THESIS

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USING THE HET-CAM TEST AND ICE TEST IN DETERMINING THE EYE IRRITATION POTENTIAL OF SOME PESTICIDES

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1. INTRODUCTION

In this chapter, we discuss the roles of pesticides in agriculture, the need for pesticide toxicity tests, the significance of eye irritation testing methods, previous and alternatively developed testing methods, and the test methods used in these studies.

1.1. Background of the Study

The world's population is expected to grow by about 2 billion people, from 7.7 billion people now to 9.7 billion people in 2050. To feed this many people, the world will need to grow more food. The majority of this growth is expected to take place in developing countries and continents like Africa; sub-Saharan Africa where I come from, is projected to double, having the fastest growth rate of 99%, while East and Southeast Asia have the slowest rate of 13% (UN, 2019). To feed this population, food production is estimated to increase by 70% to match this rate of growth (FAO, 2017). However, agricultural production is not keeping pace with population growth, particularly in Africa, where food production is not increasing at the same rate as population growth (TITTONELL and GILLER, 2013).

Agricultural production faces a number of difficulties, including political unpredictability and climate change, but the main one is crop damage from pests, diseases, weeds, and bacteria, which can result in significant crop losses and decreased yields (OERKE, 2006). A 20–40% annual yield loss is attributed to this (FAO, 2019). To address this challenge, the Food and Agriculture Organization (FAO) advocates for the use of integrated pest management (IPM) as a holistic approach to crop control (BRADER, 1979). IPM involves a series of integrated methods and monitoring, starting with cultural practices as the primary means of addressing pest and disease infestations, followed by mechanical, biological, and chemical control.

Cultural practices involve the use of crop rotation, intercropping, and other practices to reduce the incidence of pests and diseases. With mechanical control, physical barriers like nets, traps, and fences are used to keep pests from getting to crops. Biological control entails the use of natural predators and parasites to control pests, while chemical control involves the use of agricultural chemicals only as a last resort when other methods have failed (DEGUINE et al., 2021).

To limit the use of chemicals in agriculture, the EU has put in place a number of measures. All active ingredients used in pesticides must be assessed for their toxicity index and possible dangers to human health and the environment under the EU Pesticides Regulation (EC) No. 1107/2009 (EC, 2009). A further goal stated by the EU is to reduce pesticide use by 50% by 2030 through the adoption of Integrated Pest Management (IPM) techniques (EC, 2022). However, in Sub-Saharan Africa, the reverse is the case, as there is an increased use of pesticides with little or no toxicology testing and farmers have limited knowledge of the potential health risks associated with their use (ISGREN and ANDERSSON, 2020)

The wide use of agricultural chemicals to protect crops from insects, weeds, and diseases, especially in Africa, can also cause harm to non-target organisms and the environment (PIMENTEL, 2005). So, it is very important to find out how toxic these agricultural chemicals are and make sure they are not too dangerous. The degree to which a substance has the potential to harm an organism is referred to as its toxic potential (toxicity) (SULLIVAN, 2019). The level of toxicity that a substance possesses can change depending on several varied factors, including the dose, the duration of exposure, and the susceptibility of the organism (NATIONAL RESEARCH COUNCIL, 2001).

All agricultural chemicals must undergo toxicity testing before being used in the European Union, according to REACH (Registration, Evaluation, and Authorization of Chemicals in the EU) (EC, 2009). Tests to determine eye irritation are part of the toxicological tests; one of the foremost tests postulated for assessing eye irritation was the Draize test (DRAIZE et al., 1944). This test measures how much a test item irritates the eyes of albino rabbits and has mostly been used for the past 60 years to figure out how dangerous agricultural chemicals and other chemical substances are. In 1980, Henry Spira's advertisement tagged "How many rabbits does Revlon blind?" were one of the first to raise public awareness about the Draize test. Animal rights activists harshly criticized the practice and pushed for its replacement because of the irreversible effects it has on the test items (PRINSEN et al., 2017). The promotion of non-animal test methods (*in vitro*) to replace the current usage of animals (*in vivo*) in research became crucial in the years that followed (CHOKSI et al., 2019). As a result, various alternative tests have been developed to replace the use of rabbits in detecting if a chemical is likely to irritate the eyes. Eye irritation occurs when a test substance applied to the anterior surface of the eye and can result in changes that are completely reversible in less than 21 days (OECD, 2019).

The first alternative assay for eye irritation we are studying is the HET-CAM test, in which the chorioallantoic membrane (CAM) of the hen egg is exposed to test compounds and the resulting unfavourable alterations (lysis, hemorrhage, or coagulation) are observed. Technically speaking, CAM consists of arteries, capillaries, and veins as a whole tissue, similar to the tissue in a treated rabbit eye in the Draize test, and it responds to injury by entering a complete inflammatory phase (TAVASZI and BUDAI, 2007).

Secondly, the Isolated Chicken Eye (ICE) test is a popular technique for determining if agricultural chemicals and other substances might irritate the eyes. Using an isolated chicken eye, a small amount of the test chemical is applied to the cornea to check for any symptoms of irritation or injury. This method is an *in vitro* test method that can be used to classify substances as causing serious eye damage (UN GHS Category 1) or as not requiring classification for eye irritation or serious eye damage according to OECD 2018. The ICE test has reportedly been used for many years as a screening technique for potential eye irritation from agricultural chemicals, cosmetics, medications, and other compounds (KOJIMA et al., 2016). Several organizations, notably the Organization for Economic Co-operation and Development (OECD) and the European Centre for the Validation of Alternative Methodologies (ECVAM), have endorsed the use of the ICE test (ICCVAM, 2007). To ensure that the ICE test is used and interpreted correctly, the OECD created rules for it (OECD, 2018).

Since the HET-CAM and ICE tests do not employ the use of live animals, they are in line with the 3 R's (replacement, reduction, and refinement) of animal testing. In this study, we assessed the eye irritation potential of some pesticides using the *in vitro* test in comparison with the recognized *in vivo* test. To accomplish this, we used the HET-CAM and ICE assays to determine and compare the eye irritation potential of some pesticides in Hungary.

1.2. Objective of the Study

The goal of this research was to assess the use of alternative methods, their applicability as a pre-test or replacement method, and the comparison of the *in vitro* tests with the recognized *in vivo* data for primary eye irritation with some pesticides in Hungary. To accomplish this, we used the HET-CAM and ICE assays as the alternative methods to determine and compare the irritancy potential of various pesticides.

2. LITERATURE REVIEW

This chapter goes into great detail on the eye, testing procedures for eye irritation, and how *in vivo* and *in vitro* test procedures have changed through time. Lastly, we travel to sub-Saharan Africa to study pesticide usage.

2.1. The structure of the eye

The eye is a sophisticated organ made up of numerous parts that interact to facilitate vision, it is protected by a bony cavity known as the orbit. Six orbital extraocular muscles are connected to the eye. These muscles move the eye vertically, laterally, and rotationally (BOYD and TURBERT, 2023).

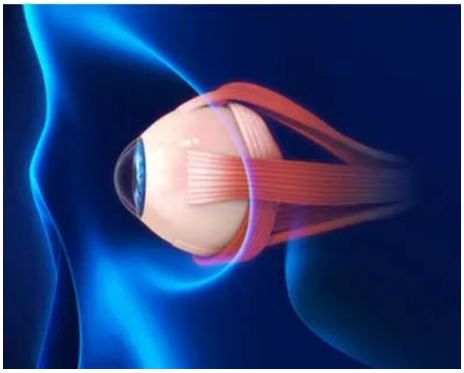

Figure 1: the eye muscles (BOYD and TURBERT, 2023)

The extraocular muscles are linked to the sclera, the white portion of the eye. This dense layer of tissue covers virtually the entirety of the eyeball's surface (BOOTE, et al., 2020). The cornea, a transparent tissue in the form of a dome that covers the front of the eye with the sclera, they are the eye's outermost layer (GUÉRIN, et al., 2021). The iris, pupil, lens, and optic nerve are the other important components of the eye. The iris is the colourful portion of the eye that regulates the quantity of light entering the eye by adjusting the size of the pupil (DAHLMANN-NOOR et al., 2014). The lens is a flexible, clear component that focuses light onto the retina and is placed behind the iris. The optic nerve delivers visual data from the

retina to the brain for processing. Sight is created by the cornea focusing light onto the retina, a small layer of tissue at the back of the eye containing photoreceptor cells responsible for sensing light and relaying visual signals to the brain (NIH, 2022).

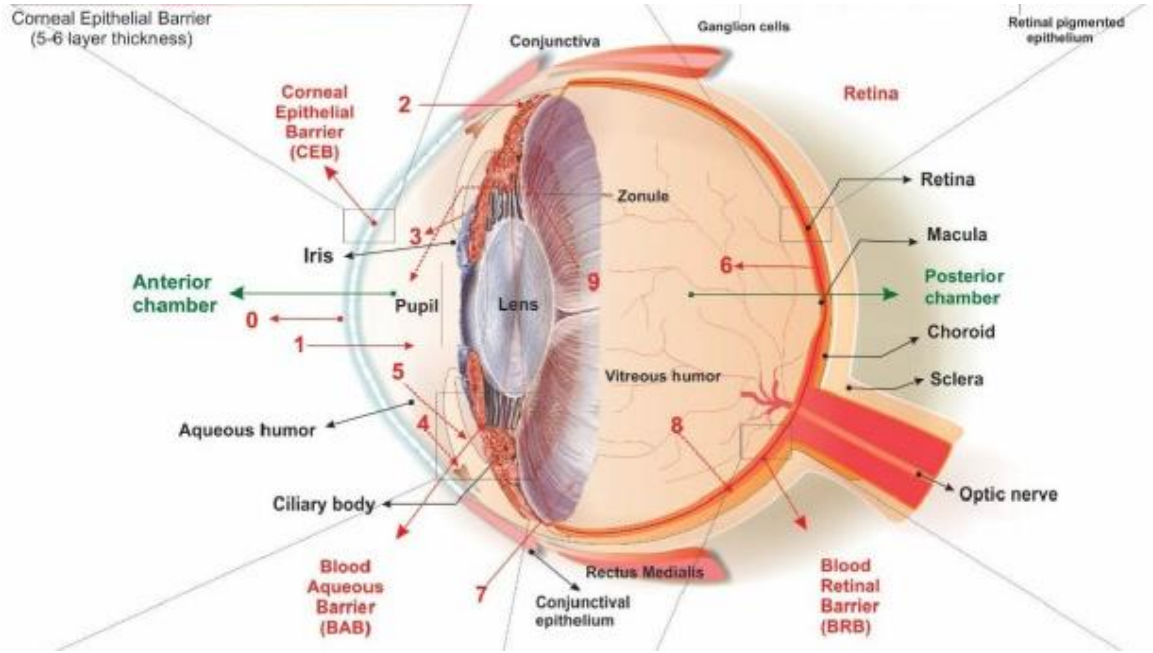

Figure 2: the Eye structure (BARAR et al., 2016)

THE CORNEA

The cornea is a highly specialized structure that is made up of five layers: the epithelium, Bowman's layer, stroma, and Descemet's membrane. The epithelium is a thin layer of cells that covers the outer surface of the cornea and acts as the first line of defence against external threats such pathogens, environmental pollutants, and mechanical damage (ESPANA and BIRK, 2020). It also contributes to maintaining corneal hydration and minimizing water loss. Bowman's layer is a very thin layer of collagen fibres that are responsible for the cornea's ability to retain its shape and structure. Collagen fibres and keratocytes, which are specialized cells responsible for preserving the cornea's transparency, make up the stroma, which accounts for approximately 90% of the cornea's thickness. The stroma is also known as the central layer of

the cornea. Descemet's membrane is a thin, elastic layer that separates the stroma from the endothelium, which is the innermost layer of the cornea that is used for managing fluid balance (ALLEN et al., 2015). Interactions between these layers are crucial to the function of the cornea. For instance, epithelium and stroma interact to maintain hydration levels, whereas stroma and endothelium interact to optimize fluid balance (BOULTON et al., 2014). Eye irritation starts affecting the outer layers of the cornea from mild cases and can progress acute cases.

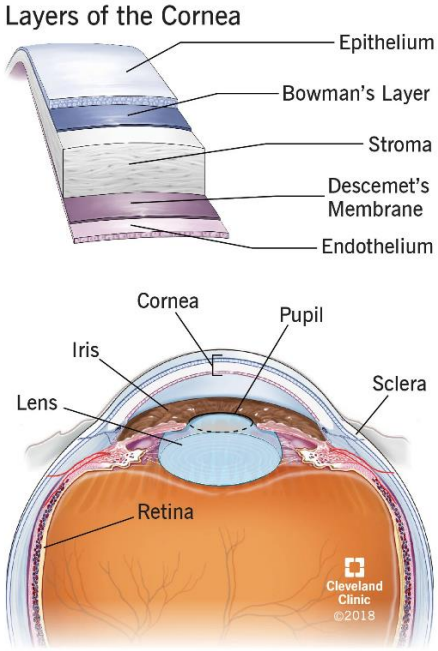

Figure 3: Structure of the cornea (CLEVELAND CLINIC, 2018)

2.2 Eye Irritation Testing

Testing of chemical substances that comes in contact with the eye for eye irritation propensity became expedient as a safety assessment measure in the 1940s due to the increased usage of chemicals in a variety of industries (cosmetics, agricultural chemicals and others). To prevent eye damage in people, it became necessary to evaluate the potential of these chemical substances to cause eye irritancy (BEYER et al., 2011). The need for eye irritation testing is driven by the potential of exposure to chemical substances, which can cause reversible or irreversible eye changes within 21 days, severe eye damage or eye loss. Regulatory bodies demand testing to guarantee the safety of consumer goods, pharmaceuticals, cosmetics, agricultural chemicals, and industrial chemicals (OECD, 2019).

DRAIZE TEST

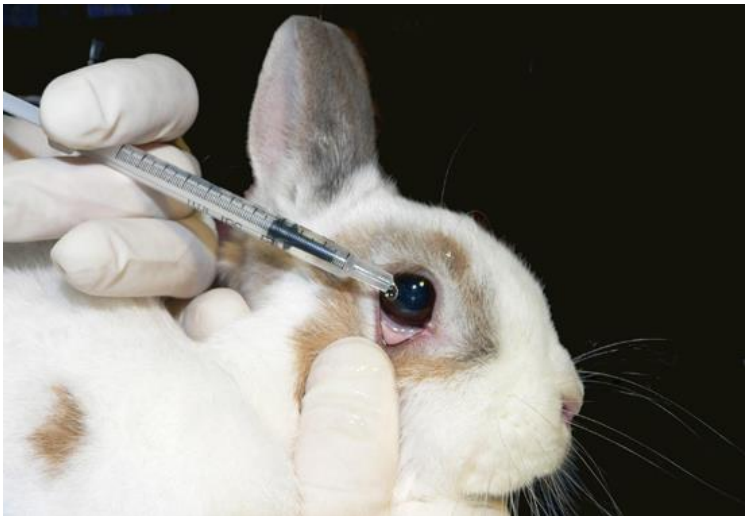

Figure 4: Draize eye irritation test (www.dw.com)

The *in vivo* approach known as the Draize test was the first significant step forward in the process of designing an assay to detect eye irritation. It was postulated by Draize et al. in 1944, the United States Food and Drug Administration US-FDA first adopted the Draize eye irritation test as part of the evaluation of the safety of foods, drugs, and cosmetics. They also

created a guidance on scoring of eye irritation, already at that time, it was recognized that subjective grading of ocular reactions posed a significant challenge (US FEDERAL REGISTER, 1961). The New Zealand white (NZW) rabbits were recommended for this test because of their large eyes, availability, fairly priced and other reasons (WILSON et al., 2015). This method involves the test material, liquids are tested in a volume of 0.1 ml, while solids (finely powdered) are tested in either 0.1 g or 0.1 ml placed directly into one of the eyes of the test rabbits and the eyelids closed for a few seconds, while the second eye served as the control (GUPTA, 2016). The effects on the rabbit's cornea, conjunctiva, and iris following the exposure to test substances were observed for one hour, 24 hours, 48 hours, and 72 hours. The ocular responses of the test eye are scored using a point system (DRAIZE et al., 1944). In order to determine the eye irritation potential of the test material, residual eye effects are recorded at regular intervals, if necessary up to approximately 3 weeks after treatment (HUHTALA et al., 2008). The most popular scoring method for the Draize test is the Maximum Average Score (MAS), the eye is checked at the chosen intervals, and any changes to the iris, conjunctiva, or cornea are noted. MAS 110 is made up of the scores for the iris (10), conjunctiva (20), and cornea (80) (LEE et al., 2017). Because of its inexpensive cost and relative ease of use, it was generally adopted and utilized for several decades despite great objections raised on its scientific validity as Rabbits produce comparatively little tears, blink less frequently, and have less sensitive ocular surfaces as compared to human eyes and its ethical acceptability (WILHELMUS, 2001). Due to the criticisms several modifications were made to the Draize test model which includes reducing the number of test animals used to administration of analgesics and anaesthetics to reduce the animal's suffering and discomfort (OECD, 2021).

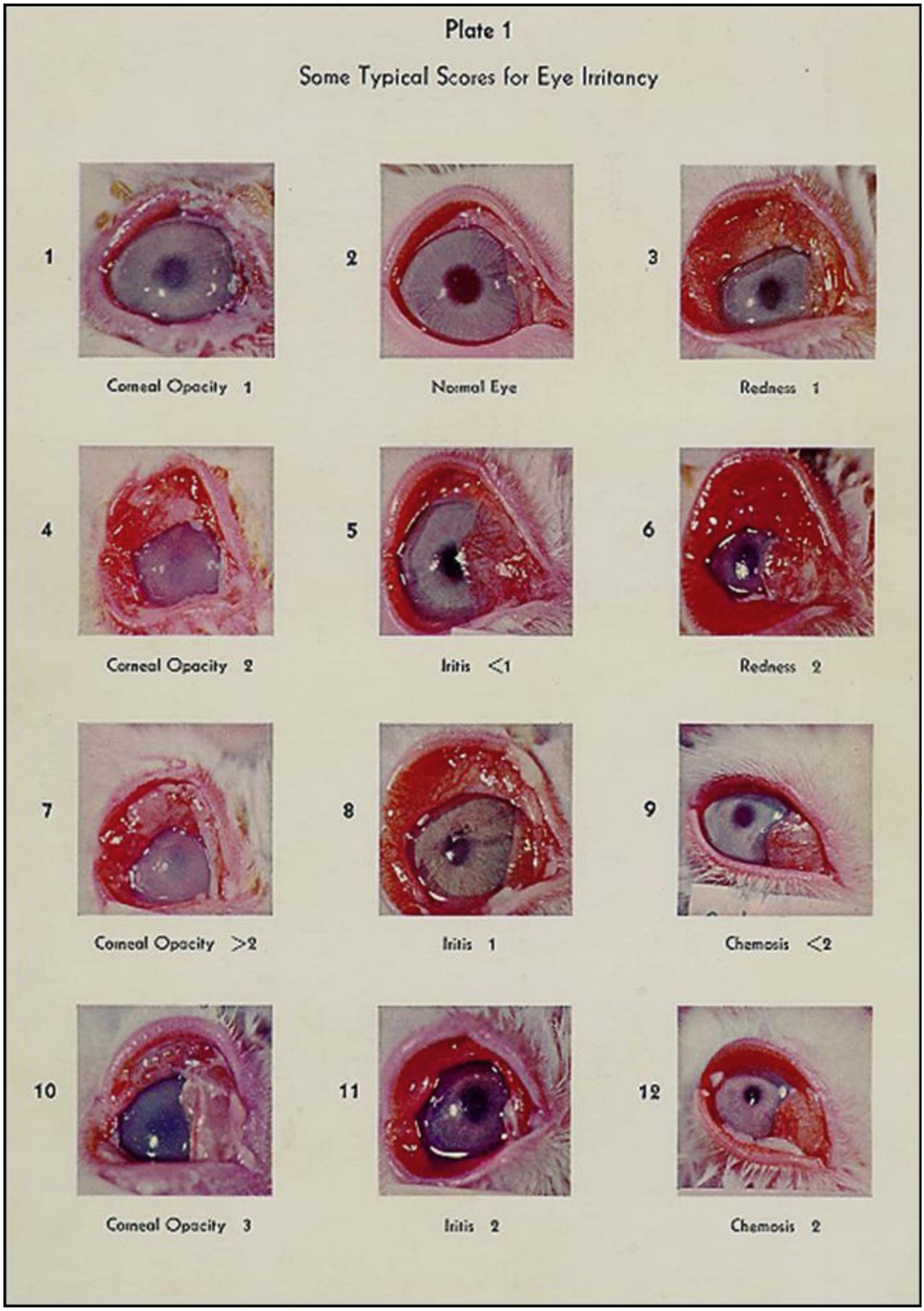

Figure 5: FDA guidance on scoring of ocular lesions; Plate 1 (FDA, 1964).

2.3 The Need and Validation of Alternative Test Methods

In the 1980s, the media raised the public's awareness on the Draize test method (PRINSEN et al., 2017), and it became the subject of criticism due to the ethical questions it raised as being inhumane and its lack of predictive value for human eye irritation (VINARDELL and MITJANS, 2008). Rabbit eyes are physically and physiologically distinct from human eyes, one of the crucial issues with the Draize test is that it cannot predict human eye discomfort (MARTINEZ et al., 2006). The Draize test is also criticized for its arbitrary interpretation of data, as various researchers may interpret the same findings in diverse ways, resulting in inconsistent and unreliable conclusions (CURREN and HARBELL, 2002). The problem has been tackled from two different angles in an effort to find a solution to it looking for alternative *in vitro* studies that focuses on the 3R (replacement, reduction, and refining of animal tests) (RUSSEL and BURCH, 1959), and second, by altering the Draize test in order to make it more ethical and predictive for people.

Validations

Animal testing for consumer product and chemical safety evaluations is progressively being replaced by *in vitro* eye irritation testing (NABARRETTI, 2022). To decrease the use of animals in testing alternative methods have been developed and validated to reduce the use of animals in testing. To guarantee its dependability and applicability for hazard detection and risk assessment, *in vitro* assays must be validated (ROVIDA et al., 2015). The European Centre for the Validation of Alternative Methods (ECVAM) developed a glossary of terms in 2007. This evaluation of the literature on the validation of *in vitro* ocular irritation testing refers to that glossary.

Validation is described as "the process of evaluating the relevance and reliability of a method or test system for a specific purpose" in the European Centre for the Validation of Alternative Methods ECVAM dictionary. In order to evaluate the predictive potential of the *in vitro* assay, validation studies often entail testing a group of chemicals with known potential for eye irritation using both *in vitro* and *in vivo* approaches (ICCVAM, 2017). The *in vitro* eye irritation tests have been the subject of numerous validation studies. The importance of standardization and quality control in assuring the accuracy and relevance of alternative methods has also been underlined by validation studies. The European Centre for the Validation of Alternative Methods (ECVAM) ad hoc working group on the use of alternatives to the rabbit Draize eye irritation test has issued guidelines on the validation, usage, and interpretation of alternative methods' results, which have been followed in the validation of the EpiOcular™ eye irritation test. These assesses alterations in the transepithelial electrical resistance of a three-dimensional human corneal epithelial tissue model, the EpiOcular™ test exhibited a 98.1% sensitivity and 72.9% specificity rate when predicting the possibility for eye irritation from chemicals (KALUZHNY et al., 2011). The Isolated Chicken Eye (ICE) test has also been approved in accordance with the OECD requirements (OECD, 2018). To detect changes in corneal opacity and permeability following exposure to a test material, the ICE test employs enucleated chicken eyes. According to OECD, 2019 the ICE test was 95 % sensitive, specificity of about 63-81% in predicting a chemical's ability to irritate the eyes.

Other *in vitro* eye irritation tests that have been approved include the rebuilt human corneal epithelium (Rhce) test, the bovine corneal opacity and permeability (BCOP) test, and the SkinEthicTM HCE eye irritation test (OECD, 2018). Silico models, such as the CAESAR model and the TOPKAT model, have been developed to predict eye irritation potential based on chemical structure and physicochemical properties (PARTHASARATHI and DHAWAN,

2018). Additionally, the HET-CAM (Hen's Egg Test - Chorioallantoic Membrane) test has been proven to be an effective technique for identifying substances that may cause ocular irritation (RIVERO et al., 2021). However, ICCVAM recommends a combination of assays with the HET-CAM test to get a more comprehensive assessment of potential eye irritation.

2.4 Alternative test methods

The ability to analyse how pesticides affect cells or tissues outside of living animals makes *in vitro* testing crucial for assessing the eye irritation of substances, the following alternative assays are discussed:

2.4.1 The HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) test

In order to evaluate the potential for eye irritation of chemicals and formulations without using live animals, Luepke in 1985 proposed the HET-CAM (Hen's Egg Test-Chorioallantoic Membrane).

Description: Fertile hen's eggs are incubated for nine days, the chorioallantoic membrane (CAM) is made visible by making a small hole in the eggshell and removing the shell membrane. The test substance is applied to the CAM, the membrane's reaction is tracked for up to five minutes for any indications of cellular or vascular reactions. The degree of vascularization and hemorrhage, as well as the presence of coagulation and lysis are all taken into account when grading the reaction's severity using an existing standardized scoring system (LUEPKE and KEMPER, 1986).

Validation: The test has been certified as valid by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM, 2010).

Classification: The irritancy potential of substances is classified as Irritant, non-irritant or severely irritant based on the (ICCVAM, 2010; Invittox protocol, 1990).

2.4.2 Isolated Chicken Eye (ICE) test

The isolated chicken eye test (ICET) was created to forecast how likely certain chemical substances are likely to irritate the eyes. The test was created to determine the degree of corneal damage and the likelihood of eye discomfort. Instead of using live animals for testing, the technique uses recently enucleated chicken eyes (PRINSEN et al., 1993). The detailed procedure is described in OECD 438 (OECD, 2018).

Description: The ICET involves removing the chicken's eye out and setting it in a modified container that keeps the cornea moist and oxygenated. Direct application of the test substance to the corneal surface of the eye allows for monitoring of any changes over time. Typically, the test measures a number of factors, including corneal opacity, swelling, and fluorescein retention. When the positive control, such as sodium hydroxide, causes severe damage to the cornea but the negative control, often saline, has no discernible effects on the cornea, the test is deemed valid (OECD, 2018).

Validation: It was validated by three bodies; European Centre for the Validation of Alternative Methods (ECVAM), Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), with the Japanese Centre for the Validation of Alternative Methods (JaCVAM) and adopted as OECD 438.

Classification: According to OECD 2018a, the ICE test method is accepted for the hazard classification and labelling of test chemicals that cause serious eye damage (UN GHS Category 1) and test chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category).

2.4.3 Bovine Corneal Opacity and Permeability (BCOP) Test

The bovine corneal opacity and permeability test, used to evaluate the potential eye irritancy of chemicals and products, which is conducted using the guidelines in OECD 437 (OECD, 2020).

Description: Bovine corneas are obtained from abattoirs that would otherwise be discarded, are isolated and stored before being exposed to test compounds, their opacity and permeability are measured, and prediction scores based on these two factors are then calculated. The cornea is incubated and exposed to the test chemical for 10 minutes, after which the opacity and permeability of the cornea are assessed. The BCOP test technique has undergone a number of modifications and improvements since it was first validated. The Organisation for Economic Co-operation and Development (OECD) updated the test guidelines in 2018 to include a required minimum of duplicates and to include more specific instructions on how to prepare and handle test materials and corneas.

Validation: It has been validated by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2010), the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Centre for the Validation of Alternative Method and adopted as test 437 (OECD, 2018).

Classification: According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations, the BCOP test method can accurately classify chemicals (both substances and mixtures) that cause serious eye damage as well as those that do not need classification. (UN, 2011; ICCVAM, 2010).

2.4.4 The Epicoliar Eye Irritation Test (EIT)

The test was designed to assess the potential eye irritation of chemicals using a three-dimensional human corneal epithelial tissue model, this is performed according to the OECD 492 guidelines (OECD, 2019).

Description: This method makes used of reconstructed human corneal epithelial (Rhce) tissue model for the epicoliar test is developed in a culture medium. The tissue's surface is treated topically with the test chemical, and it is then given a set amount of time to incubate. The tissue is rinsed to remove any remaining test ingredient after incubation, and a colorimetric assay is used to determine the cell viability (OECD, 2019). KALUZHNY et al. 2011 tested 112 test items (39 solids and 73 liquids) using the EIT and the result showed 84.8% accuracy, 72.9% specificity and 98.1% sensitivity.

Validation: It is validated and accepted as test 492 (OECD, 2019).

Classification: According to OECD, 2019 test chemicals are categorized as no category (GHS unclassified) if the tissue viability is greater than 60% and as no prediction can be made if the viability is ≤ 60%.

2.5 Sub-Sahara Africa and Agricultural Chemical Usage

The population of sub-Saharan Africa is predicted to be 1.09 billion in 2021, which is equivalent to nearly 13% of the world's population, according to the FAO 2021 data. The world bank 2021 data estimates a land area of roughly 10 million square kilometers, about 20% of the total area of the earth out of which 42.5% is used for agriculture. Around 522 million tonnes of food, comprising products from crops and livestock, were produced in 2020. This amounts to around 9% of the total food produced worldwide (FAO, 2021). Around 175 million people are directly employed by smallholder farms, which make up around 80% of all

farms in Sub-Sahara Africa. Women make up at least half of the labor force in several nations. (OECD/FAO, 2016).

I was motivated to take up this research topic by both personal experience, available research data, and the desire to take something back home after my studies that will benefit my people and the nation as a whole. Current research data indicates that in Sub-Saharan Africa, an increase in the use of agricultural chemicals has been caused by several factors, including the ever-growing population, the need to increase food production in order to attain sustainability, limited or no awareness of the risks associated with agricultural chemical usage and inadequate regulation and enforcement of existing laws (NGOWI et al., 2007). In my home country of Nigeria, however, anyone can sell, buy, and use plant protection chemicals without a plant doctor's prescription, which is in stark contrast to the requirements of obtaining and using plant protection chemicals in Hungary. According to Decree 43/2010. (IV. 23.) FVM on plant protection activity, a prescription from a plant doctor is mandatory for the purchase and use of pesticides. Additionally, despite the existing legislations few agricultural pesticides in sub-Saharan Africa have their toxicity indexes or potential dangers to human health and the environment assessed and a lot of illegal use and importations (OESTERLUND, 2014).

The use of pesticides in agriculture may have an impact on both the environment and human health. Pesticide exposure has been linked to a number of harmful health impacts, including cancer, reproductive problems, and neurological issues (TAGO et al., 2014). In addition, pesticides can contaminate soil, water, and air, which can harm ecosystems and cause environmental degradation (PIMENTEL, 2005).

3.0 MATERIALS AND METHODS

We emphasize on the types of plant protection pesticides and the approach used in evaluating eye irritation potential observing good laboratory practices.

3.1 Materials

In this study, four agricultural pesticides with the properties listed in Table 1 were applied in their original form and concentration without dilution.

Table 1: Investigated pesticides.

Product Type	**Test item**	**Physical state**	**Active ingredients**	**Concentration**
Herbicide	Esteron 60	Liquid	2,4-D acid (2-ethyl-hexyl ester)	600 g/l
Fungicide	Metkon 60	Liquid	Metconazole	60 g/l
Insecticide	Sivanto Prime	Liquid	Flupyradifurone	200g/l
Herbicide	Viballa	Liquid	Halauxifen-metil	3.0 g/l

Product Name: Esteron 60

CASRN of Active Ingredients: 1928-43-4

Applications: Post emergence weed control in maize and cereals (wheat, rye, oats, and barley) also for pastures and meadows.

Acute oral toxicity: LD_{50}, Rat, female, 3 129 mg/kg

Acute toxicity, dermal: LD_{50}, Rat, male and female, > 5 000 mg/kg

Acute toxicity, inhalation: LC_{50}, Rat, male and female, 4 h, dust/mist, > 5.63 mg/l Death at this concentration did not occur.

Product Name: Metkon 60

CASRN of active ingredients: 125116-23-6

Applications: Fungicide used to tackle fusarium infestation in cereals, and sclerotinia infestation. It can also serve a regulatory in rapeseeds. Beneficial to wheat, rapeseed, rye, barley, and triticale.

LD_{50} oral, Rat 2102 mg/kg

LD_{50} dermal, rat > 4000 mg/kg

LC_{50} Inhalation Rat (mg/l) > 9,57 mg/l/4h

Product Name: Sivanto Prime

CASRN of active ingredients: 951659-40-8

Applications: Insecticide used for soyabeans, leafy vegetables, stone fruits, pomme fruits and berries. Pest controlled includes Aphids, Colorado potato beetle, oyster shell scale, San Jose scale, whiteflies, leafhoppers, blueberry maggots and suppresses pear psylla.

LD_{50} oral female rate > 2,000 mg/kg

LC_{50} inhalation (female Rat) ca. 3.496 mg/l

Exposure time: 4 h

Determined in the form of a respirable aerosol.

LC_{50} (female Rat) ca. 13.984 mg/l

Exposure time: 1 h

Determined in the form of a respirable aerosol.

Extrapolated from the 4 hr LC_{50}.

LD_{50} acute skin toxicity (male/female combined Rat) > 2,000 mg/kg

Product Name: Viballa

CASRN of active ingredients: 943831-98-9

Applications: Weed control in sunflower

Acute toxicity, oral: LD_{50}, Rat, > 5 000 mg/kg Estimated.

Acute toxicity, Dermal LD_{50}: not determined.

Acute toxicity, inhalation: LC_{50} has not been determined.

LD_{50}, Oral Rat, > 5 000 mg/kg

Dermal LD_{50}: not determined.

LC_{50} Inhale Rat, 4 h, dust/mist, > 3.551 mg/l

3.2 Methods

In this sub section, we discuss in depth the two *in vitro* techniques used in our study to evaluate the eye irritation potential of the investigated plant protection chemicals.

3.2.1 The HET-CAM test

The HET-CAM test was conducted as described by (LUEPKE and KEMPER 1986; ICCVAM, 2010).

Equipment's required:

- Candling light
- Incubator
- Beaker
- Micropipette
- Distilled Water

- Surgical forceps
- Magnifying glass
- Stopwatch
- Volumetric flask

Solutions required:

- 0.9% sodium chloride (NaCl) in deionized/distilled water.
- 1% (w/v) sodium dodecyl sulphate (SDS) in deionized/distilled water.
- 0.1 sodium hydroxide (NaOH) in deionized/distilled water.

For the test, fresh, viable White Leghorn chicken eggs between the weights of 50 and 60 g were procured from Gallus Kft. The white Leghorn is used because this breed's eggs hatch with a very reliable and repeatable ability and do not appear to have any inherited flaws. The fertile eggs were kept in a Ragus-style incubator up until the treatment, which was regulated at a controlled temperature of 37–38 °C and 60–70% relative humidity. The daily incubator rotation prevented the embryos from sticking to the eggshell (SPIELMANN, 1991). The eggs were candled on the ninth day of incubation, and the defective ones were thrown out. The incubator was refilled with viable eggs, the big ends facing upward. They were ready for analysis on the tenth day, and the air space part was marked with a marker. I removed the shell fragment above the air space; the membrane was gently moistened with a 0.9% NaCl solution and delicately removed using surgical forceps so as not to harm the blood veins underneath.

Figure 6: Cracked egg (own image)

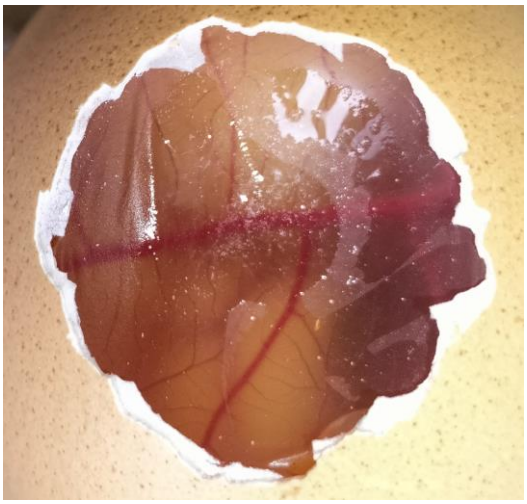

Figure 7: Moisten membrane (own image)

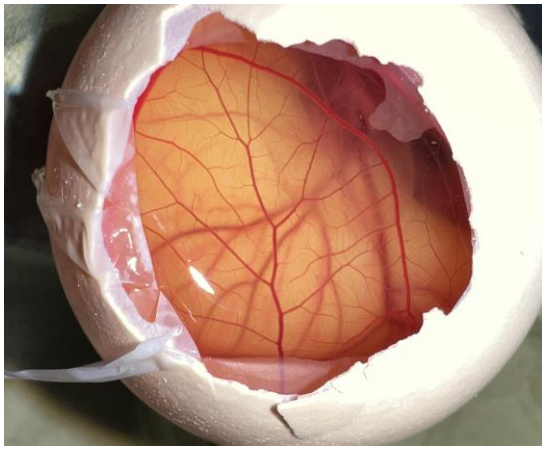

Figure 8: Removed membrane and clear CAM (own image)

For testing, only eggs with a distinct visible vascular system on the CAM were chosen. I applied each pesticide of volume 0.3 ml to the chorioallantoic membrane, and the occurrence of three endpoints (lysis, haemorrhage, and coagulation) over the course of five minutes was observed for the assessment of the pesticide's irritating potential. The appearance time of each endpoint was recorded in seconds. For each pesticide, the treatment was carried out in four duplicates on six eggs, giving a total of 24 treatments for each pesticide.

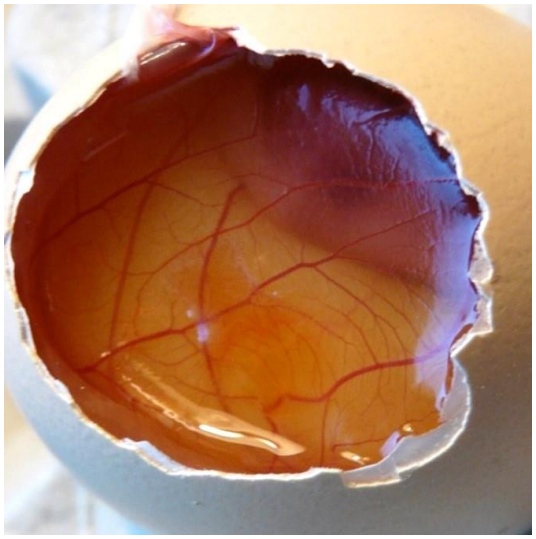

Figure 9: Occurrence of lysis (own image)

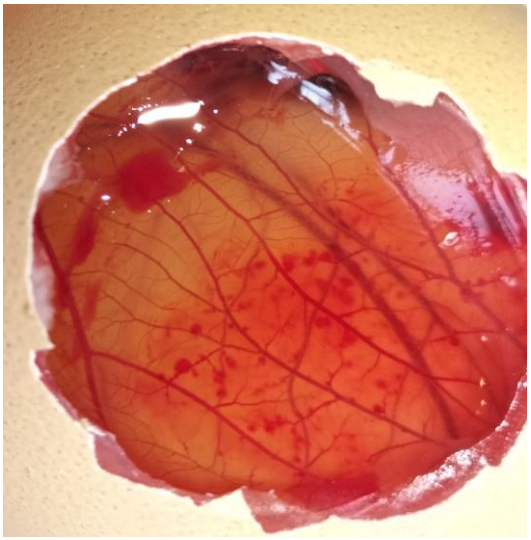

Figure 10: Occurrence of lysis and haemorrhage (own image)

Two eggs were treated with 0.9% NaCl solution as the control and two eggs were treated with 1% sodium dodecyl sulphate and 0.1 M NaOH as the standard. I used a computer program to evaluate the data, and the pesticides were then categorized using the notation suggested in Invittox (1990).

The computer program computes the irritant index (RI) using the below formula:

RI=((301-secH)/300×5) + ((301-secL)/300×7) + ((301-secC)/300×9)

Where:

RI = irritant index Sec= time in seconds H = haemorrhage

L=vascular lysis C= coagulation

The studied pesticides were grouped into three categories based on their irritation indices, as specified by (Invittox, 1990) indicated in Table 2.

Table 2: (Invittox, 1990) HET-CAM classification

Irritation index	Irritation category
0-0.9	Non irritant
1-8.9	Irritant
9-21	Severely irritant

3.2.2 Isolated Chicken Eye (ICE) test

The OECD Test Guideline 438 was followed for conducting the ICE test (OECD, 2018) which required the below tools and solutions:

Equipment's required:

- Superfusion device
- Slit lamp (Haag-Streit BQ 900, Switzerland)
- Pipette
- Beaker
- Flask
- Surgical forceps
- Surgical scissors

Solutions required:

- Negative control NaCl (9 g/l saline) solution.
- Positive control for liquids 5% Benzalkonium chloride solution.
- Fluorescein retention test Fluorescein 2 (v/v) % solution.

3.2.2.1 Chicken Eye Preparation

The eyes were gotten from roughly 7-week-old male or female ROSS 308 chickens weighing 2.5–3 kg from the nearby slaughterhouse, and the chicken heads were transported within two hours of the animals being killed. The paper in the plastic box that held the chicken heads was wet with specially produced physiological saline and transported to the location of the experiment, which was the Charles River Laboratory in Veszprém. I carefully removed the eyeballs from the chicken heads with the aid of surgical scissors and surgical forceps, after which I treated them with a drop of a 2 (v/v)% fluorescein solution before being briefly rinsed with 20 ml of physiological saline.

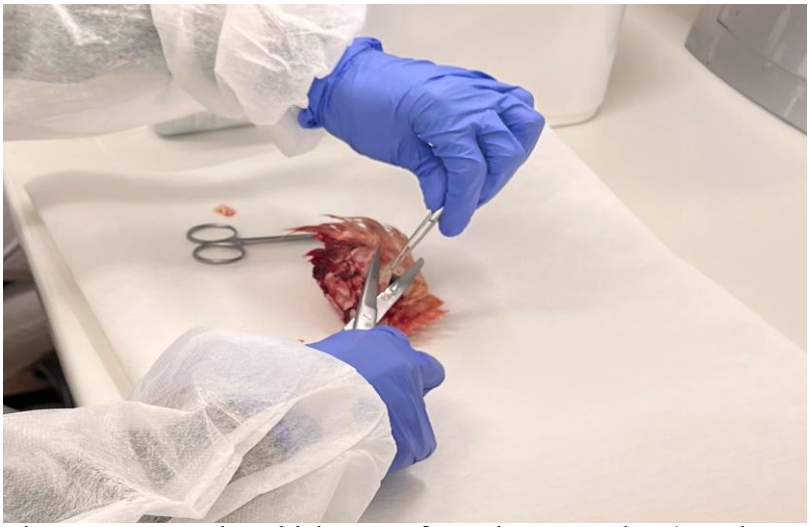

Figure11: Removing chicken eye from the eye socket (own image)

The corneal surface of eyeballs that had been fluorescein-treated under a slit lamp was checked to make sure it hadn't been harmed during the collection, delivery, or removal of the eyeballs from the chicken heads. Rejected eyes include those with significant baseline fluorescein staining (>0.5), a high corneal opacity score (>0.5), or any additional symptoms of damage during enucleation. Each test requires three eyes for the treatment group, three eyes for the positive control group, and one eye for the negative control group to meet good laboratory practice standards. To check for any ocular surface injury caused by the removal of tissue and

insertion into the eye holder, I examined fluorescein retention in the eyes when the corneal surface was unharmed (fluorescein retention ≤ 0.5 and degree of corneal opacity ≤ 0.5). Following it, a reference measurement for corneal thickness and opacity was taken on each individual eye to serve as a baseline. For each given eye, the thickness variation cannot be greater than 10% of the average value. If the chosen eyes satisfied the criteria that make them suitable for the test, we started the acclimatization of the eyes placed in the chambers of the super fusion device, as shown in Figure 12. This takes roughly 45 to 60 minutes. During acclimatization and monitoring after treatment, the temperature of the chamber was maintained between 32 ±1.5 °C.

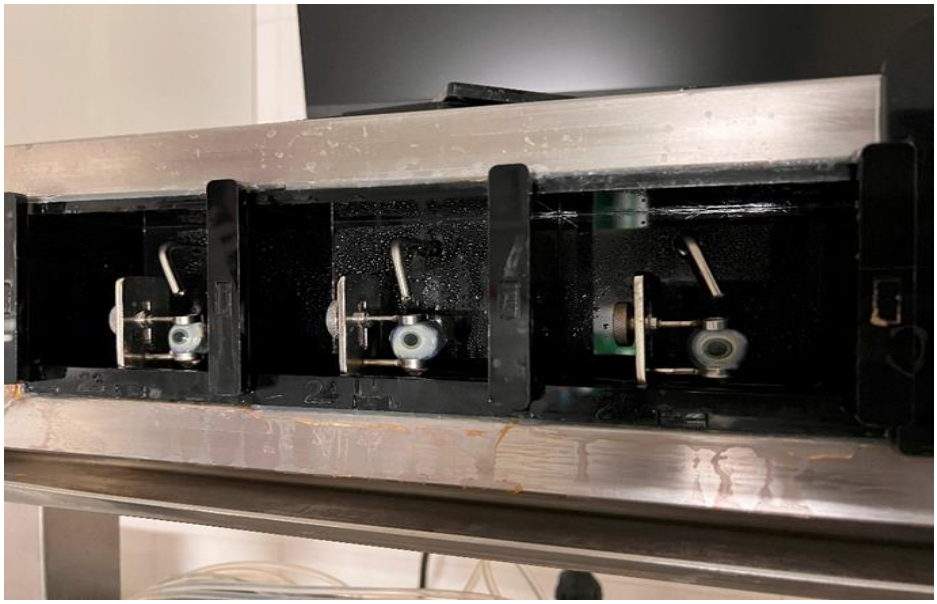

Figure 12: Chicken eyes in the superfusion device under physiological saline (own image)

3.2.2.2 Application of Pesticides Treatment

We used a micropipette to apply the test substance evenly across the whole surface of the cornea in a volume of 0.03 ml. The application time was recorded, and 10 seconds after exposure, the corneal surface was completely washed with saline solution at room temperature.

The same procedure was used to treat the positive and negative control eyes with 0.03 mL of trichloroacetic acid (30 w/v%) and NaCl (9 g/l saline) solutions, respectively.

3.2.2.3 Observation

As outcomes, we looked at fluorescein retention, corneal opacity, swelling, and morphological abnormalities, such as pitting or loosening of the epithelium. Pre-treatment (references value, t = 0) and at 30, 75, 120, 180, and 240 minutes following the post-treatment rinse were used to analyse the treated and control eyes. At each time point, the cornea's opacity and thickness were measured. Thirty minutes after the post-treatment rinse and at baseline (time = 0), fluorescein retention was evaluated twice.

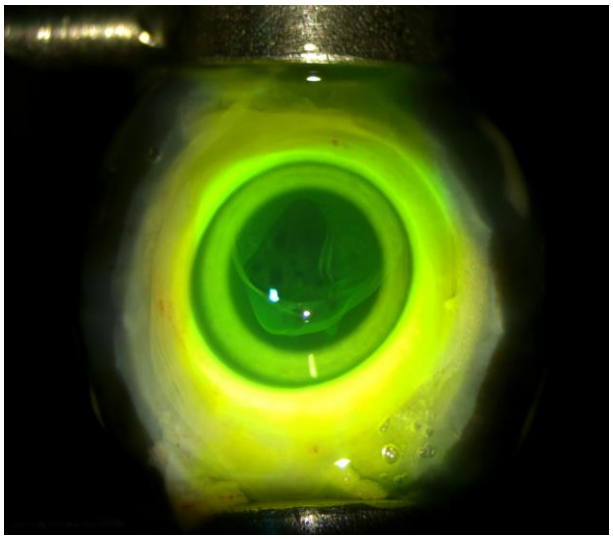

Fig 13: Loosening of the epithelium (Own image)

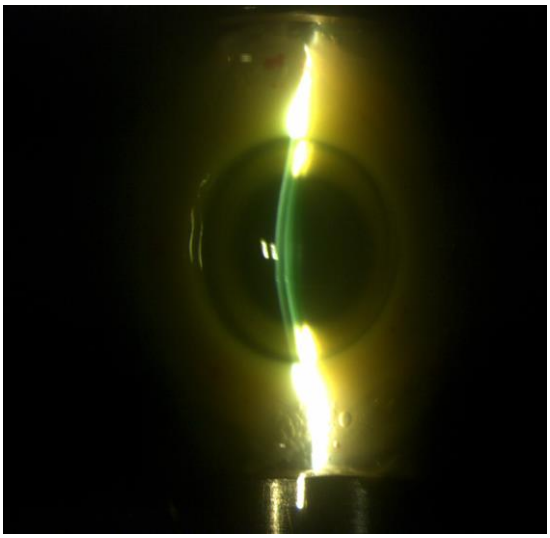

Fig 14: Cornea thickness measurement (Own image)

3.2.2.4 Evaluation

For the evaluation, using the already established classification index of the OECD (2018), we created a class for each endpoint; the results of fluorescein retention, corneal opacity, and corneal swelling were assessed separately. We aggregated the ICE classes for each endpoint

and used them to predict the *in vitro* irritation classification of each pesticide. The MESS R package software was used to analyse the data (EKSTROM, 2022). Goodman-Kruskal's rank correlation and the calculation of Cohen's kappa coefficient were used to statistically analyse the degree of agreement between classifications made using different methods (GOODMAN and KRUSKAL, 1954; COHEN, 1968).

Fluorescein Retention

At thirty minutes after treatment, we measured the mean fluorescein retention for the treated eyes and classified them in accordance with Table 3 and used Table 4 for the ICE category, which we used to determine the ICE class for each pesticide according to Table 8.

Table 3: Fluorescein retention (OECD, 2018)

Score	**Observation**
0	No fluorescein retention.
0.5	Very minor single cell staining.
1	Single cell staining scattered throughout the treated area of the cornea.
2	Focal or confluent dense single cell staining.
3	Confluent large areas of the cornea retaining fluorescein.

Table 4: Mean fluorescein retention ICE categorization (OECD, 2018)

Average fluorescein retention scores 30 minutes after treatment	**ICE category**
0.0 – 0.5	I
0.5 – 1.5	II
1.6 – 2.5	III
2.6 – 3.0	IV

Corneal Opacity

According to the findings listed in Table 5, the portion of the cornea that was most heavily opaque was used to score the cornea's opacity. For each observation time point, we determined the mean corneal opacity value for all test eyes. An ICE class was given for each test chemical based on the greatest mean corneal opacity score recorded at each given time, according to Table 6.

Table 5: Corneal opacity scores (OECD, 2018)

Score	**Observation**
0	No opacity.
0.5	Very faint opacity.
1	Scattered or diffuse areas; details of the iris are clearly visible.
2	Easily discernible translucent area; details of the iris are slightly obscured.

3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible.
4	Complete corneal opacity; iris invisible.

Table 6: ICE Opacity classification (OECD, 2018)

Maximum Average Opacity Score	**ICE category**
0.0 – 0.5	I
0.5 – 1.5	II
1.6 – 2.5	III
2.6 – 4.0	IV

Corneal Swelling

Using the equation below, we calculated corneal swelling based on percentage measurements of corneal thickness taken with an optical pachymeter on a slit-lamp microscope:

$$= \frac{\text{corneal thickness at time t } - \text{corneal thickness at time} = 0}{corneal\ thickness\ at\ time = 0} * 100$$

For each observation time point, the average percentage of corneal swelling for all the treated eyes was calculated. We assigned an ICE class to each pesticide based on the highest mean score for corneal swelling that was seen at any given time point, as shown in Table 7.

Table 7: Corneal thickness categories (OECD, 2018)

The rate of change in corneal thickness in %	**Observations**	**ICE Category**
0-5	no distortion	I
> 5-12	slight, slight distortion	II
> 12-18 (more than 75 minutes after treatment)	slight, slight distortion	II
> 12-18 (less than 75 minutes after treatment)	the degree of distortion can be well defined	III
> 18-26	the degree of distortion can be well defined	III
> 26-32 (more than 75 minutes after treatment)	the degree of distortion can be well defined	III
> 26-32 (less than 75 minutes after treatment)	severe distortion	IV
> 32	severe distortion	IV

By reading the UN GHS classification that corresponds to the combination of categories achieved for corneal swelling, corneal opacity, and fluorescein retention as indicated in Table 8, we evaluated the *in vitro* classification for each pesticide.

Please note: Less often do combinations occur.

Table 8: Overall ICE classification criteria (OECD, 2018b)

UN GHS Classification	**General Classification**	**Combinations of Three Endpoints**
No Category	Non-Irritant	3xI
		2xI, 1xII
		2xII, 1X I
Category 2/2A and 2B	Irritant	3 x II
		2 x II, 1 x III
		2xI, 1xIII**
		1 x I, 1 x II, 1 x III
		3 x III
		2 x III, 1 x I
		2 x III, 1 x II
		2 x III, 1 x IV
		2 x I, 1 x IV**
		2 x II, 1 x IV**
		1xI, 1xII, 1xIV**

		1xI, 1xIII, 1xIV**
		1 x II, 1 x III, 1 x IV*
Category 1	Severe irritant	3xIV
		2xIV, 1xIII
		2xIV, 1xII*
		2xIV, 1xI*
		Corneal opacity = 3 at 30 min (in at least 2 eyes)
		Corneal opacity = 4 at any time point (in atleast 2 eyes)
		Severe loosening of the epithelium (in at least1 eye)

4.0 RESULTS AND DISCUSSIONS

We discuss in great detail the results of these experiments, which used the *in vitro* methods HET-CAM and ICE tests, and the correlations and differences with the *in vivo* data obtained from the safety data sheet for the plant protection chemicals, which was used as a point of reference for the comparison.

4.1 Results from the HET-CAM Test

0.9% NaCl, used as a negative control, was found to be a "non-irritant" to the chorioallantoic membrane (irritation index = 0), and because 1% SDS and 0.1 N NaOH, used as positive controls, were determined to be "severely irritant" to the chorioallantoic membrane (irritation index = 11.53), the HET-CAM test was deemed to be acceptable.

The results, which are shown in Table 9, include the range of the vascular lysis and haemorrhages stated in seconds (s), the irritation index based on the computation, and the irritation category of the tested plant protection products.

Table 9: Results of the HET-CAM test

	HET-CAM			
Test item	**Lysis occurrence (seconds)**	**Haemorrhage occurrence (seconds)**	**Irritation Index**	**Irritation Category**
Esteron 60	175 - 190	-	2.77	irritant
Metkon 60	10 - 15	85 - 120	10.3	severely irritant
Sivanto Prime	21 - 30	50 - 70	10.49	severely irritant
Viballa	13 - 17	60 - 90	10.48	severely irritant

Esteron 60 was used to treat the chorioallantoic membrane, and because only vascular lysis was seen between 175 and 190 seconds after application and it had an irritation index of 2.77, it was classified as an irritant.

After treatment with Metkon 60, the CAM had lysis between 10 and 15 seconds, and they haemorrhaged between 85 and 120 seconds with an irritation index of 10.30. This was classified as severely irritating.

During the five minutes of observation, the CAM displayed lysis at 21 to 30 seconds following the application of the insecticide Sivanto Prime and haemorrhaged at 50 to 70 seconds. It also had an irritation index of 10.49, indicating that it caused severe irritation.

Lastly, following treatment with the plant protection product Viballa, vascular lysis happened in the chorioallantoic membrane between 13 and 17 seconds, while hemorrhage happened between 60 and 90 seconds, and its irritation index was computed to be 10.48. It was deemed a severe irritant.

4.2 Results from the ICE Test

In this study, the saline solution served as the negative control because it had no apparent impact on the chicken eye, whereas the positive control benzalkonium chloride was categorized as corrosive/severely irritating, according to the UN GHS Classification: (Category 1). The results of the study using the pesticides were therefore considered to be valid because the positive and negative controls produced the anticipated outcomes.

Table 10: ICE Results

Test item	ICE							
	Corneal thickness		**Corneal opacity**		**Fluorescein retention**		**Endpoints**	**Irritation category**
	Score	**Class**	**Score**	**Class**	**Score**	**Class**		
Esteron 60	15.7%	II	2.33	III	1	II	2xII, 1xIII	Category 2B (Irritant)
Metkon 60	8.3%	II	1.17	II	1.33	II	3xII	Category 2B (Irritant)
Sivanto Prime	7.0%	II	0.83	II	1.33	II	3xII	Category 2B (Irritant)
Viballa	12.6%	II	2.33	III	1.17	II	1xIII, 2xII	Category 2B (Irritant)

Following treatment with Esteron 60, slight distortion of the corneal thickness (15.7%) for more than 75 mins after the treatment occurred. We also noticed an obscured details in the iris indicating a slight change in the corneal opacity, and faint fluorescein retention were seen. The plant protection product was classified as an irritating pesticide, per the combinations of the 3 endpoints (2xII, 1xIII). Irritants pesticides are substances that, when applied to the anterior surface of the eye, cause reversible changes in the eyes, and are categorized as UN GHS Categories 2A or 2B (OECD, 2018).

In the case of eyes treated with the fungicide Metkon 60, slight corneal thickness distortion was seen for than half the time following treatment, for the corneal opacity we observed clear

visible details of the iris with a slight fluorescein retention. Based on the endpoint obtained (3xII) it is classified as an irritating fungicide Category 2B.

Furthermore, the insecticide Sivanto Prime showed faint distortion to the corneal thickness (7.0%) and sweeling, moderate corneal opacity and fluorescein retention. Thereby giving an endpoint combination and classification of (3xII) and Category 2B (Irritant) respectively.

Finally, Viballa caused moderate swelling and thickness for more than 75 minutes after treatment, easily discernible translucent area in the corneal opacity and fluorescein retention showed slight single cell staining scattered throughout the treated area of the cornea. This gave us an endpoint combination of (1xIII, 2xII) and we classified it as Category 2B (Irritant).

4.3 Results Comparison and Discussions

Using the MESS R software package, the results I obtained from the HET-CAM and ICE were compared with the already established *in vivo* data of the plant protection products. The tables below showed the summary of the irritation classifications, *in vivo* vs HET-CAM, *in vivo* vs ICE, HET-CAM vs ICE and lastly the statistical agreement and significance.

Table 11: Summary data of eye irritation category

Test item	**Irritation category**		
	***In vivo* GHS**	**HET-CAM test**	**ICE test**
Esteron 60	irritative	irritative	Irritant
Metkon 60	severely irritative	severely irritative	Irritant
Sivanto Prime	irritative	severely irritative	Irritant

Viballa	severely irritative	severely irritative	Irritant

Table 12: Irritation categories of the *in vivo* and the HET-CAM test

***In vivo* classification**	**HET-CAM test classification**			**Altogether**
	Not irritant	**Irritant**	**Severely irritant**	
Not irritant	0	0	0	0
Irritant	0	1	1	2
Severely irritant	0	0	2	2
Altogether	0	1	3	4

Table 13: *In vivo* and ICE classifications

***In vivo* classification**	**ICE test classification**			**Altogether**
	Not irritant	**Irritant**	**Severely irritant**	
Not irritant	0	0	0	0
Irritant	0	2	0	2
Severely irritant	0	2	0	2
Altogether	0	4	0	4

Table 14: HET-CAM and ICE classifications

HET-CAM classification	ICE test classification			Altogether
	Not irritant	Irritant	Severely irritant	
Not irritant	0	0	0	0
Irritant	0	1	0	1
Severely irritant	0	3	0	3
Altogether	0	4	0	4

Table 15: Statistical evaluation of the classification of eye irritation

Methods	Agreement (%)	Kendall.gamma	sig	Cohen.kappa	sig
In vivo - HET-CAM	75	0.02439	0.940717	0.5	0.248213
In vivo - ICE	50	-0.06667	0.85018	0	1
HET-CAM - ICE	25	-0.09091	0.793701	0	1

From the above tables the following deductions were made:

Compared to the *in vivo* data, the HET-CAM test results in our study demonstrated relatively high accuracy of 75%, and only 25% over estimation and no false negatives, with a 75% sensitivity, the specificity couldn't be determined because all the low number of test items. This degree of agreement concurs with (TAVASZI and BUDAI, 2006; KORMOS, 2017; TALAEI et al., 2020; BUDAI et al., 2021; IDOGWU et al., 2023). Out of the four test items used, I discovered same eye irritation classification for Esteron 60, Metkon 60 and Viballa for both HET-CAM and *in vivo* while for Sivanto Prime, HET-CAM over predicted it to be severely irritant as against an irritant.

In vivo results compared to the ICE test I observed a 50% degree of sensitivity similar to BUDAI et al. 2021 and 50% false negatives of the four-test item Esteron 60 and Sivanto Prime showed same eye irritation categories. Metkon 60 and Viballa was under predicted as irritant by ICE while being classed as severely irritant.

I noticed the least level of agreement (25%) between the HET-CAM and ICE tests (BUDAI et al., 2021), and 75% false negatives. Esteron 60 was the only test item that showed same eye irritation classifications, ICE test underestimated for Metkon 60, Viballa and Sivanto Prime as irritant plant protection production as compared to HET-CAM test. With reference to the ICE test, I can say the HET-CAM over predicted the three test items (Metkon 60, Viballa and Sivanto Prime) as severely irritant as compared to the result of the ICE test that classified it as irritating pesticides.

5.0 CONCLUSION AND RECOMMENDATIONS

The findings of my research revealed that HET-CAM and ICE tests can be effectively applied to detect the potential for eye irritation from plant protection pesticides. The results of the HET-CAM test were more in agreement (75% agreement) with those of the *in vivo* approach. Since the HET-CAM test responds to irritant substances with an inflammatory reaction similar to that produced by conjunctival tissue in rabbits, it is generally accepted that it serves as a validated ocular model for conjunctival irritation testing (BUDAI et al., 2021). The HET-CAM assay can be used to study the adverse effects of chemicals on the conjunctiva, while the ICE test can be used to thoroughly investigate corneal lesions.

Numerous benefits of the HET-CAM test include the absence of live animals, lower expenses, and fewer ethical concerns. The test is very repeatable and provides a rapid, standardized method for screening a number of chemicals or formulations. However, the HET-CAM test has limitations because it only evaluates the immediate ocular response within 5 minutes of test item application and does not provide information on the potential for delayed or cumulative effects. It cannot be used to test products that are solid or coloured.

The ICE test has advantages such as lower costs, improved reproducibility, and increased ethical acceptability. Additionally, it has been shown that the ICE test has an accuracy rate of up to 83% in predicting the possibility of ocular irritation (OECD, 2018). The test is a useful tool for early detection of potentially hazardous substances and the development of safer goods as a result. One of the biggest limitations of the ICE test is its inability to predict substantial eye irritation or injury that goes beyond the corneal surface.

Both alternative methods have the same drawback, which is that reversibility cannot be observed and is subjective.

In accordance with the 3 R's of replacement, reduction, and refinement (RUSSEL and BURCH, 1959), HET-CAM and ICE tests can be used in a tiered approach and in combination with others to evaluate eye irritation and as part of a series of experiments intended to lessen the use of animals as test subjects and reduce or completely eradicate the pain and suffering that these animals go through. These tests are a useful technique to demonstrate how likely it is that pesticides would irritate the eyes, despite the fact that they are subjective (TAVASZI and BUDAI, 2006). I strongly advise using these tests before in vivo tests, as prescribed in OECD 405.

As a young researcher from a developing nation like Nigeria, one of the most significant takeaways I've gained from this study is the importance of enacting, implementing, or revising current regulations on the distribution, usage, and knowledge of the toxicity and dangers associated with exposure to chemical products. Furthermore, in order to safeguard user health and the environment and decrease pesticide residue in agricultural products, the National Agency for Food and Drug Administration and Control (NAFDAC), the national body in charge of agrochemicals and other chemical products with just seven laboratories, may partner with independent private and public research laboratories with a wider reach in testing chemical products before they go into circulation. Due to its relative cost-effectiveness, privately funded research facilities can easily adopt the HET-CAM test. The ICE test, along with other toxicity assays, can be implemented with thorough preparation and made requisite for chemicals.

6.0 SUMMARY

Agricultural food production must grow to meet the rapidly increasing world population, estimated to reach 9.7 billion by 2050. A number of difficulties have hampered food production, including pest, disease, and bacteria infestations, and there is a growing need to overcome these difficulties through cultural, mechanical, biological, and chemical control. This led to the production of plant protection pesticides with active ingredients that can eradicate pathogen infestations. The safety of these pesticides must be ascertained before public use. Therefore, plant protection pesticides must undergo a number of toxicological tests before being authorized for use; one of these tests is the eye irritation propensity of the pesticide. For decades, the Draize test (*in vivo*), which uses rabbits, was widely accepted but became one of the most criticized test methods by animal welfare groups and the public on an ethical basis due to the harm inflicted on the test rabbits. This led to the proposition of reducing, refining, and replacing the number of animals used in eye irritation tests (RUSSEL and BURCH, 1959). A number of *in vitro* techniques, such as the HET-CAM test and the ICE test, have been developed and validated by the Organization for Economic Cooperation and Development (OECD) and utilized to examine the toxicity of suspected non-irritants, irritants, or severe irritant pesticides. In our study, a group of four pesticides were subjected to screening in order to establish *in vitro* data using the HET-CAM and ICE tests and compare them with already established *in vivo* findings obtained from the safety data sheets. According to Invittox Protocol No. 47 1990, the HET-CAM test was conducted. Similar to the rabbit's eye conjunctival tissue response to an induced test item, the chorioallantoic membrane of the hen's egg responds to injury with a full inflammatory response (BUDAI et al., 2010). White Leghorn hen eggs were used for the studies, which were incubated for 10 days at 37–38 degrees Celsius and 60–70

percent humidity. On day 10, I cracked the eggshell open to inject the 100% concentration of the test pesticides onto the chorioallantoic membrane, which was then monitored for five minutes. The irritation index was derived from the times that bleeding, vascular lysis, or coagulation occurred on the membrane and recorded to the nearest second. Four replicates of six eggs each were used for each test chemical.

Using the OECD's test guideline 438 description of the isolated chicken eye test, the eyes were gotten from chicken heads from a nearby slaughterhouse. The test items were applied in a single dose to the cornea of isolated chicken eyes and inserted into the superfusion device. The test pesticides' effects on corneal opacity, thickening, fluorescein retention, and morphological changes like loosening of epithelia were all closely monitored. Prior to treatment and beginning at roughly 30, 75, 120, 180, and 240 minutes following the post-treatment rinse, these parameters were assessed. With the exception of fluorescence retention, which was only assessed before treatment and 30 minutes after exposure to the test material, all endpoints were assessed at each of the aforementioned time periods.

In each method, positive and negative controls were used, and they produced the predicted outcomes. These methods were used to categorize the test substances as irritants or severe irritant pesticides. I compared the results with already established *in vivo* data and other literature and showed a high accuracy between *in vivo* data and that of the HET-CAM of 75%; *in vivo* and ICE have a correlation of 50%; and the least level of agreement of 25% was found between the two *in vitro* methods, HET-CAM and ICE.

I recommend the following in concurrence with other literature:

i. Though both tests are subjective, the HET-CAM test can be a useful tool for determining potential conjunctival irritation, while the ICE test can be used to study

corneal irritant effects in detail.

ii. HET-CAM and ICE tests can be said to be good tools for examining the possible eye irritation potential of pesticides, which can serve as a component of a series of tests to determine the full eye irritation potential, reduce the use of animals as test subjects, and alleviate or completely do away with the sufferings that experimental animals endure.

iii. My major takeaway from this study as a young researcher from a developing country (Nigeria), is the importance of enacting or revising regulations on chemical product usage and toxicity knowledge, which should be emphasized in Nigeria. The nation's chemical products regulatory bodies, like the National Food Drug Administration and Control (NAFDAC), should partner with private and public research laboratories with a wider reach to test pesticides using cost-effective and requisite toxicity assays, such as the HET-CAM and ICE tests.

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7.0 REFERENCES

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DECLARATION

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