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Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram

Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B) – *In Vitro* Eye Irritation Test in Isolated Chicken Eyes

Final Report

TEST GUIDELINE(S): OECD No. 438 (2018)
EC No. 2017/735, B 48. (2017)

AUTHOR(S): Balázs Orovecz, B.Sc.

COMPLETION DATE: 30 November 2021

PERFORMING LABORATORY: Charles River Laboratories Hungary Kft.
H-8200 Veszprém, Szabadságpuszta, hrsz. 028/1.,
Hungary

LABORATORY PROJECT ID: Report Number: 21/245-038CS
Study Number: 21/245-038CS
Task Number: TK0518488

SPONSOR(S): Syngenta Ltd.
Jealott's Hill International Research Centre
Bracknell, Berkshire, RG42 6EY, United Kingdom

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Company: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study has been performed in accordance with the study plan and the Principles of Good Laboratory Practice (Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17).

I, the undersigned, declare that this report constitutes a true record of the actions undertaken and the results obtained in the course of this study. By virtue of my dated signature I accept the responsibility for the validity of the data.

Signature: _____ Date: 30 November 2021

Balázs Orovecz, B.Sc.
Study Director

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Representative of Submitter/Sponsor:

_____ _____
Date

Submitter/Sponsor: Syngenta Crop Protection, LLC
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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Number: 21/245-038CS

Study Title: Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B) – *In Vitro* Eye Irritation Test in Isolated Chicken Eyes

Test Item: Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B)

This study has been inspected, and this report audited by the Quality Assurance Unit in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in written form to the study director and to management. The dates of such inspections and of the report audit are given below:

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
14 October 2021	Study Plan	14 October 2021	14 October 2021
15 October 2021	Treatment	15 October 2021	15 October 2021
22 October 2021	Draft Report	22 October 2021	22 October 2021
19 November 2021	Amendment 1 to the Study Plan	19 November 2021	19 November 2021
29 November 2021	Final Report	29 November 2021	29 November 2021

Signature: _____ Date: 30 November 2021

Eszter Sebestyén, B.Sc.
On Behalf on QA

MANAGEMENT STATEMENT

According to the conditions of the research and development agreement between Syngenta Ltd. (as Sponsor) and Charles River Laboratories Hungary Kft. (as Test Facility), the study titled "Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B) – *In Vitro* Eye Irritation Test in Isolated Chicken Eyes" was performed in compliance with the Principles of Good Laboratory Practice.

Signature: _____ Date: 30 November 2021

Balázs Tóth, Ph.D.
General Manager

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Function
Balázs Orovecz, B.Sc.	Study Director
Balázs Tóth, Ph.D.	General Manager
David J. Esdaile, M.Sc.	Director of Science and Regulatory Affairs
Kata Tóth-Gönczöl, B.Sc.	Assistant Scientist
Eszter Sebestyén, B.Sc.	Quality Assurance Unit
Carolina Vaccari, M.Sc.	Syngenta Study Manager

Study dates

Study Plan:	15 October 2021
Amendment 1 to the Study Plan:	22 November 2021
Experimental Starting Date:	15 October 2021
Experimental Completion Date:	15 October 2021
Date of Draft Report:	22 October 2021
Date of Final Report:	30 November 2021

Deviations from the Study Plan
There were no deviations from the Study Plan.

Performing laboratory test substance reference number
210563

Other
The study documents and samples:
- study plan and amendment,
- all raw data,
- sample of the test item and positive control,
- original study report and any amendments,
- correspondence
- corneas

will be archived according to the Hungarian GLP regulations and to applicable SOP's in the Archives of Charles River Laboratories Hungary Kft. (H-8200 Veszprém, Szabadságpuszta, hrsz. 028/1., Hungary). This is for a period of 15 years.

After the retention time of 15 years has elapsed all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded.

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

An *in vitro* eye irritation study of the test item difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B) was performed on isolated chicken eyes. The study used 3 eyes for test item treatment, alongside 1 negative and 3 positive control eyes. The irritation effects of the test item were evaluated according to the OECD No. 438 (25th June 2018).

After the zero reference (baseline) measurements, each eye in the treatment group was held in a horizontal position and 30 µL of difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B) was applied onto the centre of the cornea such that the entire surface of the cornea was covered. After 10 seconds exposure time, the surface was rinsed with physiological saline. The positive control eyes were treated in a similar way with 30 µL of 5% (w/v) benzalkonium chloride solution and the negative control eye was treated with 30 µL of physiological saline (0.9% (w/v) NaCl solution). Corneal thickness, corneal opacity and fluorescein retention changes were measured and any morphological effects (pitting or loosening of the epithelium) were evaluated over a four-hour observation period.

1.2 Results

Slight cornea swelling (mean = 8.1%) was observed during the four-hour observation period on all test item treated eyes. Slight cornea opacity change (severity 0.5 on two eyes and severity 1 on one eye) was observed on all test item treated eyes. Slight fluorescein retention change (severity 0.5 on two eyes and severity 1 on one eye) was noted. No other morphological effects were observed in the study.

The negative and positive control group results demonstrated that the study was valid and demonstrated the sensitivity of the assay.

1.3 Conclusion

Based on this *in vitro* eye irritation study on isolated chicken eyes with difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B), the test item is not classified as a severe irritant and not classified as non-irritant. It is concluded that further information is required for classification.

2.0 INTRODUCTION

2.1 Purpose

The enucleated eye test with isolated chicken eyes is a well validated and accepted *in vitro* test system to assess eye irritancy. It has been recognised as a valuable alternative to the Draize eye irritation test, because it represents a test system nearest to the *in vivo* test, without the need to use live animals. It can also be used as a screening tool for corrosivity/severe irritancy to avoid unacceptable effects *in vivo*. In the Isolated Chicken Eye Test (ICET) the test compound is applied in one single dose onto the cornea of isolated eyes. Chicken heads are obtained from a veterinary-inspected, commercial slaughterhouse, processing chickens for human consumption.

According to the 25 June 2018 version of the OECD No. 438 guideline, this method can provide detailed information about the effects of test items on the cornea, and can be used to identify chemicals not requiring classification for eye irritation, or for serious eye damage, as defined by the UN GHS (UN GHS non-classified or UN GHS Category 1). The test is described in OECD No. 438 and is approved by international regulatory agencies as a partial replacement for the identification of non-irritant, corrosives/severe irritants in the *in vivo* Rabbit Eye Assay (OECD No. 405).

2.2 Guidelines

The study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guidelines for Testing of Chemicals 438 (adopted on 07 September 2009, updated on 25 June 2018): “Isolated Chicken Eye Test Method for Identifying I) Chemicals Inducing Serious Eye Damage and II) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage”

- Commission Regulation (EU) 2017/735 of 14 February 2017 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

2.3 Test Facility

This study was performed in an AAALAC-accredited laboratory. The Institutional Animal Care and Use Committee (IACUC) of Charles River Laboratories Hungary Kft. monitored the conduct of the study.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The following information was provided by the Sponsor.

Name:	Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B)
Batch number:	1200767
Design code:	A23793B
Active ingredient content*:	Difenoconazole 5.45% w/w 64.0 g/L, fludioxonil 4.37% w/w 51.3 g/L, metalaxyl-M 4.31% w/w 50.6 g/L, cyclobutrifluram 21.0% w/w 247 g/L
Appearance:	Red liquid
Recertification date:	31 August 2024
Storage conditions:	Room temperature (15-25°C, ≤70% relative humidity)
Safety precautions:	Routine safety precautions (gloves, goggles, face mask, lab coat) for unknown materials were applied to ensure personnel health and safety.

*Note: No adjustment for active ingredient content was applied as agreed by the Sponsor.

A Certificate of Analysis supplied by the Sponsor is given in Appendix 1.

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

3.2 Identification and Receipt

The test item with an active ingredient content as indicated in the certificate of analysis together with all precautions required in the handling and disposal of the test item were supplied by the Sponsor. The identification of the test item was made on the basis of the information provided by Sponsor in the Pharmacy of Charles River Laboratories Hungary Kft.

3.3 Test Item Preparation

The test item was applied in its original form. A volume of 30 µL test item was applied to the entire surface of the cornea attempting to cover the cornea surface uniformly with the test substance.

3.4 Test Item Solubility

Solubility of the test item in physiological saline was tested prior to the experiment (approximately 30 µL test item in 1 mL physiological saline (Manufacturer: B. Braun Pharmaceuticals SA, Lot number: 94922Y05-1, Expiry date: 30 November 2022)). The test item dissolved in physiological saline.

3.5 Subsidiary Materials

Positive Control

Name:	Benzalkonium chloride solution, 50% (w/v) in water
Batch number:	STBH0549
CAS Number:	63449-41-2
Manufacturer:	Sigma-Aldrich Co.
Expiry date:	31 May 2022
Storage condition:	Room temperature

The 50% (w/v) benzalkonium chloride solution was mixed with distilled water (Supplier: B. Braun Pharmaceuticals SA; Batch number: 04123Y25-1, Exp. date: 30 September 2023) to achieve the final concentration of 5% (w/v). The treatment solution was prepared immediately before the experiment.

Negative Control

Name:	Physiological saline (Salsol solution, 0.9% (w/v) NaCl solution)
Manufacturer:	B. Braun Pharmaceuticals SA
Lot number:	212318142
Expiry Date:	31 May 2024
Grade:	sterile
Storage condition:	Room temperature

Fluorescein retention test

Name:	Fluorescein, 10% (w/v) solution
Lot number:	1037A
Manufacturer:	Alcon
Expiry date:	28 February 2022
Storage condition:	Room temperature

This material was mixed with physiological saline (Manufacturer: B. Braun Pharmaceuticals SA, Lot number: 94922Y05-1, Expiry date: 30 November 2022) to achieve the final concentration of 2% (w/v). The final solution was stored at room temperature (Dispensary code: S43235, Expiry date: 27 October 2021).

3.6 Chicken Heads Collection and Transport

Strain of chicken: ROSS 308
Source: TARAVIS KFT. H-9600 Sárvár, Rábasömjéni u 129., Hungary

Chicken heads were collected from a commercial abattoir after chickens (approximately 7 weeks old, mean weight: 2.4 kg) had been slaughtered for human consumption. Heads were collected by a slaughterhouse technician. After collection, the heads were inspected for appropriate quality and wrapped with paper moistened with saline, then placed in a sealed plastic box (4-5 heads/box).

The heads were immediately transported to Charles River Laboratories Hungary Kft. at ambient temperature. The heads were received at Charles River Laboratories Hungary Kft. and processed within 2 hours of collection.

3.6.1 Eyes selection

After removing the head from the plastic box, it was put on soft paper. The eyelids were carefully cut away with scissors, avoiding damaging the cornea. One small drop of 2% (w/v) fluorescein solution was applied onto the cornea surface for a few seconds and subsequently rinsed off with 20 mL physiological saline. Then the fluorescein-treated cornea was examined with a hand-held slit lamp or slit lamp microscope, with the eye still in the head, to ensure that the cornea was not damaged. If the cornea was in good condition, the eyeball was carefully removed from the orbit.

3.6.2 Preparation of eyes

The eyeball was carefully removed from the orbit by holding the nictitating membrane with surgical forceps, while cutting the eye muscles with bent scissors. Care was taken to remove the eyeball from the orbit without cutting off the optical nerve too short. The procedure avoided pressure on the eye while removing the eyeball from the orbit, in order to prevent distortion of the cornea and subsequent corneal opacity. Once removed from the orbit, the eye was placed onto damp paper and the nictitating membrane was cut away with other connective tissue. The prepared eyes were kept on the wet papers in a closed box so that the appropriate humidity was maintained.

3.6.3 Eyes examination and acclimatization time

The prepared eye was placed in a steel retainer. The cornea was positioned vertically with the eye in the correct relative position (same position as in the chicken head), taking care to avoid putting too much pressure on the eye by the retainer. Due to the relatively firm sclera of the chicken eyeball, only slight pressure was needed to fix the eye properly. The clamp with the eyeball was transferred to a chamber of the superfusion apparatus.

The retainer holding the eye was positioned in such a way that the entire cornea was supplied with physiological saline dripping from a stainless steel tube, at a rate of approximately 3-4 drops/minute or 0.1 to 0.15 mL/minute. The door of the chamber was closed except for manipulations and examinations, to maintain temperature and humidity.

The appropriate number of eyes (approximately nine to twelve) were selected. After being placed in the superfusion apparatus, they were examined again with the slit lamp microscope to ensure that they were in good condition. The focus was adjusted to clearly see the physiological saline which was flowing on the cornea surface. Eyes with a high baseline fluorescein staining (i.e. > 0.5) or corneal opacity score (i.e. > 0.5) were rejected. The cornea thickness was measured with an optical pachymeter on a slit-lamp microscope, which was set at a 0.095 mm slit-width. Any eye with cornea thickness deviating by more than 10% from the mean value for all eyes, or eyes that showed any other signs of damage were rejected and replaced.

If the selected eyes were appropriate for the test, acclimatization was started and conducted for approximately 45 to 60 minutes. The chambers of the superfusion apparatus were at a controlled temperature (32±1.5°C) during the acclimatization and treatment periods.

3.6.4 Identification

The eyes were identified by chamber number, marked on the door of the chamber.

3.6.5 The base line assessments

At the end of the acclimatization period, a zero reference measurement was recorded for corneal thickness and opacity to serve as a base line (t=0) for each individual eye. The corneal thickness of the eyes should not change by more than 5% between the -45 min and the zero time. No significant corneal thickness change (1.8%) in one eye, and no corneal thickness change (0.0%) in the other eyes were observed. Following the equilibration period, the fluorescein retention was also measured. Baseline values were required to evaluate any potential test item related effect after treatment. All eyes were considered to be suitable for the assay.

3.6.6 Treatment

After the zero reference measurements, the eye in its retainer was taken out of the chamber and placed on a layer of tissue with the cornea facing upwards. The eye was held in horizontal position, while the test item was applied onto the centre of the cornea.

The test item, difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B), was applied in a volume of 30 µL onto the entire surface of the cornea attempting to cover the cornea surface uniformly with the test substance, taking care not to damage or touch the cornea.

The positive control eyes were treated in a similar way with 30 µL of 5% (w/v) benzalkonium chloride solution. The negative control eye was treated with 30 µL of physiological saline.

One eye was treated with physiological saline, three eyes with the test item and another three with 5% (w/v) benzalkonium chloride solution.

3.6.7 Test item removal

The time of application was observed, then after an exposure period of 10 seconds from the end of the application, the cornea surface was rinsed thoroughly with 20 mL physiological saline at ambient temperature, taking care not to damage the cornea but attempting to remove all residual test item, if possible. The eye was returned to the chamber after rinsing. The time while the eye was out of the chamber was limited to the minimum required.

Note: Physiological saline (Manufacturer: B. Braun Pharmaceuticals SA, Lot number: 212318142, Expiry date: 31 May 2024) was used for rinsing.

3.6.8 Observation and assessment of corneal effects

The negative and positive control eyes as well as all test eyes were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 minutes after the post-treatment rinse. Minor variations within approximately ±5 minutes were considered acceptable.

Corneal thickness and corneal opacity were measured at all time points. Fluorescein retention was measured on two occasions, at base line (t=0) and approximately 30 minutes after the post-treatment rinse. A Haag-Streit BP 900® slit-lamp microscope was used for the measurements and was set at a 0.095 mm slit-width.

The effects were divided into four categories:
I = none
II = slight
III = moderate
IV = severe

3.6.9 Storage of corneas

At the end of the procedures, the corneas were carefully removed from the eyes and placed individually into labelled containers of preservative fluid (10% neutral buffered formalin) for potential histopathology and stored.

3.7 Evaluation

The endpoints evaluated were corneal opacity change, swelling and fluorescein retention.

Other observations which indicate damage, such as loosening of the epithelium can be taken into account in making a classification.

Results from corneal swelling, opacity change and fluorescein retention were evaluated separately to generate an Isolated Chicken Eye (ICE) class for each endpoint. The ICE classes for each endpoint were then combined to generate an Irritancy Classification for each test substance (see Table 4).

3.7.1 Corneal thickness or swelling determination

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

$$CS \text{ at time } t = \frac{CT \text{ at time } t - CT \text{ at } t=0}{CT \text{ at } t=0} \; x100$$

$$\text{Mean } CS \text{ at time } t = \frac{FECS_{(\text{at time } t)} + SECS_{(\text{at time } t)} + TECS_{(\text{at time } t)}}{3}$$

Remark:
CS = cornea swelling
CT = cornea thickness
$FECS_{(at\ time\ t)}$ = first eye cornea swelling at a given time-point
$SECS_{(at\ time\ t)}$ = second eye cornea swelling at a given time-point
$TECS_{(at\ time\ t)}$ = third eye cornea swelling at a given time-point

Note: For the calculation of Maximum Swelling, small negative numbers for swelling (0 to -5%) following application are counted as zero (scored as class I). Large negative numbers (>12% below control) are probably due to erosion and indicate a severe effect (scored as IV). Cases of values of -5% to -12% are evaluated on a case by case basis but in the absence of other findings do not indicate a severe effect (class II).

Corneal swelling was classified by the following scale:

Mean cornea swelling [%]	ICE Class
0 to 5	I
>5 to 12	II
>12 to 18 (>75 min. after treatment)	II
>12 to 18 (≤75 min. after treatment)	III
>18 to 26	III
>26 to 32 (>75 min. after treatment)	III
>26 to 32 (≤75 min. after treatment)	IV
>32	IV

The four categories are:

	ICE Class
No swelling	**I**
Slight swelling	**II**
Moderate swelling	**III**
Severe swelling	**IV**

3.7.2 Corneal opacity determination

Corneal opacity was scored using the area of the cornea that was most densely opacified. Corneal opacity was calculated according to the following formulae:

$$\Delta CO \text{ at time } t = CO \text{ at time } t - CO \text{ at } t=0$$

$$\text{Mean } \Delta CO_{max} = \frac{FECO_{max(30min \text{ to } 240min)} + SECO_{max(30min \text{ to } 240min)} + TECO_{max(30min \text{ to } 240min)}}{3}$$

Remark:
CO at time t = cornea opacity at (30, 75, 120, 180 and 240) minutes after the post-treatment rinse
CO at t=0 = base line cornea opacity
ΔCO at time t = difference between cornea opacity at t time and cornea opacity base line
FECO = first eye cornea opacity
SECO = second eye cornea opacity
TECO= third eye cornea opacity
$_{max(30min \text{ to } 240min)}$ = maximum opacity of the individual eye at 30 to 240 minutes minus base line cornea opacity of the individual eye

Using the mean value of the individual highest opacity scores (mean ΔCO_{max}), an overall maximum opacity score was given for the test item:

No opacity	**0**
Very faint opacity	**0.5**
Scattered or diffuse areas, details of iris clearly visible	**1**
Easily discernible translucent area, details of iris slightly obscured	**2**
Severe cornea opacity, no specific details of iris visible, size of pupil barely discernible	**3**
Complete cornea opacity, iris invisible	**4**

Corneal opacity is classified by the following scale:

Mean maximum opacity score	ICE Class
0.0 - 0.5	I
0.6 - 1.5	II
1.6 - 2.5	III
2.6 - 4.0	IV

The four categories are:

	ICE Class
No opacity	**I**
Slight opacity	**II**
Moderate opacity	**III**
Severe or total opacity	**IV**

3.7.3 Fluorescein retention determination

Fluorescein retention change was calculated according to the following formulae:

$$\Delta FR \text{ at time } t = FR \text{ at time } t - FR \text{ at } t=0$$

$$Mean\ \Delta FR = \frac{FEFR_{(30min)} + SEFR_{(30min)} + TEFR_{(30min)}}{3}$$

Remark:
FR at time t = fluorescein retention at 30 minutes after the post-treatment rinse
FR at t=0 = base line fluorescein retention
ΔFR at time t = difference between fluorescein retention at t time and fluorescein retention base line
FEFR = first eye fluorescein retention at 30 minutes after the post-treatment rinse minus base line fluorescein retention
SEFR = second eye fluorescein retention at 30 minutes after the post-treatment rinse minus base line fluorescein retention
TEFR = third eye fluorescein retention at 30 minutes after the post-treatment rinse minus base line fluorescein retention

The mean fluorescein retention value for all test eyes was used to give the overall fluorescein retention score for the test item:

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

Fluorescein retention is classified by the following scale:

Mean fluorescein retention score	ICE Class
0.0 - 0.5	I
0.6 - 1.5	II
1.6 - 2.5	III
2.6 - 3.0	IV

The four categories are:

	ICE Class
No fluorescein retention	**I**
Slight fluorescein retention	**II**
Moderate fluorescein retention	**III**
Severe fluorescein retention	**IV**

3.8 Validity of the Test

The results from all eyes used met the quality control standards. The negative control and positive control results were in good correlation with historic data. This experiment was considered to be valid.

Historical Control data (updated: 18 October 2021):

Negative Control: Physiological saline (0.9% w/v NaCl solution)

Observation	Min. Value	Max. Value
Maximum corneal swelling at up to 75 min	-3.2%	3.7%
Maximum corneal swelling at up to 240 min	-4.8%	3.7%
Maximum corneal opacity change	0.00	0.50
Fluorescein retention change	0.00	0.50
Number of cases	621	

Positive Control: 5% (w/v) Benzalkonium chloride

Observation	Min. Value	Max. Value
Maximum corneal swelling at up to 75 min	-7.9%	28.3%
Maximum corneal swelling at up to 240 min	-10.7%	52.8%
Maximum corneal opacity change	2.50	4.00
Fluorescein retention change	1.50	3.00
Number of cases	1079	

4.0 RESULTS AND DISCUSSION

The mean values of the treated eyes for maximum corneal thickness, corneal opacity and fluorescein retention changes are given below. The conclusion on eye irritancy was based on the OECD guideline No. 438 quantitative assessments. Details of data interpretation for overall Isolated Chicken Eye Class are given in Table 4.

4.1 Test Item

Observation	Value	ICE Class
Mean maximum corneal swelling at up to 75 min	4.6%	I
Mean maximum corneal swelling at up to 240 min	8.1%	II
Mean maximum corneal opacity change	0.67	II
Mean fluorescein retention change	0.67	II
Other Observations	None	
Overall ICE Class	3xII	

Based on this in vitro eye irritation study in isolated chicken eyes with difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B), the test item is not classified as a severe irritant and not classified as non-irritant. It is concluded that further information is required for classification.

4.2 Positive Control

Observation	Value	ICE Class
Mean maximum corneal swelling at up to 75 min	14.2%	III
Mean maximum corneal swelling at up to 240 min	25.7%	III
Mean maximum corneal opacity change	4.00	IV
Mean fluorescein retention change	3.00	IV
Other Observations	Severe loosening of epithelium was observed in all three eyes at 30 minutes after the post-treatment rinse.	
Overall ICE Class	1xIII 2xIV	

The positive control, 5% (w/v) benzalkonium chloride solution, was classified as severely irritating.

4.3 Negative Control

Observation	Value	ICE Class
Maximum corneal swelling at up to 75 min	0.0%	I
Maximum corneal swelling at up to 240 min	0.0%	I
Maximum corneal opacity change	0.00	I
Fluorescein retention change	0.00	I
Other Observations	None	
Overall ICE Class	3xI	

The negative control, physiological saline (Salsol solution, 0.9% w/v NaCl solution), was classified as non-irritating.

4.4 Morphological Examination

In the positive control group, severe loosening of epithelium was observed in all three eyes at 30 minutes after the post-treatment rinse.

5.0 CONCLUSIONS

Based on this *in vitro* eye irritation study on isolated chicken eyes with difenoconazole/fludioxonil/metalaxyl-m/cyclobutrifluram FS (A23793B), the test item is not classified as a severe irritant and not classified as non-irritant. It is concluded that further information is required for classification.

6.0 REFERENCES

1. OPPTS 870.2400 (EPA 712-C-98-195) August 1998
2. OECD Guidelines for the Testing of Chemicals 438 (25th June 2018)
3. EU Commission Regulation (EC) No 1272/2008 (16th December 2008) on CLP
4. REGULATION (EC) No 1907/2006 on REACH (30 December 2006)
5. Commission Regulation (EU) 2017/735 of 14 February 2017 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
6. Directives: 67/548/EEC and 1999/45/EC
7. INVITTOX (1994) Protocol 80: Chicken Enucleated Eye Test (CEET).
8. Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17.
9. United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Ninth revised edition, UN New York and Geneva (2021)

7.0 DISTRIBUTION OF THE FINAL REPORT

Sponsor: 1x PDF file and 1x Word file will be uploaded to the collaboration website.

Archive: 1x original, bound

TABLES SECTION

TABLE 1 (A23793B) Individual Data for Test Item, Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS

Study Code:	21/245-038CS														Strain:	ROSS 308								
Date of Exposure:	15 October 2021														Test Item:	Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B)								
Chamber number ↓	Corneal thickness (instrument units)														Corneal opacity score							Fluorescein retention		
Relative observation time (min) →	-45	0	Change	30	change at 30	75	change at 75	Max change up to 75	120	change at 120	180	change at 180	240	change at 240	Max change up to 240	0	30	75	120	180	240	Max Δ Opac	0	30	Δ Flu ret
14	58	58	0.0%	60	*3.4%*	61	*5.2%*	*5.2%*	64	*10.3%*	64	*10.3%*	64	*10.3%*	*10.3%*	0	0.5	0.5	0.5	0.5	0.5	*0.5*	0	0.5	*0.5*
15	58	58	0.0%	58	*0.0%*	60	*3.4%*	*3.4%*	60	*3.4%*	60	*3.4%*	61	*5.2%*	*5.2%*	0.5	1	1	1	1	1	*0.5*	0	1	*1.0*
16	56	57	1.8%	58	*1.8%*	60	*5.3%*	*5.3%*	60	*5.3%*	61	*7.0%*	62	*8.8%*	*8.8%*	0	0.5	0.5	0.5	1	1	*1.0*	0	0.5	*0.5*
Mean values:					*1.7%*		*4.6%*	*4.6%*		*6.4%*		*6.9%*		*8.1%*	*8.1%*							*0.67*			*0.67*

Note: No morphological effect was observed.

TABLE 2 Individual Data for the Positive Control, 5% (w/v) Benzalkonium Chloride Solution

Study Code:	21/245-038CS														Strain:	ROSS 308									
Date of Exposure:	15 October 2021														Positive Control:	5% (w/v) Benzalkonium chloride solution									
Chamber number ↓	Corneal thickness (instrument units)														Corneal opacity score							Fluorescein retention		
Relative observation time (min) →	-45	0	Change	30	change at 30	75	change at 75	Max change up to 75	120	change at 120	180	change at 180	240	change at 240	Max change up to 240	0	30	75	120	180	240	Max Δ Opac	0	30	Δ Flu ret
17	61	61	0.0%	69	*13.1%*	70	*14.8%*	*14.8%*	71	*16.4%*	72	*18.0%*	77	*26.2%*	*26.2%*	0	3	4	4	4	4	*4.0*	0	3	*3.0*
18	62	62	0.0%	68	*9.7%*	70	*12.9%*	*12.9%*	72	*16.1%*	76	*22.6%*	78	*25.8%*	*25.8%*	0	4	4	4	4	4	*4.0*	0	3	*3.0*
19	60	60	0.0%	66	*10.0%*	69	*15.0%*	*15.0%*	70	*16.7%*	73	*21.7%*	75	*25.0%*	*25.0%*	0	4	4	4	4	4	*4.0*	0	3	*3.0*
Mean values:					*10.9%*		*14.2%*	*14.2%*		*16.4%*		*20.8%*		*25.7%*	*25.7%*							*4.00*			*3.00*

Note: Severe loosening of epithelium was observed in all three eyes at 30 minutes after the post-treatment rinse.

TABLE 3 Individual Data for the Negative Control, Physiological Saline (Salsol Solution 0.9% w/v)

Study Code:	21/245-038CS														Strain:	ROSS 308									
Date of Exposure:	15 October 2021														Negative Control:	Physiological saline (Salsol solution, NaCl 0.9% w/v)									
Chamber number ↓	Corneal thickness (instrument units)														Corneal opacity score								Fluorescein retention		
Relative observation time (min) →	-45	0	Change	30	change at 30	75	change at 75	Max change up to 75	120	change at 120	180	change at 180	240	change at 240	Max change up to 240	0	30	75	120	180	240	Max Δ Opac	0	30	Δ Flu ret
20	62	62	0.0%	62	*0.0%*	62	*0.0%*	*0.0%*	62	*0.0%*	62	*0.0%*	62	*0.0%*	*0.0%*	0	0	0	0	0	0	*0.00*	0	0	*0.00*

Note: No morphological effect was observed.

TABLE 4 Assessment of the General *In Vitro* Eye Irritancy and Regulatory EC/GHS Classification (2021)

The following table is used to identify the probable eye irritancy potential of test items. In the case where the result indicates Non-irritant, or Corrosive/Severely Irritating, then the test item can be classified as one of these two cases. In all other cases the probable level of irritancy can be reported, but a regulatory *in vivo* rabbit eye irritation test is required for regulatory classification and labelling purposes.

UN GHS Classification	**Combinations of the three ICE Classes**
No Category	**3×I** **2×I, 1×II** **2×II, 1×I**
No prediction can be made	**Other combinations**
Category 1	**3×IV** **2×IV, 1×III** **2×IV, 1×II*** **2×IV, 1×I*** **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 epithelium (in at least 1 eye)**

Remark: *: combinations of categories less likely to occur

APPENDICES SECTION

APPENDIX 1 Certificate of Analysis

Syngenta Crop Protection, LLC
Analytical and Product Chemistry
Greensboro, NC 27409

Certificate of Analysis

A23793B

Batch ID 1200767 (GP210610)

Test Substance Name:	CGA169374/CGA173506/CGA329351/SYN549522 FS (062.51/049.93/050.05/250.08)
Common Name:	Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (062.51/049.93/050.05/250.08)
Material ID:	A23793B
Batch ID:	1200767
Other ID:	GP210610
Source:	Syngenta Crop Protection LLC., 410 Swing Road, Greensboro, NC 27409, US

Chemical Analysis

AI	% w/w	g/L
Difenoconazole	5.45	64.0
Fludioxonil	4.37	51.3
Metalaxyl-M	4.31	50.6
Cyclobutrifluram	21.0	247

Identity of the Active Ingredients: Confirmed

Methodology Used for Characterization: LC, mass spectrometry, oscillating density meter.

The Active Ingredient(s) content is within the FAO limits.

Isomer Assay

Analyte	Isomer	% w/w
CGA329351	D-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	4.15
CGA351920	L-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	0.15

COA Number: USGR210208

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Physical Analysis

Analyte	Value	Units
Density	1.174	g/cm3

Appearance: red liquid

Storage Temperature: <30°C

Re-certification Date:End of Aug/2024

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.

The stability of this test substance will be determined concurrently through reanalysis of material held in inventory under GLP conditions at Syngenta Crop Protection, LLC, Greensboro, NC.

This Certificate of Analysis is summarizing data from a study that has been performed in compliance with Good Laboratory Practices per 40 CFR Part 160. Raw data, documentation, protocols, any amendments to study protocols and reports pertaining to this study are maintained in the Syngenta Crop Protection Archives in Greensboro, NC.

Study Number: USGR210208

Authorization: Sherry Perine

Sherry C Perine

Sherry Perine

Analytical and Product Chemistry Department

Aug 24, 2021

Date

COA Number: USGR210208

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APPENDIX 2 GLP Certificate

OGYÉI Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet

Hatósági Ellenőrzési Főosztály

1051 Budapest, Zrínyi utca 3.
Levélcím: 1372 Postafiók 450
Tel.: +36 1 886 9300, Fax: +36 1 886 9460
E-mail: ogyei@ogyei.gov.hu
Web: www.ogyei.gov.hu

Ref. no: OGYÉI/-29520-2/2021
Admin.: Dr. Szaller Zoltán

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

It is hereby certified that the test facility

Charles River Laboratories Hungary Kft.

H-8200 Veszprém, Szabadságpuszta

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic or terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation, analytical and clinical chemistry, pathology studies, preparation of microscopic tissue sections, reproduction toxicology, in vitro studies, inhalation toxicology, and contract archiving

in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.

Date of the inspection: **07-11 May 2018.**

This certificate is valid up to 11th of May, 2022.

Dr. Lukács Ferenc József
Digitálisan aláírta: Dr. Lukács Ferenc József
Dátum: 2021.05.06 13:04:14 +02'00'

Dr. Ferenc Lukács
Head of Inspectorate

Note: Translation of the text of the certificate in the header: ("Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet") - ("National Institute of Pharmacy and Nutrition"); (“Hatósági Ellenőrzési Főosztály”) - (Inspectorate Division) and at the signature: (“Digitálisan aláírta”) - (Digitally signed); (“Dátum”) - (“Date”).