chromatography with the spiked sample extract covering the same distance (Rf) as the standard 2,4-D acid. The target analyte's retention time also confirmed its presence in the spiked samples during HPLC analysis.

Extraction recovery

The recovery of 2,4-D from the determined in accordance with the method described in the previous section. The recoveries (mean) of 2,4-D from water and soil were found to be (80–88%). The precision of the method was confirmed by how the recovery rates were close to each other while the accuracy of the method was established by how close the recovery rates were to the true value in this case where the true value was 100mg.

Table 7: Recoveries of 2,4-D from the spiked soil and water samples

Experiment #	*Soil % recoveries*	*Water % recoveries*
1	*80.0± 3.1*	*83.0± 2.7*
2	*84.0± 1.2*	*88.0± 2.3*
3	*86.0± 3.5*	*84.0± 0.6*
4	*86.0± 1.2*	*84.0± 2.7*

The method's overall performance was acceptable in recovery (80–88%) **Table 7** and precision. **Figure 24** presents the recovery of water and soil samples. Based on the table and

graph, percentage recoveries were within an acceptable range. This indicates that the method developed was valid.

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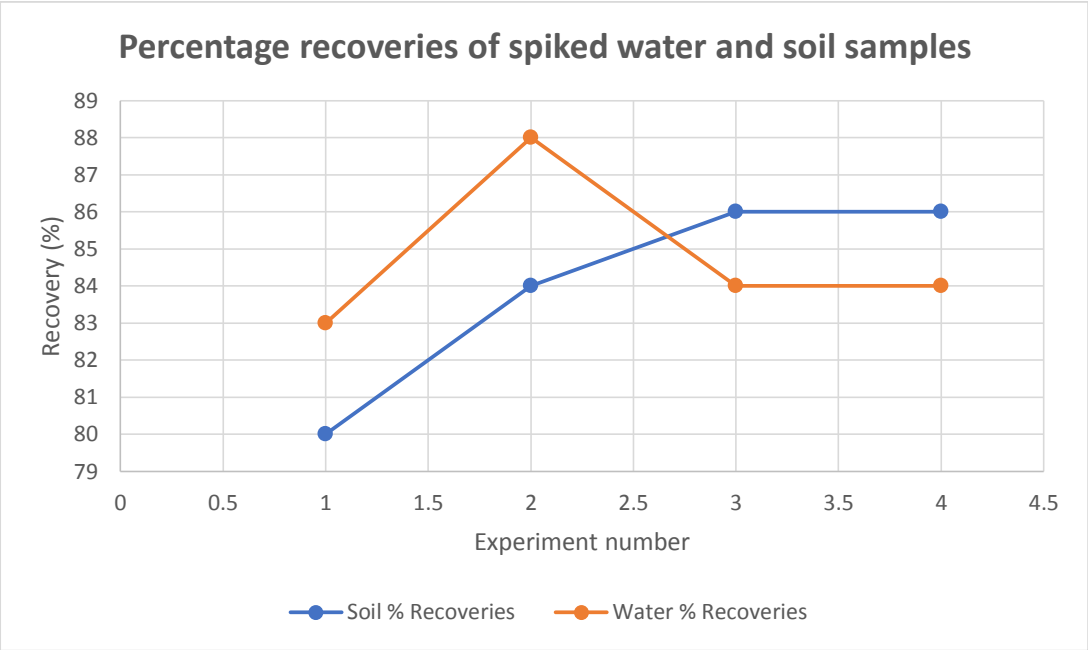


Figure 24: Percentage recoveries of spiked soil and water samples

Linearity and Limits of Qualification

To determine whether the detection method holds a linear response between amount of analyte and acquired response from the HPLC, calibration curves with pure compounds were produced. The linearity of the method is considered satisfying (0.95- 1.00). This requirement was fulfilled as shown in **figure 25**. The r^2-values are, according to the validation guidelines. These results support the results from the recovery experiments as it confirms the accuracy of the method. **Table 8** illustrates the concentration and the area obtained after the standards were analysed with HPLC. The detector response of 2,4-D acid was linear within the given range, with correlation coefficients (R^2) = 0.9996 (**Figure 25).** The LODs and LOQs which were calculated manually by taking noise to signal ratio of the lowest concentration of 2,4-D were 0.45 µg/mL and 2 µg/mL, respectively, which are generally comparable to the methods reported in most of the literature. The lower limit of quantification were calculated when the signal to noise ratio is 10:1 (S/N = 10)

Table 8: Area and concentration of standards

Concentration (mg/L)	Area (A.U.)
10	96.13
20	189.4
40	398.4
60	583.2
80	796.9
100	979.2

A calibration curve was plotted to verify the method's linearity. The graph **(Figure 25)** illustrates the curve by plotting the area obtained during the HPLC analysis against the concentration of each standard.

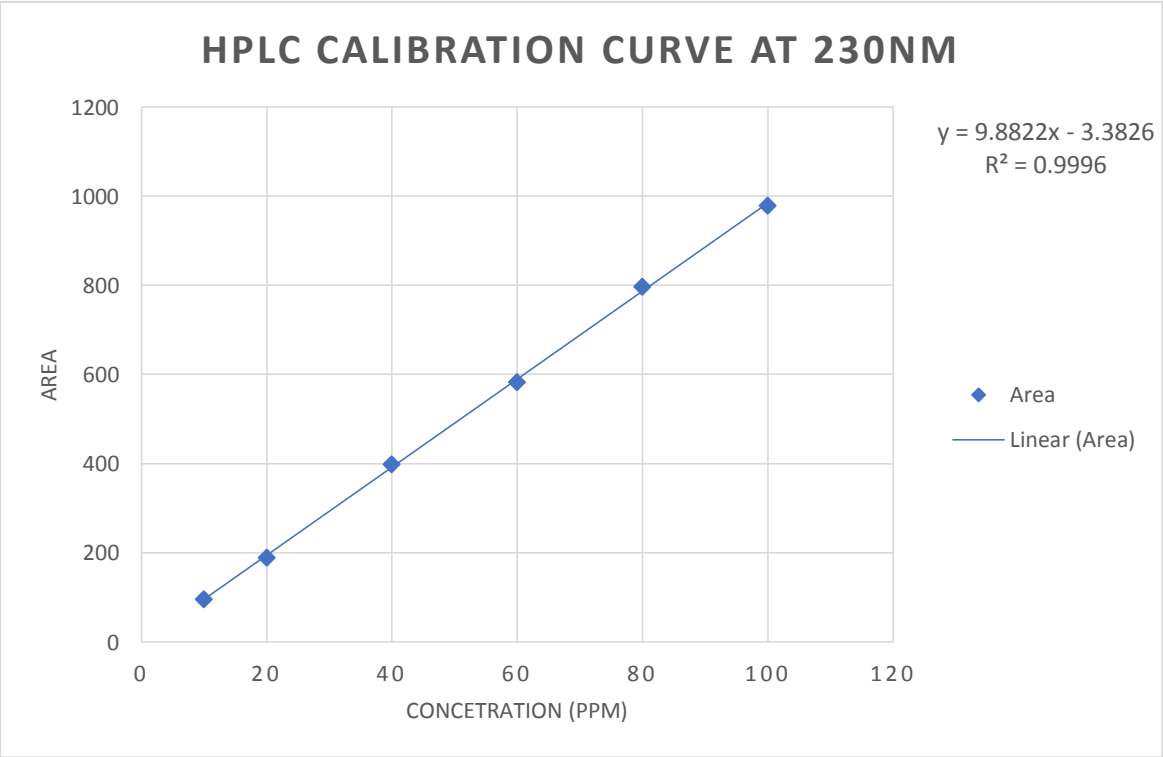


Figure 25: HPLC calibration curve of the 2,4-D acid standards

Specificity

Representative chromatograms for blank 2,4-D standard and blank 2,4-D spiked with 2,4-D obtained were well resolved at retention times of (2.7 ± 0.3) min. No endogenous interference was found at the retention times of 2,4-D, indicating that the developed method is specific for 2,4-D.

Repeatability or Reproducibility

Repeatability of the method was verified under optimum conditions on the same day and on different days. Intra-day repeatability of both retention times and peak areas was obtained by measuring different injections (n=4) from the same sample, while the inter-day repeatability was obtained by measuring different injections (n=4) from the same samples over five different days. As can be observed in the **table 9**, repeatability for retention time, expressed as recovery rates, was in the accepted range. Therefore, good repeatability results were obtained for both retention times and peak areas.

4.2 Stability of 2,4 D acid in water

2 g of 2,4-D acid was spiked into 2 L lake water to check its stability. The spiked water was extracted for a period of 45 days. **Table 9** illustrates the percentage recoveries obtained in different days of extraction.

Table 9: Percentage recoveries of spiked 2,4-D in water

Days	*Time intervals (hours)*	*Amount extracted (mg)*
1	0	80.0
2	24	74.3
3	48	72.7
4	72	73.8
5	96	73.9
6	120	73.8
7	144	72.3
8	168	72.1
9	192	75.3

10	216	72.1
15	341	72.1
20	480	72.4
30	720	72.3
40	960	72.5
45	1080	72.3

The data above was used to plot a graph. In order to obtain a line graph that clearly illustrates the relationship between the time and percentage yield, the variables were first converted into log.

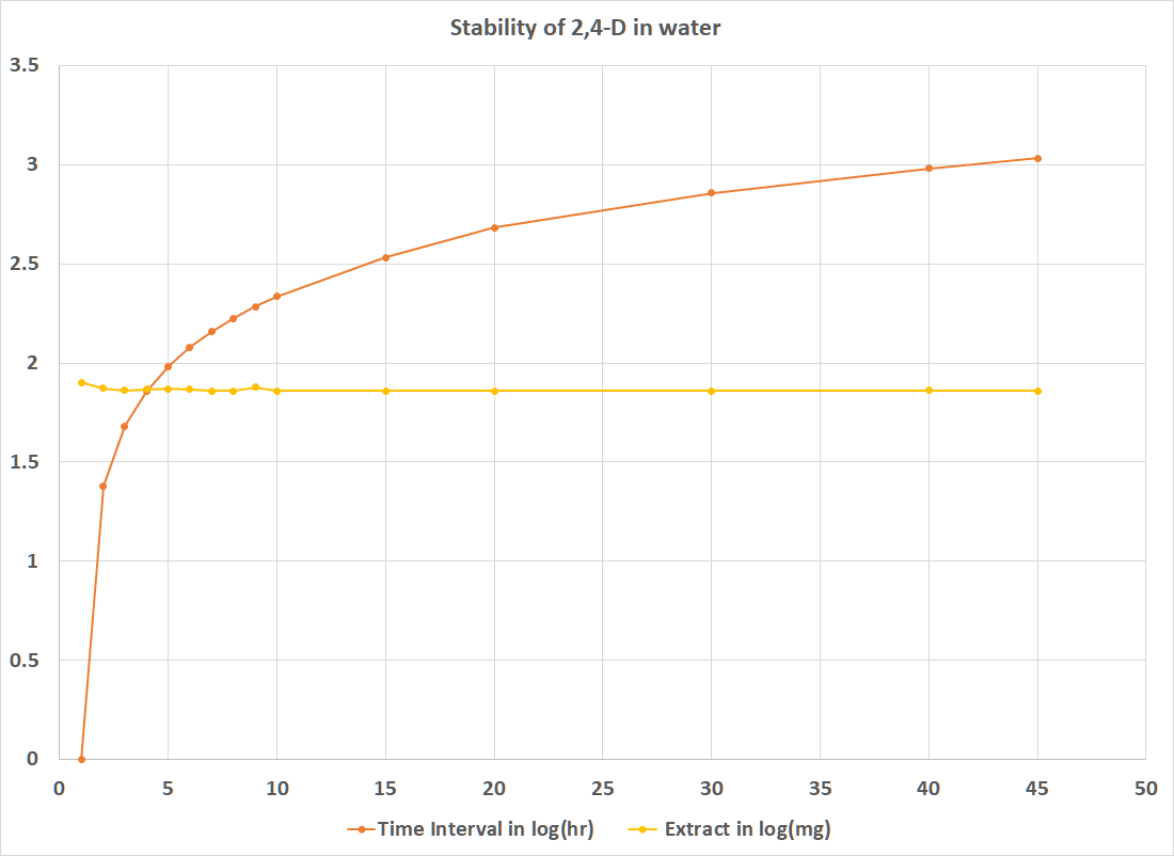


Figure 26: Percent Recovery of spiked water at different time intervals

As illustrated in **Figure 26**, the percentage recovery remained constant as the number of hours increased. The only difference was in the recovery rate on the first day of extraction, 80 percent. This can be explained by the possibility that there was a compound in the water

which reacts with 2,4-D, and the increase in recovery yield at the zeroth hour indicated that the reaction had not yet begun. However, once the reaction was complete, no compound was available to react with the 2,4-D, resulting in the observed constant percentage yield obtained from the 24 h. to 1080 h. extraction. 2,4-D can enter the environment via effluents and spills generated during its manufacture and transportation and via direct application as a weed control agent. It is primarily removed from the environment via biodegradation, with the formation of 2,4-dichlorophenol as an intermediate. With a half-life of less than one day, 2,4-D is removed from the atmosphere via photooxidation and rainfall. 2,4-D has a half-life in soil ranging from 4–7 days in most soil types to up to 6 weeks in acidic soils. 2,4-D degrades rapidly in water, although some may be photolyzed near the surface. Under aerobic conditions, half-lives in water range from 1 to several weeks; under anaerobic conditions, half-lives can exceed 120 days. There is no reason to believe that 2,4-D will accumulate in bottom sediments and mud. It does not bioaccumulate in aquatic or terrestrial organisms, except for some algae, due to its rapid degradation. The results of this study are inconsistent with Karanth's (2005) reported 10-day stability of 2,4-D in water. However, the results agreed with Toft (2003), except that in his report, stability for more than 30 days is expected under anaerobic conditions, which was not the case in this study. Although this study was conducted in an aerobic environment, stability of more than 30 days was obtained. Thus, it is possible that the reported 10-day stability of 2,4-D is for the amine salt rather than the acid form and that the methods used in those studies targeted the amine form of 2,4-D, resulting in the conclusion above. However, in aquatic environment with pH less than 6, the amine salt has higher possibility to exist in acidic form. This is exactly why this study targeted both 2,4-D amine and acid forms at the same time.

The extracts from the different days were analysed with HPLC at wavelength 230 nm in order to confirm their identity.

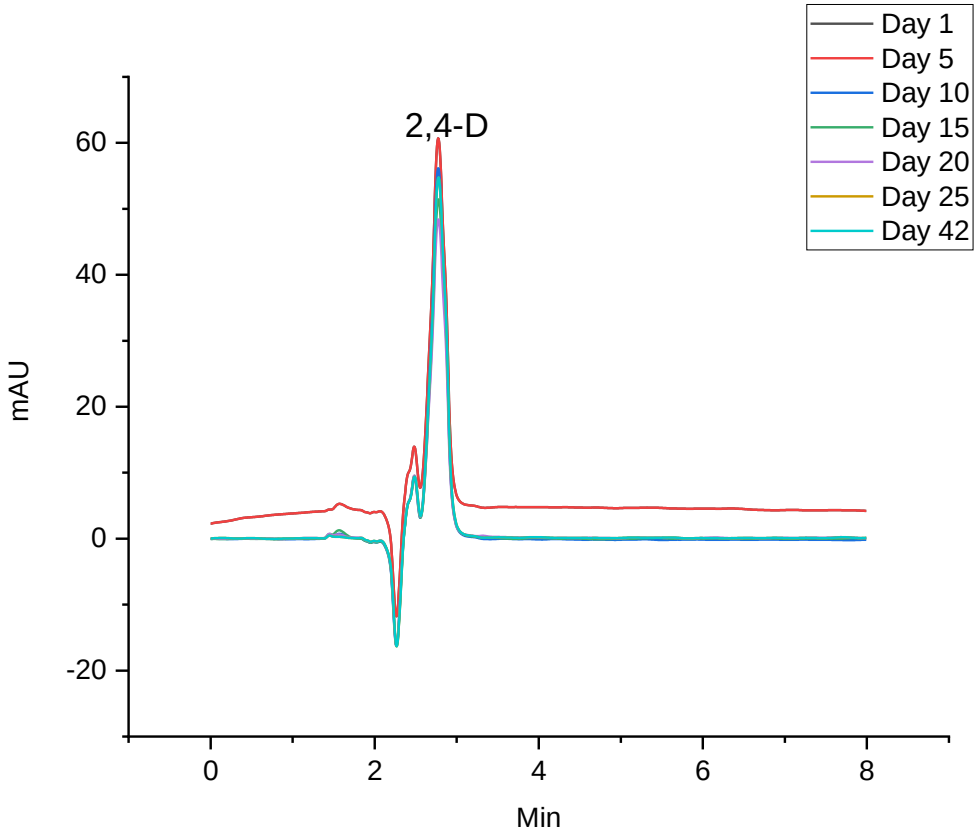


*Figure 27**: A chromatogram graph showing 2,4-D acid in the spiked wate***

4.3 Detection and determination of 2,4-D in water, soil and sediment by using HPLC

The proposed HPLC-DAD method was applied for selective and quantitative extraction and determination of 2,4-D acid in the water, soil and sediment samples collected from Ziway. The extracts of the samples were analyzed to determine the concentration of 2,4-D. Peak identification was performed by comparing the retention times of analytes to those of pure standard of 2,4-D acid. Quantitative determination of 2,4-D acid was done after constructing calibration of standard working solutions of 2,4-D acid.

Analytical method was developed to check the presence of the 2,4-D pesticide residues in the water, sediment and soil samples (**Table 2)**. Following similar procedure, the presence of 2,4-D in the samples was conducted. **Table 10** shows the results obtained when the soil, water and sediment samples were analysed with HPLC.

Table 10 : Levels of 2,4-D acids at different sites

Table 10.1 samples from Wafiko and Sher Site

Number of samples	***Sampling site***	***Type of sample***	***Concentration level*** *(mg/L)*	***Mass of 2,4-D per 100ml of water (mg)***
1	**Wafiko and Sher Site**	Water sample	142.57	1.43
2			96.68	0.97
3			167.69	1.68
4			128.21	1.28
5			143.44	1.43
6			94.46	0.94

Table 10.2 samples from Bochessa Site

				Mass of 2,4-D per 100g of soil (mg)
1	**Bochessa Site**	Soil sample	75.60	0.76
2			68.22	0.68
3			131.88	1.32
4			98.44	0.98
5			111.76	1.14
6			95.58	0.96

Table 10.3 samples from Kontola Site

1	**Kontola site**	Soil sample	below the detection limit (BDL)
2			BDL
3			BDL

4			BDL
5			BDL
6			BDL

Table 10.4 samples from Kontola Site

1	***Wafiko and Sher site***	*Sediment sample*	*BDL*
2			BDL
3			BDL
4			BDL
5			BDL
6			BDL

From the tables above, it can be noted that in the soil samples taken from the Bochesa site **(Table 10.2)**, 2,4-D was detected of which measured concentrations range between 68.22 mg/L to 131.88 mg/L. For the water samples collected from Wafiko and Sher site **(Table 10.1),** significant amount of 2,4-D was observed which ranges of 94.46 mg/L to 167.7 mg/L. However, there was no 2,4-D observed in the sediment samples collected from the same site **(Table 10.4)**. The findings on sediment can be attributed to the fact that 2,4-D is polar hence most likely stays dissolved in water. As such, in a lake it can only accumulate in the sediment if and only if the lake is saturated with the compound which is a rare case. Likewise, no 2,4-D was detected in the soil samples obtained from Kontola site (**Table 10.3**). By the time soil samples were collected, the farm land was already cultivated with cabbage and that was an indication that farmers in the area are well aware of where to apply the herbicide. Cabbage is grouped under broad-leave plants which can easily be affected by 2,4-D which is effective to control broad-leave weeds.

The United States Environmental Protection Agency (US EPA) set the maximum allowable level of 2,4-D at 70 µg/L (0.07 mg/L), in water for human consumption. Similarly, the European Union (EU) set the maximum residue levels, for individual pesticide at 0.1 µg/L

and 0.5 µg/L for mixtures of pesticides (Chen et al. 2015). However, in Ethiopia, there is no established maximum pesticide residue limit for drinking water except DDT (Berhanu et al., 2020).

Based on the findings of this study, 2,4-D was detected in the water sample of Wafiko and Sher site with a range of 94.46 mg/L to 167.7 mg/L and in the soil samples from Bochesa site 68.22 mg/L and a maximum of 131. 88 mg/L which is higher than the limit set by EPA (Chen et al. 2015) and the EU. Even if the water from this source is not used for drinking purposes, however, the findings of this study could be used as a warning alarm for the need of continuous monitoring program in order to protect the environmental deterioration, and minimizing human and animal health risks possibly caused by future accumulation of the pesticide residues in the study areas.

Typical HPLC chromatograms for the samples are shown **(Figure 28 and 29**). The peak at the retention time 2.7 min corresponds to 2,4-D acid. From the chromatograms, other organic compounds which were not identified in this research were also observed. There is a high probability that these are also some pesticides that are used in the area.

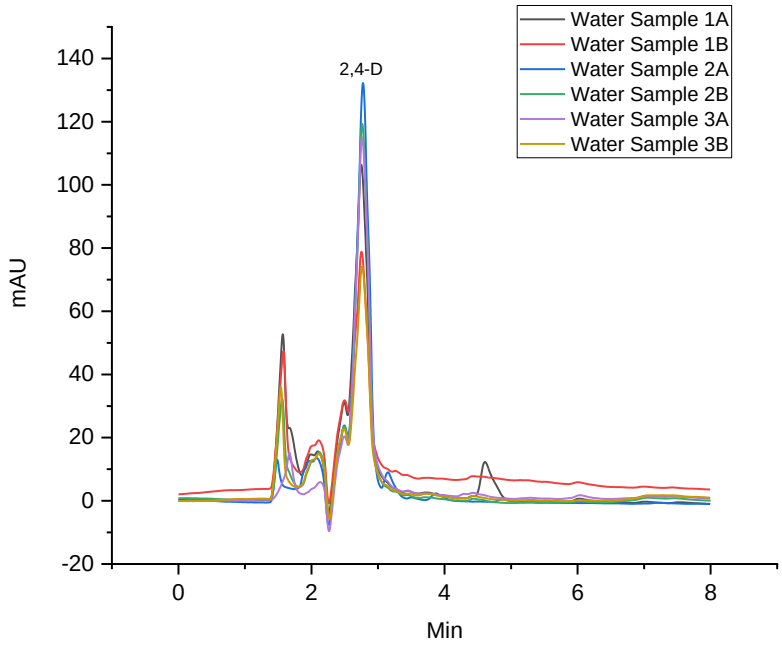


Figure 20: Chromatograms of the water samples from the Wafiko and Sher Site

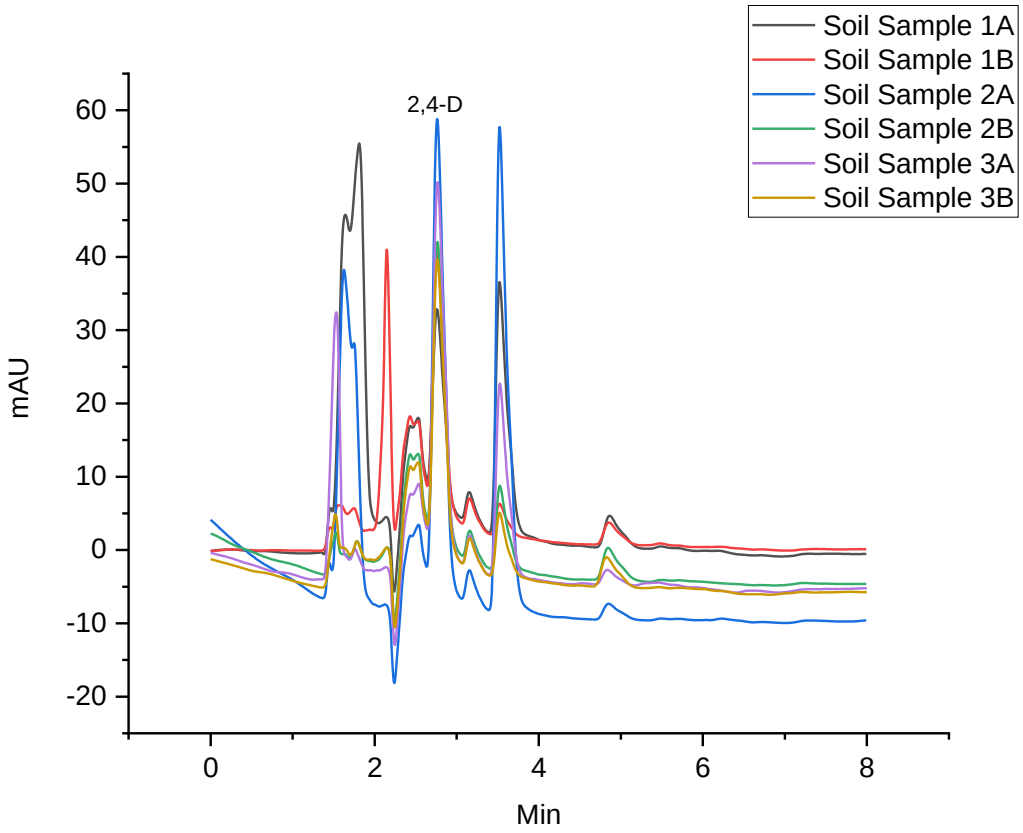


Figure 21: Chromatograms of soil samples from Bochessa site

4.4 Comparison of the levels of 2,4-D from our studies with other studies

In a study conducted by Park et al. (2011) in Korea, 2,4-D was detected in four samples from paddy fields in Hampyung and Sun-chang counties, which farm many paddy field areas for rice. Two of the samples came from Hampyung (12.8 and 6.18 mg/L) county, while the other two came from Sunchang (3.55 and 24.0 mg/L) county. In another study conducted by De Amarante et al. (2003), 2,4-D was detected only up to the 15th day after the herbicide application in a eucalyptus field. The study by Park et al. agrees with our findings that 2,4-D is detectable at higher levels in areas where it is primarily used due to accumulation over time. However, the study by De Amarante et al. contradicts our findings. The availability of additional physical parameters such as metals, organic matter, and pH in the soil where this experiment was conducted could be attributed to the findings.

5. CONCLUSIONS

The method developed herein proved successful, evidencing superior and reliable selectivity, sensitivity, accuracy and repeatability. This analytical method could detect 2,4-D and other organic compounds in other matrices. During method development, various parameters affecting the target analytes' chromatographic separation and extraction efficiencies were evaluated and the optimum conditions were established. Under these conditions, the method was found to be linear over wide concentration ranges with the coefficient of determination of 0.9996; exhibited acceptable precision (%RSD ≤ 5%) and satisfactory relative recoveries ranging from 88–100%. Employing the optimized experimental parameters, trace level extraction followed by HPLC-UV determination of the target analytes in the samples collected from the Ethiopian rift valley were successfully achieved. The results indicated that 2,4-D was detected in water from Wafiko and Sher site and soil samples from the Bochessa site in a higher amount compared to UA EPA and EU maximum allowable level of 2,4-D. Based on the experimental findings and the inference from the comparison with other literature, it can be generalized that the currently developed method is simpler, cheap, rapid and reliable for selective and quantitative extraction of trace level 2,4-D residues and other chemical pollutants, possessing similar physicochemical properties from contaminated samples of different origins. On nature of the compound in water, the acid form of 2,4-D was confirmed to be stable for more than a month with recovery ranges of 73.46±2.00%.

6. Recommendations

Large amounts of pesticides applied to farmland are released into the environment. These agrochemicals are degraded rapidly in soil, water, and sediments, but a few are persistent in the environment and bioaccumulate, which have a major impact on environmental quality and lead to serious consequences. The determination of pesticide residues in the environment is necessary for ensuring that human exposure to these contaminants does not exceed acceptable levels for health. Therefore, this study recommends that:

- Analytical methods should move toward higher levels of automation and portability bearing in mind their benefits for analyte determination in general and 2,4-D monitoring in particular.
- Attention should also be paid to the removal or replacement of harmful chemicals in the developed methodologies. Thus, within the context of green analytical chemistry, researchers should consider with greater conviction the removal or replacement of harmful chemicals by greener alternatives. This aspect should not be limited to the application of the analytical method, but to every aspect related to it. Thus, features such as the preparation of advanced materials to be used in organic compounds analytical systems should be carefully considered.

- In this field, reproducible analytical methods are required to allow the effective separation, selective identification, and accurate quantification of pesticide analytes at low levels in the environmental samples, as fast as possible without impairing method properties such as recovery, accuracy, sensitivity, selectivity, and specificity. The search for more sustainable methods is, in fact, of much importance to avoid the troubling situation that methods devoted to the determination organic compounds in environmental samples may significantly aggravate environment issues.

- The government should institute programs that curb the effect of population growth on land resources, the programs can include; Promote the use of natural fertlizers for example wastes rather than the organic chemicals which have proved to be hazardous not just to the environmental but also to people.
- The government should establish water monitoring points in areas where 2,4-D is used to help in identifying the levels of pollution and management.

- There should be a clear working and chain of communication system among the Ministry of Health, Ministry of Agriculture, and Environmental Protection Authority about how to control the situation.
- Famers should be aware of the long-run effect of 2,4-D on health.

REFERENCE

Abate Jote, C. (2019). A Review of 2,4-D Environmental Fate, Persistence and Toxicity Effects on Living Organisms. *Organic & Medicinal Chemistry International Journal*, *9*(1). https://doi.org/10.19080/omcij.2019.09.555755

Agmas, B., & Adugna, M. (2020). Attitudes and practices of farmers with regard to pesticide use in NorthWest Ethiopia. *Cogent Environmental Science*, *6*(1). https://doi.org/10.1080/23311843.2020.1791462

Alvarez-Rivera, G., Bueno, M., Ballesteros-Vivas, D., Mendiola, J. A., & Ibañez, E. (2019). Pressurized liquid extraction. *Liquid-Phase Extraction*, 375–398. https://doi.org/10.1016/B978-0-12-816911-7.00013-X

Amani, V., Roshan, S., Asgharinezhad, A. A., Najafi, E., Abedi, H., Tavassoli, N., & Lotfi Zadeh Zhad, H. R. (2011). Determination of 2,4-D in environmental samples by three phases directly suspended LPME combined with HPLC-UV. *Analytical Methods*, *3*(10), 2261–2267. https://doi.org/10.1039/c1ay05150d

Amiri, M. J., Bahrami, M., Beigzadeh, B., & Gil, A. (2018). *RESEARCH ARTICLE A response surface methodology for optimization of 2 , 4-dichlorophenoxyacetic acid removal from synthetic and drainage water : a comparative study*. 34277–34293.

Atalay, A., & Hwang, K. J. (1999). Extractability of 2,4-D, dicamba and MCPP from soil. *Water, Air, and Soil Pollution*, *114*(1–2), 155–170. https://doi.org/10.1023/A:1005005122760

Baggiani, C., Giraudi, G., Giovannoli, C., Trotta, F., & Vanni, A. (2000). *Chromatographic characterization of molecularly imprinted polymers binding the herbicide 2 , 4 , 5-trichlorophenoxyacetic acid*. *883*, 119–126.

Bajoub, A., Pacchiarotta, T., Hurtado-fernández, E., Olmo-garcía, L., & Carrasco-pancorbo, A. (2016). Comparing two metabolic profiling approaches (liquid chromatography and gas chromatography coupled to mass spectrometry) for extra-virgin olive oil phenolic compounds analysis : A botanical classification perspective. *Journal of Chromatography A*, *1428*, 267–279. https://doi.org/10.1016/j.chroma.2015.10.059

Barba, S., Carvela, M., Villaseñor, J., Rodrigo, A., & Cañizares, P. (2019). *Fixed-bed biological barrier coupled with electrokinetics for the in situ electrobioremediation of 2*

, *4-dichlorophenoxyacetic acid polluted soil*. *June*. https://doi.org/10.1002/jctb.6079

Behbahani, M., & Najafi, F. (2014). *Coupling of solvent-based de-emulsification dispersive liquid – liquid microextraction with high performance liquid chromatography for simultaneous simple and rapid trace monitoring of 2 , 4-dichlorophenoxyacetic acid and 2-methyl-4-chlorophenoxyacetic ac*. 2609–2618. https://doi.org/10.1007/s10661-013-3564-x

Berhanu, A., Okonkwo, J. O., Berhanu, T., Daso, A. P., Olukunle, O. I., & Leta, S. (2020). Ecological risk assessment of organochlorine pesticides and polychlorinated biphenyls in water and surface sediment samples from Akaki River catchment , central Ethiopia. *Emerging Contaminants*, *6*, 396–404. https://doi.org/10.1016/j.emcon.2020.11.004

Bilal, M., Adeel, M., Rasheed, T., Zhao, Y., & Iqbal, M. N. (2019). *Emerging contaminants of high concern and their enzyme-assisted biodegradation – A review*. *124*(October 2018), 336–353. https://doi.org/10.1016/j.envint.2019.01.011

Brito, G. M., Roldi, L. L., Schetino, M. Â., Checon Freitas, J. C., & Cabral Coelho, E. R. (2020). High-performance of activated biocarbon based on agricultural biomass waste applied for 2,4-D herbicide removing from water: adsorption, kinetic and thermodynamic assessments. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *55*(9), 767–782. https://doi.org/10.1080/03601234.2020.1783178

Brodowska, M. S. (2020). *Muszyński 2020_Article_Occurrence And Transformation Of Phenoxy acid.pdf*. 1276–1293.

Cairns, W. R. L., Ranaldo, M., Hennebelle, R., Turetta, C., Capodaglio, G., Ferrari, C. F., Dommergue, A., Cescon, P., & Barbante, C. (2008). Speciation analysis of mercury in seawater from the lagoon of Venice by on-line pre-concentration HPLC-ICP-MS. *Analytica Chimica Acta*, *622*(1–2), 62–69. https://doi.org/10.1016/j.aca.2008.05.048

Chau, N. D. G., Sebesvari, Z., Amelung, W., & Renaud, F. G. (2015). *Pesticide pollution of multiple drinking water sources in the Mekong Delta , Vietnam : evidence from two provinces*. 9042–9058. https://doi.org/10.1007/s11356-014-4034-x

Chen, X., Zhang, H., Wan, Y., Chen, X., & Li, Y. (2018). Determination of 2,4-Dichlorophenoxyacetic acid (2,4-D) in rat serum for pharmacokinetic studies with a

simple HPLC method. *PLoS ONE*, *13*(1), 1–10. https://doi.org/10.1371/journal.pone.0191149

Chu, J. jun, Ding, Y., & Zhuang, Q. jia. (2006). Invasion and control of water hyacinth (Eichhornia crassipes) in China. *Journal of Zhejiang University. Science. B.*, *7*(8), 623–626. https://doi.org/10.1631/jzus.2006.B0623

Çipa, M., & Kauffmann, J. (2018). Critical Reviews in Analytical Chemistry Chromatographic Methods for the Determination of Emerging Contaminants in Natural Water and Wastewater Samples : A Review Chromatographic Methods for the Determination of Emerging Contaminants in Natural Water and . *Critical Reviews in Analytical Chemistry*, *0*(0), 1–27. https://doi.org/10.1080/10408347.2018.1496010

De Amarante, O. P., Brito, N. M., Dos Santos, T. C. R., Nunes, G. S., & Ribeiro, M. L. (2003). Determination of 2,4-dichlorophenoxyacetic acid and its major transformation product in soil samples by liquid chromatographic analysis. *Talanta*, *60*(1), 115–121. https://doi.org/10.1016/S0039-9140(03)00113-9

Dehghani, M., Nasseri, S., & Karamimanesh, M. (2014). *ENVIRONMENTAL HEALTH Removal of 2 , 4-Dichlorophenolyxacetic acid (2 , 4-D) herbicide in the aqueous phase using modified granular activated carbon*. 1–10.

Dimpe, K. M., & Nomngongo, P. N. (2016). Trends in Analytical Chemistry Current sample preparation methodologies for analysis of emerging pollutants in different environmental matrices. *Trends in Analytical Chemistry*, *82*, 199–207. https://doi.org/10.1016/j.trac.2016.05.023

Ding, W., Liu, C., & Yeh, S. (2000). *Analysis of chlorophenoxy acid herbicides in water by large-volume on-line derivatization and gas chromatography – mass spectrometry*. *896*, 111–116.

EPA, U. S. E. P. A. (2007). EPA Method 1699 2007 pesticides in Water, Saoil, Sediment, Biosolids, and Tissue by HRGC-HRMS. *U.Epa*, *December*, 1–96. https://www.epa.gov/sites/production/files/2015-10/documents/method_1699_2007.pdf

Farre, M. (2013). *Analysis of emerging contaminants in food*. *43*. https://doi.org/10.1016/j.trac.2012.12.003

Fournier, M., Echeverría-sáenz, S., & Mena, F. (2018). *Risk assessment of agriculture impact*

on the Frío River watershed and Caño Negro Ramsar wetland , Costa Rica. 13347–13359. https://doi.org/10.1007/s11356-016-8353-y

Ganeshpure, P. A., George, G., & Das, J. (2007). Application of triethylammonium salts as ionic liquid catalyst and medium for Fischer esterification. *Arkivoc*, *2007*(8), 273–278. https://doi.org/10.3998/ark.5550190.0008.821

Ganjali, M. R., Rezapour, M., Norouzi, P., & Faridbod, F. (2015). *Liquid-Phase Microextraction*. 1625–1658.

Garabrant, D. H., & Philbert, M. A. (2002). Review of 2,4-dichlorophenoxyacetic acid (2,4-D) epidemiology and toxicology. *Critical Reviews in Toxicology*, *32*(4), 233–257. https://doi.org/10.1080/20024091064237

Geissen, V., Mol, H., Klumpp, E., Umlauf, G., Nadal, M., van der Ploeg, M., van de Zee, S. E. A. T. M., & Ritsema, C. J. (2015). Emerging pollutants in the environment: A challenge for water resource management. *International Soil and Water Conservation Research*, *3*(1), 57–65. https://doi.org/10.1016/j.iswcr.2015.03.002

Gul, P., Shahzad, K., Mahwash, A., & Gul, M. (2020). Herbicide thiencarbazone‐methyl pedospheric disposition through sorption and degradation mechanisms in heterogenous soils. *Environmental Earth Sciences*, 1–19. https://doi.org/10.1007/s12665-020-09179-w

Han, D., Jia, W., & Liang, H. (2010). Selective removal of 2,4-dichlorophenoxyacetic acid from water by molecularly-imprinted amino-functionalized silica gel sorbent. *Journal of Environmental Sciences*, *22*(2), 237–241. https://doi.org/10.1016/S1001-0742(09)60099-1

Hinshaw, J. V. (2003). Solid-phase microextraction. *LC-GC Europe*, *16*(12), 803–807. https://doi.org/10.1007/978-3-662-49054-9_2843-1

Hu, C., He, M., Chen, B., & Hu, B. (2015). Simultaneous determination of polar and apolar compounds in environmental samples by a polyaniline / hydroxyl multi-walled carbon nanotubes composite-coated stir bar sorptive extraction coupled with high performance liquid chromatography. *Journal of Chromatography A*, *1394*, 36–45. https://doi.org/10.1016/j.chroma.2015.03.046

Islam, F., Wang, J., Farooq, M. A., Khan, M. S. S., Xu, L., Zhu, J., Zhao, M., Muños, S., Li, Q. X., & Zhou, W. (2018). Potential impact of the herbicide 2,4-dichlorophenoxyacetic

acid on human and ecosystems. *Environment International*, *December*, 332–351. https://doi.org/10.1016/j.envint.2017.10.020

Jergentz, S. (2005). *Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina*. *61*, 817–826. https://doi.org/10.1016/j.chemosphere.2005.04.036

Jin, X., Wang, Y., Jin, W., Rao, K., Giesy, J. P., Hollert, H., Richardson, K. L., & Wang, Z. (2014). Ecological risk of nonylphenol in China surface waters based on reproductive fitness. *Environmental Science and Technology*, *48*(2), 1256–1262. https://doi.org/10.1021/es403781z

Karanth, S. (2005). *Chlorophenoxy Herbicides*. 569–571.

Kashyap, S. M., Pandya, G. H., Kondawar, V. K., & Gabhane, S. S. (2005a). Rapid analysis of 2,4-D in soil samples by modified Soxhlet apparatus using HPLC with UV detection. *Journal of Chromatographic Science*, *43*(2), 81–86. https://doi.org/10.1093/chromsci/43.2.81

Kashyap, S. M., Pandya, G. H., Kondawar, V. K., & Gabhane, S. S. (2005b). *Rapid Analysis of 2 , 4-D in Soil Samples by Modified Soxhlet Apparatus Using HPLC with UV Detection*. *43*(February), 81–86.

Kim, J., Choi, K., & Chung, D. S. (2012). Sample preparation for capillary electrophoretic applications. In *Comprehensive Sampling and Sample Preparation* (Vol. 3, Issue 2). Elsevier. https://doi.org/10.1016/B978-0-12-381373-2.00110-1

Kodamatani, H., Yamasaki, H., Sakaguchi, T., Itoh, S., Iwaya, Y., Saga, M., Saito, K., Kanzaki, R., & Tomiyasu, T. (2016). Rapid method for monitoring N-nitrosodimethylamine in drinking water at the ng/L level without pre-concentration using high-performance liquid chromatography-chemiluminescence detection. *Journal of Chromatography A*, *1460*, 202–206. https://doi.org/10.1016/j.chroma.2016.07.014

Kung, C., Hou, C., Wang, Y., & Fu, L. (2019). Sensors and Actuators B : Chemical Microfluidic paper-based analytical devices for environmental analysis of soil , air , ecology and river water. *Sensors & Actuators: B. Chemical*, *301*(August), 126855. https://doi.org/10.1016/j.snb.2019.126855

Ledoux, M. (2011). Analytical methods applied to the determination of pesticide residues in

foods of animal origin . A review of the past two decades. *Journal of Chromatography A*, *1218*(8), 1021–1036. https://doi.org/10.1016/j.chroma.2010.12.097

Li, Honghong, Feng, Y., Li, X., & Zeng, D. (2018). Analytical Confirmation of Various Herbicides in Drinking Water Resources in Sugarcane Production Regions of Guangxi , China. *Bulletin of Environmental Contamination and Toxicology*, *0*(0), 0. https://doi.org/10.1007/s00128-018-2324-6

Li, Huizhen, Wei, Y., Lydy, M. J., & You, J. (2014). Inter-compartmental transport of organophosphate and pyrethroid pesticides in South China : Implications for a regional risk assessment. *Environmental Pollution*, *190*, 19–26. https://doi.org/10.1016/j.envpol.2014.03.013

López-Bascón-Bascon, M. A., & Luque de Castro, M. D. (2019). Soxhlet extraction. *Liquid-Phase Extraction*, 327–354. https://doi.org/10.1016/B978-0-12-816911-7.00011-6

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S., & Wang, X. C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment*, *473–474*, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

Macirella, R., Madeo, G., Sesti, S., Tripepi, M., & Bernab, I. (2020). *Chemosphere Exposure and post-exposure effects of chlorpyrifos on Carassius auratus gills : An ultrastructural and morphofunctional investigation*. *251*. https://doi.org/10.1016/j.chemosphere.2020.126434

Martín-Pozo, L., de Alarcón-Gómez, B., Rodríguez-Gómez, R., García-Córcoles, M. T., Çipa, M., & Zafra-Gómez, A. (2019). Analytical methods for the determination of emerging contaminants in sewage sludge samples. A review. *Talanta*, *192*(June 2018), 508–533. https://doi.org/10.1016/j.talanta.2018.09.056

Matias, C. A., Vilela, P. B., Becegato, V. A., & Paulino, A. T. (2019). *Adsorption Kinetic , Isotherm and Thermodynamic of 2 , 4-Dichlorophenoxyacetic Acid Herbicide in Novel Alternative Natural Adsorbents*. 1–13.

Meng, Y., Liu, W., Liu, X., Zhang, J., Peng, M., & Zhang, T. (2021). A review on analytical methods for pharmaceutical and personal care products and their transformation products. *Journal of Environmental Sciences (China)*, *101*, 260–281.

https://doi.org/10.1016/j.jes.2020.08.025

Merkle, S., Kleeberg, K., & Fritsche, J. (2015). Recent Developments and Applications of Solid Phase Microextraction (SPME) in Food and Environmental Analysis—A Review. *Chromatography*, *2*(3), 293–381. https://doi.org/10.3390/chromatography2030293

Mohanty, S. (2000). Modeling of liquid-liquid extraction column: A review. *Reviews in Chemical Engineering*, *16*(3), 199–248. https://doi.org/10.1515/REVCE.2000.16.3.199

Mohanty, S. S., & Jena, H. M. (2019). Journal of Water Process Engineering A systemic assessment of the environmental impacts and remediation strategies for chloroacetanilide herbicides. *Journal of Water Process Engineering*, *31*(June), 100860. https://doi.org/10.1016/j.jwpe.2019.100860

Mormeta, B. N. (2017). *Occupational risks and health effects of pesticides in three commercial farming systems in Ethiopia* (Issue June 2017).

Obeng, A., Osafo, S., Dede, S., & Kwaansa-ansah, E. E. (2018). Science of the Total Environment Distribution and risk assessment of banned and other current-use pesticides in surface and groundwaters consumed in an agricultural catchment dominated by cocoa crops in the Ankobra Basin , Ghana. *Science of the Total Environment*, *633*, 630–640. https://doi.org/10.1016/j.scitotenv.2018.03.129

Papadakis, E., Tsaboula, A., Vryzas, Z., Kotopoulou, A., Kintzikoglou, K., & Papadopoulou-mourkidou, E. (2018). Science of the Total Environment Pesticides in the rivers and streams of two river basins in northern Greece. *Science of the Total Environment*, *624*, 732–743. https://doi.org/10.1016/j.scitotenv.2017.12.074

Park, J. Y., Choi, J. H., Abd El-Aty, A. M., Kim, B. M., Park, J. H., Choi, W. J., & Shim, J. H. (2011). Development and validation of an analytical method for determination of endocrine disruptor, 2,4-D, in paddy field water. *Biomedical Chromatography*, *25*(9), 1018–1024. https://doi.org/10.1002/bmc.1559

Pena-pereira, F., Bendicho, C., Pavlović, D. M., Díaz-álvarez, M., Pan, Y., Cooper, J., Yang, Z., Pospiskova, K., Segundo, M. A., & Psillakis, E. (2020). Jo ur na l P re of. *Analytica Chimica Acta*. https://doi.org/10.1016/j.aca.2020.11.040

Petrie, B., Barden, R., & Kasprzyk-Hordern, B. (2015). A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and

recommendations for future monitoring. *Water Research*, *72*(0), 3–27. https://doi.org/10.1016/j.watres.2014.08.053

Pintado-herrera, M. G., González-mazo, E., & Lara-martín, P. A. (2012). *Environmentally friendly analysis of emerging contaminants by pressurized hot water extraction – stir bar sorptive extraction – derivatization and gas chromatography – mass spectrometry*. https://doi.org/10.1007/s00216-012-6453-1

Prado-lu, J. L. Del. (2015). *Insecticide Residues in Soil , Water , and Eggplant Fruits and Farmers ' Health Effects Due to Exposure to Pesticides*. 53–62. https://doi.org/10.1007/s12199-014-0425-3

Rasheed, T., Bilal, M., Nabeel, F., Adeel, M., & Iqbal, M. N. (2019). *Environmentally-related contaminants of high concern : Potential sources and analytical modalities for detection , quanti fi cation , and treatment*. *122*(September 2018), 52–66. https://doi.org/10.1016/j.envint.2018.11.038

Rashid, A., Nawaz, S., Barker, H., Ahmad, I., & Ashraf, M. (2010). Development of a simple extraction and clean-up procedure for determination of organochlorine pesticides in soil using gas chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1217*(17), 2933–2939. https://doi.org/10.1016/j.chroma.2010.02.060

Rezaee, M., Assadi, Y., & Hosseini, M. M. (2006). *Determination of organic compounds in water using dispersive liquid – liquid microextraction*. *1116*, 1–9. https://doi.org/10.1016/j.chroma.2006.03.007

Ryu, H., Li, B., Guise, S. De, Mccutcheon, J., & Lei, Y. (2020). Recent progress in the detection of emerging contaminants PFASs. *Journal of Hazardous Materials*, *October*, 124437. https://doi.org/10.1016/j.jhazmat.2020.124437

Sajid, M. (2018). Trends in Analytical Chemistry Dispersive liquid-liquid microextraction coupled with derivatization : A review of different modes , applications , and green aspects. *Trends in Analytical Chemistry*, *106*, 169–182. https://doi.org/10.1016/j.trac.2018.07.009

Sankoh, A. I., Whittle, R., Semple, K. T., Jones, K. C., & Sweetman, A. J. (2016). An assessment of the impacts of pesticide use on the environment and health of rice farmers in Sierra Leone. *Environment International*, *94*(September), 458–466.

https://doi.org/10.1016/j.envint.2016.05.034

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11), 1–16. https://doi.org/10.1007/s42452-019-1485-1

Shen, J., & Shao, X. (2005). A comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco. *Analytical and Bioanalytical Chemistry*, *383*(6), 1003–1008. https://doi.org/10.1007/s00216-005-0078-6

Shin, E., Choi, J., El-aty, A. M. A., & Khay, S. (2011). *Simultaneous determination of three acidic herbicide residues in food crops using HPLC and confirmation via LC-MS / MS*. *2010*(September 2010), 124–135. https://doi.org/10.1002/bmc.1513

Stefanakis, A. I., & Becker, J. A. (2015). A review of emerging contaminants in water: Classification, sources, and potential risks. *Impact of Water Pollution on Human Health and Environmental Sustainability, September*, 55–80. https://doi.org/10.4018/978-1-4666-9559-7.ch003

Tamru, S., Minten, B., Alemu, D., & Bachewe, F. (2017). The Rapid Expansion of Herbicide Use in Smallholder Agriculture in Ethiopia: Patterns, Drivers, and Implications. *European Journal of Development Research*, *29*(3), 628–647. https://doi.org/10.1057/s41287-017-0076-5

Thiel, N. A., Sachett, A., Schneider, S. E., Garbinato, C., Decui, L., Eichwald, T., Conterato, G. M. M., Latini, A., Piato, A., & Siebel, A. M. (2020). Exposure to the herbicide 2,4-dichlorophenoxyacetic acid impairs mitochondrial function, oxidative status, and behavior in adult zebrafish. *Environmental Science and Pollution Research*, *27*(36), 45874–45882. https://doi.org/10.1007/s11356-020-10497-6

Thorstensen, C. W., Lode, O., & Christiansen, A. L. (2000). *Development of a Solid-Phase Extraction Method for Phenoxy Acids and Bentazone in Water and Comparison to a Liquid - Liquid Extraction Method*. 98–100.

Toft, P. (2003). Background document for preparation of WHO Guidelines for drinking-

water quality. *2,4-D in Drinking-Water*, 1–8.

Tor, A., Aydin, M. E., & Özcan, S. (2006). Ultrasonic solvent extraction of organochlorine pesticides from soil. *Analytica Chimica Acta*, *559*(2), 173–180. https://doi.org/10.1016/j.aca.2005.11.078

Tran, A. T. K., Hyne, R. V, & Doble, P. (2007). *Determination of commonly used polar herbicides in agricultural drainage waters in Australia by HPLC*. *67*, 944–953. https://doi.org/10.1016/j.chemosphere.2006.11.002

Vashisht, D., Kumar, A., Mehta, S. K., & Ibhadon, A. (2020). Analysis of emerging contaminants: A case study of the underground and drinking water samples in Chandigarh, India. *Environmental Advances*, *1*(May), 100002. https://doi.org/10.1016/j.envadv.2020.100002

Wang, X., Yu, J., Wu, X., Fu, J., Kang, Q., & Shen, D. (2016). Biosensors and Bioelectronics A molecular imprinting-based turn-on Ratiometric fl uorescence sensor for highly selective and sensitive detection. *Biosensors and Bioelectronic*, *81*, 438–444. https://doi.org/10.1016/j.bios.2016.03.031

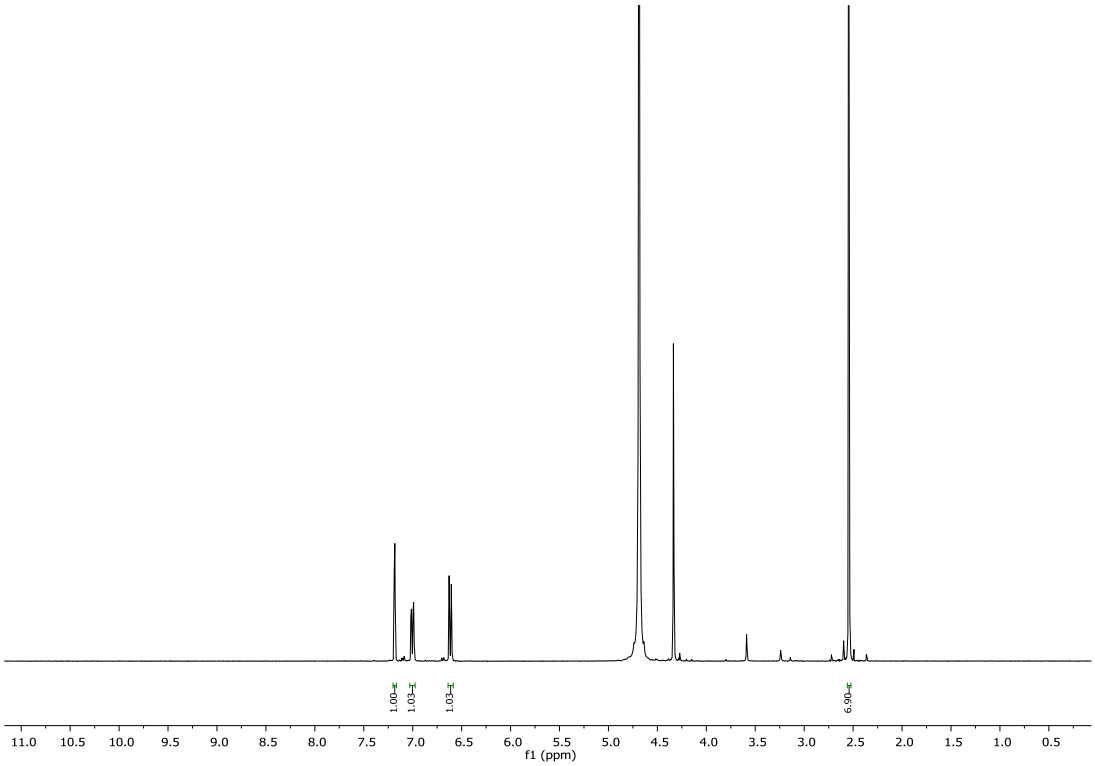
Wells, M. J. M., & Yu, L. Z. (2000). Solid-phase extraction of acidic herbicides. *Journal of Chromatography A*, *885*(1–2), 237–250. https://doi.org/10.1016/S0021-9673(00)00206-5

Wu, J., Zhang, L., & Yang, Z. (2010). A review on the analysis of emerging contaminants in aquatic environment. *Critical Reviews in Analytical Chemistry*, *40*(4), 234–245. https://doi.org/10.1080/10408347.2010.515467

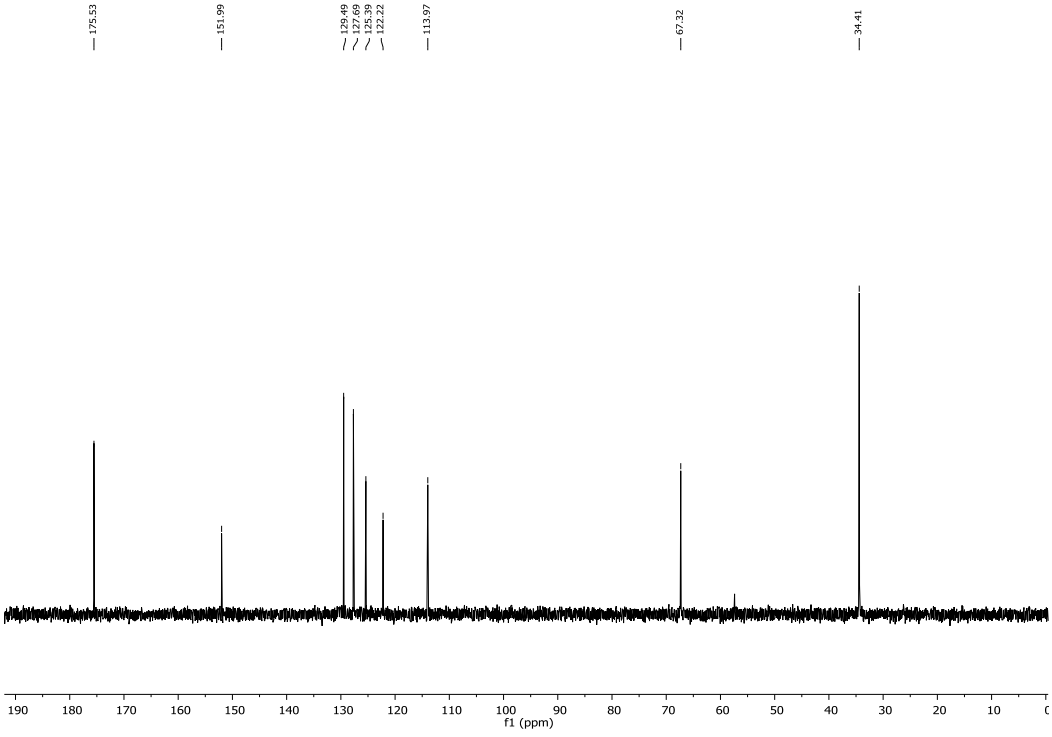
Xi, Y., Mallavarapu, M., & Naidu, R. (2010). Adsorption of the herbicide 2,4-D on organo-palygorskite. *Applied Clay Science*, *49*(3), 255–261. https://doi.org/10.1016/j.clay.2010.05.015

Zhou, H. (2020). *Dechlorination of 2 , 4-dichlorophenoxyacetic acid using biochar-supported nano-palladium / iron : Preparation , characterization , and influencing factors*. *July*, 1–15. https://doi.org/10.1002/aoc.6010

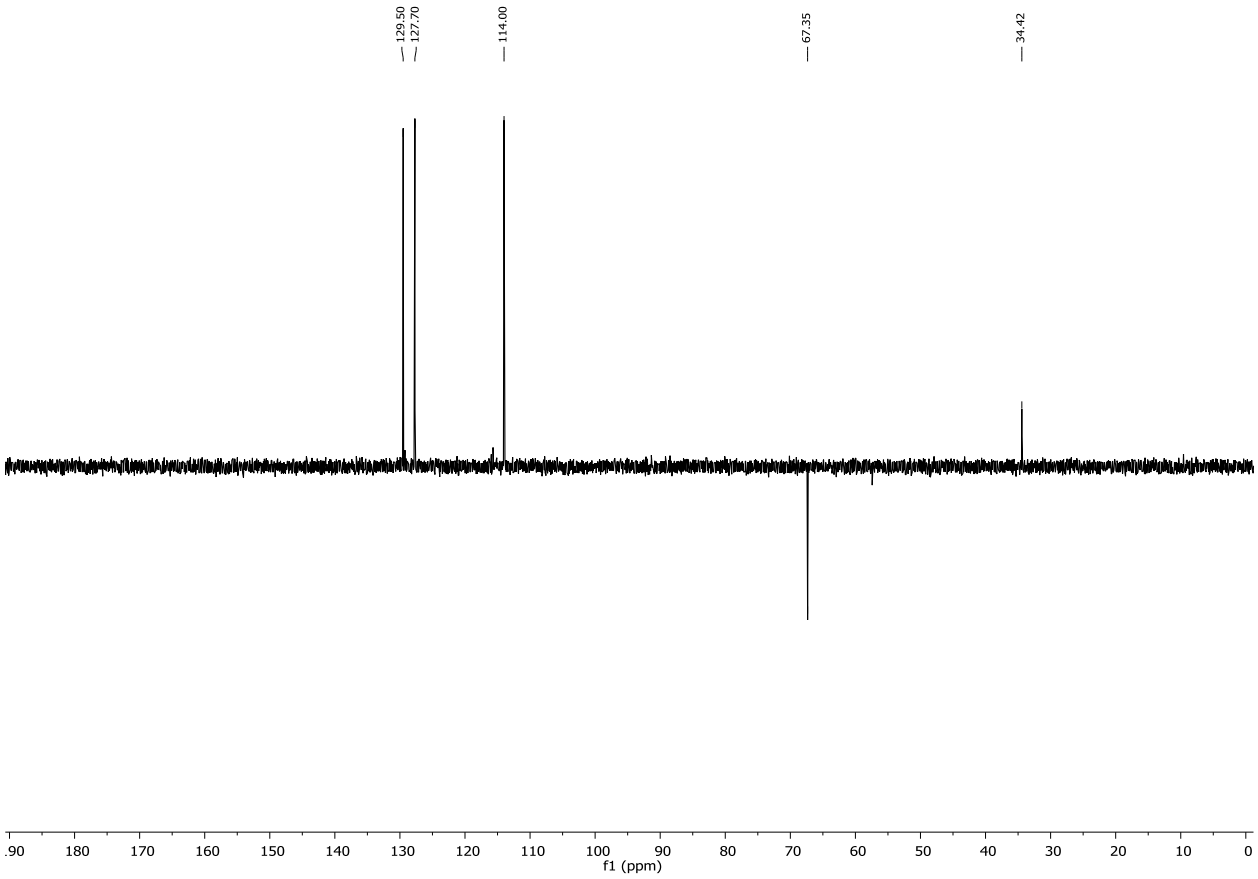
APPENDIX



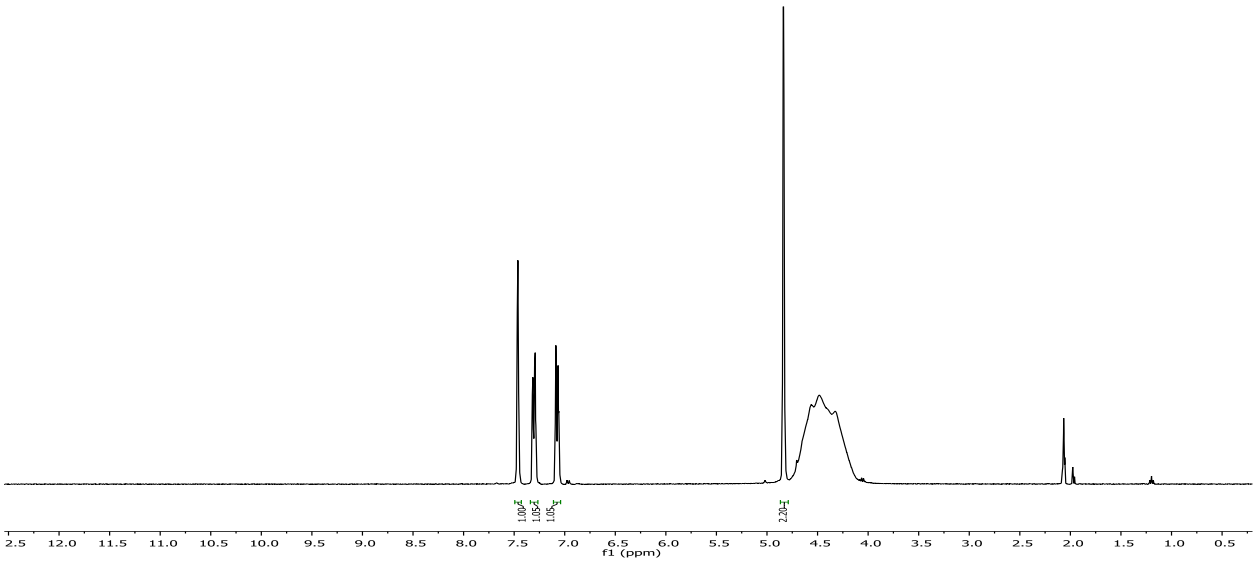
Appendix i : Proton NMR of crude 2,4-D amine salt



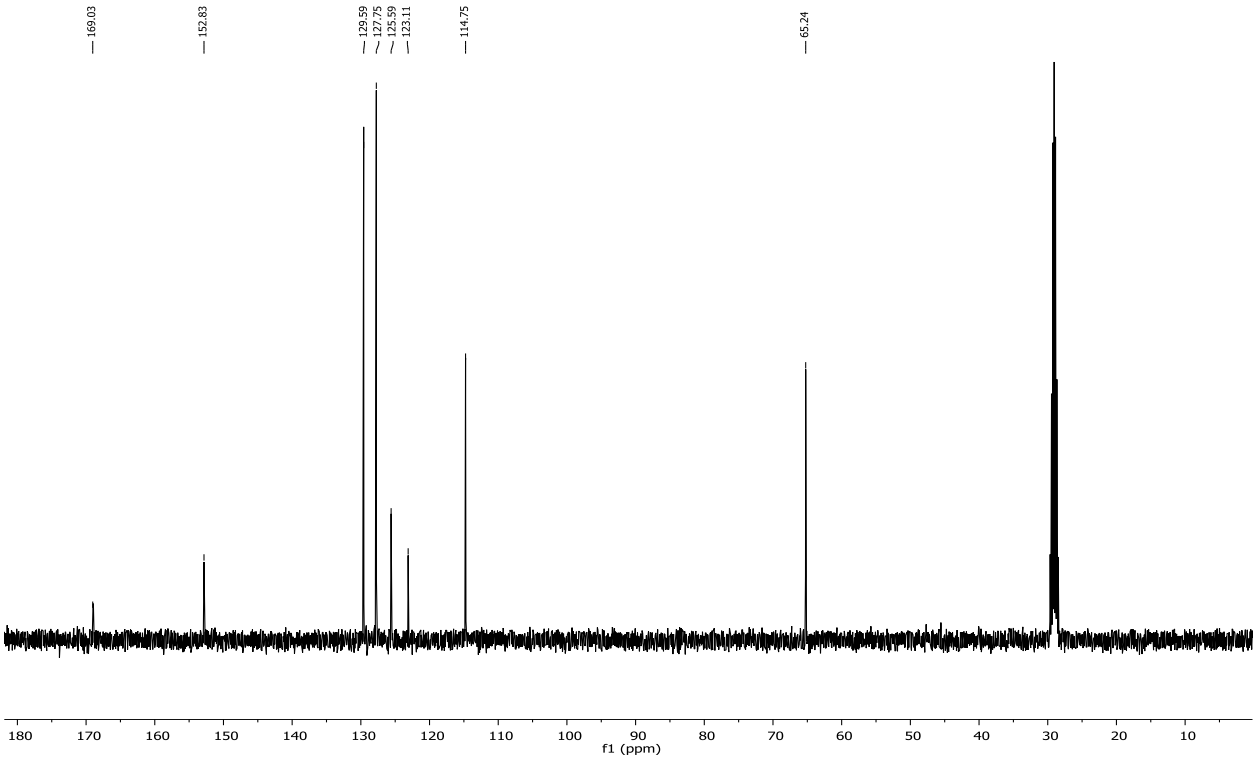
Appendix ii: Carbon NMR of crude 2,4-D amine salt



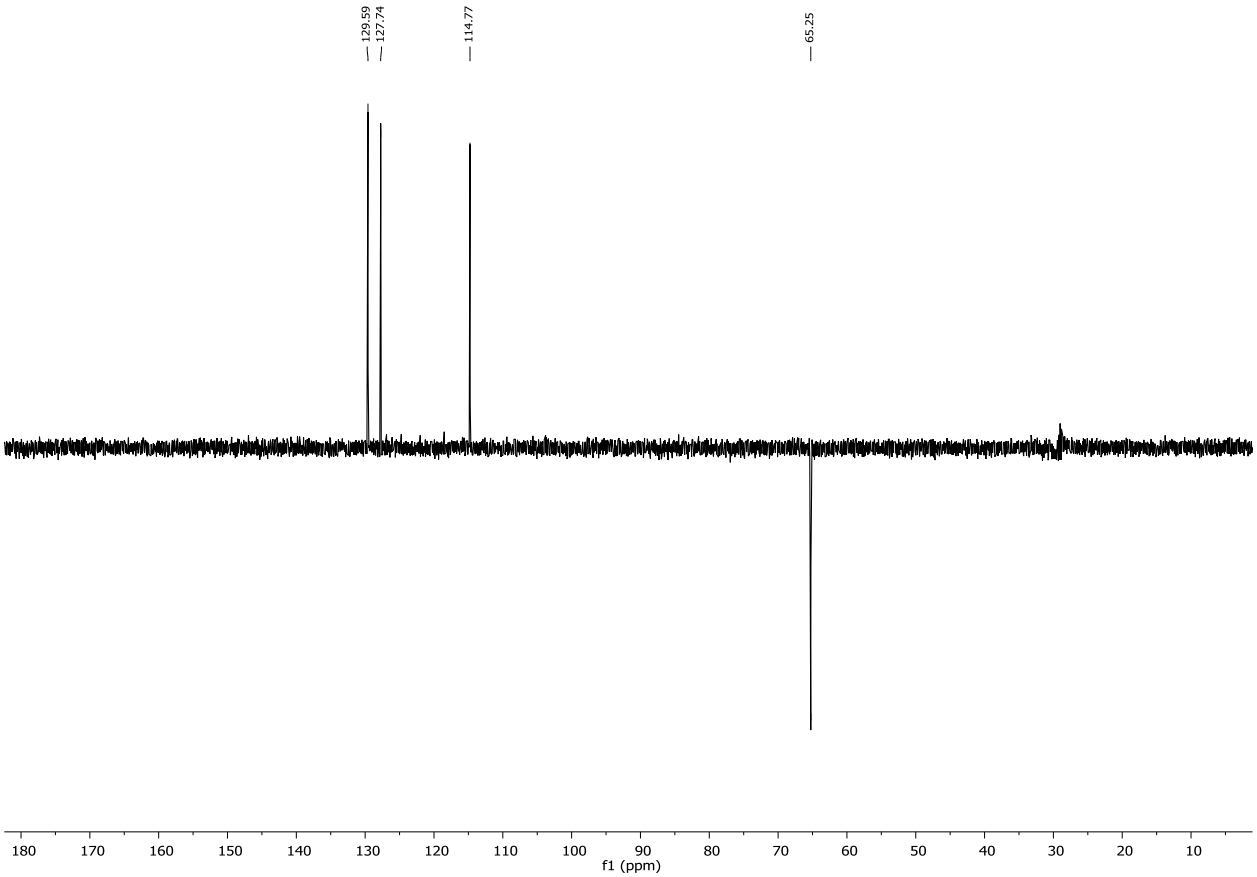
Appendix iii: DEPT-135 NMR of crude 2,4-D amine salt



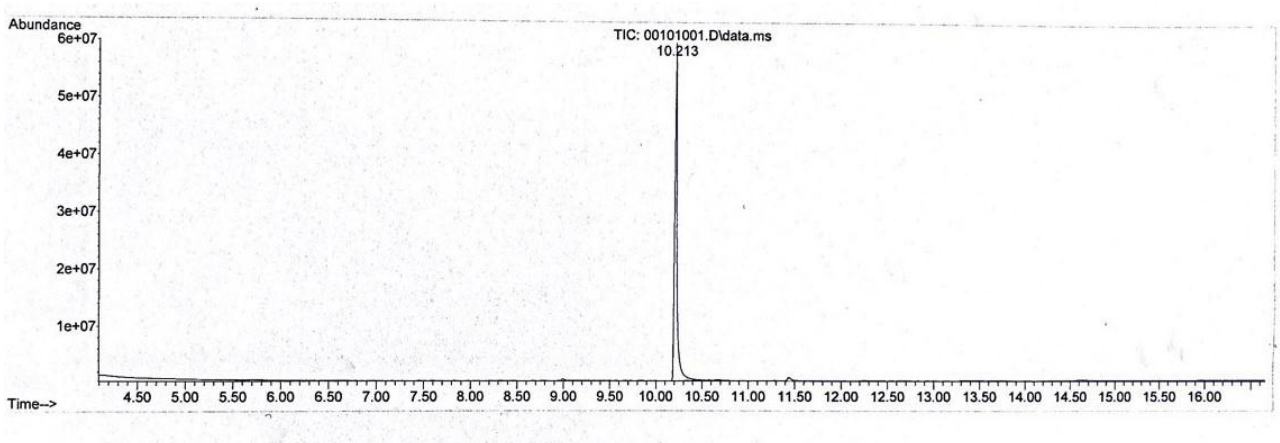
Appendix iv: Proton NMR of recrystallized 2,4-D amine salt



Appendix v: Carbon NMR of recrystallized 2,4-D amine salt



Appendix vi: DEPT-135 NMR of recrystallized 2,4-D



Appendix vii: Chromatogram of the standard ester