In-House Method Validation of the Herbicide Cyhalofop-Butyl by HPLC and its In-Vivo Toxicological Investigations

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By

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Dedication

I would like to extend my deepest gratitude to my dearest father **Mr. Ijaz Hussain** and Mother **Mrs**. **Ghazala Perveen**, their unwavering support and encouragement have been invaluable throughout this journey. To my father, whose wisdom, strength, and relentless belief in me have provided a solid foundation upon which I have built my ambition. To my father, whose patience and constant support have been my anchor, allowing me to navigate the challenges of this endeavor with confidence and determination. This achievement reflects their immense contributions and sacrifices, and I am profoundly thankful for their steadfast presence and support.

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Komal Zehra

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List of Abbreviations

OECD	Organization for Economic Co-operation and Development
GHS	Globally Harmonized System
ID	Inner Diameter
OD	Optical Density
PDI	Primary Dermal Irritation
DT	Dermal Toxicity
LD50	Median Lethal Dosage
LC50	Median Lethal Concentration
Ppm	Parts Per Million
mg/kg	Milligrams per Kilogram
Ml	Milliliters
HPLC	High Performance Liquid Chromatography
UV	Ultraviolet
RSD	Relative Standard Deviation
LOD	Limit of Detection
LOQ	Limit of Quantification
R2	Coefficient of Determination
CRM	Certified Reference Material

ABSTRACT

A growing population necessitates more food. More crops need to be raised by farmers. Because weeds prevent agricultural growth, they should remain in control. One strategy for managing weed growth is the application of a specialized chemical known as an herbicide. This study focused on the analytical validation and toxicological evaluation of the aryloxy phenoxy propionate (cyhalofop butyl). This was accomplished by the application of analytical methods, High-Pressure Liquid Chromatography. The purpose of this effort was to create a solid analytical framework for cyhalofop butyl quantification and detection, ensuring validation parameters and toxicological effects. The cyhalofop butyl spray, mobile phase (80% methanol+ 20% water), and standard solution were prepared. The methodology followed OECD standards and GHS criteria for toxicological evaluation. The validation parameters accuracy, precision, limit of quantification (LOQ), linearity and range, limit of detection (LOD), robustness, and uncertainty were evaluated to validate the HPLC method. The HPLC with column (150 mm x 4.6 mm (ID) packed with 5µm C18), Detector (UV-visible), injection size (20µl), Wavelength (254 nm), and Flow rate (1 ml/min) were used. The result showed that HPLC had high selectivity, sensitivity, and repeatability, evidenced by low relative standard deviation. Additionally, the outcome demonstrated good linearity of the calibration curve ($R^2>0.995$). Toxicological results in rats showed that cyhalofop butyl had moderate acute inhalation (category 3) toxicity. It was non-irritant to eye irritation, non-sensitizing nature to the skin, and had no oral toxicity. In the context of agricultural measurement techniques, the validated outcome showed that the herbicide analysis could yield precise and accurate results. With this understanding, farmers can use it responsibly to protect the environment and themselves.

Keywords: Aryloxy Phenoxy Propionate, Acetyl-CoA carboxylase, Herbicide, Environmental Impact, Toxicological Investigation.

CHAPTER 1

INTRODUCTION

1.1 Background

There will be more than nine billion people in the world by the year 2050. There has to be a 70–100% increase in global food production to meet the needs of this population. There are socioeconomic concerns, abiotic factors, and biotic factors as obstacles to agricultural production. For farmers in both rich and poor nations, weeds pose the greatest biological issues to crop yields. While plant diseases caused by fungus and bacteria, etc.) Animal pests (e.g., insects, rats, nematodes, mites, birds, etc.) are less problematic, weeds provide the greatest risk of agricultural production loss. The sunshine, water, nutrients, and space that crops need are all used by weeds [1].

Modern agriculture would not be the same without herbicides, which have transformed the way farmers control weeds to enhance crop harvest agriculture [2]. A significant development in this field occurred with the introduction of APP herbicides in the 1970s [3]. The dynamic component, aryloxy phenoxy propionate, specifically blocks the plant catalyst acetyl-CoA carboxylase (ACC). This substance is important in the synthesis of fatty acid (an important component of the cell membrane) [4]. Herbicides can be used in a wide range of crops, including maize, rice, and pastures, without harming the crops themselves, they have become essential tools for controlling resistant weed species [5]. Using the same herbicide over and over can lead to the growth of safe weed species, making weed control efforts more difficult and necessitating new definitions and systems. Furthermore, discussions about herbicides have primarily focused on safety and environmental issues. Research and administrative investigation keep on minimizing their effect on water quality, and human well-being [6, 7].

1.1.1 Green Revolution, Problem, and Herbicides

The Green Revolution, which began in the 1940s and lasted until the late 1970s, aimed at preventing hunger and food insecurity in developing nations [8].This initiative focused on many aspects of agriculture, such as the protection of better crop varieties, the adoption of new irrigation methods, the use of more fertilizer, and the increase in

pesticide use [9]. Pesticides aim to destroy a variety of pests including fungi, weeds, rodents, and insects [10]. These chemicals may be either naturally occurring or man-made [11]. The word "pesticide" may refer to a broad class of chemicals such as herbicides, which kill weeds. Herbicides have mostly been used in agriculture to suppress weeds in row crops, orchards, and vineyards. They find particular utility in the cultivation of several crops, including corn, maize, citrus, bananas, coffee, and sugarcane, among many others. Nursery crops and the cultivation of Christmas trees are two other examples of agricultural uses [12, 13].

1.1.2 Aryloxy Phenoxy Propionate Herbicide

In the area of current agribusiness, as the globe develops, weeds are often the enemy. Substance herbicides altered agricultural agreements by providing a practical way to manage these weeds [14, 15]. Among these, aryloxy phenoxy propionate herbicides have emerged as a basic chemical in this case. Because they could selectively target a wide range of weed species without harming crops. APP herbicides, which were developed in the 1970s, changed the way weeds were controlled [16].

1.1.3 Toxic Effect of APP

The aryloxy phenoxy propionates are effective in controlling grass weeds selectively while having little impact on crops. Herbicides include several harmful ingredients, including aryloxy phenoxy propionates, and glyphosate [17]. The aromatic carboxylic acid subfamily known as aryloxy phenoxy propionates inhibits the chloroplast fatty acid biosynthesis enzyme acetyl coenzyme A carboxylase (ACCase). Destroying the membranes is another way that aryloxy phenoxy propionate works. Ion mobility is most inhibited by membranes that are depolarized [18]. Herbicides like aryloxy phenoxy propionates kill plants by inhibiting their ability to make fatty acids. This stops the plants from growing and eventually causes chlorosis and necrosis.

A lot of people who work with human cells report experiencing gastrointestinal issues including nausea, vomiting, and stomach discomfort. Carboxylic acid subfamily aryloxy phenoxy propionate is probably to blame for the corrosive effects seen in the stomach in this instance [19].

1.1.4 Acetyl-CoA Carboxylase (ACCase) Enzyme

Acetyl-CoA carboxylase (ACCase) is a target of herbicides due to its role in the metabolism of fatty acids. It facilitates the conversion of acetyl-CoA to malonyl-CoA

via the ATP-dependent carboxylation reaction and is a biotin-dependent enzyme. Herbicides that block ACCase belong to three chemical groups: Phenoxy propionates with aryloxy propionates (FOPs), cyclohexanodiones (DIMs), and phenyl pyrazoles (DENs) [20, 21].

Herbicides designed to inhibit ACCase aim to kill plants by blocking their production pathways. ACCase herbicides are advantageous because they have a low soil toxicity level, and effectively control a wide variety of grass weeds [22-24]. They are used extensively to control weeds in many crops [25].

1.1.5 Cyhalofop Butyl

In 72 nations, herbicide-resistant weeds have emerged and affected 97 different agricultural products, including rice [23]. Among countries, the United States, Australia, and Canada have the greatest levels of resistance. As of right now, 47 different species have shown resistance to ACCase inhibitors [26, 27]. Following an emergency, ACCase herbicides such as cyhalofop butyl and metamifop are useful for weed control in rice and cereal crops. There is a higher chance of resistance since most Indonesian lowland rice farmers use high dosages of pesticides repeatedly without rotating their crops [28]. Most people who plant rice utilize cyclafop butyl. Herbicides are used to help get rid of plants that aren't necessary for the rice fields to develop [29]. These unwanted plants, or weeds, consume the food and sunlight that the rice crop needs to grow strong and healthy. The rice plant is compromised in the presence of these weeds. The farmers use cyhalofop butyl to get rid of these weeds without harming the rice plants [30]. Using these herbicides at the suggested dosage is crucial. It has potentially harmful effects associated with human life or the environment like any other chemical. The ACCase, in physiological conditions, metabolizes carboxylic acids with long aliphatic chains. These carboxylic acids play a crucial role in many biological processes.

- Production of energy,
- Integral part of cell membranes,
- Organelle membranes,
- Control of hormone levels [31].

The biotin-carboxyl carrier protein (BCCP), biotin carboxylase (BC), and

carboxyltransferase (CT, including subunits α and β) are the three functional domains that make up the enzyme (Figure 1).

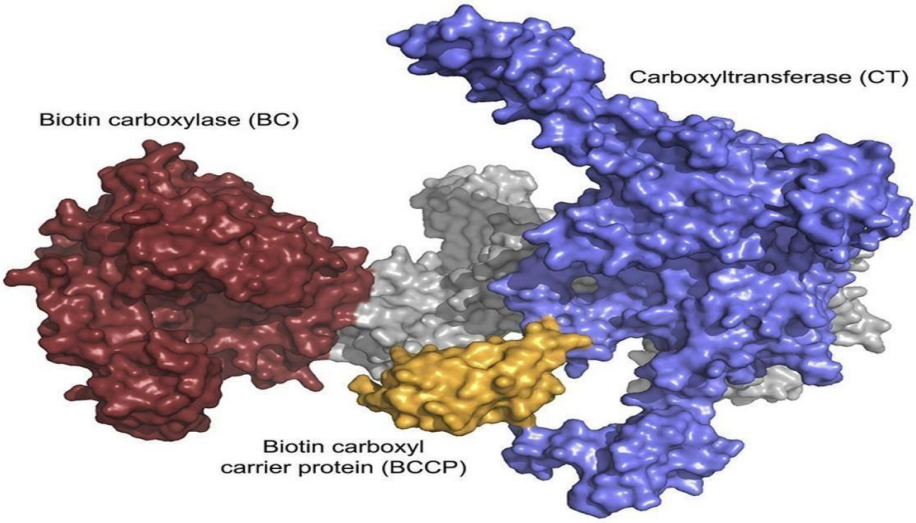

Figure 1.1: The eukaryotic ACCase domains—biotin carboxylase, carboxyltransferase (CT), and biotin carboxyl carrier protein, and in this tertiary structure view of wheat (T. aestivum) [32].

1.2 Problem Statement

The desire for more food production must be balanced, which is a difficulty for modern agriculture. The other challenge is the use of different herbicides to minimize crop losses. But since the usage of herbicides has increased. Ecology is not the only thing affected by the use of herbicides. Weeds lead to a significant yield reduction, affecting food security and economic stability. Development of new herbicides or their functions has always been a challenge. The main goal of this thesis was to determine the toxicity of the cyhalofop butyl belonging to the class of aryloxy phenoxy propionates. The effects of herbicides were examined with the use of the analytical method HPLC-DAD. The validation and toxicity investigation were studied.

1.3 Aims and Objective

- Utilized high-pressure liquid chromatography technique to observe and measure the herbicide's toxic effects,
- Validated the analytical methods ensuring accuracy and precision.
- Investigated toxicological level of Cyhalofop-butyl herbicide through different toxicity test.

CHAPTER 2

LITERATURE REVIEW

Herbicides play an important role in modern agriculture by managing weeds that compete with crops for nutrients, water, and sunlight and they use extra nutrients. Chemicals used to destroy or control plants are known as herbicides. The practice of selective weed management in agriculture was first proposed in 1896 in France. In the last year, herbicides have been the most rapidly expanding category. Inorganic salts including sulfuric acid, calcium cyanamide, sodium arsenite, kainite, sodium chlorate, carbon bisulfide, and sodium arsenite were created between 1908 and 1989 [33]. Selective and non-selective weed control agents were developed between 1930 and 1940. These agents included boron compounds, thiocyanates, dinitrophenols, ammonium sulfate, and specific mineral salts [34]. A new era in herbicide technology began in 1941 with the compound 2, 4-D (2, 4- dichlorophenoxyacetic acid). 2, 4-D was an excellent herbicide [35, 36]. Most perennial grasses and many perennial broadleaf weeds were effectively controlled with the advent of the non-selective herbicide glyphosate in the late 1970s. Synthesis of related compounds is a common process in current herbicide development. This is done when chemists find new organic compounds with phytotoxic qualities [37]. The 1980s saw a shift in herbicide. Numerous studies have focused on the aryloxy phenoxy propionate (APP) herbicide mechanism of action, which includes inhibiting acetyl-CoA carboxylase (ACCase) [38, 39]. In agricultural systems, this herbicide is the primary method of weed control [40]. IWM joins different techniques to oversee weeds actually, intending to keep their effect beneath financially harmful levels [34, 39]. ACCase, or acetyl-CoA carboxylase, assumes a basic part in plant development by managing the unsaturated fats, which are fundamental for creating plant development chemicals, lipids, and optional metabolites. Fatty acid synthesis can be disrupted by herbicides that target ACCase, resulting in an imbalance of these essential compounds. This influences plant development. How these herbicides work includes their development through the plant's xylem and phloem tissues to come to the meristematic area, where they enter the cell walls of weeds. This infiltration causes tissue harm and sap spillage, hindering different metabolic pathways and obstructing the development of new leaves. Commonly, in no less than seven days of herbicide application, impacted plants give

indications of a decrease in well-being and advancing to leaf chlorosis [41]. The enzymatic examination has been exhibited while cutthroat inhibitors of the ACCase substrate, meaning they block the protein's dynamic site. They are also noncompetitive inhibitors of $HCO3^{-}$, Mg^{+2}, and ATP, indicating a more complex inhibitory action than simply blocking the active site. This literature review explores the herbicides and previous work on them and their method validation and their effects on humans and environment [42]. HPLC-DAD are the common type of detector used in HPLC. DAD operates based on the absorption of light by sample components. It uses a diode array to measure the absorbance of the eluting compounds across a range of wavelengths simultaneously [43]. Analytical methods such as High-Performance Liquid Chromatography (HPLC) are used to isolate and measure individual substances in a mixture. Medications, food analysis, environmental monitoring, and many other areas make extensive use of it [44]. Principle of High-performance liquid chromatography (HPLC) deals with differential interactions of the sample components with a stationary phase, which is typically a particle- packed column. A mobile phase, which is a liquid solvent.

- **The Stationary Phase**: is used to determine the elution timing of the various components of the sample, which are introduced into the column during injection.
- **Colum:** which is responsible for separation.
- **Mobile Phase:** This is the solvent that feeds the sample into the column.
- **Detector:** Finds and measures individual components [44].

The method validation start in the pharmaceutical industry in the 1970s to ensure the safety, efficacy, and quality of drug product on human health and on environment. Validation involves the important parameters such as accuracy, precision, specificity, linearity, range, and robustness. ICH guideline Q2 (R1) established in 2005 are used to validate drugs [45].

Johnson and his co-workers show the importance of recovery and inter-day variability measurements in 2010. Linearity was explained by Brown in 2012, and show that analytical results are directly proportional to the analyte concentration **[**46, 47].

Taylor and his co-workers use HPLC to validate an HPLC method for detecting

pesticide residues in water in 2016. Experiment was involved spiking water samples of known concentrations of pesticides and recorded recovery rates was of 95-105%, which was showing high accuracy. Repeatability was recorded with an RSD below 2% and it was a good precision value for a system [48].

UV-Visible spectroscopy also can be used for validating pharmaceutical compounds. Green and Patel use UV-Visible method for quantifying active pharmaceutical ingredients in 2015. The method show linearity over a concentration range of 1 to 50 µg/mL with a correlation coefficient value of 0.999. Robustness was also tested that was involved varying the wavelength and solvent, and it was showing minimal impact on results [49].

Kim and his teammates use Immunoassay Techniques to validate an enzyme linked immunosorbent assay for detecting cancer biomarkers in 2019. Specificity tests was conducted and they show no cross-reactivity with non-target proteins. Sensitivity was recorded by determining the LOD at 0.5 ng/mL, and it ensure the method suitability for clinical diagnostics [50].

Carter and Hill use GC-MS for identifying volatile organic compounds in air samples in 2020. The results of detection limit was low as 0.01 ppm. The method was confirmed with recovery rates above 90% and it is a good recovery for a method [51]. The literature on validation show that validation is Important in ensuring quality and accuracy in various industries. All methods such as chromatography, spectroscopy, immunoassays, and mass spectrometry are validated with rigorous experimental methods, demonstrating their reliability and applicability. The use of animals in toxicity testing start in 20th century when safety concerns about food was risen. Toxicological tests are conducted to check safety and potential hazards of substances which is a most important parameter in pharmaceutical development, environmental science, and food safety. These tests are conducted by following the OECD guidelines. Aim of these testing in animals is to check parameters such as dose-response relationships, lethal dose (LD50), there organ-specific toxicities [52].

Smith evaluate toxicological effect of a Novel Pesticide Formulation in 2020. They use Sprague-Dawley rats for this purpose. Different doses was used for this purpose such as 5, 50, 300, and 2000 mg/kg. The pesticide was given orally to rats at in different concentration with increasing doses. Symptoms of lethargy, reduced motor

activity, and slight tremors were noted at highest dose level of 2000 mg/kg and LD50 was between 300 and 2000 mg/kg. These effects was recovered and was not for long term [53].

Johnson and his co-workers check toxicity of a Pharmaceutical Compound in 2019 On Wister rats and they use doses 0, 10, 50, 250 mg/kg. After repeated of 28 days oral dose application no mortality was recorded but rats in the highest dose group 250 mg/kg demonstrate mild liver enlargement, as indicated by histopathological changes and slight alterations in liver enzymes were also recoded. No Adverse Effect was found to be 50 mg/kg/, and liver effects observed at the higher dose [54].

Lee and his co-workers check Toxicity of an Experimental Drug on Sprague-Dawley rats in 2021.They use different doses such as 0, 10, 50, and 100 mg/kg. During this period of the study rats were dosed from days 6 to 15 of gestation. At the highest dose fetal resorptions were observed and some fetuses exhibited malformations, including cleft palate and skeletal anomalies. It was mild toxic to use but results was not adverse [55]. Robinson evaluate the Carcinogenic Potential of a Chemical Additive in 2022 by utilizing Fischer rats and mice under different doses 0, 10, 50, and 250 mg/kg/day (rats) 0, 20, 100, and 500 mg/kg/day (mice) the results of the study demonstrate an increased incidence of liver tumors at high-dose level in male rats and lung tumors in high-dose in male mice. No any tumors were observed in females at any dose [56]. Toxicological testing following the OECD guidelines provides a strong safety of chemicals and pharmaceuticals. Acute toxicity studies are common types of tests that show the effects of single exposure to a substance with a period of over to 24-48 hours. The main aim of the study is to evaluate LD50 which is the amount of chemical that causes the death of 50% of the test animals. Davies conduct a study to evaluate the acute toxicity of a new pharmaceutical agent by utilizing the animals which names was Wister rats in 2018. The animals were exposed to different levels of doses of the chemical that was to be tested. Dose from 5 mg/kg to 2000 mg/kg were tested. The results of the study was that the highest dose caused lethargy and respiratory depression, and the LD50 was determined to be around 1000 mg/kg in Wister rats [57].

CHAPTER 3

METHODOLOGY

In this chapter cyhalofop butyl was analyze and its methods validation were studied to ensure the reliability. Toxicological evaluation was carried out to verify that the procedures and materials utilized are safe for the environment and human health. It examined the toxicological effects of the herbicide cyhalofop butyl by GHS criteria and OECD guidelines [58].

3.1 Chemicals for Identification

The list of chemicals for method validation of cyhalofop butyl is given in the table.

Table 3.1: List of the chemicals

Item No.	Reagent/Media	Purity	Company
1	Methanol	HPLC Grade	Sigma-Aldrich
2	Water	HPLC Grade	Sigma-Aldrich
3	Ortho Phosphoric Acid 85% (Analytical Grade)	Analytical Grade	Sigma-Aldrich
4	Cyhalofop Butyl Std. of Known Purity	Known Purity	Sigma-Aldrich

3.2 Equipment for Identification

Table 3.2: List of the equipment

No.	Equipment	Function
1	HPLC-DAD	Separates and quantifies components in a sample.
2	Analytical Balance	Measures mass with high precision.
3	Ultrasonic Bath	Cleans samples or equipment using high-frequency Sound waves.

3.2.2 Preparation of Makeup Phase

Took 80% Methanol and added 20% Water in a suitable measuring cylinder. Filtered it with the 0.45µm filter paper, and degas in an ultrasonic bath.

3.2.3 Identification of Cyhalofop Butyl

The retention periods of the standards and samples were precisely matched to ensure accuracy, providing a maximum variation of 3%. By using methanol and acidic water as the mobile phase and reverse-phase chromatography with UV detection at 254 nm, the Quality Control Department was able to analyze cyhalofop butyl. The standard and sample retention periods were similar within a range of +3%.

3.2.4 Preparation of Mobile Phase

2.94 grams of phosphoric acid were dissolved in a 25-milliliter volumetric flask, and the flask was then filled with water to the appropriate level to create a 10% phosphoric acid solution. The 10% phosphoric acid solution was then added to 100 milliliters of an 80:20 methanol water mixture in a measuring cylinder. The mobile phase was prepared by filtering the solution through 0.45µm filters and degassing it in a Picture showing the Mobile phase.

Figure 3.1: Filtration assembly.

3.2.5 Preparation of Standard Solution

Approximately 0.01±0.0025 gm of cyhalofop butyl standard was weighed and added in a 25 mL volumetric flask. To achieve the required volume, the makeup phase was then added to achieve the desired volume, and it was properly mixed and dissolved in an ultrasonic bath.

3.2.6 Preparation of Sample Solution

A sample of cyhalofop butyl weighing 0.01±0.0025 grams was put in a 25 mL volumetric flask. After adding enough makeup phase to reach a 25 mL total volume, the mixture was swirled in an ultrasonic bath until it completely dissolved.

Figure 3.2: HPLC Company Ageint

Table 3.3: HPLC condition for the analysis.

Parameter	Specification
Column	150 mm x 4.6 mm (ID) packed with 5µm C18
Detector	DAD
Wavelength	254 nm
Injection Size	20 µl
Flow Rate	1 ml/min

3.2.7 Operation

The sample was injected and a standard solution was used when the baseline was stabilized. Three readings were taken for the standard and two for the samples. Following the Horwitz principle, the standard deviation was ≤1.3%.

%age purity was calculated by following formula.

$$\%\text{Age purity (w/w)} = \frac{\text{Average Peak Area of sample X Wt. of Std. X Purity of Std.}}{\text{Average Peak Area of Std. X Wt. of Sample}}$$

3.2.8 Cautions/Safety Requirements

1. The filtered sample was always used in the HPLC.
2. The HPLC system was cleaned with a suitable solvent for a minimum of 30 minutes before it was turned off.
3. Acid was always added to water, rather than adding water to acid.

3.3 Validation Procedure

Validation was performed by following the ICH guidelines. The following parameters were used in the validation method [45].

3.3.1 Selectivity and Specificity

Sample mixture of cyhalofop butyl was prepared in which one was oil dispersion and other was SC suspension Concentrate. These two phases were prepared in two forms one with mixture and other was sole formation. They were analyze to determine selectivity. Recovery was calculated in both phases. Calculation was done by following the formula.

Recovery calculation

Recovery (%) = (Concentration in Sole Sample ÷ Concentration in mixture) ×100

Cyhalofop butyl in OD

Recovery = 12.69% / 12.65% × 100 = **100.32%**

Calculated Cyhalofop butyl in SC

Recovery = 15.11% / 15.04 % × 100 = **100.47%**

By following ICH guidelines it given value was in the range of (±20%).

3.3.2 Repeatability

Ten solution were prepared of cyhalofop butyl and were injected into HPLC system under chromatographic conditions. Ten readings were taken to determine its repeatability and calculation was by following steps.

Step 1

Mean calculation

Mean= ∑ Readings ÷ Number of Readings

12.68

=12.75+12.67+12.63+12.68+12.67+12.74+12.65+12.63+12.65 / 10

Mean was calculated and it was 12.68%.

Step 2

Calculation of Standard Deviation (SD)

$$\mathrm{SD} = \frac{\sqrt{\sum(\mathrm{Reading} - \mathrm{Mean})2}}{\mathrm{n} - 1}$$

SD was calculated and it was 0.0412.

Calculated ∑ (Reading-Mean) 2 was equal to 0.0155 and was divided by no of readings.

=0.0155 / 9

Sequare root of 0.0155 / 9 was equal to approximately 0.0412.

SD≃0.0412.

Step 3

Calculation of RSD

RSD (%) = (SD÷ Mean) ×100

=0.0412/12.68×100

RSD= 0.3248%.

Relative standard deviation was calculated and its calculated value was 0.3248%.

According to guidelines value of SD and RSD was below to 2%.

3.3.3 Reproducibility

Reproducibility was determined by two analyst under same conditions but after an

interval of time. Concentration of cyhalofop butyl was measured ten times to check its reproducibility. And their calculation was done by following formulas.

Step 1

Calculation of average for Each Analyst

$$\text{Average} = \sum \text{Readings} \div \text{Number of Readings}$$

Calculated value for **Analyst-1 was 12.68%.**

Calculated value for **Analyst-2 was 12.67%.**

Step 2

Calculation of Standard Deviation (SD) for Each Analyst

$$\text{SD} = \frac{\sqrt{\sum(\text{Reading} - \text{Mean})2}}{\text{N} - 1}$$

- Calculated ∑ (Reading-Mean) 2 for analyst-1 was **0.0155.**
- Calculated ∑ (Reading-Mean) 2 for analyst-2 was **0.0361.**
- N-1 for both analyst was 9.
  - **SD for analyst 1**

$$\text{SD} = \frac{\sqrt{0.0155}}{9}$$

$$\text{SD1} = 0.0412$$

  - **SD for Analyst-2**

$$\text{SD} = \frac{\sqrt{0.0361}}{9}$$

$$\text{SD2} = 0.0631$$

Calculated values of SD was less than two which are acceptable value.

Step 3

T test

T test was performed to check its difference between two analysts.

$$t = (V1 - V2) / \sqrt{((SD1)2/n + (SD2)2/n)}$$

V1-V2 are the averages.

- SD1 =0.0412
- SD2=0.0631
- N is numbers of readings

$$= 12.68 - 12.67 / \sqrt{(0.0412)2/10 + (0.0631)2/10}$$

After calculated them t value was equal to 0.42 and this values was less than given t value.

3.3.4 Method Limit of Detection and Limit of Quantification

Eight solutions of different concentration in ppm of cyhalofop Butyl were prepared to determine its LOD and LOQ. Its average SD and LOD and LOQ was calculated after taken readings of eight solutions.

Step 1

Calculation of Average

Average = sum of all readings ÷ number of readings

Average =47+48+48+48+48+48+48+47 / 8

= 382/8 **= 47.71**

Calculated average value was 47.71ppm.

Step 2

SD Calculation

Its SD was calculated by following formula

$$SD = \frac{\sqrt{\sum(xi - \text{average})2}}{N - 1}$$

- X_i are its individual readings.

- Calculated average was 47.7 by adding these values into the formula calculated ∑ (xi-average) 2 was equal to 1.5.
- N-1 was equal to 7.
- Taking square root of 1.5 / 7 its SD was equal to 0.3674.

Step 3

LOD is typically calculated as follow

$$LOD = 3 \times SD$$

$$LOD = 3 \times 0.3674$$

$$= 1.1022 \text{ ppm}$$

It was rounded as 1.21ppm.

LOQ is typically calculated as:

$$LOQ = 10 \times SD$$

$$LOQ = 10 \times 0.3674$$

$$= 3.674 \text{ ppm}$$

It was rounded to 3.67.

3.3.5 Calibration Curve and Linearity

Standard solutions of different concentration of cyhalofop butyl such as 20.02, 50.04, 100.08, 150.12, 200.16, 300.24, 400.32, 600.48, and 800.64 ppm were prepared. These standard solutions were injected into HPLC. A calibration cure was plotted with concentration on x axis and standard area on y axis. Its R^2 was calculated and a linear curve was observed.

Established the relationship between concentration and area the following equation was used

$$Y = mx + c$$

Y is standard area. X is standard concentration in ppm, m is slop and c is intercept.

Formula used for calculate its slop

$$m = N \sum x\, y - \sum x \sum y / N \sum x^2 - (\sum x)$$

By adding values m was 67.7

Formula used for intercept

$$c = \sum y - m \sum x / N$$

- By adding values c was -5251.3.
- Y is standard area.
- X is standard concentration in ppm.
- N is numbers of readings.
- All values put in $Y = mx + c$.

$$Y = 67.7\, x - 5251.3$$

R2 values was calculated from regression equation as following.

$$R2 = 1 - SS\ res \div SS\ tot$$

This formula was used to calculate R2 in this SS residue was residue calculation which was calculated from

$$SS\ res = \sum (yi - \hat{y}i)$$

$\hat{Y}i$ = predicted values calculated from adding x value in regression equation

Yi= these are observed values

BY adding these values correlation coefficient was calculated

SS tot= calculation of total sum

Calculated Correlation coefficient was

3.3.6 Linearity and Method Working Range

From calibration curve its expected results was noted to check linearity. Its observed results was experimentally noted by injected it into the HPLC. HPLC calculate its concentration by compare it with peak area. A graph was plotted. Expected and

observed results are calculated due to check method accuracy. Linearity was calculated by following equation.

$$Y = mx + c$$

From this equation its correlation coefficient was calculated.

Residue was calculated by this formula

$$\text{Residual} = \text{Observed value} - \text{Expected value}$$

The calculated difference was not significant and close to expected value.it confirms the accuracy of method.

Method working range was calculated from its linearity. All linearity value was its working range. 20 ppm to 800 ppm was its working range it was linear in this range.

3.3.7 Correlation Coefficient

From linearity its R^2 value was calculated. In this expected and observed residue results difference was noted. By following the guidelines its tolerance range was (±10%). R^2 value approximately close to 1.00 indicate a good positive linear relationship between expected and observed results.

3.3.8 Recovery

Recovery was calculated by following formula

$$\text{Recovery (\%)} = (\text{Expected Result} \div \text{Observed Result}) \times 100$$

Calculated recovery was within the range of 80-120%. It indicate a good recovery of a method.

3.3.9 Ruggedness and Robustness Study

To determine its Ruggedness and Robustness it's the flow rate was adjusted from 1mL/min to 1.2mL/min. goal was to ensure its accuracy under different conditions. Its difference was calculated.

Formula

$$\text{Result Difference (\%)} = \text{Result (\% at 1.2 mL/min)} - \text{Result (\% at 1 mL/min)}$$

According to guidelines its tolerance range was ±10%. Calculated value for this

parameter was in this given range and it was passed.

3.4 Toxicology Test

These test was performed by following the OECD guidelines [52].

3.4.1 Acute Eye Irritation Test

3.4.1.1 Procedure: Use of Topical Anesthetics and Systemic Analgesics

We used the following strategies to reduce pain and suffering during retinal safety testing [59].

1. The test chemical (TCA) was applied an hour after applying buprenorphine.
2. A topical pain reliever was applied five minutes before TCA.
3. Meloxicam and buprenorphine were given eight hours after TCA.
4. Meloxicam was then taken every 12 to 24 hours.
5. Rescue analgesia (giving extra pain relief) was given right after TCA if topical anesthesia and preemptive analgesia were not enough. If necessary, other approaches could be used.

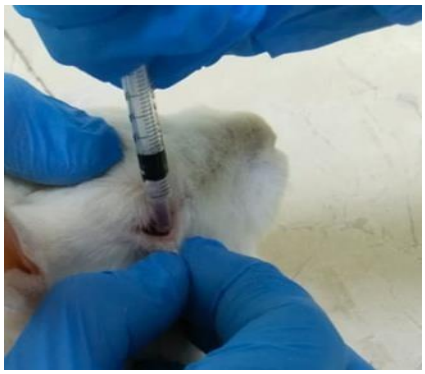

Figure 3.3: Application of Sample in Eyes

3.4.1.2 Principle of the In vivo Test

After injecting anesthesia and pain medication, the test chemical was inserted into one eye while the other eye served as a control. To determine whether eye irritation was reversible, it was examined at specific times. Animals in severe pain were gently put down, and experiments on other animals were stopped.

3.4.1.3 Test Procedure for Eye Irritation

Eye drops without preservatives were used five minutes before the eye test, and

buprenorphine (0.01 mg/kg) was administered one hour before to reduce pain. Meloxicam (0.5 mg/kg) and buprenorphine (0.01 mg/kg) were administrated eight hours later. Until the condition improved, buprenorphine was taken every 12 hours and meloxicam every 24 hours. The dosage of buprenorphine was increased to 0.03 mg/kg shortly after the test and again every 8 hours if necessary if the discomfort continued.

3.4.1.4 Irrigation

Cleaning an animal's eyes for 24 hours after a chemical test was not advised unless the eyes were solid or inflamed. Details were noted if there was an intention to wash afterward.

3.4.1.5 Dosage Level

Liquids: Used 0.1 mL; did not spray directly.

Solids: Used up to 100 mg; rinsed after an hour.

Aerosols: Collected before use; sprayed at a distance of 10cm.

3.4.1.5 Initial Test (In vivo serious Eye Damage/Eye Irritation Test using one animal)

One animal was used for in vivo testing to determine severity and reversibility. Further testing was avoided to safeguard animal welfare Significant eye damage was discovered during the test, and the need for the operation was confirmed by the differences in findings between individual animals.

3.4.1.6 Confirmatory Test

One animal was tested first in the experiment, and it did not exhibit any significant eye injury. After testing a second animal, we discovered some irritation, therefore we halted the experiment. No additional testing was required because the results of the second test were sufficient to categorize the danger.

3.4.2 Method of Acute Oral Toxicity

3.4.2.1 Application of Acute Oral Toxicity

With this procedure, animals were given the test chemical one at a time over 48 hours. The decision about each dose depended on how the animal reacted to the previous treatment. The second dose increased when the animal survived, if it did not survived

then the dose was decreased. The dosage changes, whether they were made higher or lower, adhered to a predetermined formula. Testing continued until either the LD50 (50% of animals died at this dose) was found or the upper limit (between 2000 and 5000 mg/kg) was achieved without resulting in death [60].

3.4.2.2 Procedure: Administration of Doses

A stomach tube or an intubation cannula was used to administer the test chemical in a single dose; alternatively, it could be divided into smaller doses and given over 24 hours. Before being given the dose, the animals had fasted. The body weight of each animal during this time was noted to determine the necessary dosage based on body weight.

Figure 3.4: Application of Sample into mouth

3.4.2.3 Principle of Limit Test

Utilizing up to five animals and doses of 2000 or, in certain situations, 5000 mg/kg, the Limit Test was a methodical procedure. Safety was prioritized by the sequential plan, which increased statistical power and attempted to reject the test if the compound's LD50 was near the maximum dose. When a compound's LD50 were close to the upper limit dose, then classification accuracy declines.

3.4.2.4 Principle of Main Test

One dose at a time, spaced 48 hours apart, was given to the animals; the timing was modified according to the onset, severity, and persistence of toxic effects. It was best to delay starting treatment until the animal was thought to have a high chance of

survival. Maintaining a constant time interval streamlined test procedures, but when the interval varied in the middle, there was no need to modify probability ratios or dose calculations. The initial dose was determined by estimating the dose-response curve's slope and LD50. The antilog of 1 divided by the predicted dose-response slope was the constant dose escalation factor that was used during the test. In the absence of fatality data, 175 mg/kg was the starting dose.

3.4.3 Acute Dermal Toxicity

3.4.3.1 The Procedure of Dermal Toxicity

To stop the animals from consuming the chemical during a 24-hour skin test, the test spot was covered. The mild restriction was used if necessary, but the animals were not made immobile. It was ensured that no animals shared the chemical by keeping them in different cages. Solid substances were hydrated to improve skin contact. After the test, any remaining chemicals were washed off, and the animals were put back in the group housing unless they required separation due to stress or extreme reactions [61].

3.4.3.2 Dose Level

Each dose was tested on two animals in acute dermal toxicity tests. The toxicity was likely significant (LD50 < 200 mg/kg) or unknown. One animal was initially employed at 200 mg/kg for dose-range research because there was not enough information available regarding a test chemical. Two additional animals were used in the primary study in light of those findings. The correctness of this approach had been statistically verified. The same procedure was used in cases when some information was provided but permission could not be acquired, and different starting doses (e.g., 50, 1000, or 2000 mg/kg) may be selected. Depending on the toxicity indicators, a minimum of 48 hours was spent between testing each animal. Before testing the next animal, it was ensured that the prior one was stable. After dosing, every animal was monitored for at least 14 days.

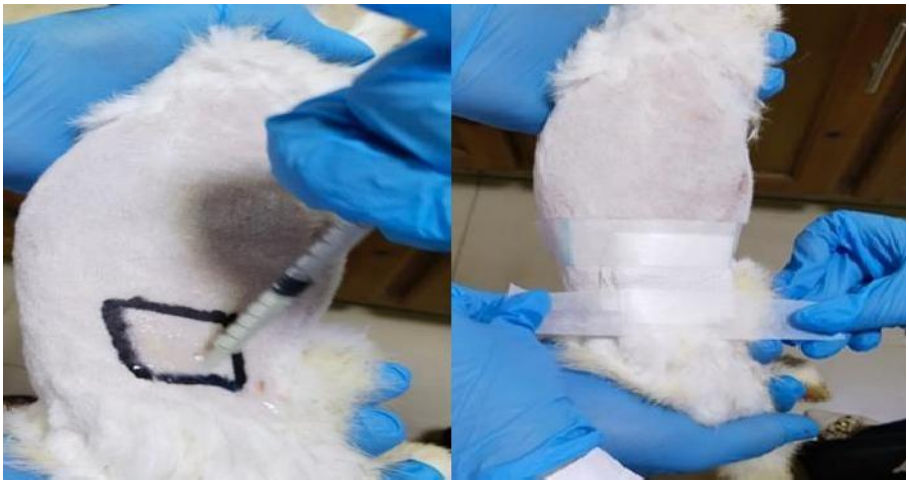

Figure 3.5: Application of Sample on skin

3.4.3.3 Confirmatory Test

Animals were monitored daily for 14 days following the dose in toxicity studies, with particular attention paid to the first 30 minutes and the first 24 hours. Changes in skin, fur, and behavior were important indicators to look out for. Every animal had a record, and any that were very sick were put down in a compassionate manner. It was necessary to record the time of death. Additionally, treatment sites were examined for irritation 24, 48, and 72 hours following exposure.

3.4.4 Acute Dermal Sensitization Test

3.4.4.1 Preparation of Animal

Rabbit fur was clipped 24 hours before the test, and then the back fur of the rabbit was trimmed. Only healthy rabbits were utilized, and skin damage was avoided. Areas where hair was dense were not used. The rabbits were kept in separate cages. The room's temperature was set at 20°C (± 3°C). A humidity level of 30% to 70% was maintained, preferably between 50% and 60%. Twelve hours of daylight and twelve hours of darkness were provided. Unlimited drinking water and consistent lab diets were offered [62].

3.4.4.2 Procedure

Applied the test chemical to a tiny (6 cm^2) patch of skin, then covered it with a gauze patch that was taped in place. Applied it to the gauze first as it was a liquid/paste. The animal couldn't get to the patch, which remained in contact with the skin. To improve interaction, undiluted liquids and mildly wet solids were used. After four hours or so,

water or a solvent was used to get rid of any remaining chemicals without damaging the skin.

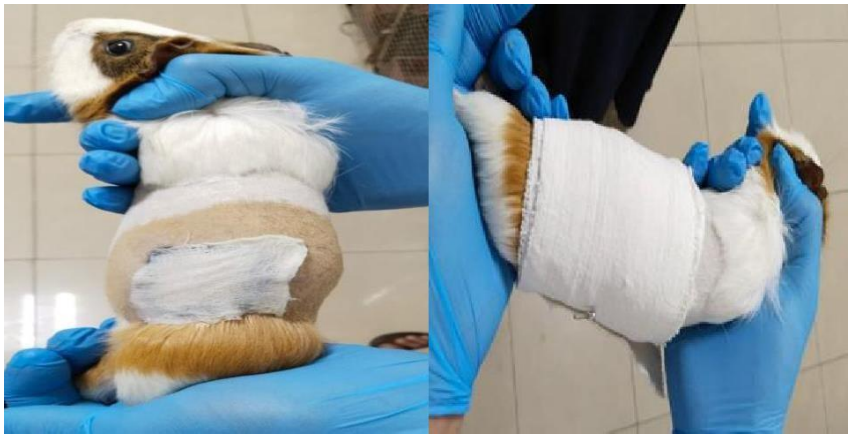

Figure 3.6: Application of Sample on Skin

3.4.4.3 Dose Level

On the test site, a dose of 0.5 mL of liquid or 0.5 g of solid or paste was applied.

3.4.4.4 Initial Test (In vivo Dermal Irritation/Corrosion Test using one animal)

A single animal could have had up to three skin patches evaluated when more information was needed. It remained in place for three minutes for the first patch, an hour for the second, and four hours for the third testing ceased at the first indication of corrosion, and the animal was observed for fourteen days after no corrosion was seen. Applying a single patch for four hours was used for compounds that were not anticipated to cause corrosion but may have irritated.

3.4.4.5 Confirmatory Test

Two additional animals could be examined with a single patch for four hours to ensure there was no irritation if the first test revealed no harmful impact. Two more animals could be tested simultaneously or consecutively if discomfort was discovered during the first test. One patch could be applied to two or three animals for four hours in exceptional circumstances, the initial test was not conducted. No more testing was necessary if two animals exhibited the same response. Uncertain findings might have required more animal testing.

3.4.5 Test for Inhalation Toxicity

3.4.5.1 Initial Consideration

The testing facility had to collect important information, such as the chemical's identity and structure, physical and chemical characteristics, and any prior toxicity test findings, before beginning toxicity research on the chemical. Potential human exposure and data from related substances had to be taken into account before utilizing a chemical. This information could have helped establish the proper starting concentration and confirm that a mixture satisfied regulatory requirements [63].

3.4.5.2 Conditions Required for Test 1

1. Kept the temperature at 22 ± 3ºC.
2. Maintained a minimum of 30%, ideally between 45 and 65%, and never more than 70%, unless cleaning was being done.
3. Used artificial light with a 12-hour cycle of light and dark. Provided regular lab meals and limitless water for drinking.
4. Gave them as much food as they desired or ad libitum. Sorted animals into groups based on their areas of focus, ensuring that there were enough animals in each cage to allow for easy observation**.**

3.4.5.3 Principle of Inhalation Toxicity

The ideal temperature for an animal room was 22 ± 3ºC, with a humidity level of 30 to 70%. There were 12 hours of darkness and 12 hours of light. Animals were marked for identification after a minimum of five days of acclimatization to the lab. They were given unrestricted access to food and water, and group cage animals were used for observation. Depending on the test material, whole-body or head/nose-only procedures were utilized. It was ensured that the breathing zone's airflow, temperature, and humidity were within the advised ranges by checking them frequently. These rules enhanced the accuracy of experiments while preserving animal welfare.

3.4.5.4 Procedure of Toxicity

When testing a chemical, the sighting study established the beginning concentration and the most sensitive sex. For at least four hours, a male and a female animal were tested together. The primary investigation was then conducted on the more sensitive

sex. Following at least a week of observation, the chemical was categorized using particular guidelines depending on the findings.

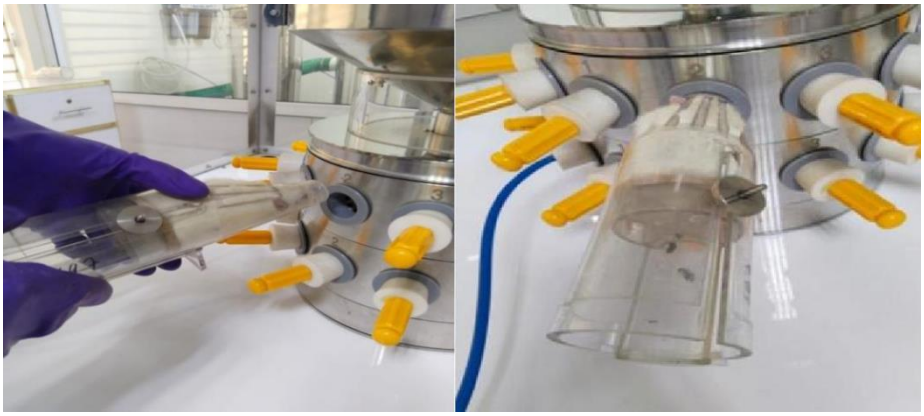

Figure 3.7: Application of Sample in Nasal cavity

3.4.5.5 Limit Test

For substances that were thought to be non-toxic based on related compounds, the limit test was employed. When toxicity was anticipated or unknown, a primary test was required. They began with 20 mg/l for liquids, 5 mg/l for aerosols, and 20,000 ppm for gases for the limit test. Aerosols were to be as tiny as possible to breathe in, ideally at 2 mg/L. Excessive levels were only checked when required, and Category 5 testing was to be avoided unless it was absolutely essential for human health.

3.4.6 Test for Skin Sensitization

3.4.6.1 The General Idea of Guinea Pig Sensitization Tests

The medicine was first injected through the skin or applied externally (induction exposure) to test animals. They were given a challenge dose after a 10- to 14-day rest period to allow an immune response to develop. During induction, the test animals' skin reactions were compared with those of control animals, which received the same challenge dose and underwent a sham treatment. This procedure was used to identify whether the chemical had the potential to cause skin sensitization [64].

3.4.6.2 Guinea Pig Maximization Test (GPMT)

Both male and female guinea pigs in good health were employed; the females were either postpartum or had already given birth. The animals were kept at 20°C (±3°C), with a 12-hour light/dark cycle, 30–70% humidity, and unlimited use of food and water with vitamin C. Before and after the test, they were weighed, their hair was

carefully removed, and they were given time to adjust for five days.

3.4.6.3 Dose Level

The induction test chemical concentration should cause mild to moderate skin irritation and be safe for the body." It should be the maximum dosage for the challenge without causing any irritation. This can be determined with the use of a modest pilot research involving two to three animals. Animals treated with FCA may also be utilized.

3.4.6.4 Methods

Five control and ten treatment animals were used. On day 0, the test chemical and adjuvant were injected into the treated animals, while the vehicle and adjuvant were given to the controls. Treatment animals received the test chemical topically on days 6–8, while controls received the vehicle. The animals were kept in dressings for 48 hours.

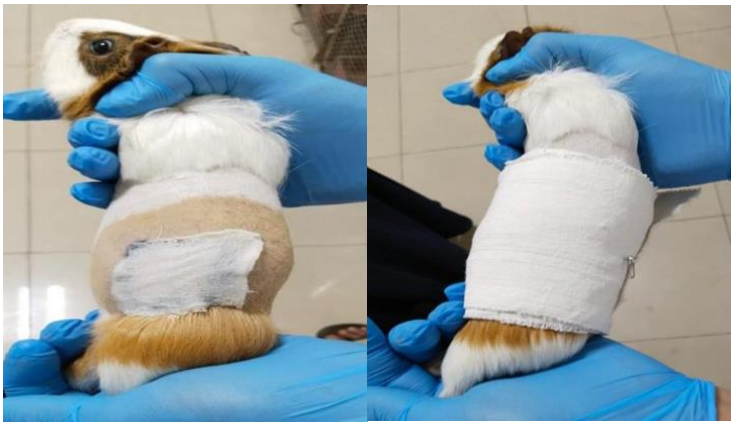

Figure 3.8: Application of Sample in Skin

3.4.6.5 Observation and Results

Clean and trim the problem area 21 hours after removing the patch (shave or depilate if necessary). 3-observe and document the skin reaction using the grading system three hours later (48 hours after the patch application). Make another observation and record it 24 hours after the first one (72 hours after the patch).

CHAPTER 4

RESULTS

In this chapter results of identification validation and toxicological tests were demonstrated.

4.1 Blank Usage

The system automatically sets it to zero before each injection.

4.2 Reference Standards

The standard identification of cyhalofop butyl had a purity of 96%.
Table 4.1: Standards Information.

Standard identification	**Purity**
Cyhalofop butyl	96.00%

4.3 Selectivity and Specificity

Sample mixtures including Active ingredients were analyzed with their sole formation to determine the method selectivity. The data is displayed in Table 4.2. The table showed that the content was analyzed with a good recovery level without nutrient interference in the mixture as per formulation. This test passed.

Table 4.2: Evaluation of method selectivity.

Products	**Results in Mixture**	**Mean Result in Sole Sample**	**Recovery (±20%)**	**Remarks**
Cyhalofop butyl (OD)	12.65%	12.69%	100.32%	Pass
Cyhalofop Butyl (SC)	15.04%	15.11%	100.47%	Pass

4.4 Repeatability

Relative Standard Deviation (RSD) was used as the repeatability metric, and it was qualified with the phrase "repeatability". With a relative standard deviation of ±0.3248% for cyhalofop butyl, which was much less than 2 the results from 10

repetitions indicated agreement. Table 4.3 indicates that the process is repeatable. So, the parameter is classified as pass.

Table 4.3: Repeatability Measurement

Sr.No	Observations	Cyhalofop butyl
1	Reading 1	12.68
2	Reading 2	12.75
3	Reading 3	12.67
4	Reading 4	12.63
5	Reading 5	12.68
6	Reading 6	12.67
7	Reading 7	12.74
8	Reading 8	12.65
9	Reading 9	12.63
10	Reading 10	12.65
	Average	12.68
	SD	0.0412
	RSD	0.3248

4.5 Reproducibility

Table 4.4: Reproducibility Measurement.

Sr.No	Observations		
		Analyst-1 (%)	**Analyst-2 (%)**
1	Reading 1	12.68	12.65
2	Reading 2	12.75	12.69
3	Reading 3	12.67	12.76
4	Reading 4	12.63	12.58
5	Reading 5	12.68	12.63
6	Reading 6	12.67	12.68
7	Reading 7	12.74	12.71
8	Reading 8	12.65	12.57
9	Reading 9	12.63	12.64
10	Reading 10	12.65	12.74
	Average	12.68	12.67
	SD	0.0412	0.0631

Table 4.4 explains the high degree of agreement between independent B findings that were acquired using the same methodology on the same test material but under various circumstances (differing operators, environments, and time intervals).

T-Test

Table 4.5: t = (V1-V2) / (SQRT ((SD1^2/10) + (SD2^2/10)).

t-Test	**Cyhalofop butyl**	
	12.68	12.67
SD	0.04	0.06
(SD)2/10	0.00017	0.00040
(SQRT((S.D)2/10)+(SD)2/10)	0.024	
V1-V2	0.01	
T	0.420	

4.6 Method Limit of Detection (LOD)

The minimal application of a material that was detectable and reported with 99% confidence that the analyte concentration was higher than was known as the method limit of detection (LOD), which was established by analysis of a sample in a specific matrix containing the analyte. The study LOD was determined to be 1.21ppm peak. Formulation samples were analyzed using the Eurachem laboratory guide technique. Both the LOD and LOQ (limit of quantification) values were determined using the data from 10 samples in Table 4.6.

4.7 Method Limit of Quantification (LOQ)

The lowest level of analyte that could be identified with satisfactory performance was known as the LOQ. For calculating the LOQ, the analyte concentration that corresponded to the obtained standard deviation at low levels was multiplied by a factor, kg, which was often set at 10. The LOQ for cyhalofop butyl in this investigation was 3.67 ppm (Table 4.6).

Table 4.6: Assessment of the Quantification and Detection Limits.

Sr. No	Observations	Cyhalofop butyl (ppm)
1	Reading 1	47
2	Reading 2	48
3	Reading 3	48
4	Reading 4	48
5	Reading 5	48
6	Reading 6	48
7	Reading 7	48
8	Reading 8	47
11	**Average**	47.71
12	**SD**	0.3674
LOD		1.21
LOQ		3.67

4.8 Calibration Curve

A sample concentration was calculated using a 9-point calibration curve by measuring the sample's area using HPLC and working standard conditions. Table 4.7 and Figure 4.1 provided the data and calibration curve for cyhalofop butyl. The R^2 value of the curve, which was higher than 0.995, was 0.9995. Good behavior and predictability were shown by an R^2 value greater than 0.995.

Table 4.7: Calibration of Cyhalofop butyl.

Sr. No	Standard Concentration(ppm)	Standard Area
Blank	0	0
Std.1	20.02	1272.431
Std.2	50.04	2498.6982
Std.3	100.08	4969.7319
Std.4	150.12	7360.4858
Std.5	200.16	9749.8125
Std.6	300.24	14657.3799
Std.7	400.32	18511.3379
Std.8	600.48	28667.6855
Std.9	800.64	36758.7227

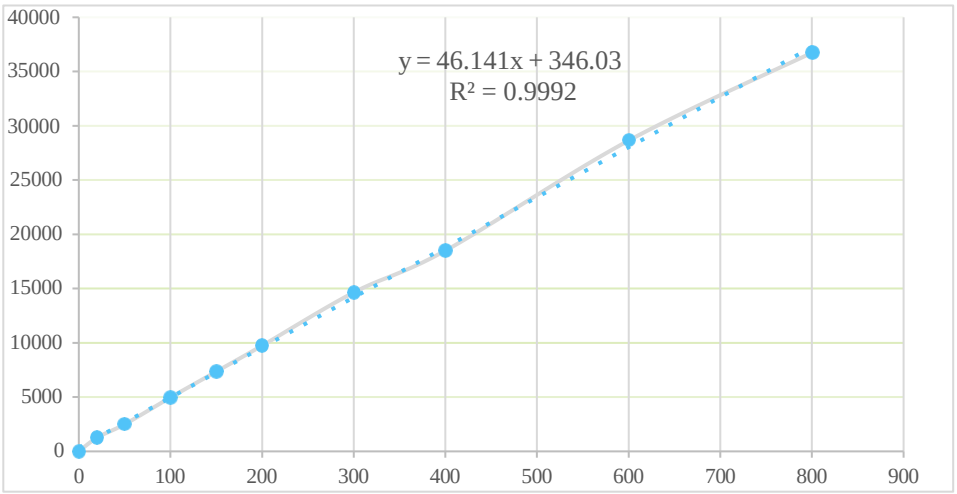

Figure 4.1: Polynomial Curve of Cyhalofop butyl.

4.9 Linearity

A dependent variable has a linear relationship with one or more independent variables.

The concentration and sample standard area have a linear relationship, as seen in Figure 4.4. We require an R2 value larger than 0.995 for our analysis, and our R2 value of 0.9992 satisfies this condition. The residuals are displayed in Table 4.8 and Figures 4.2 and 4.3. While any patterns could imply nonlinearity, their random distribution close to zero supports linearity.

Table 4.8: Expected and Observed Readings.

Cyhalofop butyl		
Expected Result	**Observed Result**	**Residual**
1	0.96	0.04
2.5	2.55	-0.05
5	4.98	0.02
12.6	12.65	-0.05
15	15.1	-0.1

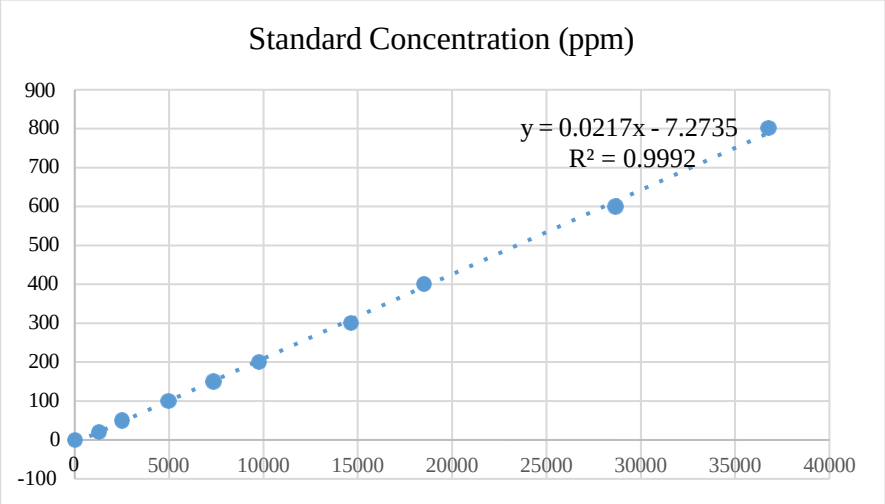

Figure 4.2: Linear Curve of Cyhalofop butyl.

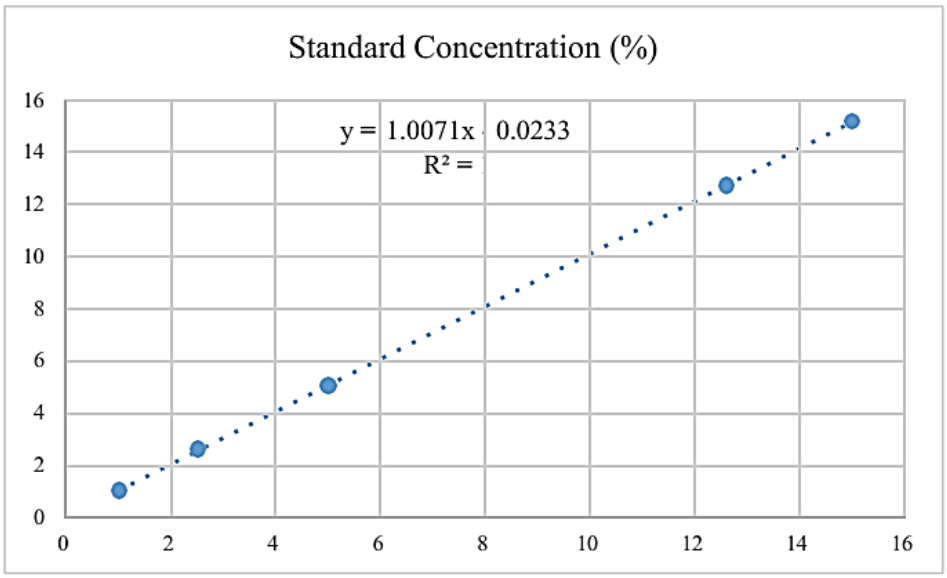

Figure 4.3: Expected and Observed Relationship.

4.10 Method Working Range

It is necessary to determine the technique's working range for the product matrix. This is due to the fact that interferences may result in non-linear responses and the capacity of the method to extract or recover the analyte may change depending on the sample matrix. Since all tested product samples are shown in Figure 4.8. This showed a linear relationship, the whole range is regarded as the methods working range. Therefore, the range for cyclofop butyl was considered to be 20 ppm to 800 ppm.

4.11 Correlation Coefficient

In Table 4.9 and Figure 4.3, R^2=1 indicates a very high correlation between the observed and (actual) values. Because of these correlations, it can be stated that the method performance in terms of the correlation coefficient was outstanding. As a result, this parameter was rated as pass.

Table 4.9: Analysis Verification

Cyhalofop butyl					
Sr. No	**Expected Result**	**Observed Result**	**Difference**	**Tolerance range for verification (±10%)**	**Remarks**
1	1	0.96	0.04	0.90%-1.1%	Pass
2	2.5	2.55	-0.05	2.25%-2.75%	Pass
3	5	4.98	0.02	4.5%-5.5%	Pass
4	12.6	12.65	-0.05	11.34%-13.86%	Pass
5	15	15.1	-0.1	13.5%-16.5%	Pass

4.12 Recovery

The method performance was assessed within a given range of criteria, namely within ±20% of recovery, and the recovery was documented in Table 4.10 When the procedure was tested at the lowest, medium, and highest concentrations, the results demonstrated that it was valid in this regard.

Table 4.10: Evaluation of Recovery of cyhalofop butyl.

Sr. No	**Cyhalofop butyl**				
	Expected Result	**Observed Result**	**Recovery**	**Range (±20%)**	**Verification**
1	1	0.96	96.00%	80% - 120%	Verified
2	2.5	2.55	102.00%	80% - 120%	Verified
3	5	4.98	99.60%	80% - 120%	Verified
4	12.6	12.65	100.40%	80% - 120%	Verified
5	15	15.1	100.67%	80% - 120%	Verified

4.13 Ruggedness (Robustness)

In an analytical procedure "ruggedness" or "robustness" is defined as " the ability to be unaffected by slight variations in procedure parameters that are purposeful." An indication of the method's dependability of the method provided by ruggedness during

normal usage. The matrix flow rate of cyhalofop butyl Analysis has been changing from the standard flow rate in this study. The variances in cyhalofop butyl findings are shown in Table 4.11. These were within the specified tolerance level, which was 10%.

Table 4.11: Ruggedness and Robustness Study.

Sr. No	**Cyhalofop butyl**			
	Result (%) at Flow Rate-1ml/min	**Result (%) at Flow Rate-1.2ml/min**	**Result Difference (%)**	**Tolerance range for Verification (±10%)**
1	12.68	12.70	-0.16	Pass
2	12.75	12.69	0.47	Pass
3	12.67	12.75	-0.63	Pass
4	12.63	12.72	-0.71	Pass
5	12.68	12.66	0.16	Pass
6	12.67	12.77	-0.79	Pass
7	12.74	12.70	0.31	Pass
8	12.65	12.62	0.24	Pass
9	12.63	12.71	-0.63	Pass
10	12.65	12.70	-0.40	Pass

4.14 Summary of Criteria

In this table all results of validation was summarized. Validation parameters was summarized with their ranges.

Table 4.12: Summary of Criteria

Sr. No.	Validation Parameter	Criteria	Result	Remarks
1	**Blank**	-	-	Autozero
2	**CRM**	Available	Complies	Pass
3	**Repeatability**	RSD <2	0.3248%	Pass
4	**Reproducibility**	t value < 2.262	0.420	Pass
5	**LOD**	Was calculated	1.21	Pass
6	**LOQ**	Was calculated	3.67	Pass
7	**Calibration curve**	$R^2 > 0.995$	R^2=0.9995	Pass
8	**Linearity**	$R^2 > 0.995$	R^2=0.9992	Pass
9	**Method working range**	Was calculated	20 ppm – 800 ppm	Pass
10	**Correlation Coefficient**	$R^2 > 0.995$	R^2=1	Pass
11	**Recovery**	In range of 80 – 120%	96%-102%	Pass
12	**Selectivity**	Recovery > 95%	100.32%	Pass
13	**Robustness**	Result difference < ±10%	-0.16 to 0.47%	Pass

4.15 Toxicological Investigations

The "six-pack" of animal tests for acute toxicity is used to determine acute systemic toxicity through three exposure routes: skin and ocular irritation/corrosion, and skin sensitization. (EPA's NAMs Work Plan, June 2020).

1. Result of Acute Dermal Irritation test

2. Result of Acute Dermal Toxicity test
3. Result of Acute Inhalation Toxicity test
4. Result in Acute Eye Irritant toxicity test
5. Result of Acute Skin Sensitization Test
6. Result of Oral inhalation toxicity Test

4.15.1 Result of Acute Dermal Irritation Test

An Acute Dermal Irritation Test on cyhalofop butyl is described in Table 4.14. The findings of the test, which was completed in accordance with OECD 404 recommendation, showed that the product is not unpleasant.

Table 4.13: Showing the acute dermal irritation test on cyhalofop butyl.

Report ID:	JAT/D1/40823
Quantity sampled	100 ml
Physical Nature	OD
Test Animal	Albino Rabbits
Result	0 "Non Irritant"
Test Duration	14 Days
Dose used	0.5ml

Table 4.14: Tabulation response data.

Time after removal of a patch	**Erythema**	**Edema**	**Total PDI Score**
30-60 min	0	0	Erythema = 0 Edema= 0
24 Hrs	0	0	
48 Hrs	0	0	
72 Hrs	0	0	PDI score=0

Table 4.15: Showing GHS report data.

Category	Description	Mean value at the end of the observation period
Category 2 (Irritant)	Erythema or edema score < 4.0	Throughout the duration of the observation period, inflammation continues.
Category 3 (Mild Irritant)	Erythema or edema score < 2.3	Not Applicable
No clinical signs	No Erythema, No Edema	Not Applicable

4.15.2 Result of Acute Dermal Toxicity

Six male Wister rats were used in the acute dermal toxicity test, which evaluated the possible toxicity of cyhalofop butyl by applying it to their skin. The test was conducted using a 100 ml oil-dispersible sample in accordance with OECD 402 standards.

Table 4.16: Result of acute dermal toxicity test.

Report ID	JAT/DT/40823
Quantity Sampled	100 ml
Physical Nature	OD
Product	Cyhalofop Butyl
Guideline Followed	OECD 402
Temp	22+3°C
Humidity	30-70%
Duration	12 hour Dark and 12 hour Light
Test Animal	Wister Rats
Number of Animals	6
Sex	Male

Table 4.17: Tabulation response data for acute dermal toxicity.

Sr. No	Number of animals exposed	Dose Level (mg/Kg)	Mortality
1	2	200	0
2	2	1000	0
3	2	2000	0

A 12-hour light and dark cycle, a temperature of 22 ± 3°C, and humidity levels were all maintained for the study's animals ranging from 30 to 70%. Cyhalofop butyl did not exhibit acute skin toxicity under the given conditions, as seen by the test animals' lack of notable side effects throughout the trial. The tested parameters imply that the chemical is safe for skin exposure.

4.15.3 Results for Acute Inhalation Toxicity

According to test data, two out of five rats exposed to a 10 mg/L inhalation dosage died from cyhalofop butyl.

Table 4.18: Result for acute inhalation toxicity test.

Report ID	JAT/IT/40823
Customer Detail	Lab
Quantity Sampled	100 ml
Physical Nature	OD
Product	Cyhalofop Butyl
Guideline Followed	OECD 433
Temp	22+3°C
Light Duration	12 hour Light
Humidity	30-70%
Test Animal	Wister Rats
Number of Animals	15
Sex	Male

Table 4.19: Tabulation data for acute inhalation toxicity.

Sr. No	Number of animals Exposed	Dose Level (mg/L)	Mortality
1	5	0.5	0/5
2	5	2	0/5
3	5	10	2/5

A material is placed in Category 3 according to the GHS criteria for acute inhalation toxicity if the LC50 is more than 2 mg/L and below or equivalent to 10 mg/L. As a result, the report determined that cyhalofop butyl acute inhalation toxicity is present under Category 3.

By OECD 433 recommendations, 15 male Wister rats were used in the acute inhalation toxicity test for cyhalofop butyl. A 100 ml sample was evaluated in a controlled environment 12-hour light cycle and a 22±3 temperature°C, and a humidity of 30-70%. The tested parameters imply that the chemical is safe for human beings.

4.15.4 Result of Acute Eye irritant

1. Body weight changed,10±5 grams and outcome
2. Not an irritant to the eyes

The mean scores for corneal opacity (0.58), iritis (0.91), redness (1.41), and chemosis (1.08) were all below the GHS criterion levels for ocular irritation, according to the data. Thus, the investigation comes to the conclusion that cyhalofop butyl does not cause eye irritation.

Table 4.20: Results for acute eye irritant.

Quantity Sampled	100 ml
Physical Nature	OD
Product	Cyhalofop Butyl
Guideline Followed	OECD 405
Temp	20±3°C
Light Duration	12 hour Light
Humidity	30-70%
Test Animal	Albino Rabbits
Number of Animals	3
Sex	Male

Table 4.21: Tabulation of response for eye irritant.

Animal No.	**Corneal Opacity Score**	**Iritis Score**	**Redness Score**	**Chemosis Score**
1	0.75	0.75	1.25	1.0
2	0.5	1.5	1.5	1.25
3	0.5	0.5	1.5	1.0
Mean Score	0.58	0.92	1.42	1.08

By OECD 405 criteria, in a controlled setting (20±3°C, 12 hours of light, and 30–70% humidity), three male albino rabbits were given 100 ml of the substance to test. In order to provide accurate data for safety assessments, the test was designed to evaluate possible eye irritation under controlled circumstances. The tested parameters imply that the chemical is safe.

4.15.5 Results of Skin Sensitization Test

In these tables results of skin sensitization are label.

Table 4.22: Result of skin sensitization test.

Report ID	JAT/SS/40823
Customer Detail	Lab
Quantity Sampled	100 ml
Physical Nature	OD
Test Method	Buhler's method
Section	Toxicology
Test	Skin Sensitization Test
Product	Cyhalofop Butyl
Guideline Followed	OECD 406
Temp	20±3°C
Light Duration	12 hour Light
Humidity	30-70%
Induction Dose	17%
Challenge dose	6%
Test species	Guinea pigs
Feed and water	Mineral water and feed are given ad libitum

The animals shaved skin was rubbed with a 17% concentration of the test material. Following an induction period, a separate area of the shaved skin was handled with a 6% concentration of the test chemical. Over 27 days, the animals were watched for any indications of cutaneous irritation or sensitization. The test animals did not exhibit any positive reactions, suggesting that cyhalofop butyl did not result in skin sensitization. Cyhalofop butyl is therefore categorized as a "Non-Sensitizer" by the skin sensitization criteria specified in the Globally Harmonised System (GHS). This indicates that, under typical usage circumstances, it is unlikely to result in an allergic skin reaction in people.

Table 4.23: Tabulation table for skin sensitization test.

Patch No.	(Incidences of positive effect/animal)	Control Group	Experimental Group
1 (Day 1)	Not Reported	0	0
2 (Day 6)	Not Reported	0	0
3 (Day 13-15)	Not Reported	0	0
4 (Day 27)	Not Reported	0	0

The OECD guideline 406 was followed in the skin sensitization test of cyhalofop butyl, which used guinea pigs, using Buhler's method. Under controlled conditions20 ± was the temperature at which the test was performed. 3 °C, 12 hours of light exposure, and a humidity range of 30 to 70%. 17% was the induction dose and 6% was the challenge dose. To ensure the guinea pigs comfort throughout the study, they were given mineral water and unlimited food. The test outcomes are crucial for assessing the products possible skin sensitization effects.

4.15.6 Result of Acute Oral Test

Cyhalofop butyl did not result in skin sensitization. Cyhalofop butyl is therefore categorized as a "Non-Sensitizer" in accordance with the skin sensitization criteria specified in the Globally Harmonised System (GHS). This indicated that, under typical usage circumstances, it is unlikely to result in an allergic skin reaction in people.

Table 4.24: Table for acute oral toxicity.

Report ID	JAT/OT/40823
Quantity Sampled	100 ml
Physical Nature	OD
Section	Toxicology
Test	Acute Oral Toxicity Test
Product	Cyhalofop Butyl
Guideline Followed	OECD 425
Test Animal	Wister Rats
Number of Animals	5
Sex	Female

Table 4.25: Tabulation table for acute oral toxicity.

Sr. No	**Number of animals Exposed**	**Dose Level (mg/Kg)**	**Mortality**
1	5	175	0/1
2	5	550	0/1
3	3	2000	0/3

In accordance with OECD 425 recommendations, an acute oral toxicity test was performed on the insecticide cyhalofop butyl. Five female Wister rats were used in the investigation, and a 100 cc sample was used to test for toxicity. The optical density (OD) of the sample is a measure of its physical characteristics. The experimental setup, test settings, and particular parameters that were followed throughout the toxicity assessment are all included in this table. This information is vital for assessing the product's safety and any dangers.

CHAPTER 5

DISCUSSION

Chapter 5 provides an overview and discussion of the methods used for the analysis of aryloxy phenoxy propionates, with the focus on cyhalofop butyl. The validation process was designed to evaluate various parameters to ensure the accuracy, precision, and reliability of the analytical method. Toxicological investigation were also conducted on cyhalop butyl providing a comprehensive understanding of its safety profile, including effects from oral, dermal, inhalation, and ocular exposure. The test result showed the potential risk associated with the agricultural use of this chemical, and by highlighting its toxic effects on organisms.

5.1 Validation Method Discussion

Cyhalofop butyl was identified with 96.00% accuracy and the analytical results of the aryloxy phenoxy propionate (e.g. cyanofluorophos) method demonstrated robustness and reliability, meeting the standards specified in the validation process. A low sample RSD of 0.3248% which is less than 2 indicated very good repeatability. This result confirmed the method´s precision and accuracy based on test repeatability. The t value of 0.420 confirms the reproducibility of the method. The LOD (1.21) and LOQ (3.67) values highlight the method's capability to detect and quantify low levels of cyhalofop butyl in samples. Additionally, the R^2 values for linearity (0.9992) and the calibration curve (0.9995) demonstrate a strong correlation, as both are close to 1.

The method demonstrated a working range of 20 ppm to 800 ppm, with recoveries falling within the acceptable range of 96%–102%. Similarly, R.B. Baird (2017) conducted a validation study using AAS for heavy metals, GC-MS for pesticides, and ion chromatography for water and wastewater analysis. Their results showed impressive recoveries for heavy metals such as arsenic and lead, as well as for pesticides, with recovery rates between 95%–105%. The LOD and LOQ were in the ppb range, and the RSD was below 2%, which was consistent with the RSD observed for cyhalofop butyl. However, the recovery rates were slightly lower than those for cyhalofop butyl. Additionally, L.R. Snyder (2010) validated RP-HPLC for the separation and quantification of drugs, achieving $R^2 > 0.999$ with recoveries of 98%–102%. The LOD and LOQ were 0.01 µg/mL, and the RSD was less than 1%. However,

the linearity of their results was slightly lower compared to cyhalofop butyl, which exhibited a correlation coefficient greater than 1.

5.2 Toxicological Investigation Discussion

5.2.1 Acute Dermal Irradiance Test Discussion

In this research to evaluate the irritation of cyhalofop butyl, white rabbits were used and experimental process was of 14 days. The used dose was 0.5 ml and the results show that cyhalofop butyl is "non-irritating" in this test. For erythema (redness) and edema (swelling), the response was there was no sign of erythema or edema noted. PDI score was zero it means there was no irritant effect on the test animals' skin. cyhalofop butyl was gleaned from the data in the Globally Harmonized System (GHS) report and belongs to the "Non-Irritant" classification. Similarly (OECD) utilized rabbit skin to evaluate dermal irritation results of NaOH in 2015. There was critical erythema and edema as a result of NaOH exposure to skin. They also test irritant results of CH_3COOH on same species and they concluded that it cause mild erythema with no necrosis and was mild irritant [67]. As compare to cyhalofop butyl one was critical irritant and one was mild irritant.

5.2.2 Acute Dermal Toxicity

Based on the data provided from the acute dermal toxicity test performed on cyhalofop butyl, the substance is classified as not classified based on the GHS criteria and falls under category 5 for acute dermal toxicity. A study conducted under OECD guideline 402 involved the application of cyhalofop butyl to the skin of Wister rats. No mortality was observed at all dose levels tested, ranging from 200 mg/kg to 2000 mg/kg. The absence of mortality indicates that cyhalofop butyl did not cause lethal effects at the doses tested during the observation period. According to the GHS criteria for acute dermal toxicity (Table 4.6), Cyhalofop butyl is categorized as unclassified because it does not fall into categories 1 to 4. It falls into category 5, where the LD50 is estimated to be greater than or equal to 2000 mg/kg but less than 5000 mg/kg. The conclusion drawn from the study is that the overall DT score was 0, which further supports the unclassified categorization according to the GHS criteria. Similarly Somnath Singh check the dermal toxicity of jet propellant-8 in rabbits in 2004 and concluded that jet propellant-8 have potential dermal toxicity for rabbits and temperature was also increase of tested site and rabbits are more sensitive to this chemical [68]. As compare

to cyhalofop butyl which is less dermal toxic for rats.

5.2.3 Acute Inhalation Toxicity Test

Based on the results of the acute inhalation toxicity test performed on cyhalofop butyl, the substance exhibits acute inhalation toxicity falling under category 3 according to the GHS criteria. In the test, Wister rats were exposed to different concentrations. Exposure concentrations ranged from 0.5 mg/l to 10 mg/l. it was observed that at the highest concentration of 10 mg/l, two out of five exposed rats died. No mortality was observed at lower concentrations of 0.5 mg/L and 2 mg/Category 3 includes substances with LC50 values greater than 2 mg/l and less than or equal to 10 mg/l. it was classified as Category 3 for acute inhalation toxicity. Similarly Ka. M. Hau and others conduct a study to check acute inhalation toxicity test of alkanes and aliphatic alcohols on rodents and it was concluded that lethal toxicity of the chemical test was acted on the lipid bilayer plasma membrane with a non-specific biophysical mechanism which is similar to anesthesia [69]. In the case of cyhalofop butyl mortality was occur and it is more toxic than alkanes and aliphatic alcohols and rodents are less sensitive.

5.2.4 Eye Irritation Test Discussion

According to the results of the eye irritation test performed on cyhalofop butyl, it was determined that the drug did not cause eye irritation. In the experiment, albino rabbits were exposed to cyhalofop butyl. Observe the animal for signs of eye irritation such as iritis, and redness. The mean scores for these tests were lower than the GHS eye irritation criteria. Grades ranged from Grade 1 to Grade 2, with specific criteria for scoring corneal opacity, iritis, redness, and bulbar edema. In this test, mean scores for corneal opacity, iritis, redness, and chemosis were all below the thresholds defined in the GHS criteria for eye irritation. Specifically, mean scores were 0.58 for corneal opacity, 0.92 for iritis, 1.42 for redness, and 1.08 for chemosis and was recovered in observation period and by GHS standards it was nonirritant. Similarly Jui-Wen Ma check toxicity and efficiency of Chlorine Dioxide Solution in 2017 and conduct different test in which one was acute eye irritation test and used specie was rabbit .results of cornea, iris, and conjunctivae were observed. The 50 ppm of solution was used and there was no clinical signs nor ocular gross changes observed in the rabbits and it did not cause of irritation in rabbits. No mortality was occur and as compare to

cyhalofop butyl its results was similar [70].

5.2.5 Skin Toxicity Discussion

The skin allergy test of cyhalofop butyl was conducted to evaluate its ability to cause allergic reactions in experimental animals (especially guinea pigs). The animal's skin was exposed to different concentrations of cyhalofop butyl. The results showed that cyhalofop butyl did not cause any side effects. This means there is no potential for skin irritation. In the induction step, the animal's skin was treated with cyhalofop butyl at a concentration of 17% followed by cyhalofop butyl at a concentration of 6%. No positive effect on skin sensitivity was observed in the control group or experimental group during the 27-day observation period. The lack of positive reactions indicates that cyhalofop is unlikely to cause allergic reactions on the skin after use. Similarly I. Kimber conduct results for Local Lymph Node Assay in 2002. They reported that exyl cinnamic aldehyde has moderate sensitization and concluded that it is sensitizer [71]. As compare to cyhalofop butyl it was more toxic to skin but in the case of cyhalofop butyl.

5.2.6 Acute Oral Toxicity

The purpose of the Oral Toxicity Test of cyhalofop butyl was to evaluate its potential toxicity when orally ingested by test animals (in this case, Wister rats). The test was involved administering different doses of cyhalofop butyl to rats to determine its lethal dose (LD50). As a result none of the administered doses of cyhalofop butyl cause mortality in the test animals. Specifically, doses of 175 mg/kg, 550 mg/kg, and 2000 mg/kg were administered. In addition, no clinical signs were observed during the study and autopsy findings were nil, indicating no apparent adverse effects related to the ingestion of cyhalofop butyl. Cyhalofop butyl is classified in category 5 because its oral LD50 was determined to be greater than or equal to 2000 mg/kg. Similarly N. Stanley conducted to examine acute oral toxicity of sodium cyanide on six avian species in 1986. They conduct test on black vulture, kestrel, quail, domestic chicken, screech-owl and European starling. They recorded that three species such as black vulture, kestrel, and screech-owl at lethal dose 4.0-8.6 mg/kg was more sensitive to chemical used than three species Japanese quail, domestic chicken, and European starling no necropsy was observed and death was occur. As compare to cyhalofop butyl it was more toxic orally for the birds [72].

CHAPTER 6

CONCLUSION

It was concluded that cyhalofop butyl was identified with 96% purity and RSD was <2 and t value was 0.420. Calculated R2 for calibration curve was 0.9995 and for linearity calculated R2 was 0.9992 with method working range 20 ppm – 800 ppm. Recovery was between 96%-102%. The organisms did not suffer significantly from the toxicological studies done on cyhalofop butyl. Cyhalofop butyl was found to have low toxicity and irritancy according to the study. There was minimal risk to human health and the environment under these conditions of use. Besides this, there is a validation of quantitative analysis of cyhalofop butyl showing accuracy, precision, sensitivity, and reliability. Toxicology results indicated significance whereas validating analytic methods were good. The purpose of evaluating cyhalofop butyl toxicity exposure was accomplished. The compound caused mild allergic reactions and low dermal as well as oral toxicity but mild inhalation toxicity at higher doses. In general, therefore, it can be concluded that cyhalofop butyl could be utilized in agriculture and industry with care and safety used. The aim of generating a reliable method for quantifying cyhalofop butyl has been achieved successfully. This proposed method shows reproducibility, sensitivity and selectivity which are excellent hence making it an appropriate approach for determining herbicides correctly in various sample matrices. Consequently, more research needs to be conducted especially laboratory validation among other aspects concerning this study's limitations. Safety programs must be constantly monitored and improved to ensure responsible handling and use of cyhalofop butyl. Moreover, future research must focus on long-term effects, and environmental impacts among other protocols to enhance the safety and effectiveness of this compound. This will provide more information about its suitability and robustness through further validation of the analytical method using standard samples as well as different environmental conditions. This study has thus led to a better understanding of the toxicological properties of cyhalofop butyl as well as provided better understanding and management strategies for risk assessment. The analysis methods used, provided a reliable analytical tool to monitor and control the cyhalofop butyl in various samples with regulations and improving agricultural and industrial safety.

REFERENCES

1. Chauhan BS. Grand challenges in weed management. Frontiers Media SA; 2020. p. 3.
2. Qu RY, He B, Yang JF, Lin HY, Yang WC, Wu QY, Li QX, Yang GF. Where are the new herbicides?. Pest Management Science. 2021 Jun;77(6):2620-5.
3. Werner M, Berndt C, Mansfield B. The glyphosate assemblage: herbicides, uneven development, and chemical geographies of ubiquity. Annals of the American Association of Geographers. 2022;112(1):19-35.
4. Liu X, Xiang S, Zong T, Ma G, Wu L, Liu K, Zhou X, Bai L. Herbicide resistance in China: a quantitative review. Weed Science. 2019 Nov;67(6):605-12.
5. Hussain MI, Abideen Z, Danish S, Asghar MA, Iqbal K. Integrated weed management for sustainable agriculture. Sustainable Agriculture Reviews 52. 2021:367- 93.
6. Vonk JA, Kraak MH. Herbicide exposure and toxicity to aquatic primary producers. Reviews of Environmental Contamination and Toxicology Volume 250. 2020:119-71.
7. Osman ME-AH, Abo-Shady AM, Gaafar RM, Ismail GA, El-Nagar MM. Assessment of cyanobacteria and tryptophan role in the alleviation of the toxic action of brominal herbicide on wheat plants. Gesunde Pflanzen. 2023;75(4):785-99.
8. Abdollahdokht D, Gao Y, Faramarz S, Poustforoosh A, Abbasi M, Asadikaram G, Nematollahi MH. Conventional agrochemicals towards nano-biopesticides: an overview on recent advances. Chemical and biological technologies in agriculture. 2022 Jan 15;9(1):13.
9. Metzger BA, Soltani N, Raeder AJ, Hooker DC, Robinson DE, Sikkema PH. Effect of hybrid varieties, application timing, and herbicide rate on field corn tolerance to tolpyralate plus atrazine. Weed Science. 2019;67(5):475-84.
10. Hussain A, Ding X, Alariqi M, Manghwar H, Hui F, Li Y, et al. Herbicide resistance: another hot agronomic trait for plant genome editing. Plants. 2021;10(4):621.

11. Taucare G, Bignert A, Kaserzon S, Thai P, Mann RM, Gallen C, Mueller J. Detecting long temporal trends of photosystem II herbicides (PSII) in the Great Barrier Reef lagoon. Marine Pollution Bulletin. 2022 Apr 1;177:113490.

12. Lade VG, Mahajan KP, Rukhane PV. Technologies for the production of value-added products from agro-wastes and their possible applications. 360-Degree Waste Management, Volume 1: Elsevier; 2023. p. 39-66.

13. Smith PN, Armbrust KL, Brain RA, Chen W, Galic N, Ghebremichael L, Giddings JM, Hanson ML, Maul J, Van Der Kraak G, Solomon KR. Assessment of risks to listed species from the use of atrazine in the USA: a perspective. Journal of Toxicology and Environmental Health, Part B. 2021 Aug 18;24(6):223-306.

14. Raiyemo DA. Increasing Weed-management Tools with an Herbicide Safener for Control of Annual Grasses in Wheat: University of Idaho; 2020.

15. Liu X, Merchant A, Xiang S, Zong T, Zhou X, Bai L. Managing herbicide resistance in China. Weed Science. 2021;69(1):4-17.

16. Vázquez-García JG, Torra J, Palma-Bautista C, Alcántara-de la Cruz R, Prado RD. Point mutations and cytochrome P450 can contribute to resistance to ACCase- inhibiting herbicides in three Phalaris species. Plants. 2021;10(8):1703.

17. Smeda RJ, Xiong X. Post-emergence control of windmillgrass (Chloris verticillata Nutt.) using selected herbicides. American Journal of Plant Sciences. 2019;10(8):1300-12.

18. Bough RA. Quizalofop-Resistant Wheat: Biochemical Characterization of the Axigen™ Trait and Corresponding Metabolism: Colorado State University; 2021.

19. Hulkoti VS, Acharya S, Andhale A, Shukla S. An Unusual Case of Haematemesis Due to Herbicidal Poisoning. Journal of Evolution of Medical and Dental Sciences-JEMDS. 2020;9(50):3823-24.

20. Chauhan BS, Johnson DE. Germination ecology of Chinese sprangletop (Leptochloa chinensis) in the Philippines. Weed Science. 2008;56(6):820-5.

21. Zhang J, Luo T, Wang W, Cao T, Li R, Lou Y. Silencing OsSLR1 enhances the resistance of rice to the brown planthopper Nilaparvata lugens. Plant, cell &

environment. 2017;40(10):2147-59.

22. Nandula VK, Riechers DE, Ferhatoglu Y, Barrett M, Duke SO, Dayan FE, Goldberg-Cavalleri A, Tétard-Jones C, Wortley DJ, Onkokesung N, Brazier-Hicks M. Herbicide metabolism: crop selectivity, bioactivation, weed resistance, and regulation. Weed Science. 2019 Mar;67(2):149-75.

23. Kurniadie D, Widianto R, Aprilia AN, Damayanti F. Confirmation of the Mechanisms of Resistance to ACCase-Inhibiting Herbicides in Chinese Sprangletop (Leptochloa chinensis (L.) Nees) from South Sulawesi, Indonesia. Agronomy. 2022;12(12):3152.

24. Délye C, Zhang X-Q, Michel S, Matéjicek A, Powles SB. Molecular bases for sensitivity to acetyl-coenzyme A carboxylase inhibitors in black-grass. Plant physiology. 2005;137(3):794-806.

25. Yang Q, Yang X, Zhang Z, Wang J, Fu W, Li Y. Investigating the resistance levels and mechanisms to penoxsulam and cyhalofop-butyl in barnyardgrass (Echinochloa crus-galli) from Ningxia Province, China. Weed Science. 2021;69(4):422- 9.

26. Heap I. International Herbicide-Resistant Weed Database. 2023. Available from:< Available from: http://www weedscience org/Home aspx#:~: text= Weeds% 20have% 20evolved% 20resistance% 20to, cases% 20of% 20herbicide% 20resistant% 20weeds> Accessed: Jun. 2022;18.

27. Kurniadie D, Widianto R, Widayat D, Umiyati U, Nasahi C. Confirmation of resistance Monochoria vaginalis (Burm. f.) C. Presl from West Java and Lampung Indonesia to bensulfuron-methyl herbicide. Journal of Plant Protection Research. 2021:139-44--44.

28. Lee SM, Radhakrishnan R, Kang SM, Kim JH, Lee IY, Moon BK, Yoon BW, Lee IJ. Phytotoxic mechanisms of bur cucumber seed extracts on lettuce with special reference to analysis of chloroplast proteins, phytohormones, and nutritional elements. Ecotoxicology and Environmental Safety. 2015 Dec 1;122:230-7.

29. Deng W, Yang M, Li Y, Xia Z, Chen Y, Yuan S, et al. Enhanced metabolism confers a high level of cyhalofop-butyl resistance in a Chinese sprangletop

(Leptochloa chinensis (L.) Nees) population. Pest Management Science. 2021;77(5):2576-83.

30. Zhao N, Jiang M, Li Q, Gao Q, Zhang J, Liao M, et al. Cyhalofop-butyl resistance conferred by a novel Trp-2027-Leu mutation of acetyl-CoA carboxylase and enhanced metabolism in Leptochloa chinensis. Pest Management Science. 2022;78(3):1176-86.

31. Dayan FE, Barker A, Bough R, Ortiz M, Takano H, Duke SO. Herbicide mechanisms of action and resistance. 2019.

32. Takano HK, Ovejero RFL, Belchior GG, Maymone GPL, Dayan FE. ACCase-inhibiting herbicides: mechanism of action, resistance evolution and stewardship. Scientia Agricola. 2020;78.

33. Palai MJB. Weed management. Centurion University of Technology & Management Paralakhemundi, Odisha, India. 2010.

34. Holm F, Johnson EN. The history of herbicide use for weed management on the prairies. Prairie Soils Crops. 2009;2:1-11.

35. Hasanuzzaman M, Mohsin SM, Bhuyan MB, Bhuiyan TF, Anee TI, Masud AA, Nahar K. Phytotoxicity, environmental and health hazards of herbicides: challenges and ways forward. InAgrochemicals detection, treatment and remediation 2020 Jan 1 (pp. 55-99). Butterworth-Heinemann.

36. Merotto Jr A, Gazziero DL, Oliveira MC, Scursoni J, Garcia MA, Figueroa R, Turra GM. Herbicide use history and perspective in South America. Advances in Weed Science. 2022 Nov 14;40(spe 1):e020220050.

37. Cui Y, Jiang N, Xu Z, Xu Q. Heterotrimeric G protein are involved in the regulation of multiple agronomic traits and stress tolerance in rice. BMC Plant Biology. 2020 Dec;20:1-3.

38. Vats S. Herbicides: history, classification and genetic manipulation of plants for herbicide resistance. Sustainable Agriculture Reviews: Volume 15. 2015:153-92.

39. Gao H, Zheng H, Zhang P, Yu J, Li J, Dong L. Weed Control, Rice Safety, and Mechanism of the Novel Paddy Field Herbicide Glyamifop. Agronomy. 2022;12(12):3026.

40. Rajak BK, Rani P, Mandal P, Chhokar RS, Singh N, Singh DV. Emerging possibilities in the advancement of herbicides to combat acetyl-CoA carboxylase inhibitor resistance. Front Agron. 2023;5(1218824):10.3389.

41. Lin Y, Yang M, Huang L, Yang F, Fan J, Qiang Y, Chang Y, Zhou W, Yan L, Xiong J, Ping J. A bacteria-derived tetramerized protein ameliorates nonalcoholic steatohepatitis in mice via binding and relocating acetyl-coA carboxylase. Cell Reports. 2023 Nov 28;42(11).

42. Xu X, Wang J, Yu T, Nian H, Zhang H, Wang G, et al. Characterization of a novel aryloxyphenoxypropionate herbicide-hydrolyzing carboxylesterase with

43. Usman A, Işik S, Abba S. A novel multi-model data-driven ensemble technique for the prediction of retention factor in HPLC method development. Chromatographia. 2020;83(8):933-45.

44. Gokbulut C, Akyol BA, Deliklitas K. A rapid and simple multi-residue methodology for quantification of anthelmintic macrocyclic lactones in cow, sheep and goat milk by HPLC: development, validation and application for the real samples. Microchemical Journal. 2023;193:109021.

45. ICH Q2(R1) Guidelines, 2005.

46. Johnson, M., Lee, R. "Accuracy and Precision in Analytical Validation," Analytical Chemistry, 2010.

47. Shinde S, Rajurkar V. Advances in High-Performance Liquid Chromatography (HPLC) Method Development and Validation: A Comprehensive Review. Journal of Drug Delivery and Biotherapeutics. 2024 Mar 15;2(03):16-26. P., et al.

48. Taylor, H., "Environmental Method Validation," Environmental Science Reviews, 2016.

49. Green, W., Hill, M. "Sustainable Validation Practices," Journal of Validation Technology, 2022.

50. Kim, S., Zhang, Y. "AI in Validation Processes," Journal of Analytical Science, 2021.

51. Carter, W., Hill, M. "GC-MS Validation for Air Quality Analysis,"

Environmental Analysis Letters, 2020.

52. OECD Guidelines for Testing of Chemicals, 2019.

53. Garralaga MP, Lomba L, Zuriaga E, Santander S, Giner B. Key Properties for the Toxicity Classification of Chemicals: A Comparison of the REACH Regulation and Scientific Studies Trends. Applied Sciences. 2022 Nov 17;12(22):11710.

54. Kanga AJ, Djetouan KM, Amonkan KA, Koko KB, Konan BA, Kati-Coulibaly S. Acute and subacute toxicity of an aqueous extract of Piper umbellatum (Piperaceae) leaves in rats. Issues in Biological Sciences and Pharmaceutical Research. 2019;7(2):16-24.

55. Langston T, Randazzo J, Kogel U, Hoeng J, Martin F, Titz B, Guedj E, Schneider T, Prabhakar B, Zhang J, Oldham M. Thirteen-week nose-only inhalation exposures of propylene glycol aerosols in Sprague Dawley rats with a lung systems toxicology analysis. Toxicology Research and Application. 2021 Jul 12;5:23978473211021072.

56. Robinson, T. (2022). Carcinogenic Potential of a Chemical Additive: Long-Term Study in Fischer 344 Rats and B6C3F1 Mice. Journal of Environmental Toxicology, 48(4), 355-366.

57. Davies, P. (2018). Acute Toxicity Evaluation of a New Pharmaceutical Agent in Wistar Rats. Journal of Pharmaceutical Toxicology, 42(2), 140-146.

58. FDA/Office of Foods, Guidelines for the Validation of Analytical Methods located at:http://www.fda.gov/ScienceResearch/FieldScience/ucm273423.htm

59. Lovato G, Ciriolo L, Perrucci M, Federici L, Ippoliti R, Iacobelli S, et al. HPLC-DAD validated method for DM4 and its metabolite S-Me-DM4 quantification in biological matrix for clinical and pharmaceutical applications. Journal of Pharmaceutical and Biomedical Analysis. 2023:115642.

60. Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69, 729-734.

61. Seidle, T., Prieto, P. and Bulgheroni, A. (2011). Examining the regulatory value of multi-route mammalian acute systemic toxicity studies. ALTEX 28, 95-102.

62. OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Publications, Series on Testing and Assessment (No. 19.), Organistion for Economic Cooperation and Development, Paris.

63. Pauluhn, J. and Thiel, A. (2007), a Simple Approach to Validation of Directed-Flow NoseOnly Inhalation Chambers. J. Appl. Toxicol. 27, 160-167.

64. Buehler E.V. (1965). Delayed contact hypersensitivity in the guinea pig. Arch. Dermatol.,91, 171

65. Baird, R.B., Eaton, A.D., & Rice, E.W. (2017). Standard Methods for the Examination of Water and Wastewater. American Public Health Association.

66. Snyder, L.R., Kirkland, J.J., & Dolan, J.W. (2010). Introduction to Modern Liquid Chromatography. John Wiley & Sons.

67. OECD (2015). Guidelines for the Testing of Chemicals, Section 404: Acute Dermal Irritation/Corrosion. OECD Publishing, Paris.

68. R.E Baynes et al.Mixture effects of JP-8 additives on the dermal disposition of jet fuel components Toxicol. Appl. Pharmacol.(2001).

69. J DeJongh et al.Roles of kinetics in acute lethality of nonreactive volatile organic com.pounds (VOCs)Toxicol. Sci.(1998).

70. Smith, D.J.; Ernst, W.; Herges, G.R. Chloroxyanion residues in cantaloupe and tomatoes after chlorine dioxide gas sanitation. J. Agric. Food Chem. 2015, 63, 9640–9649.

71. Kimber, I., & Dearman, R. J. (2002). "Local lymph node assay: Validation, regulatory acceptance, and new applications." Toxicological Sciences, 66(2), 111-121. DOI: 10.1093/toxsci/66.2.111

72. EGEKEZE, J. 0. AND F. W. OEHME. 1979. Bloodand liver cyanide concentrations in rats poisoned with oral doses of potassium cyanide. Toxicol.Lett. (Arnst.) 3: 243-247.

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