THESIS

Svetlana Kazarova
MSc. Plant Protection

KESZTHELY
2023

Hungarian University of Agriculture and Life Science

Georgikon Campus

MSc. Plant Protection

TESTING OF PESTICIDES FOR EYE IRRITATION EFFECT WITH HET-CAM TEST AND ON ISOLATED CHICKEN EYE TEST

Primary Supervisor: Eva Kormos

Assistant professor

Author: **Svetlana Kazarova**

HB6BV7

Institute: **Plant Protection**

KESZTHELY
2023

Table of Contents

1. INTRODUCTION

The world population has reached 8 billion and agricultural land reduction has forced increased use of chemicals to ensure a good yield. According to studies, pests can cause 20% to 40% direct crop yield loss (Bag et al., 2017; Sultana et al., 2018) as well as indirect effects with short and long-term consequences (Savary et al., 2012). Pesticides are widely used to control insects, fungi, weeds, rodents, and nematodes (Bernardes et al., 2015). The earliest recorded use of insecticides was 4500 years ago by Sumerians who used natural compounds to control pests (Unsworth, 2010). Then after 1870, people started using inorganic synthetic materials to control pests. Development and consumption of pesticides increased during World War II because of the urgency to improve crop production and control diseases. After the 1940s synthetic pesticide consumption increased food production (Unsworth, 2010). Global pesticide production increased 11% annually, from 0.2 million tons in the 1950s to more than 5 million tons by 2000 (Carvalho, 2017). Out of three billion kilograms of pesticides sued annually , only 1% are used effectively to control targeted pests (Unsworth, 2010), while a large amount of remaining pesticides reach non-targeted animals or cause environmental pollution and affect human health (Hernández et al., 2013).

Pesticides are among the few toxic substances that are intentionally released into the environment by humans. Although the goal is only to reduce pests, exposure to these chemicals can be harmful to humans as well (Sarwar, 2016). When used incorrectly or with insufficient precautions, chemicals can cause various harmful effects if they get into the eyes. Toxic substances in the eyes can cause corneal opacity, severe and permanent eye damage, even blindness, and other damages. Before industrialization, eye problems were either caused by physical trauma or by disease associated with malnutrition, or bacterial infections (Prinsen et al., 2017). In the 1930s, cosmetic use caused serious eye damage. In response, the Food and Drugs Administration (FDA) developed a Draize eye test to check the toxicity of chemicals (Callabrese, 1987).

The Draize eye test was a simple, straightforward test that provided useful information about chemical toxicity. However, it is inhumane to the animals causing pain and even permanent eye damage. In 1980, the controversial nature of this chemical testing method was exposed by Animal rights associations, which lead to the requirement for alternate testing methods. Since then, scientists have developed several *in vitro, in vitro,* and *ex vivo* tests such as Hen's Egg Test (HET) and the isolated chicken eye (ICE) test.

Hen's egg test focuses on the effect of tested substances on the chorioallantoic membrane (CAM) (Budai et al., 2021), therefore, it is also called the HET-CAM test. CAM is a complete tissue and easy to research (Leighton et al., 1985). The irritancy potential of test substances can be determined by observing changes CAM of the hen egg. The test substance is applied directly to the chorioallantoic membrane of a fertilized chicken egg when nerve tissues and pain perception have not yet developed which causes an inflammatory reaction on the conjunctival blood vessels (Anderson and Russell, 1995). The observations include time to hemorrhage, lysis, and coagulation (Derouiche and Abdennour, 2017). The developers of the HET-CAM test reported that the effects of the test substances on CAM will be identical to the rabbit's eye, hence, to the human eye (Luepke and Kemper, 1986). This test has been reported to predict accurately the non-irritant nature of test substances and to distinguish between irritating and non-irritating substances (Schrage et al., 2010; Scheel et al., 2011).

Isolated chicken eye (ICE) is another widely used test that uses corneal swelling, opacity, and fluorescein retention to determine the irritation effect of the test substance. Corneal swelling has been recognized as an accurate endpoint for both *in vitro* and *in vivo* corneal injury assessment (Burton, 1972; Burton et al., 1981). The corneal opacity observed in the ICE test provides information about corneal damage that is directly related to corneal damage observed during the Draize eye test. Moreover, fluorescein retention shows corneal permeability, which indicates corneal surface damage (Schutte et al., 2009). ICE test has been officially adopted as Test Guideline 438. It is recommended to use for chemicals that do not need classification (GHS No

category), and for chemicals that can cause serious eye damage (OECD, 2019). Therefore, both these tests i.e. HET-CAM and ICE test were used in this study to determine the irritation potential of four pesticides.

1.1. Objective of the Study

The objective of this study was to determine the toxicity potential of four pesticides (Prosaro, Tilmor, Zantara and Kideka) widely used in Hungary. The aim of the study also included assessing and establishing alternate methods to study the harmful effects of pesticides. Therefore, we used HET-CAM and ICE tests to determine the irritation potential of under-study pesticides and then compared them.

2. REVIEW OF LITERATURE

2.1. World population and agriculture

According to the United Nations, the world population reached 8 billion in 2022. This is expected to grow to 8.5 billion by 2030, 9.7 billion by 2050, and reach 11.2 billion people by the end of the century. Such rapid growth is associated with rising demand for resources. Population growth is an interesting dynamic that affects human life in many ways. The rapid increase in population is intensifying the pressure on agricultural productivity. In the last 25 years (1995-2020), the human population has increased in access to 2 billion (Figure 1). To feed the ever-increasing population, an increase in agricultural productivity is also desired.

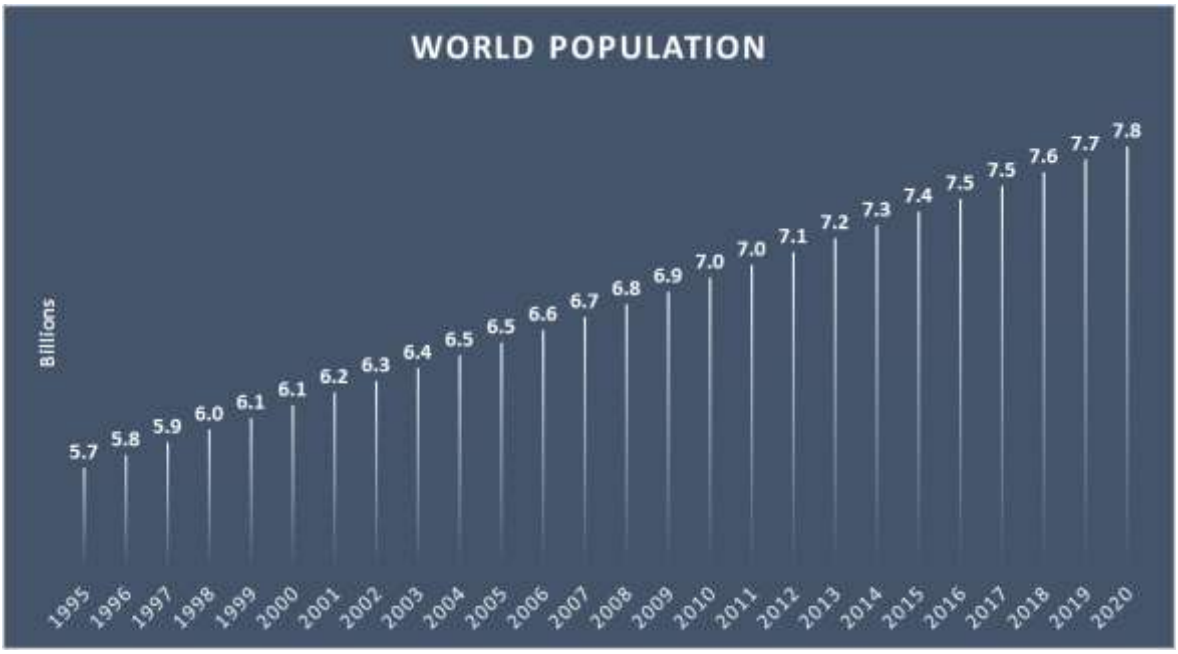

Figure 1 Increase in human population since 1995 (Source: UN)

2.2. Use of pesticides and its effects

Pesticides include insecticides, herbicides, fungicides, rodenticides, nematicides, and molluscicides (Bernardes et al., 2015). It is generally accepted that pesticides play an important role in agricultural development because they can reduce the losses of agricultural products and improve the affordable yield and quality of food.(Aktar et al., 2009; Fenik et al., 2011; Strassemeyer et al., 2017). Therefore, pesticides used in the last 25 years increased by 1 million tonnes. The use of pesticides can harm non-target organisms, both animals and the environment. The person who releases it into the environment can also be poisoned. Among other poisonings, contact with the eyes may occur, where symptoms ranging from mild conjunctivitis to severe eye damage may appear.

2.3. Anatomy of the human eye

The eye is one of the most complicated organs in the human body. It consists of three layers; outer, middle, and inner (Figure 2). The outer layer protects the eye, the middle layer controls the amount of light reaching the retina, and the inner layer process the light. The outer layer consists of the cornea and sclera that are connected by limbus. The function of the cornea is to protect the eye from structural damage and infection along with refracting and transmitting light to the lens. The sclera is covered by a translucent mucous membrane called the conjunctiva that covers the visible part of the sclera. The sclera itself is a connective tissue coat that protects the eye from internal and external forces (Willoughby et al., 2010). Behind the outer layer is the middle layer which consists of the iris, the ciliary body, and the choroid. The iris regulates the amount of light reaching the retina by regulating pupil size. The function of the ciliary body is to regulate the power and form of the lens, while the choroid supplies oxygen and nutrients to the outer retinal layers. The inner layer of the eye consists of the retina which is connected to a

network of neurons that captures and processes the incoming light. The aqueous, vitreous, and lens are the three translucent structures that comprise the ocular layers.

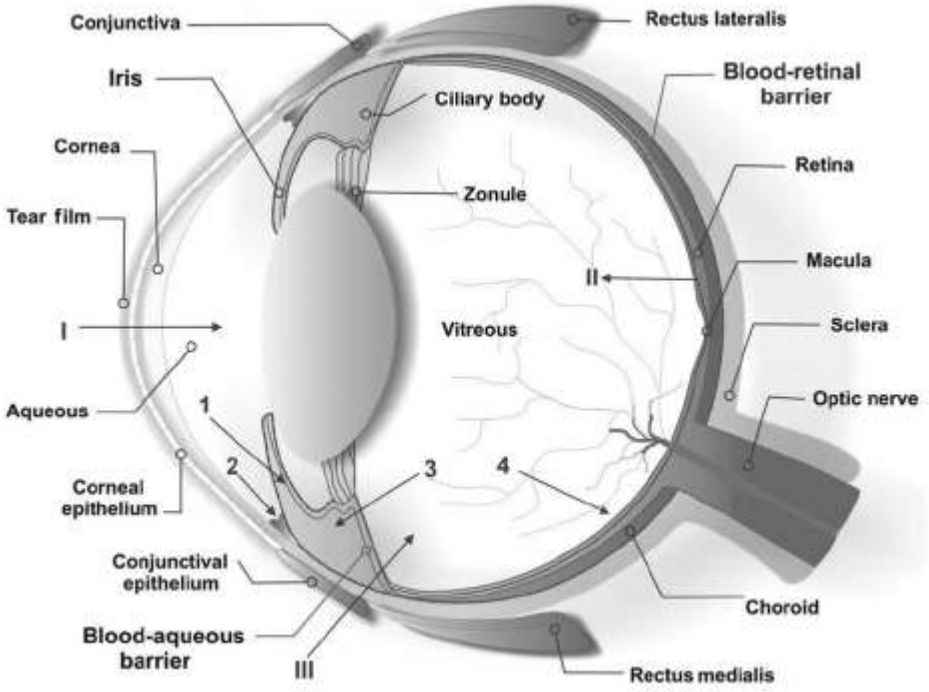

Figure 2 Eye structure (Jaleh et al., 2009)

The cornea is present in front of the iris and pupil. It is the transparent tissue covering the front portion of the eye. It has five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium (Figure 3). The cornea is the most densely innervated tissue in the body (Bonini et al., 2003), where innervation first occurs at 5 months of gestation (Kitano, 1957). Most corneal nerves are sensory nerves (Müller et al., 2003) that originate from the ophthalmic division of the trigeminal ganglion (Marfurt et al., 1989). In an adult human eye, the cornea has a vertical diameter of 10.5 mm, a horizontal diameter of 11.5 mm, and a constant curvature throughout life (Rüfer et al., 2005). An optic zone of 4 mm diameter is situated in the center of the cornea, anterior to pupil, in photopic condition. This optic zone is involved in the refractive function of the cornea. The peripheral cornea is different from the central cornea in terms of physiology and pathology. The branches of the anterior ciliary arteries form arcades at the limbus that supply the peripheral cornea (Van Buskirk, 1989).

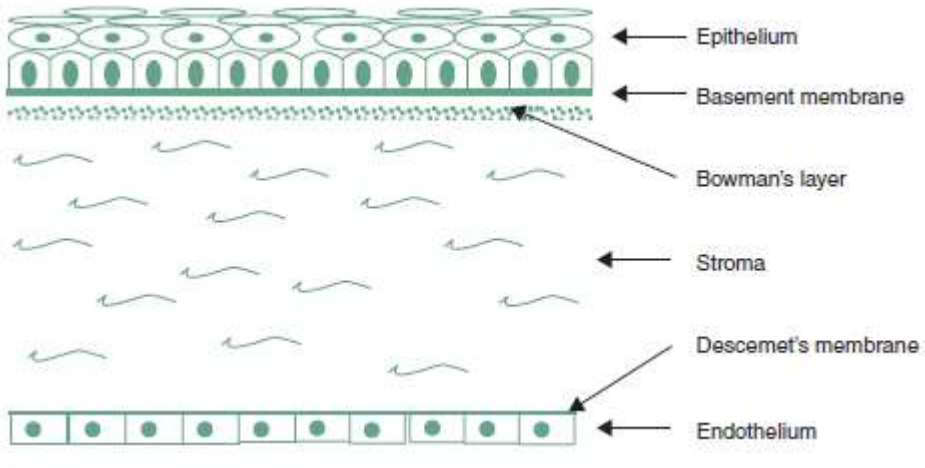

Figure 3 Affected corneal layers during irritation (Wilson et al., 2015)

A tear film covers the corneal epithelium. The corneal epithelium consists of 2-3 layers of superficial cells, a layer of basal cells, and 2-3 layers of wing cells. The tear film is composed of a lipid layer and a water-mucous layer. The mucous layer is on the inner side and interacts with the epithelial cells that allow the tear film to spread with each blink. The function of the tear film is to protect the corneal surface from chemical toxicity, foreign body damage, and microbial invasion, and smooth out the micro-irregularities of the epithelium surface (Willoughby et al., 2010).

However, exposure to chemicals such as pesticides can irritate the eyes. Due to the structure of the eye, eye irritation can affect several regions. Usually, the damage starts from the cornea, but stronger irritation can also injure the deeper eye layers. The damage to the eyes depends upon the irritation capacity of the substance. A mildly irritating substance may damage the outer layer i.e. corneal surface only, while, mild to moderate irritating substances can damage the epithelium and stromal surface, and severely irritating substances can damage the deeper parts or the entire depth of the stroma (Figure 4) (Maurer et al., 2002). Cornea differs in wound healing from other body tissues because it does not contain any blood vessels and its metabolism is very slow. Maurer et al. (2002) pointed out that the degree and persistence of eye irritation can be assessed in the first hours of treatment by observing corneal damage.

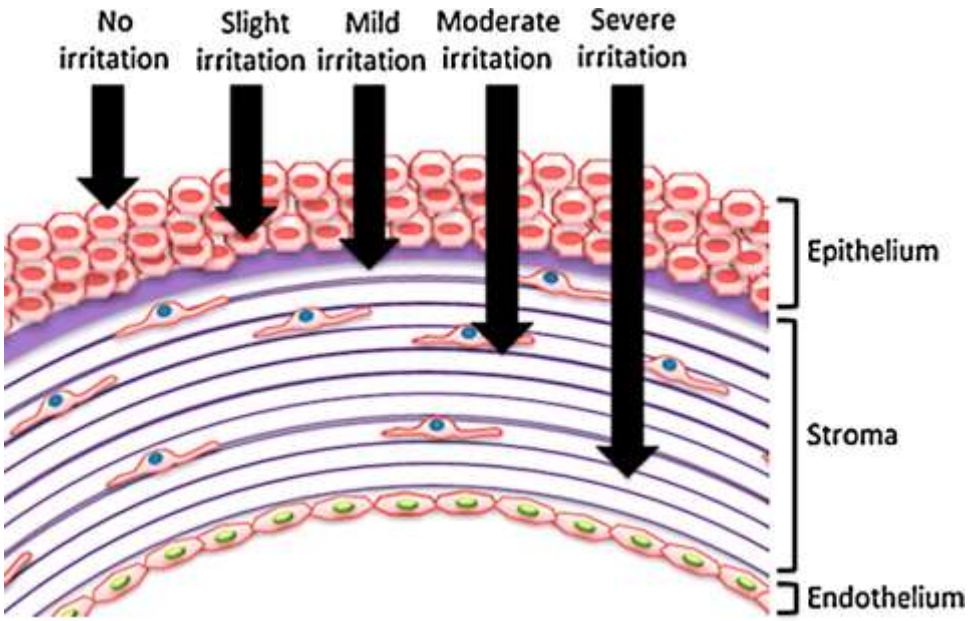

Figure 4 the substances penetrating the deepest into the cornea cause the most severe irritation (Wilson et al., 2015)

For safe usage of chemicals such as pesticides, it is important to test the products and/or their active ingredients for eye irritation. So that farmers and the general public can be assured of their safety, or warned of the dangers associated with the product. Therefore, eye irritation tests are necessary to ensure that the risks associated with products meet suitable safety criteria and are clearly labeled.

2.4. Tests to study effects on eyes

2.4.1. Draize testing

Since the 18th century, live animals are being used to test the effects of products on the eyes (Wilhelmus, 2001). In the 1930s, cosmetic use caused serious eye damage. In response, the Food and Drugs Administration (FDA) developed a Draize eye test to check the effect of chemicals on eyes (Callabrese, 1987). In this test, New Zealand white rabbits were used because of their easy availability, inexpensiveness, large eyes, described anatomy, and ease to handle (Wilhelmus, 2001).

The Draize test requires the application of 0.1 ml liquid test substance or 0.1 g solid test substance onto the cornea and conjunctival sac of an alive rabbit. Treatment is applied to one eye of the rabbit while the other eye remains untreated and serves as a control (Draize et al., 1944). According to earlier Draize eye protocol, at least six rabbits were used per test, however, it was modified to use three or fewer rabbits in case serious eye damage is expected. Rabbits with severe effects are humanely euthanized. The application and delivery of analgesics and anesthetics to reduce pain have also been included in the latest guidelines. Treatment is applied for 72 hours and observations regarding irritation, redness, cloudiness, swelling, hemorrhage, discharge, and blindness are recorded at pre-determined intervals for up to 21 days (Huhtala et al., 2008). Based on these observations, tested chemicals are classified as ranging from non-irritating to severely irritating. Animals are recommended to euthanize or removed for the experiment in case of severe irritation or pain is observed (OECD, 2002). This test can be used to identify both reversible and irreversible ocular effects (Barile, 2010).

Earlier, eye irritation was observed as the "maximum average score" (MAS) (Huhtala et al., 2008), where, many countries developed their scoring system. It led to multiple classifications of a single chemical in different countries. To tackle this problem, the United Nations (UN) proposed a unified classification system called Globally Harmonized System (GHS) (Secretariat, 2013). The GHS is based upon averaged single tissue observations which can account for the reversibility of the observed chemical effects (Eskes et al., 2005). The GHS was adopted in 2002 and published in 2003 (Silk, 2003). Tested substances are classified into three groups: (1) No category, non-irritating substances are placed in this group; (2) Category 1, substances that cause irreversible damage to the eyes are placed in this group; (3) Category 2, substances that cause reversible damage to the eyes are placed in this group (Figure 5). Category 2 substances are further classified into two groups i.e. category 2A and 2B. Substances that cause reversible eye irritation within 21 days are placed in category 2A, while those that cause reversible eye irritation within 7 days are placed in category 2B.

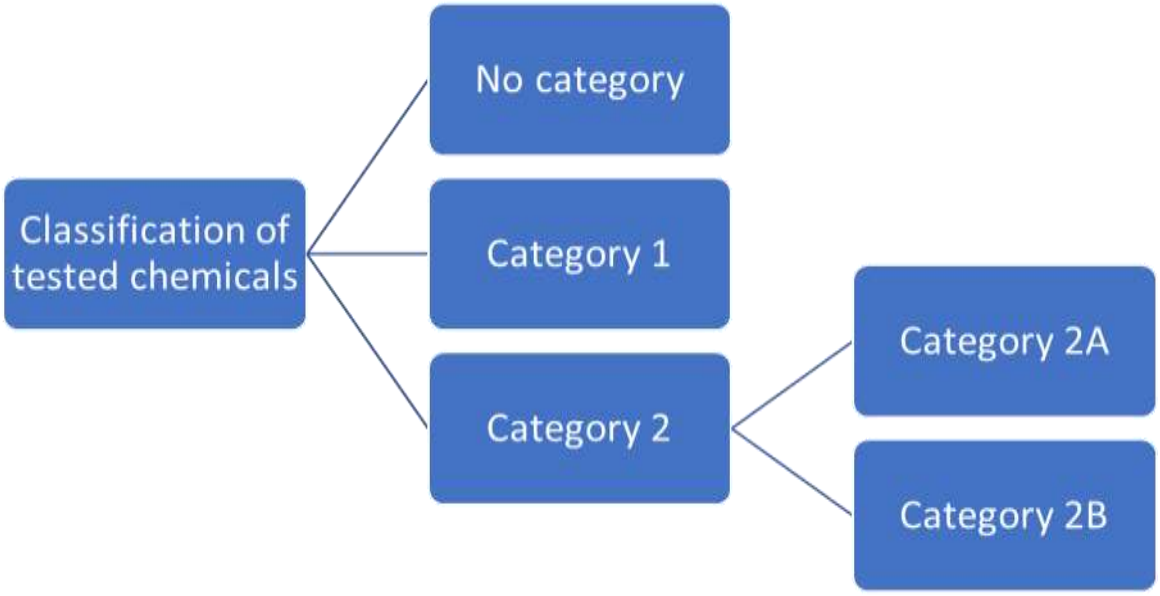

Figure 5 GHS classification of chemicals tested by Draize eye test

Despite adopting the unified classification system (GHS), the Draize test is often criticized for multiple reasons including:

- Time-consuming (21 days)
- Lack of repeatability
- Insufficient test chemical application relevance (Davila et al., 1998)
- High dosages (Curren and Harbell, 2002)
- Over-prediction of human responses to the chemicals (Jester et al., 2001)
- Non-defined standardized test substance exposure time (Prinsen, 2006)
- provide very little information about the primary or secondary responses in the cornea, iris, or conjunctiva (Maurer et al., 2002)
- Structural, physiological, and biochemical differences between human and rabbit eyes (Huhtala et al., 2008).

Moreover, eye irritation test is also criticized by animal rights and welfare activists because it is inhumane. To resolve the above-mentioned problems, scientists have continuously attempted to find an alternative *in vitro, in vitro,* and *ex vivo* tests. Some of the most used tests are as follows:

1. *In vivo* tests
    a. Low-volume eye irritation test
    b. Human data
2. *Ex vivo* tests
    a. Organotypic methods
        i. Isolated rabbit eye
        ii. Isolated chicken eye
        iii. The Bovine Cornea opacity Permeability
        iv. The Porcine Cornea opacity Permeability
    b. Non-ocular organotypic methods
        i. Hen's egg test
3. *In vitro* tests
    a. Cytotoxicity assessment
    b. Corneal epithelial models
    c. Corneal equivalents
4. *In silico* models

2.4.2. Organotypic methods

To minimize the usage of live animals, Burton et al., (1981) introduced enucleated eye tests using isolated eyes of rabbits that have been sacrificed for food purposes or other research purposes (Wilson et al., 2015). The isolated rabbit eye (IRE) test was originally developed to test severe irritants (Guo et al., 2012). Enucleated eye tests aim to maintain the normal physiological and biochemical function of the isolated eye or cornea for the short-term experiment (Barile, 2010). The neat application of test substances in these methods is more industry relevant and faithfully

represents accidental exposure (Reader et al., 1990). Opacitometric and spectroscopic methods are usually used in these protocols to assess the changes in the cornea followed by histological analysis. The endpoints include corneal opacity (Barile, 2010), fluorescein retention (Prinsen and Koëter, 1993), corneal swelling, and histological analysis (OECD, 2018). The effect of test substances is determined by the initial injury that correlates to the extent of cell death (Jester et al., 2001). Since its introduction, IRE has been extensively evaluated by international regulatory bodies (Guo et al., 2012), yet, it has not been considered adequately valid for ocular irritation classification.

Contrary to IRE, the isolated chicken eye (ICE) test is widely accepted to be accurate and reliable for assessing the effect of test substances on eyes (Prinsen, 1996). The IRE test was introduced by Prinsen and Koëter (1993). The protocol involves the collection of the isolated chicken head from a slaughterhouse. The isolated eye is placed in a clamp in such a way that the cornea is in a vertical position. The eye is transferred to a superfusion apparatus (Maurer et al., 2002) (Figure 6i). Eyes are then equilibrated for up to 1 h (Figure 6ii), followed by baseline thickness and opacity measurement. The eye is then placed horizontally and the specified quantity of test substance is applied for 10 s (Figure 6iii). Hypertonic saline is then used to rinse the cornea (Figure 6iv) and the eye is returned to the superfusion chamber (Figure 6v). The observation related to opacity, tissue swelling, fluorescein retention, and changes to the tissue surface are recorded to assess the effect of test substance (Wilson et al., 2015).

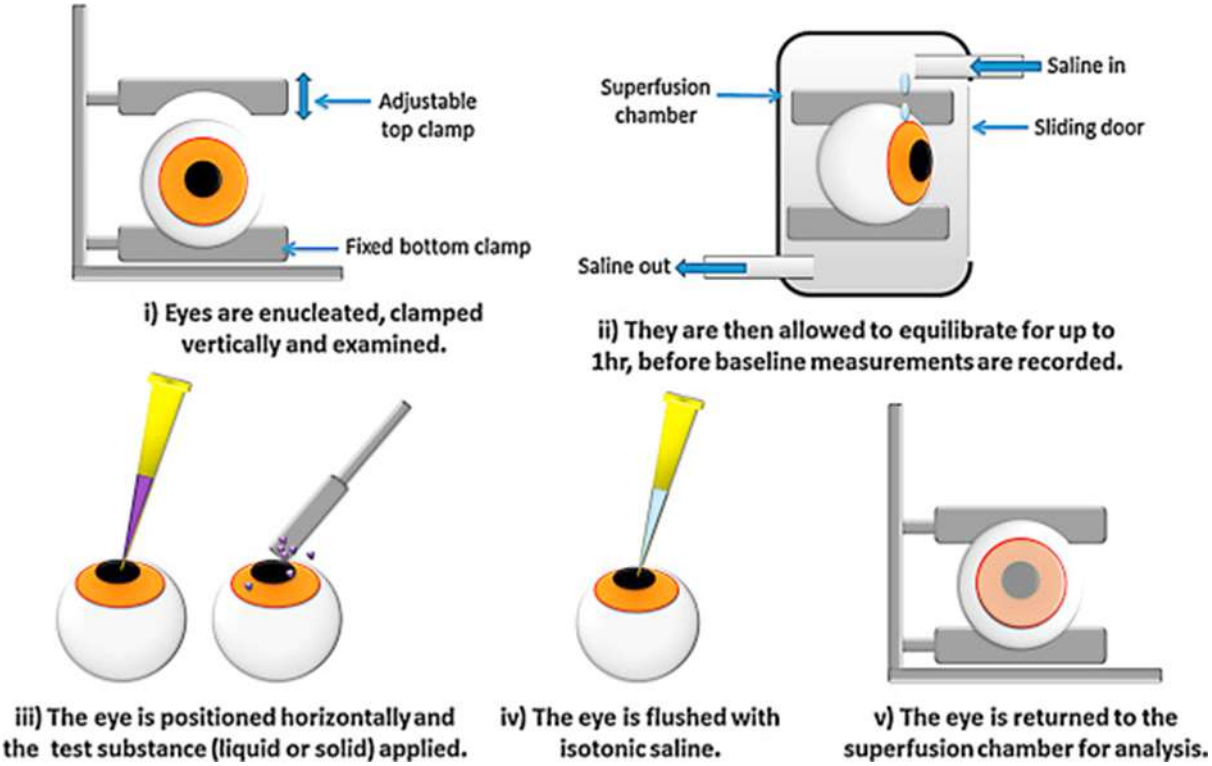

Figure 6 Schematic representation of Isolated Chicken Eye (ICE test) (Wilson et al., 2015)

Recently, the ICE test was re-evaluated that confirmed that this test is scientifically sound and can be used to identify GHS no category (non-irritants) substances as well as GHS category 1 substances (causing irreversible damage).ICE has an accuracy of 82% while identifying non-irritant substances, while 86% while identifying substances causing irreversible damage to eyes (OECD, 2018). Solids, liquids, gels, and emulsions can all be tested by ICE test, while, gases and aerosols have yet to be assessed with this method. Moreover, ICE cannot be used to classify substances causing reversible damage (GHS category 2, 2A, and 2B). However, so far, no ex vivo or *in vitro* is capable of classifying substances in this category.

2.4.3. Non-ocular organotypic models

The chorioallantoic membrane (CAM) vascular assay was proposed by Luepke and Kemper (1986). It is also known as the Hen's egg test (HET), CAM assay, or Hühner-embryonen test on chorioallantoic membrane (HET-CAM). The chorioallantoic membrane is the vascularized respiratory membrane within the fertilized chicken egg membrane. The vasculature and inflammatory process of CAM is similar to the conjunctival tissue of rabbit eyes. This test

provides information about the potential effect of the test substance in conjunctiva and coagulation that can be used to determine potential damage to the cornea (NICEATM-ICCVAM, 2006). Although the CAM test is also an *ex vivo* test it differs from other *ex vivo* tests since they have the addition of vasculature (Curren and Harbell, 2002; Barile, 2010).

The HET-CAM protocol involves removing the eggshell to expose CAM (Figure 7i), followed by test substance application (Figure 7ii), which is rinsed afterward (Figure 7iii), and changes to the membrane morphology are observed and scored (Figure 7iv). The observations mostly include time to hemorrhage, vasoconstriction, and coagulation (Vinardell and Mitjans, 2008). Other observations include injection, dilation, and lysis (Spielmann, 1995; Gettings et al., 1996; Macián et al., 1996). The irritation scoring may vary depending on the classification system being used. Experimental conditions such as incubation time, relative humidity, replications, breed of hen, egg selection criteria, egg rotation, method of opening the eggshell, the quantity of test substance used, use of exposure times, and positive/negative controls may vary depending upon the protocol used. These variations lead to problems regarding intra-laboratory reproducibility.

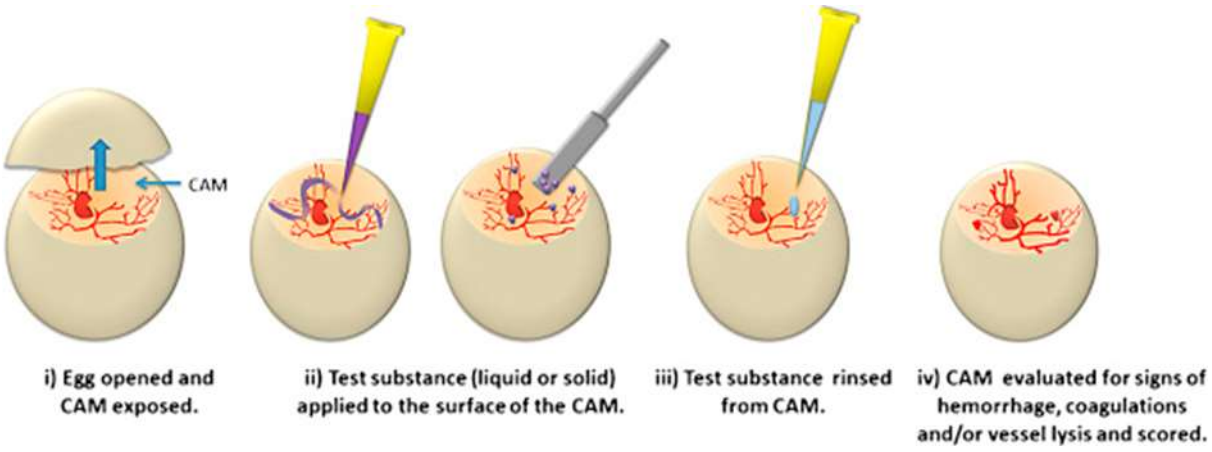

Figure 7 Schematic representation of Hen's egg test (HET) (Wilson et al., 2015)

3. MATERIALS AND METHODS

3.1. Test Materials

Four pesticides were tested to study their eye irritation effect on the chicken eyes. The tested pesticides were:

1. Prosaro (fungicide)
2. Tilmor (fungicide)
3. Zantara (fungicide)
4. Kideka (herbicide)

The eye irritation effect was tested by HET-CAM (Hen's Egg Test - Chorioallantoic Membrane) test and isolated chicken eye (ICE) test. The details of the pesticides used in the experiment are given below.

3.1.1. Prosaro

Prosaro is a protective and curative fungicide that offers a very broad spectrum of disease control. It is used in different formulations in different parts of the world. Prosaro 420-SC (suspension concentrate) is used widely internationally. In Europe, Prosaro 250-EC (emulsifiable concentrate) is widely used that contains prothioconazole (125 g L^{-1}) and tebuconazole (125 g L^{-1}) as active ingredients. In Europe, Prosaro 250-EC is used to control diseases in oat (*Avena sativa*), rye (*Secale cereal*), triticale (x Triticosecale), barley (*Hordeum vulagre*), and wheat (*Triticum* aestivum) but in Hungary, it is mostly used in wheat crops.

Toxicological information

Acute oral toxicity	LD50 (Rat) > 2500 mg kg^{-1}
Acute inhalation toxicity	LC50 (Rat) > 5.153 mg L^{-1}

Inhalation Duration of exposure:	4 hours
Acute dermal toxicity:	LD50 (Rat) > 4000 mg kg^{-1}
Skin corrosion/irritation:	Irritating to skin (Rabbit)
Serious damage/irritation eye	Irritating to eyes (Rabbit)

3.1.2. Tilmor

Tilmor 240-EC is a systematic fungicide that is used to control and suppress diseases in oats, barley, and wheat (durum, spring, and winter). Tilmor presents preventive, curative, and sometimes eradicative properties, which give it a wide spectrum of activity. Tilmor contains the highly effective active substance prothioconazole together with the established active substance tebuconazole. Tilmor 240-EC has been reported to effectively control botch (glume, leaf, net, spot), rusts (crown, leaf, stripe, stem), and tan spot while it suppresses fusarium head blight (in wheat). In Europe, Tilmor is approved for disease control in rapeseed.

Toxicological information

Acute oral toxicity	LD50 (Rat) > 2500 mg kg^{-1}
Acute inhalation toxicity	LC50 (Rat) > 4.969 mg L^{-1}
Inhalation Duration of exposure:	4 hours
Acute dermal toxicity:	LD50 (Rat) > 2000 mg kg^{-1}
Skin corrosion/irritation:	Irritating to skin (Rabbit)
Serious damage/irritation eye	Irritating to eyes (Rabbit)

3.1.3. Zantara

Zantara is a systematic emulsifiable concentrate fungicide for the control of fungal diseases. It has a protective and curative mode of action. Zantara contains bixafen (50 g L^{-1}) and tebuconazole (166 g L^{-1}) as active substances. Zantara acts by preventing the development of fungal spores and healing by blocking the available latent infection in cultures and preventing their further development and distribution. It is used for disease control/prevention in cereals, particularly wheat, and barley.

Toxicological information

Acute oral toxicity	LD50 > 2000 - 5000 mg kg^{-1}
Acute inhalation toxicity	LC50 (Rat) > 5.03 mg L^{-1}
Inhalation Duration of exposure:	4 hours
Acute dermal toxicity:	LD50 (Rat) > 2000 mg kg^{-1}
Skin corrosion/irritation:	Irritating to skin (Rabbit)
Serious damage/irritation eye	Irritating to eyes (Rabbit)

3.1.4. Kideka

Kideka is a selective herbicide with acropetal and basipetal translocation of the active substance. The active substance is mesotrione 10% (10 g L^{-1}), which is absorbed through the leaf mass and the roots. Mesotrione inhibits the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the conversion reaction of tyrosine into plastoquinone and α tocopherol. A deficiency of plastoquinone leads to the cessation of the carotenoid biosynthesis process in plants, which causes bleaching of leaves and then meristem tissue necrosis in sensitive plants.

Toxicological information

Acute oral toxicity	LD50 > 2000 mg kg^{-1}
Acute inhalation toxicity	not applicable
Acute dermal toxicity:	LD50 (Rat) > 2000 mg kg^{-1}
Skin corrosion/irritation:	Not irritating
Serious damage/irritation eye	Irritating to eyes (Rabbit)

3.2. Test methods

The *in vivo* data were taken from the MSDS (Safety Data Sheet), and the HET-CAM and ICE tests were compared with them.

Two test methods (HET-CAM test and ICE test) were used to observe the eye irritation effect of understudy pesticides. The details of the tests used are as follows:

3.2.1. HET-CAM (Hen's Egg Test - Chorioallantoic Membrane) test

The following tools are required to conduct the HET-CAM test:

- Incubator
- Candling light
- Surgical forceps
- Dentist's rotating saw blade
- Deionized/Distilled Water
- Micropipette

- Beaker
- Volumetric flask
- Stopwatch
- Magnifying glass

The following solutions are also required for the HET-CAM test:

- 0.9% (w/v) sodium chloride (NaCl) in deionized/distilled water (JT Baker, USA)
- 1% (w/v) sodium dodecyl sulfate (SDS) in deionized/distilled water (Acros Organics, Belgium)
- 0.1 N sodium hydroxide (NaOH) in deionized/distilled water(VWR Prolabo, Germany)

Hen eggs were obtained from premises of Gallus Kft. Infertile eggs were removed and only eggs with high fertility indices (White Leghorn) were used in the experiment. Selected eggs were incubated in Ragus type incubator, where the temperature was maintained at 37 - 38 ˚C and relative humidity was controlled at 50-70%. To prevent the embryo from sticking, the eggs were rotated several times a day (Spielmann et al., 1997). On the 9th day of the experiment, CAM development was observed and inadequately developed eggs were taken out. The next day (Day 10), the eggs with developed CAM were used for the experiment.

During the experiment, the eggshell was cut and removed with surgical forceps above the air chamber. 0.9% sodium chloride (NaCl) solution was used to moisten the membrane followed by gently pulling up the membrane with surgical forceps as shown in Figure 08. Understudy pesticides were applied on the CAM in a constant volume of 0.3 ml at 37 ˚C. Positive control (0.1 N sodium hydroxide and 1% sodium lauryl sulfate) and negative control (saline solution NaCl 0.9%) was also set to determine the validity of the test. For each test substance, six eggs were used. After the application of test substances, the membrane, blood vessels, and albumen were observed for 5 minutes. The time of appearance of each irritant effect (haemorrhage,

vasoconstriction, and coagulation) was recorded in seconds using a stopwatch. The index describing the irritancy potential was calculated using the following formula (Spielmann et al., 1996):

$$Irritationindex = (301 - H) \times \frac{5}{300} + (301 - L) \times \frac{7}{300} + (301 - C) \times \frac{9}{300}$$

Where H is the time at which haemorrhage appeared (s), L is the time at which vascular lysis first occurred (s), and C is the time at which coagulation of protein or blood was first noted (s). Based on the irritation index, the test substances were classified into three categories shown in Table 1.

Table 1 Irritation categories for the HET-CAM test (ICCVAM 2006)

Irritation index	**Irritation category**
0-0.9	not irritating
1-8.9	Irritant
9-21	severely irritating

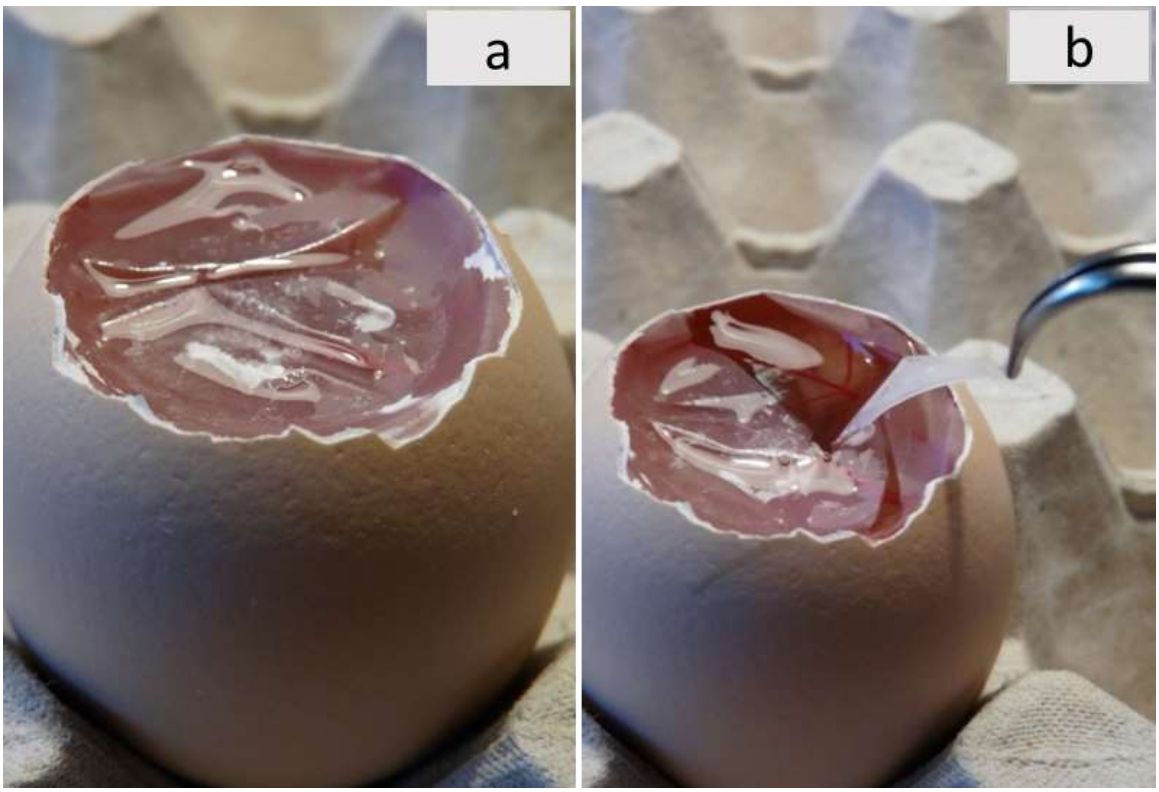

Figure 8 (a) Moistened membrane and (b) removal of membrane during HET-CAM test (Kormos, 2017)

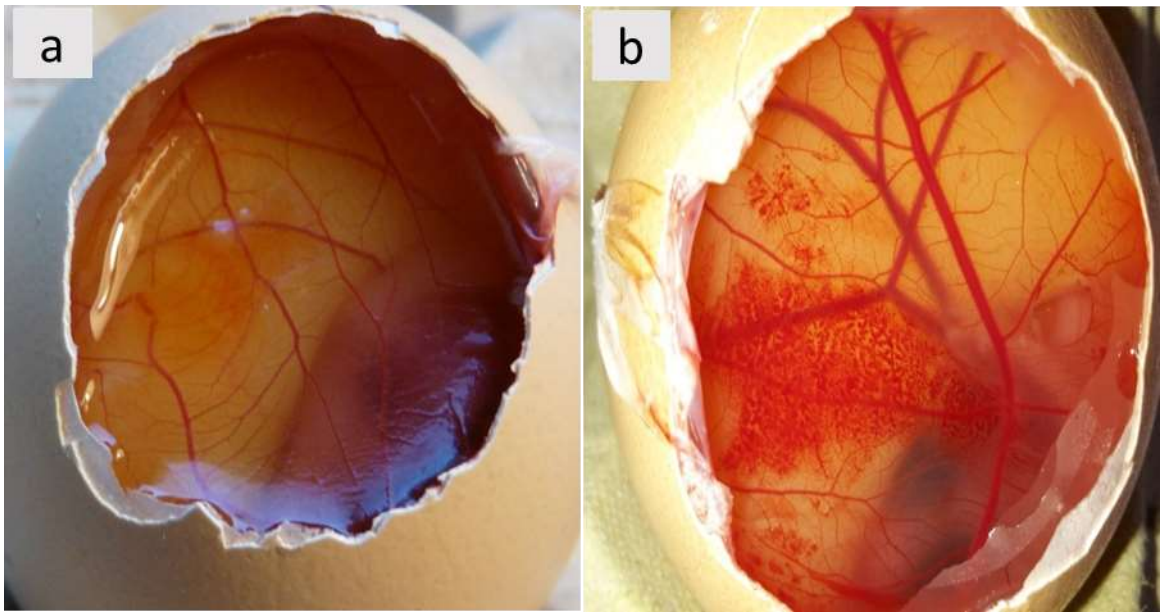

Figure 9 (a) Lysis of the membrane and (b) Haemorrhage of the membrane (Kormos, 2017)

3.2.2. ICE (Isolated Chicken Eye) test

The isolated chicken eye test is also used to determine the irritation effect of pesticides. Based on the results of this test, positive test items can be classified as ocular corrosives or severe irritants (UN GHS/CLP Category 1) while negative test items can be classified as not requiring classification and labeling (UN GHS/CLP No Category) without further testing.

The isolated chicken eye test for under-study pesticides was performed according to OECD guidelines 438. The following tools and solutions were used to conduct the ICE test:

- Superfusion device
- Slot lamp (Haag-Streit BQ 900, Switzerland)
- Pipette
- Beaker
- Flask
- Surgical forceps
- Surgical scissors
- Negative control sodium chloride (9 g/L saline) solution (lach:ner)
- Positive control Trichloroacetic acid 30% (w/v) solution (Sigma-Aldrich)
- Fluorescein retention test Fluorescein 2% (v/v) solution (Sigma-Aldrich)

3.2.2.1. Collection of chicken head

The experiment was conducted at TOXI-COOP Toxicology Research Center Ltd. in Balatonfüred, where COBB 500 or ROSS 308 chickens were used for the study. The chickens were slaughtered in a nearby local slaughterhouse so that chicken heads can be transferred to the research center at the earliest convenience. Since the eyes must be placed in a superfusion apparatus that provides isolated conditions within 2 hours of slaughter. It is important for the test that the chicken head maintains the proper biochemical and physiological function of the

cornea during the study. Therefore, chicken heads from the slaughterhouse were transported in a plastic box containing paper moistened with physiological saline prepared for this purpose.

3.2.2.2. Preparation of chicken eye

After chicken head collections, chicken eyes were prepared for the test. Surgical forceps were used to grab the eyelid for lifting from the surface of the cornea, followed by the removal of the eyelid using surgical scissors. Care was taken while removing the eyelid so that the cornea was not injured.

A slit lamp and fluorescein solution were used to check if the cornea was damaged during the transportation of chicken heads or the removal of the eyelid. A drop of a 2% fluorescein solution was added to the surface of the thus-released cornea, which was washed after a few seconds with 20 ml of physiological saline. Fluorescein-treated eyes were examined with a slit lamp to verify the corneal integrity. Eyes that showed high baseline fluorescein staining (i.e., >0.5) or corneal opacity score (i.e., >0.5), or any additional signs of damage after enucleation were rejected.

Twenty-eight eyes were used in total for the ICE test to observe the irritation effect of understudy pesticides. Seven eyes (three eyes for positive control, one eye for negative control, and three eyes for test substance) were used per test substance.

To remove the eye from the eye socket, eye muscles were cut with surgical scissors by pulling the conjunctiva outward without damaging the cornea. The highlighted eyeball was cleaned of excess tissue, leaving a visible piece of the optic nerve (Figure 10).

Figure 10 Removing the eye from the eye socket

The collected eyes were then placed in the stainless steel clamp (Figure 11) of a superfusion device in such a fashion that the cornea was vertical. The surface of the corneas was continuously moistened with dripping saline.

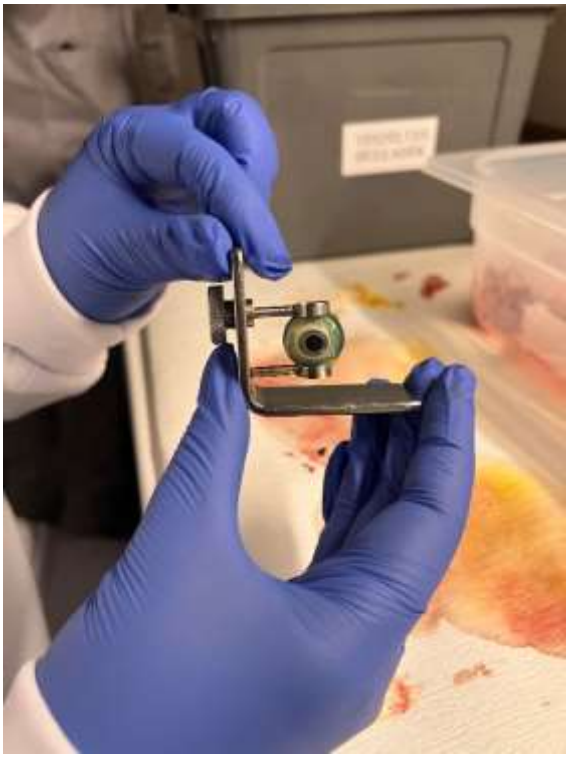

Figure 11 Vertical placement of eye in stainless steel clamp

Fluorescein retention was again performed after placing the eyeball in the ventricle, to check if there was any damage to the corneal surface during tissue removal and placement in the steel clamp. I then examined the degree of corneal opacity in the eyes. Eyeballs were removed in case the cornea was injured. After confirming the condition of the cornea, the corneal thickness was also measured. The difference in thickness for a given eye must not exceed 10% of the mean value. When these conditions were met, we started acclimatizing the eyes in the superfusion device, which took 45-60 minutes. The chamber temperature was kept between 32 ± 1.5 ° C during both acclimatization time and post-treatment observation.

The baseline assessments

Baseline values are required to evaluate any potential test item-related effects after treatment.

3.2.2.3. Application of test substance

30 µl of the test substance was applied to the cornea in such a way that the entire surface of the cornea was covered. The test substances were allowed to stay on the cornea for 10 seconds, and then the cornea surface was rinsed thoroughly using 20 ml isotonic saline at ambient temperature.

3.2.2.4. Observations:

The data regarding corneal thickness and corneal opacity of all eyes (control and test) was recorded pre-treatment and at approximately 30, 75, 120, 180, and 240 minutes after the post-treatment rinse. The fluorescein retention was also measured on two occasions i.e. at baseline (t=0) and 30 minutes after the post-treatment rinse. Observations related to morphological effects such as pitting or loosening of the epithelium were also made.

Corneal swelling

Corneal swelling was determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope. Corneal swelling is expressed as a percentage and is calculated using the following formula:

$$Cornealswelling = \left(\frac{Cornealthicknessatt_1 - Cornealthicknessatt_0}{Cornealthicknessatt_0}\right) \times 100$$

Where, t_1 is corneal thickness measured 30 minutes after rinsing the treatment, and t_0 is corneal thickness measured at baseline.

The mean percentage of corneal swelling for all observation time points was calculated for all test eyes. Based on the highest mean score for corneal swelling, as observed at any time point, an ICE Class was assigned for each test chemical (Table 2).

Table 2 Corneal thickness categories (OECD, 2018)

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

Corneal opacity

An area of the cornea that was densely opacified by test substances was used to determine corneal opacity. As per instructions described in Table 3, the corneal opacity is scored. The mean corneal opacity value for all examined eyes is calculated for all observation time points. Based on the highest mean score for corneal opacity, as observed at any time point, an ICE class is assigned for each test chemical(Table 3).

Table 3 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 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; the size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

Fluorescein retention

Fluorescein retention is evaluated at the 30-minute post-treatment rinse according to the scores shown in Table 4. The mean fluorescein retention value of all test eyes is then calculated for the 30-minute observation time point and used to assign an ICE class for each test chemical (Table 4).

Table 4 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

The *in vitro* classification for a test chemical is assessed by the UN GHS classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention as described in Table 5.

Table 5 Overall ICE classification criteria (OECD, 2018)

UN GHS Classification	**Combinations of Three Endpoints**
No Category	3xI 2xI, 1xII 2xII, 1X I
No prediction can be made	Other combination
Category 1	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 at least 2 eyes) Severe loosening of the epithelium (in at least 1 eye)

Note: *Combinations are less likely to occur.

Statistical analysis

Correlation among methods were analyzed with Goodman-Kruskal's gamma (Goodman and Kruskal, 1954).Calculations were performed by using R statistical language (R Core Team, 2023) and the gkgamma() function from the MESS package (Ekstrøm, 2022).

Cohen's Kappa test was used to measure inter-rater reliability. In calculation of Weighted Kohen's Kappa the non-agreements of the methods were weighted by the square of the difference between the categories.

4. RESULTS AND DISCUSSION

The *in vivo* data came from the safety data sheets (MSDS).

4.1. Results from the HET-CAM test

HET-CAM test was used to study the irritation potential of three fungicides and one herbicide. Results showed that Kideka herbicide caused the lysis on the membrane after 19 s, which was followed by bleeding at 180th s. The irritation index was calculated to be 6.75. Therefore, this herbicide was categorized as an irritant (Table 6).

Application of Prosaro fungicide also caused lysis of chorioallantoic membrane (CAM) after 15 s. However, no bleeding was observed. With a calculated irritation index of 6.7, Prosaro was also classified as an irritant (Table 6).

After treatment with Tilmor fungicide, lysis was observed between 17-40 s. Bleeding was not observed in all cases and the irritation index was calculated to be 6.89. Therefore, this fungicide was categorized as an irritant (Table 6).

During the application of Zantara pesticide, lysis was observed between 16-27 s, followed by bleeding between 18-140 s. Based on the calculated irritation index of 8.48, this fungicide was also categorized as an irritant (Table 6).

Table 6 Time of lysis, bleeding, and Irritation index of pesticides calculated from HET-CAM test

Test item	Time to lysis (s)	Time to bleeding (s)	Irritation index
Kideka	From 19 s	180 s	6.75
Prosaro	From 15 s	No bleeding	6.7
Tilmor	From 17 to 40 s	No bleeding	6.89
Zantara	From 16 to 27 s	80-140 s	8.48

We compared the results of the HET-CAM test with *in vivo* data, which showed a 75% match (Table 07). The influence of the physical and chemical properties of the test materials on the evaluation and the subjectivity of evaluation are some of the disadvantages of the HET-CAM test. Therefore, we also included the ICE test to determine the effect of under-study pesticides.

Table 7 *Comparison of the categories of the in vivo data and HET-CAM test*

Test item	***In vivo* data**	**HET-CAM**
Kideka	severely irritative	irritative
Prosaro	irritative	irritative
Tilmor	irritative	Irritative
Zantara	irritative	Irritative

4.2. Results from the Isolated Chicken Eye test

The negative and positive controls were evaluated based on corneal thickness, corneal opacity, and fluorescein retention. The results showed that negative control always falls in the category of non-irritant chemicals, while positive control always falls in the category of severe eye irritant chemicals. Therefore, the test performed to categorize the under-study chemicals can be considered valid.

Kideka herbicide caused 13.3% corneal thickness after 30 minutes OF application. The corneal thickness increased to 18.3% at 120 mins and 29.7% at 240 mins. Treatment with Kideka also caused corneal opacity in all three eyes. At 120 min, severe opacity was observed in only two eyes, however, at 240 mins, no details of the iris or pupil were visible in all three eyes. Fluorescein retention showed single-cell staining in two eyes and confluent dense cell staining in one eye. Severe loosening of the epithelium was observed in one eye. Based on corneal thickness, opacity, and fluorescein retention observations, Kideka was classified as an irritant (Table 08).

Prosaro insecticide was also classified as an irritant (Table 08). The observations included a 20.5% increase in corneal thickness at 120 mins and severe distortion (change in corneal thickness >32%) at 240 mins. Prosaro treatment also caused severe opacity in two eyes, while, one eye had a visible translucent area. Fluorescein retention was also observed on large areas of the cornea in two eyes and confluent dense single-cell staining in one eye. Severe loosening of the epithelium was observed in each tested eye.

Tilmor pesticide increased the corneal thickness of treated eyes by 26.7%. Severe corneal opacity was observed in two eyes in response to the Tilmor application, while details of the iris were slightly obscured in one eye. Fluorescein retention showed single cell staining in two treated eyes while large corneal area staining in one eye. Based on the observations, Tilmor pesticide was classified as an irritant. Severe loosening of the epithelium was observed in each tested eye (Table 08).

The application of Zantara also caused an increase in corneal thickness in all three eyes. Mid-point observation (120 mins) showed an average increase of 22.8% that reached 32.4% at 240 mins. It also caused severe corneal opacity in treated eyes. No specific details of the iris or the size of the pupil were visible. High fluorescein retention was also observed in all treated eyes with confluent single-cell staining in two eyes and confluent large area staining in one eye. Zantara only caused slight epithelium loosening in one eye. Based on the endpoints obtained, the Zantara pesticide is classified as an irritant (Table 08).

Table 8 Observation recorded during Isolated Chicken Eye test

Test item	Corneal thickness		Corneal Opacity		Fluorescein retention		Irritation category
Kideka	29.7%	III	2	III	1.33	II	Category 2A
Prosaro	35.8%	IV	2.67	IV	2.67	IV	Category 1
Tilmor	26.7%	IV	2.67	IV	2.17	III	Category 1
Zantara	32.4%	IV	3	IV	2.33	III	Category 1

The statistical analysis showed that no significant correlation was observed between the studied tests (Table 09). Cohen's Kappa was used to measure the inter-rater reliability of studied tests. No significant differences were observed between *in vivo* and HET – CAM test, and HET – CAM and ICE test. Only a significant difference was observed between *in vivo* and ICE tests (Table 10) because *In vivo* method rated each pesticide as more severe by one level compared to ICE. However, the correlation between them was not significant, since in each pair of pesticides the same one was rated as more severe.

Table 9 Correlation analysis

Methods	Kendall gamma	sig
***In vivo* – HET-CAM**	0.11	0.76
***In vivo* – ICE**	-0.09	0.83
HET-CAM – ICE	-0.2	0.64

Table 10 Cohen's Kappa test

Methods	Cohen's kappa	sig
***In vivo* – HET-CAM**	0	1
***In vivo* - ICE**	0.27	0.04
HET-CAM - ICE	0	1

5. CONCLUSION AND RECOMMENDATION

The present study was conducted with the aim of assessing and developing alternate methods to determine irritation potential of pesticides. The irritation potential of four pesticides i.e. Prosaro, Tilmor, Zantara and Kideka was evaluated using two *in vitro* methods i.e. HET-CAM and isolated chicken eye (ICE) test. Both tests are cheaper and faster than the Draize eye irritation test. The use of eggs in HET-CAM test and eyes of slaughtered animals in ICE test, reduces the use of animals in *in vivo* tests. Both test models also correctly described the irritation potential of tested pesticides. We concluded that the data are well correlated. These results are in line with the results of (Bagley et al., 1992, 1994; Jírová et al., 2014; Kormos, 2017), who reported that the HET-CAM test provides the lowest rate of false results along with valuable results related to the conjunctiva. HET-CAM test is cheaper, faster, and has adequate sensitivity. However, both tests have their disadvantages. The observations in HET-CAM test are subjective and may reduce the reliability of the results. Moreover, testing of solid and colored chemicals with HET-CAM test may be difficult because of their physical and chemical properties. On the other hand, limited time sustainability of the chicken eyes is a big disadvantage of ICE test. Moreover, ICE test does not take conjunctival and iris injuries into consideration. Therefore, no single *in vitro* test is enough to replace traditional *in vivo* testing method. However, combination of different *in vitro* methods can be used to study the full irritation potential of chemicals. Both studied tests are suitable for refining *in vivo* tests. It is recommended to include both HET-CAM test and ICE test before performing an *in vivo* test according to OECD 405.

6. SUMMARY

Toxicological eye irritation tests are an important part of the licensing process for plant protection products. With the help of these, it is possible to know exactly the harmful effects on the eyes. For decades since 1944, Draize's primary eye irritation test was the only method to accurately determine these harmful effects. However, this procedure can be painful for the experimental animal and is ethically highly questioned by animal protection organizations, so in accordance with the 3R rule based on Russel and Burch (1953), alternative *in vitro* methods aimed at induction have appeared one after another. Today, several such methods based on isolated eyes or tissue cultures are accepted by the OECD, but none of them can cover the entire irritation potential, namely cannot reliably indicate all three GHS categories. GHS categories are health hazards, physical hazards, and environmental hazards. Health hazards are threats to human health (e.g. breathing or vision), while physical hazards harm the body (e.g. skin corrosion) and environmental hazards include pollution that harms human health.

In this study, we used the HET-CAM test and ICE test to assess the irritation potential of plant protection agents. We compared the results from the observed changes caused by pesticides with the *in vivo* data, with the aim of which was to see how close the two methods are to each other and to expand the data in this direction.

The *in vivo* data were taken from the safety data sheet of the products. The HET-CAM test was performed based on Invittox Protocol No. 47. For the HET-CAM test, hatched hen eggs were carefully opened on the 10th day so that the chorioallantoic membrane (CAM) could be observed. The four plant protection products Tilmor (fungicide), Prosaro (fungicide), Zantara (fungicide), and Kideka (herbicide) used were applied in 100% concentration in the HET-CAM test.

The results showed that the HET-CAM test classified all under-study pesticides as an irritant. The comparison between HET-CAM and *in vivo*, data showed a 75% match. We reached a

similar result as (Bagley et al., 1994; Jírová et al., 2014), who established during their research that the HET-CAM test provides the lowest rate of false results and also provides valuable results related to the conjunctiva.

For the ICE test, heads of slaughtered chickens were used. The test substances were classified as irritant or severe irritant following the observations related to corneal swelling, corneal opacity, and fluorescein retention. Understudy pesticides were applied directly onto the cornea of the isolated chicken eye in a single dose. The observations were recorded pre-treatment, and 30, 75, 120, 180, and 240 mins after treatment. Positive and negative controls used showed the expected results each time. The results showed that the ICE test classified all test substances as an irritant. Three pesticides were determined to be category 1 irritants while Kideka was assessed to be a category 2 irritant. These results correspond to the available information about the tested herbicides, so these studies with the isolated chicken eye are considered to be successful.

Based on the results obtained, the results of the two *in vitro* tests converge well. Both tests are cheaper, faster, and ethical. Based on the comparison with *in vivo* data, both tests prove to be suitable as pre-test methods and for the acceptance of certain categories. Therefore, I recommend the usage of ICE and HET-CAM tests as pre-test methods. However, further studies are needed to cover the full irritant potential

7. BIBLIOGRAPHY

Aktar Md.W., Paramasivam M., Sengupta D., Purkait S., Ganguly M. and Banerjee S. 2009. Impact assessment of pesticide residues in fish of Ganga river around Kolkata in West Bengal. Environ Monit Assess. **157**: 97–104.

Anderson D. and Russell T. 1995. status of alternative methods in toxicology. Royal Society of Chemistry,

Bag M., Yadav M. and Mukherjee A. 2017. Changing Disease Scenario with Special Emphasis on False Smut of Rice. SATSA Mukhapatra- Annual Technical issue. **21**: 219–224.

Bagley D.M., Bruner L.H., de Silva O., Cottin M., O'Brien K.A.F., Uttley M. and Walker A.P. 1992. An evaluation of five potential alternatives in vitro to the rabbit eye irritation test in vivo. Toxicology in Vitro. **6**: 275–284.

Bagley D.M., Waters D. and Kong B.M. 1994. Development of a 10-day chorioallantoic membrane vascular assay as an alternative to the draize rabbit eye irritation test. Food and Chemical Toxicology. **32**: 1155–1160.

Barile F.A. 2010. Validating and troubleshooting ocular in vitro toxicology tests. Journal of Pharmacological and Toxicological Methods. **61**: 136–145.

Bernardes M.F.F., Pazin M., Pereira L.C. and Dorta D.J. 2015. Impact of Pesticides on Environmental and Human Health. Toxicology Studies - Cells, Drugs and Environment, InTech,

Bonini S., Rama P., Olzi D. and Lambiase A. 2003. Neurotrophic keratitis. Eye. **17**: 989–995.

Budai P., Kormos É., Buda I., Somody G. and Lehel J. 2021. Comparative evaluation of HET-CAM and ICE methods for objective assessment of ocular irritation caused by selected pesticide products. Toxicology in Vitro. **74**: 105150.

Burton A.B.G. 1972. A method for the objective assessment of eye irritation. Food and Cosmetics Toxicology. **10**: 209–217.

Burton A.B.G., York M. and Lawrence R.S. 1981. The in vitro assessment of severe eye irritants. Food and Cosmetics Toxicology. **19**: 471–480.

Callabrese E.J. 1987. Available at: https://scholar.google.com/scholar_lookup?title=Principles%20of%20Animal%20Extrapolation&publication_year=1987&author=E.J.%20Calabrese.

Carvalho F.P. 2017. Pesticides, environment, and food safety. Food and Energy Security. **6**: 48–60.

Curren R.D. and Harbell J.W. 2002. Ocular Safety: A Silent (In Vitro) Success Story. Altern Lab Anim. **30**: 69–74.

Davila J.C., Rodriguez R.J., Melchert R.B. and Acosta D. 1998. Predictive Value of in Vitro Model Systems in Toxicology. Annual Review of Pharmacology and Toxicology. **38**: 63–96.

Derouiche M.T.T. and Abdennour S. 2017. HET-CAM test. Application to shampoos in developing countries. Toxicology in Vitro. **45**: 393–396.

Draize J.H., G. W. and H.O. C. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. **82**: 377–390.

Ekstrøm C.T. 2022. MESS: Miscellaneous Esoteric Statistical Scripts. .

Eskes C., Bessou S., Bruner L., Curren R., Harbell J., Jones P., Kreiling R., Liebsch M., McNamee P., Pape W., Prinsen M.K., Seidle T., Vanparys P., Worth A. and Zuang V. 2005. Eye irritation. Altern Lab Anim. **33 Suppl 1**: 47–81.

Fenik J., Tankiewicz M. and Biziuk M. 2011. Properties and determination of pesticides in fruits and vegetables. TrAC Trends in Analytical Chemistry. **30**: 814–826.

Gettings S.D., Lordo R.A., Demetrulias J., Feder P.I. and Hintze K.L. 1996. Comparison of low-volume, draize and in vitro eye irritation test data. I. Hydroalcoholic formulations. Food and Chemical Toxicology. **34**: 737–749.

Goodman L.A. and Kruskal W.H. 1954. Measures of Association for Cross Classifications*. Journal of the American Statistical Association. **49**: 732–764.

Guo X., Yang X.F., Yang Y., Hans R., Cai J.H., Xue J.Y., Tan X.H., Xie X.P., Xiong X.K. and Huang J.M. 2012. Prediction of Ocular Irritancy of 26 Chemicals and 26 Cosmetic Products with Isolated Rabbit Eye (IRE) Test. Biomedical and Environmental Sciences. **25**: 359–366.

Hernández A.F., Gil F., Lacasaña M., Rodríguez-Barranco M., Tsatsakis A.M., Requena M., Parrón T. and Alarcón R. 2013. Pesticide exposure and genetic variation in xenobiotic-metabolizing enzymes interact to induce biochemical liver damage. Food and Chemical Toxicology. **61**: 144–151.

Huhtala A., Salminen L., Tähti H. and Uusitalo H. 2008. Corneal Models for the Toxicity Testing of Drugs and Drug Releasing Materials. pp. 1–24.

Jaleh B., Masoud A., Seyed Abdolreza M.T. and Yadollah O. 2009. Ocular drug delivery; impact of invitro cell culture models. .

Jester J.V., Li L., Molai A. and Maurer J.K. 2001. Extent of initial corneal injury as a basis for alternative eye irritation tests. Toxicology in Vitro. **15**: 115–130.

Jírová D., Kejlová K., Janoušek S., Bendová H., Malý M., Kolářová H. and Dvořáková M. 2014. Eye irritation hazard of chemicals and formulations assessed by methods in vitro. .

Kitano S. 1957. Available at: https://scholar.google.com/scholar_lookup?journal=Jpn+J+Ophthalmol&title=An+embryologic+study+of+the+human+corneal+nerves&author=S+Kitano&volume=1&publication_year=1957&pages=48-55&issn=0021-5155&.

Kormos É. 2017. PhD thesis, University of Pannonia

Leighton J., Nassauer J. and Tchao R. 1985. The chick embryo in toxicology: an alternative to the rabbit eye. Food and Chemical Toxicology. **23**: 293–298.

Luepke N.P. and Kemper F.H. 1986. The HET-CAM test: An alternative to the draize eye test. Food and Chemical Toxicology. **24**: 495–496.

Macián M., Seguer J., Infante M.R., Selve C. and Vinardell M.P. 1996. Preliminary studies of the toxic effects of non-ionic surfactants derived from lysine. Toxicology. **106**: 1–9.

Marfurt C.F., Kingsley R.E. and Echtenkamp S.E. 1989. Sensory and sympathetic innervation of the mammalian cornea. A retrograde tracing study. Investigative Ophthalmology and Visual Science. **30**: 461–472.

Maurer J.K., Parker R.D. and Jester J.V. 2002. Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. Regulatory Toxicology and Pharmacology. **36**: 106–117.

Müller L.J., Marfurt C.F., Kruse F. and Tervo T.M. 2003. Corneal nerves: structure, contents and function. Experimental eye research. **76**: 521–542.

NICEATM-ICCVAM N. 2006. ICCVAM test method evaluation report (TMER): in vitro cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity testing. .

OECD 2002. Test No. 405: Acute Eye Irritation/Corrosion. .

OECD 2018. Available at: https://scholar.google.com/scholar_lookup?title=Test%20No.%20438%3A%20isolated%20chicken%20eye%20test%20method%20for%20identifying%20ocular%20corrosives%20and%20severe%20irritants&publication_year=2009&author=OECD.

Prinsen M.K. 1996. The chicken enucleated eye test (CEET): a practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. Food and Chemical Toxicology. **34**: 291–296.

Prinsen M.K. 2006. The Draize Eye Test and in vitro alternatives; a left-handed marriage?. Toxicology in Vitro. **20**: 78–81.

Prinsen M.K., Hendriksen C.F.M., Krul C.A.M. and Woutersen R.A. 2017. The Isolated Chicken Eye test to replace the Draize test in rabbits. Regulatory Toxicology and Pharmacology. **85**: 132–149.

Prinsen M.K. and Koëter H.B.W.M. 1993. Justification of the enucleated eye test with eyes of slaughterhouse animals as an alternative to the draize eye irritation test with rabbits. Food and Chemical Toxicology. **31**: 69–76.

R Core Team 2023. Available at: https://www.r-project.org/.

Reader S.J., Blackwell V., O'Hara R., Clothier R.H., Griffin G. and Balls M. 1990. Neutral red release from pre-loaded cells as an in vitro approach to testing for eye irritancy potential. Toxicology in Vitro. **4**: 264–266.

Rüfer F., Schröder A. and Erb C. 2005. White-to-white corneal diameter: normal values in healthy humans obtained with the Orbscan II topography system. Cornea. **24**: 259–261.

Sarwar M. 2016. Indoor risks of pesticide uses are significantly linked to hazards of the family members. Cogent Medicine. **3**: 1155373.

Savary S., Ficke A., Aubertot J.-N. and Hollier C. 2012. Crop losses due to diseases and their implications for global food production losses and food security. Food Sec. **4**: 519–537.

Scheel J., Kleber M., Kreutz J., Lehringer E., Mehling A., Reisinger K. and Steiling W. 2011. Eye irritation potential: Usefulness of the HET-CAM under the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). Regulatory Toxicology and Pharmacology. **59**: 471–492.

Schrage A., Gamer A.O., van Ravenzwaay B. and Landsiedel R. 2010. Experience with the HET-CAM Method in the Routine Testing of a Broad Variety of Chemicals and Formulations. Altern Lab Anim. **38**: 39–52.

Schutte K., Prinsen M.K., McNamee P.M. and Roggeband R. 2009. The isolated chicken eye test as a suitable in vitro method for determining the eye irritation potential of household cleaning products. Regulatory Toxicology and Pharmacology. **54**: 272–281.

Secretariat U.N.E.C. for E. 2013. Globally harmonized system of classification and labelling of chemicals (GHS). United Nations Publications,

Silk J.C. 2003. Development of a globally harmonized system for hazard communication. International Journal of Hygiene and Environmental Health. **206**: 447–452.

Spielmann H. 1995. HET-CAM Test. pp. 199–204. In: O'Hare S. and Atterwill C.K. (eds), In Vitro Toxicity Testing Protocols, Humana Press, Totowa, NJ.

Spielmann H., Liebsch M., Kalweit S., Moldenhauer F., Wirnsberger T., Holzhütter H.-G., Schneider B., Glaser S., Gerner I., Pape W.J.W., Kreiling R., Krauser K., Miltenburger H.G., Steiling W., Luepke N.P., Müller N., Kreuzer H., Mürmann P., Spengler J., Bertram-Neis E., Siegemund B. and Wiebel F.J. 1996. Results of a Validation Study in Germany on Two in Vitro Alternatives to the Draize Eye Irritation Test, the HET-CAM Test and the 3T3 NRU Cytotoxicity Test. Altern Lab Anim. **24**: 741–858.

Spielmann H., Liebsch M., Moldenhauer F., Holzhütter H.-G., Bagley D.M., Lipman J.M., Pape W.J.W., Miltenburger H., de Silva O., Hofer H. and Steiling W. 1997. IRAG Working Group 2: CAM-based assays. Food and Chemical Toxicology. **35**: 39–66.

Strassemeyer J., Daehmlow D., Dominic A.R., Lorenz S. and Golla B. 2017. SYNOPS-WEB, an online tool for environmental risk assessment to evaluate pesticide strategies on field level. Crop Protection. **97**: 28–44.

Sultana H., Mannan M.A., Kamal M.M., Quddus K.G. and Das S. 2018. Management of brinjal shoot and fruit borer using selected botanicals. Bangladesh Journal of Agricultural Research. **43**: 431–440.

Unsworth J. 2010. Available at: https://agrochemicals.iupac.org/index.php?option=com_sobi2&sobi2Task=sobi2Details&catid=3&sobi2Id=31.

Van Buskirk E.M. 1989. The anatomy of the limbus. Eye. **3**: 101–108.

Vinardell M.P. and Mitjans M. 2008. Alternative Methods for Eye and Skin Irritation Tests: An Overview. Journal of Pharmaceutical Sciences. **97**: 46–59.

Wilhelmus K.R. 2001. The Draize Eye Test. Survey of Ophthalmology. **45**: 493–515.

Willoughby C.E., Ponzin D., Ferrari S., Lobo A., Landau K. and Omidi Y. 2010. Anatomy and physiology of the human eye: effects of mucopolysaccharidoses disease on structure and function–a review. Clinical & Experimental Ophthalmology. **38**: 2–11.

Wilson S.L., Ahearne M. and Hopkinson A. 2015. An overview of current techniques for ocular toxicity testing. Toxicology. **327**: 32–46.

Available at:
https://scholar.google.com/scholar_lookup?title=The%20dangers%20of%20pesticides%20associated%20with%20public%20health%20and%20preventing%20of%20the%20risks&publication_year=2015&author=M.%20Sarwar.

Available at:
https://scholar.google.com/scholar_lookup?title=Impact+of+Pesticides+on+Environmental+and+Human+Health&author=Bernardes,+M.F.F.&author=Pazin,+M.&author=Pereira,+L.C.&author=Dorta,+D.J.&publication_year=2015&pages=195%E2%80%93233.

STUDENT DECLARATION

Signed below, Svetlana Kazarova (HB6BV7), student of the Georgikon Campus of the Hungarian University of Agriculture and Life Science, at the MSc Course of plant protection declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

Confidential data are presented in the thesis: yes no

Date: 2023 April 27

S Kazarova

_____

Student

STATEMENT ON CONSULTATION PRACTICES

As a supervisor of Svetlana Kazarova (HB6BV7), I here declare that the final master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

I recommend the final master's thesis to be defended in a final exam.

The document contains state secrets or professional secrets: yes no

Place and date: Keszthely, 2023 April 26

Dr. Komos Éva
Internal supervisor