Product Safety Labs

SYN549522

SYN549522 FS (A22417C) - Local Lymph Node Assay (LLNA) in Mice

Final Report

DATA REQUIREMENT(S): OECD 429 (2010)
EPA 870.2600 (2003)
EC No. 640/2012, B.42 (2012)

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COMPLETION DATE: April 26, 2019

PERFORMING LABORATORY: Product Safety Labs
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LABORATORY PROJECT ID: Report Number: 49380
Study Number: 49380
Task Number: TK0317076

SPONSOR(S): Syngenta Crop Protection, LLC
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VOLUME 1 OF 1 OF STUDY

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study meets the requirements U.S. EPA GLP: Pesticide Programs (FIFRA): 40 CFR Part 160, 1989, which are compatible with OECD Principles of GLP (as revised in 1997): ENV/MC/CHEM(98)17, OECD, Paris, 1998, Japanese Ministry of Agriculture, Forestry and Fisheries: No. 23-Syouan-5173, 2 February, 2012, and EC Directive 2004/10/EC, Official Journal of the European Union, L50/44, Feb. 20, 2004.

Specific information related to the characterization of the test substance as received and tested is the responsibility of the study Sponsor (see Test Substance section).

I, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.

Jennifer Durando, BS
Study Director, Product Safety Labs

04/26/2019
Date

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

The Product Safety Labs' Quality Assurance Unit has reviewed this final study report to assure the report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

QA activities for this study:

QA Activity	Performed By	Date Conducted	Date Findings Reported To Study Director And Management
Protocol review	M. Zakrzewski; B. Simms	May 1, 2018[1]; Jan 30, 2019	May 1, 2018; Jan 30, 2019
Critical phase inspection: *Day 3 sample preparation for preliminary groups*	M. Zakrzewski	Dec 14, 2018	Dec 14, 2018
Raw data audit	B. Simms	Jan 30, 2019	Jan 30, 2019
Draft report review	B. Simms	Jan 30, 2019	Jan 30, 2019

Final report reviewed by:

Barbara Simms
Quality Assurance Auditor
Product Safety Labs

04/25/2019
Date

[1] PSL's "generic" protocol used for this study was reviewed by the Quality Assurance group on this date.

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Jennifer Durando, BS	Study Director
Monique Inforzato, BS	Syngenta Study Monitor
Shannon Stevens, BS	Primary Scientist
Amber Norton, BS	Scientist
Xiomara Portuguez, BS	Scientist
Matthew Sorber, BS	Scientist

Study dates

Study initiation date: November 26, 2018
Experimental start date: December 12, 2018
Experimental termination date: January 8, 2019

Deviations from the Guidelines

None

Amendments to Final Protocol

None

Deviations from Final Protocol

None

Retention of samples

The test substance is retained for at least 3 months following submission of the final report, unless otherwise specified by the Sponsor. All remaining test substance will be returned to the Sponsor or properly disposed. Records of sample disposition are maintained by Product Safety Labs (PSL).

Other

Information on care of the test system, equipment maintenance and calibration, storage, usage, and disposition of the test substance, and all other records that would demonstrate adherence to the protocol will be maintained. Facility records which are not specific to the subject study will be maintained by the testing facility and archived according to PSL SOP.

The original signed final report and electronic copies (in Microsoft Word and pdf) of the final report, including the signed QA and GLP Compliance pages will be sent to the Sponsor. A copy of the signed report, together with the protocol (P327 SYN) and all raw data generated at PSL, is maintained in the PSL Archives in Notebook No. 49380: pages 1-72. PSL will maintain these records for a period of at least five years. After this time, the Sponsor will be offered the opportunity to take possession of the records or request continued archiving by PSL.

Any electronic raw data generated is maintained on-site in accordance with GLP archiving procedures.

Performing laboratory test substance reference number

181105-1H

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

A local lymph node assay (LLNA) was conducted with mice to examine the dermal sensitization potential for SYN549522 FS (A22417C).

Two concentrations (25% and 50%) of the test substance w/w in 1% Pluronic® L92 Surfactant w/w in distilled water (1% Pluronic® L92), the neat test substance (100%) and the vehicle alone were topically applied to sixteen healthy test mice (4 mice/group) for three consecutive days. Three days after the last application, 250 µL of sterile phosphate buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine was injected intravenously via the tail vein of each mouse. Approximately five hours later, all animals were euthanized via an overdose of inhaled Isoflurane and the draining (auricular) lymph nodes were harvested and prepared for analysis in a scintillation counter. The results are presented in disintegrations per minute per mouse (DPM/mouse). The ears of each animal were also evaluated for erythema and edema prior to each application and again on Day 6, prior to the ^{3}H-methyl thymidine IV injection.

The sensitivity of the procedure was validated using recent historical positive control data (Study 48612). A positive control group (four animals) was maintained under the same environmental conditions and treated in the same manner as the test and vehicle control animals. The positive control group animals were treated with a 25% (w/w) mixture of alpha-hexylcinnamaldehyde (HCA), purity ≥ 95%, in 1% Pluronic® L92.

1.2 Results

A table summarizing the sensitization results noted is found below:

	Mean DPM	Stimulation Index[1]
Group 1 - Vehicle Control	2498.85	–
Group 2 - 25% Test Substance	3134.72	1.25
Group 3 - 50% Test Substance	3877.80	1.55
Group 4 - 100% Test Substance	6723.38	2.69

1.3 Conclusion

Based on the results of this study, SYN549522 FS (A22417C) is not considered to be a contact dermal sensitizer in the LLNA. Proper conduct of the LLNA was confirmed via a positive response with 25% Hexyl Cinnamic Aldehyde (HCA), a moderate contact sensitizer.

[1] The stimulation index is derived by dividing the DPM of each experimental group by the DPM of the vehicle control group. A stimulation index of greater than or equal to 3.0 generally indicates a positive response.

2.0 INTRODUCTION

2.1 Purpose

This study was conducted to determine the potential for SYN549522 FS (A22417C) to elicit a dermal sensitization reaction.

2.2 Regulatory Guidelines

The procedures as described in this protocol are based on the most recent version of the following testing guidelines:

- OECD Guidelines for the Testing of Chemicals, Test No. 429 (2010)
- U.S. EPA Health Effects Test Guidelines, OPPTS 870.2600 (2003)
- Commission Regulation (EU) No 640/2012, B.42 (2012) amending Regulation (EC) No 440/2008

2.3 Test Facility

This study was conducted at Product Safety Labs' test facility at 2394 US Highway 130, Dayton, New Jersey 08810. In the opinion of the Sponsor and the Study Director, this study did not unnecessarily duplicate any previous work.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance was identified as: SYN549522 FS (A22417C)
A22417C
Batch ID SMU8IP001

It was received on November 5, 2018, and was further identified with PSL Reference Number 181105-1H. The test substance was stored at room temperature. Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained by the Sponsor.

Characterization of the test substance was provided to PSL by the Sponsor (see Appendix 1):

Composition: SYN549522 (498 g/L), 41.4% w/w (a mixture of SYN547386 and SYN548941):
SYN547386 (448 g/L), 37.2% w/w
SYN548941 (50.0 g/L), 4.16% w/w

Physical Description: Red liquid

Stability: Test substance was expected to be stable for the duration of testing.

Recertification Date: End of October 2021

3.2 ^{3}H-methyl Thymidine

^{3}H-methyl Thymidine, Lot Nos.: 201812 and 201901 were received on December 12, 2018 and January 4, 2019, respectively and stored refrigerated. Documentation of the methods of synthesis, fabrication, or derivation is retained by PerkinElmer, Inc., Boston, MA.

The following information related to the characterization of the radioisotope was provided on the Technical Data Sheet:

Specific Activity: 20 Ci/mmol

Molecular Weight: 242

Radioactive Concentration: 37 MBq/mL; 1.0 mCi/mL

Radiochemical Purity: > 97% (HPLC)

Thymine Content: < 0.5%

Expiration Dates: January 12, 2019 and February 4, 2019

3.3 Experimental Design

3.3.1 Animals

Species/Strain: Mouse, CBA/J

Number of Animals: 17

Number of Groups: 5

Number of Animals per Group:
Preliminary Irritation: 1
Test (3 groups): 4 per group
Vehicle (Negative) Control: 4

Sex: Female, nulliparous and non-pregnant.

Age: Preliminary Animal: Young adult (9 weeks)

Age/Body Weight: Test and Control Animals: Young adult (10 weeks)/18.6-21.3 grams at experimental start.

Source: Received from Envigo RMS Inc. on December 5, 2018 (Preliminary Irritation Animal) and on December 19, 2018 (Test Control Group and Test Group Animals).

3.3.2 Husbandry

Housing: The animals were individually housed in plastic solid bottom cages during the dosing and resting phase of the study. After final weighing until sacrifice, animals were housed in their respective dose groups in plastic cages with bedding. Enrichment (e.g., nesting material) was placed in each cage. Bedding in the plastic, solid bottom cages was changed at least once per week. All caging conformed to the size recommendations in the most recent *Guide for the Care and Use of Laboratory Animals* (Natl. Res. Council, 2011).

Animal Room Temperature: 19-21ºC

Animal Room Relative Humidity: 36-55%

Animal Room Air Changes: 13/hour. Airflow measurements are evaluated regularly and the records are kept on file at PSL.

Photoperiod: 12-hour light/dark cycle

Acclimation Period: 7 or 14 days

3.3.3 Food and feeding

Food: Envigo Teklad Global 16% Protein Rodent Diet® #2016. The diet was available *ad libitum*.

Water: Filtered tap water was supplied *ad libitum*.

Contaminants: There were no known contaminants reasonably expected to be found in the food or water at levels which would have interfered with the results of this study. Analyses of the food and water are conducted regularly and the records are kept on file at PSL.

3.3.4 Identification

Cage: Each cage was identified with a cage card indicating at least the study number, identification, and sex of the animal.

Animal: Each animal was marked with a color code and given a sequential animal number assigned to study 49380, which constituted unique identification. Only the sequential animal number is presented in this report.

3.4 Preparation of Test Substance

The test substance as received (neat) was mixed well prior to use. Solubility testing conducted by PSL indicated that the test substance was soluble in 1% Pluronic® L92. All preparations were mixed well prior to dosing.

3.5 Preliminary Toxicity Testing

One mouse was treated with the test substance at the maximum concentration suitable for application (100%) dilution. The ears of the mouse were evaluated for erythema and edema immediately prior to dosing on Days 1, 2, 3, and on Day 6 according to the scoring system described in Table 15. Body weight measurements were taken on Days 1 and 6. Ear thickness measurements were taken on Day 1 (pre-dose), Day 3 and Day 6.

Twenty-five μL of the test substance was applied to the dorsum of both ears of the mouse once per day for three consecutive days. Application was done using an appropriate size micropipette to accurately deliver 25 μL. The dose was gently spread as evenly as possible over the dorsal surface of the ear using the disposable pipette tip. No treatment was made on Days 4 and 5. On Day 6, each site was evaluated for local reactions (erythema & edema).

The animal was observed daily for signs of toxicity. The Study Director used this data in conjunction with any pre-existing data to select the three concentrations to be tested. The test substance at 25% and 50% (w/w) mixtures in 1% Pluronic® L92 and the test substance at 100% were selected for test.

3.6 Selection of Animals/Dose Levels

Prior to dosing, the animals were weighed and the ears were checked for any abnormalities or clinical signs of diseases or injury. Sixteen healthy, naive female mice without pre-existing ear irritation were selected and distributed (four mice per group) into the following groups:

Group #	Purpose	Concentration (% w/w)
1	Vehicle Control	0
2	Test Substance	25
3	Test Substance	50
4	Test Substance	100

Concentrations were selected based on toxicity, solubility, irritancy, and viscosity.

3.7 Sample Preparation

Concentrations of 25%, 50% and 100% were selected for the main test based on results of the preliminary screening test. Dilutions of the test substance were prepared as w/w mixtures in 1% Pluronic® L92. The vehicle control, 1% Pluronic® L92 was also prepared. All dosage preparations were freshly prepared on the day of application.

3.8 Test Substance Application

Beginning on Day 1, a quantity of 25 µL of the appropriate test substance concentration or the vehicle alone was applied to the dorsum of both ears of each mouse once per day for three consecutive days (Days 1, 2, and 3) using a micropipette. During application, the material was gently spread as evenly as possible over the dorsal surface of the ear using the micro-pipette tip.

3.9 Dermal Scoring

Prior to each application (Days 1, 2, and 3) and on Day 6, the ears were evaluated for erythema and edema according to the modified Draize scoring system (Draize, Woodard, & Calvary, 1944; see Table 15).

3.10 Ear Thickness Measurements

Duplicate measurements of each animal's ears were made using a micrometer. The measurements were made at the apex of the pinna. Measurements were taken on the preliminary screen animal on Day 1 (pre-dose), Day 3 and Day 6. The % ear swelling was calculated for each ear using the following equation:

$$\% \text{ Ear swelling} = \frac{(B - A)}{A} \times 100\% \text{ where:}$$

A = ear thickness measurement on Day 1 (mm x 10^{-2})

B = ear thickness measurement on Day 3 or 6 (mm x 10^{-2})

3.11 ^{3}H-methyl Thymidine Injections

On Day 6 of the study (three days after the final topical application) 250 µL of sterile phosphate buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine was injected intravenously via the tail vein of each mouse.

3.12 Lymph Node Assessment

Approximately five hours after the injection, all test and control mice were euthanized via overdose of inhaled Isoflurane and the draining auricular lymph nodes from all animals were excised. The lymph nodes were evaluated for each individual mouse. A single cell suspension of lymph node cells (LNC) was prepared in PBS by gently massaging the lymph nodes between the frosted ends of two microscope slides over a collection vessel. The slides were then rinsed briefly with PBS into the vessel. The contents of the vessel were transferred to a centrifuge tube and washed with an excess of PBS and centrifuged for approximately 10 minutes at 1800 rpm, with an RCF[1] of 489G. This process was carried out twice. In both cases, the supernatant was decanted and discarded following each centrifugation. After the second wash, 5 mL of the 5% trichloroacetic acid (TCA) in distilled water was then added to

[1] Relative Centrifugal Force.

the sediment and the tube was vortexed briefly. The DNA was then precipitated in the 5% TCA in distilled water at approximately 4°C overnight (approximately 18 hours).

Following the overnight precipitation of the DNA, the tubes were centrifuged again for approximately 10 minutes at 1800 rpm and the supernatant was discarded. The resulting precipitate was re-suspended using 1 mL of the 5% TCA in distilled water and transferred to 10 mL of scintillation fluid. Incorporation of ^{3}H-methyl thymidine was measured by β-scintillation counting and expressed as disintegrations per minute, minus background DPM.

3.13 Clinical Observations

All test, control and preliminary mice were observed for signs of mortality, gross toxicity, and/or behavioral changes daily (see Tables 4 and 9). The preliminary mouse was euthanized via CO_2 inhalation and all test and control mice were euthanized via overdose of inhaled Isoflurane (an anesthetic) on Day 6.

3.14 Body Weights

Individual body weight of the preliminary animal was recorded on Day 1 (initial) shortly before test substance application and prior to sacrifice on Day 6. Individual body weights of test and control animals were recorded on Day 1 (initial) shortly before test substance application and prior to IV injections of ^{3}H-methyl thymidine on test Day 6.

3.15 Evaluation

The mean and standard deviation of the DPM values were calculated for each dose group. A stimulation index (SI) was derived for each experimental group by dividing the mean DPM of each experimental group by the mean DPM of the vehicle control group. Any test substance that produces an SI $\geq$ 3 in the LLNA is normally considered "positive" for dermal sensitization potential (Kimber et al., 1994).

The EC3 value was not calculated since all dose levels induced a stimulation index of less than 3.0.

3.16 Historical Positive Control Validation Study

The procedures used in this study were validated using alpha-hexylcinnamaldehyde, purity ≥ 95% (HCA) as the positive control substance, namely 25% (w/w) mixture of HCA in 1% Pluronic® L92. The most recent validation, PSL Study # 48612, was performed by PSL between August 8 and August 14, 2018. A copy of the signed report, together with the protocol and all raw data generated at PSL, are maintained in the PSL Archives in Notebook No. 48612: pages 1-45. This test was conducted at the Dayton Facility with CBA/J mice from Envigo RMS following procedures similar to those described in Sections 3.8 through 3.15. The results obtained from this testing are presented below.

Historical Vehicle Control Group – 1% Pluronic® L92: No dermal irritation was observed for any of the historical vehicle control group sites.

Historical Positive Control Group – 25% (w/w) HCA in 1% Pluronic® L92: Very slight erythema (score of 1) was evident at five historical control sites on Day 2, at all sites on Day 3, and seven sites on Day 6. Slight edema (score of 1) was present at three sites on Day 3 and at two sites on Day 6. Desquamation was present at five sites on Day 6.

Number of positive control sites with dermal irritation

Day	Erythema				Edema	
	Very slight (score of 1)	Well-defined (score of 2)	Moderate to Severe (score of 3)	Severe (score of 4)	Slight (score of 1)	Marked (score of 2)
2	5/8	0/8	0/8	0/8	0/8	0/8
3	8/8	0/8	0/8	0/8	3/8	0/8
6	7/8	0/8	0/8	0/8	2/8	0/8

The positive control (HCA) at 25% produced a dermal sensitization response in mice (SI = 3.61).

3.17 Statistical Analysis

Statistical analysis was performed on the DPM values. Significance was judged at $p < 0.05$. The treated groups and negative vehicle control group were compared using a One-Way Analysis of Variance (ANOVA), followed by comparison of the treated groups to control by Dunnett's t-test for multiple comparisons (INSTAT Biostatistics, Graph Pad Software, San Diego, CA). Outlier analysis was conducted using Grubbs' test (Grubbs, 1969).

4.0 RESULTS AND DISCUSSION

Preliminary irritation body weights, testing scores, ear thickness measurements and individual in-life observations are presented in Tables 1-4. Individual body weights for vehicle, test, and historical positive control animals are presented in Table 5-6. Individual dermal irritation scores are presented in Table 7-8. Individual in-life observations are presented in Table 9-10. Individual DPM values are presented in Table 11-12. A summary of results for vehicle control, test, and historical positive control animals is presented in Table 13-14. The Draize Primary Skin Irritation Scoring System is presented in Table 15. The Certificate of Analysis is presented in Appendix 1.

All animals appeared active and healthy throughout the study. One mouse from the vehicle control and two mice in the test substance groups lost body weight during the study; however, all other animals gained body weight during the study.

Group 1 (Vehicle Control – 1% Pluronic® L92): No dermal irritation was observed for any of the vehicle control group sites.

Group 2 (25% Test Substance in 1% Pluronic® L92): No dermal irritation was observed for any of the test group sites.

Group 3 (50% Test Substance in 1% Pluronic® L92): No dermal irritation was observed for any of the test group sites.

Group 4 (100% Test Substance in 1% Pluronic® L92): No dermal irritation was observed for any of the test group sites.

Treatment of mice with 25%, 50% and 100% of SYN549522 FS (A22417C) resulted in stimulation index values of 1.25, 1.55 and 2.69, respectively. As a stimulation index (SI) of less than 3.0 was observed in all the treatment groups, the test substance was not considered positive for a dermal sensitization potential.

5.0 CONCLUSIONS

Based on these findings and on the evaluation system used, SYN549522 FS (A22417C) is not considered to be a contact dermal sensitizer in the LLNA.

The positive response observed in the historical positive control validation study with 25% Hexyl Cinnamic Aldehyde (HCA), validated the test system used in this study (see Section 3.16).

6.0 REFERENCES

Boverhof, D. R., Wiescinski, C. M., Botham, P., Lees, D., Debruyne, E., Repetto-Larsay, M., Ladics, G., Hoban, D., Gamer, A., Remmele, M., Wang-Fan, W., Ullmann, L. G., Mehta, J., Billington, R. & Woolhiser, M. R. (2008). Interlaboratory validation of 1% Pluronic® L92 surfactant as a suitable, aqueous vehicle for testing pesticide formulations using the murine local lymph node assay. *Toxicol. Sci*. 105(1), 79-85.

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Ryan, C.A., Cruse. L.W., Skinner, R.A., Dearman, R.J., Kimber, I., & Gerberick, G.F. (2002). Examination of a vehicle for use with water soluble materials in the murine local lymph node assay. *Food Chem. Toxicol.*, 40, 1719-1725.

TABLES SECTION

TABLE 1 Preliminary Irritation Group Body Weights

Animal No.	Sex	Body Weight (g)	
		Day 1	Day 6
Group 1P - 100%			
3680	F	19.2	18.6

TABLE 2 Preliminary Irritation Group Testing Scores

Erythema/Edema

Animal No.	Sex	Day							
		1		2		3		6	
		Left	Right	Left	Right	Left	Right	Left	Right
Group 1P - 100%[1]									
3680	F	0/0	0/0	0/0[2,3]	0/0[2]	0/0[2,3]	0/0[2,3]	0/0[2,3]	0/0[2,3]

[1] 25 µL of the test substance was applied as received to each ear (50 µL total).
[2] Test substance residue at the dose site.
[3] Hair loss at and exceeding the dose site.

TABLE 3 Preliminary Irritation Group Ear Thickness Measurements (mm)

Erythema/Edema

Preliminary Animal (Left Ear)

Dose Level	Group No.	Animal No.	Day 1 1st	Day 1 2nd	Mean Thickness Day 1	Day 3 1st	Day 3 2nd	Mean Thickness Day 3	% Change Days 1-3	Day 6 1st	Day 6 2nd	Mean Thickness Day 6	% Change Days 1-6
100% Test Substance	1P	3680	0.24	0.23	0.24	0.26	0.27	0.27	12.50%	0.26	0.27	0.27	12.50%

Preliminary Animal (Right Ear)

Dose Level	Group No.	Animal No.	Day 1 1st	Day 1 2nd	Mean Thickness Day 1	Day 3 1st	Day 3 2nd	Mean Thickness Day 3	% Change Days 1-3	Day 6 1st	Day 6 2nd	Mean Thickness Day 6	% Change Days 1-6
100% Test Substance	1P	3680	0.23	0.23	0.23	0.27	0.27	0.27	17.39%	0.28	0.28	0.28	21.74%

TABLE 4 Preliminary Irritation Group Individual In-life Observations

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3680	F	1P	100	Active and healthy	x	x	x	x	x	x

TABLE 5 Individual Body Weights

Animal No.	Group	Sex	Day 1 (g)	Day 6 (g)
3601	1 Vehicle Control (1% Pluronic® L92)	F	18.7	19.3
3602		F	18.6	19.2
3603		F	19.9	20.6
3604		F	20.5	20.3
3605	2 25% Test Substance in 1% Pluronic® L92	F	20.7	21.7
3606		F	18.8	19.7
3607		F	20.7	20.3
3608		F	19.0	19.8
3609	3 50% Test Substance in 1% Pluronic® L92	F	18.9	20.0
3610		F	18.8	19.5
3611		F	21.3	20.2
3612		F	19.2	19.9
3613	4 100% Test Substance	F	20.1	20.2
3614		F	19.6	20.8
3615		F	20.4	21.2
3616		F	19.5	20.1

TABLE 6 Individual Body Weights Historical Positive Control Validation Study[1]

Animal No.	Group	Sex	Day 1 (g)	Day 6 (g)
3701	1 Vehicle Control (1% Pluronic® L92)	F	21.7	21.0
3702		F	20.6	20.6
3703		F	22.7	22.3
3704		F	24.6	23.6
3705	2 Positive Control (25% HCA in 1% Pluronic® L92)	F	24.3	23.4
3706		F	24.4	24.4
3707		F	24.2	23.3
3708		F	23.1	22.5

[1] PSL Study # 48612, testing was performed by PSL between August 8 and August 14, 2018.

TABLE 7 Individual Dermal Irritation Scores

Group 1 – Vehicle Control[1]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left	Right	Left	Right	Left	Right
3601	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3602	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3603	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3604	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Group 2 – 25% Test Substance[2]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left	Right	Left[3]	Right[3]	Left[3]	Right[3]
3605	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3606	F	0/0	0/0	0/0[3]	0/0	0/0	0/0	0/0	0/0
3607	F	0/0	0/0	0/0	0/0[3]	0/0	0/0	0/0	0/0
3608	F	0/0	0/0	0/0	0/0[3]	0/0	0/0	0/0	0/0

[1] 25 µL of 1% Pluronic® L92 was applied to each ear (50 µL total).
[2] 25 µL of the test substance was applied as a w/w mixture in 1% Pluronic® L92 to each ear (50 µL total).
[3] Red staining at the dose site(s).

TABLE 7 Individual Dermal Irritation Scores (Continued)

Group 3 – 50% Test Substance[1]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left[2]	Right[2]	Left[2]	Right[2]	Left[2]	Right[2]
3609	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3610	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3611	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3612	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Group 4 – 100% Test Substance[3]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left[2]	Right[2]	Left[2,4]	Right[2,4]	Left[2,4]	Right[2,4]
3613	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3614	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3615	F	0/0	0/0	0/0	0/0[4]	0/0	0/0	0/0	0/0
3616	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

[1] 25 µL of the test substance was applied as a w/w mixture in 1% Pluronic® L92 to each ear (50 µL total).
[2] Test substance residue at the dose site(s).
[3] 25 µL of the test substance was applied as received to each ear (50 µL total).
[4] Hair loss at and exceeding the dose site(s).

TABLE 8 Individual Dermal Irritation Scores Historical Positive Control Validation Study[1]

Group 1 – Vehicle Control[2]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left	Right	Left	Right	Left	Right
3701	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3702	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3703	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
3704	F	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Group 2 – Positive Control[3]

Erythema/Edema

Animal No.	Sex	Days							
		1		2		3		6	
		Left	Right	Left	Right	Left	Right	Left	Right
3705	F	0/0	0/0	1/0	0/0	1/0	1/0	1/0[4]	1/0[4]
3706	F	0/0	0/0	0/0	1/0	1/0	1/1	1/1[4]	1/1[4]
3707	F	0/0	0/0	1/0	1/0	1/0	1/1	0/0	1/0
3708	F	0/0	0/0	1/0	0/0	1/1	1/0	1/0[4]	1/0

[1] PSL Study # 48612, testing was performed by PSL between August 8 and August 14, 2018.
[2] 25 µL of 1% Pluronic® L92 was applied to each ear (50 µL total).
[3] 25 µL of a 25% w/w mixture of HCA in 1% Pluronic® L92 was applied to each ear (50 µL total).
[4] Desquamation at the dose site.

TABLE 9 Individual In-life Observations

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3601	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3602	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3603	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3604	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x

TABLE 9 Individual In-life Observations (Continued)

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3605	F	2	25% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3606	F	2	25% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3607	F	2	25% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3608	F	2	25% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x

TABLE 9 Individual In-life Observations (Continued)

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3609	F	3	50% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3610	F	3	50% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3611	F	3	50% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x
3612	F	3	50% Test Substance in 1% Pluronic® L92	Active and healthy	x	x	x	x	x	x

TABLE 9 Individual In-life Observations (Continued)

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3613	F	4	100% Test Substance	Active and healthy	x	x	x	x	x	x
3614	F	4	100% Test Substance	Active and healthy	x	x	x	x	x	x
3615	F	4	100% Test Substance	Active and healthy	x	x	x	x	x	x
3616	F	4	100% Test Substance	Active and healthy	x	x	x	x	x	x

TABLE 10 Individual In-life Observations Historical Positive Control Validation Study[1]

Animal Number	Animal Sex	Group	Dose Conc. (%)	Observation	Day of Observation (x=observation is present)					
					1	2	3	4	5	6
3701	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3702	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3703	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3704	F	1	Vehicle Control (1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3705	F	2	Positive Control (25% HCA in 1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3706	F	2	Positive Control (25% HCA in 1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3707	F	2	Positive Control (25% HCA in 1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x
3708	F	2	Positive Control (25% HCA in 1% Pluronic® L92)	Active and healthy	x	x	x	x	x	x

[1] PSL Study # 48612, testing was performed by PSL between August 8 and August 14, 2018.

TABLE 11 Individual and Mean DPM Values[1]

Background:	59.38						
Group	Animal #	DPM	DPM minus background[2]	Group Mean DPM	Std. Dev	SI[3]	SI ≥3
1 Vehicle Control (1% Pluronic® L92)	3601	1953.58	1894.20	2498.85	483.48	-	-
	3602	2475.05	2415.67				
	3603	3116.83	3057.45				
	3604	2687.44	2628.06				
2 25% Test Substance in 1% Pluronic® L92	3605	2705.31	2645.93	3134.72	575.01	1.25	No
	3606	2950.89	2891.51				
	3607	4021.58	3962.20				
	3608	3098.63	3039.25				
3 50% Test Substance in 1% Pluronic® L92	3609	3799.57	3740.19	3877.80	573.60	1.55	No
	3610	3184.68	3125.30				
	3611	4435.61	4376.23				
	3612	4328.86	4269.48				
4 100% Test Substance	3613	5766.62	5707.24	6723.38	824.13	2.69	No
	3614	6793.54	6734.16				
	3615	6785.66	6726.28				
	3616	7785.20	7725.82				

[1] Disintegrations per minute.
[2] Values analyzed for outliers, Grubbs, 1969.
[3] Stimulation Index = Average DPM of Test Substance/Average DPM of Vehicle.

TABLE 12 Individual and Mean DPM Values[1] Historical Positive Control Validation Study[2]

Background:	49.33						
Group	Animal #	dpm	dpm minus background[3]	Group Mean DPM	Std. Dev	SI[4]	SI ≥3
1 Vehicle Control (1% Pluronic® L92)	3701	3231.06	3181.73	2339.90	741.13	-	-
	3702	2105.27	2055.94				
	3703	2703.12	2653.79				
	3704	1517.47	1468.14				
2 Positive Control (25% HCA in 1% Pluronic® L92)	3705	9696.03	9646.70	8437.47	1944.06	3.61	Yes
	3706	9978.40	9929.07				
	3707	8551.25	8501.92				
	3708	5721.50	5672.17				

[1] Disintegrations per minute.
[2] PSL Study # 48612, testing was performed by PSL between August 8 and August 14, 2018.
[3] Values analyzed for outliers, Grubbs, 1969.
[4] Stimulation Index = Average dpm of Test Substance/Average dpm of Vehicle.

TABLE 13 Stimulation Index

Group		Group Mean DPM	SI	Sensitization Response
Vehicle Control	1	2498.85	-	N/A
25% Test Substance	2	3134.72	1.25	Not a Sensitizer
50% Test Substance	3	3877.80*	1.55	Not a Sensitizer
100% Test Substance	4	6723.38**	2.69	Not a Sensitizer

N/A= Not Applicable

* Statistically significant difference from vehicle control, $p < 0.05$, by Dunnett's Multiple Comparisons Test.

** Statistically significant difference from vehicle control, $p < 0.01$, by Dunnett's Multiple Comparisons Test.

TABLE 14 Stimulation Index Historical Positive Control Validation Study[1]

Group		Group Mean DPM	SI	Sensitization Response
Vehicle Control	1	2339.90	–	N/A
Positive Control (25% HCA)	2	8437.47***	3.61	Positive – valid study

N/A= Not Applicable

*** Significant to control, $p < 0.0011$ by unpaired t-test

[1] PSL Study # 48612, testing was performed by PSL between August 8 and August 14, 2018.

TABLE 15 Primary Skin Irritation Scoring System[1]

Evaluation of Skin Reactions	Value
Erythema and eschar formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation:	
No edema	0
Slight edema (barely perceptible)	1
Marked edema (swelling is obvious)	2

[1] Modified from a published method (Draize, et al., 1944).

APPENDICES SECTION

APPENDIX 1 Certificate of Analysis

syngenta

Syngenta Crop Protection AG
GLP Testing Facility WMU
Analytical Development & Product Chemistry
Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

A22417C
SYN549522 FS (500)
SMU8IP001

Batch Identification	**SMU8IP001**
Other Batch ID	1058463
Product Code	**A22417C**
Other Product Code(s)	SYN549522 FS (500)
Chemical Analysis (Active Ingredient content)	
– **Identity of the Active Ingredient(s)***	**confirmed**
– **Content of SYN549522***	**41.4 % w/w corresponding to 498 g/l**
– **Content of SYN547386***	**37.2 % w/w corresponding to 448 g/l**
– **Content of SYN548941***	**4.16 % w/w corresponding to 50.0 g/l**
	The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	HPLC, chiral HPLC, oscillating density meter
Physical Analysis	
– **Appearance**	**red liquid**
– **Density***	**1203 kg/m³**
Stability:	
– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of October 2021**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.
This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP.
Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection AG, Switzerland.

Study number of batch characterization: CHMU180671
Study number(s) of batch recertification: ---

Authorization: 17-Oct-2018

Daniel Jenniches
Analytical Development & Product Chemistry

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