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GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*)

i2LResearch Ltd
Capital Business Park
Wentloog
Cardiff CF3 2PX
UK

Dr Pavel Foltan
June 2015

- Chemicals Regulation Directorate Accredited
- GLP (Good Laboratory Practice) Compliant

Compliance statement

The study was conducted in compliance with the Czech GLP compliance programme (Act No. 350/2011 Coll. and Decree No. 163/2012 Coll. as amended), which follows the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (revised, 1997).

This report represents a true and accurate record of all data obtained.

Signed…….. Date……..
Dr Pavel Foltan
Study Director

Approved by…… Date……..
Dr Peter M^{c}Ewen
Chief Executive Officer

All raw data and a copy of the final report will be archived at i2LResearch Ltd for a period of ten years. At the end of this period all data relating to this report will either be retained by i2LResearch Ltd for a further disclosed period of time, or passed on to the sponsor.

Report circulated to: Organic Laboratories, Inc. (1 copy)

i2LResearch Ltd (1 copy)

Quality assurance statement

Study code: 15/141
Study title: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*)

Study director: Pavel Foltan

The conduct of this study was audited on the dates given below. The report has been audited to ensure that it accurately describes the methods used and that the reported results accurately reflect the raw data of the study.

Date of audit	Date of QA report to Study Director	Date of QA Report to Management	Phase audited
20th April 2015	**20th April 2015**	**20th April 2015**	Protocol
6th May 2015	**7th May 2015**	**7th May 2015**	Critical phase
3rd June 2015	**3rd June 2015**	**3rd June 2015**	Study folder
3rd June 2015	**3rd June 2015**	**3rd June 2015**	Draft report

Quality Assurance:

Signed…….. Date……..
Jan Okrouhlik
Quality assurer

Contents

Page No.

Study Information

GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*)

Testing organization	i2LResearch Ltd Capital Business Park Wentloog Cardiff, CF3 2PX UK
Testing facility	i2LResearch Ltd Lipova 1789/9 Ceske Budejovice, 37005 Czech Republic
Sponsor	Organic Laboratories, Inc. 2963 SE Dominica Terrace Stuart, FL 34997, USA

i2LResearch Ltd Study code:	15/141
Study start date:	8th April 2015
Experimental start date:	6th May 2015
Experimental end date:	10th May 2015
Study end date:	Draft report
Study Director:	Pavel Foltan
Primary personnel:	Jitka Zelova, Tereza Bad'urova
Certification:	GLP certificate no. 100003/ENV/11

Test items and reference items:

Product	Active Ingredient	content of a.i. nominal	Batch/lot number	Expiry Date	i2L code	storage conditions	description
Organocide® 3-in-1 Garden Spray	sesame oil	5.0% w/w	N/A	14.4.2017*	1504142	ambient	1L white plastic bottle with labels

Product	Active Ingredient	content of a.i. nominal	Batch/lot number	Expiry Date	i2L code	storage conditions	description
Organocide™ 3-in-1 Garden Spray	sesame oil	5.0% w/w	N/A	14.4.2017*	1504143	ambient	1L white plastic bottle with labels

(i2LResearch default expiry date following receipt of product)*

Summary

A GLP laboratory study was conducted to assess the acute contact toxicity of two 5% sesame oil products products, Organocide® 3-in-1 Garden Spray (1) and Organocide™ 3-in-1 Garden Spray (2), to honey bees (*Apis mellifera*). The study design followed EPA (2012) test guidelines. 4 µL of each product was topically applied to adult bee workers at a single rate:

(1) 3oz of the product per gallon of water (2.34 % productV/V)

(2) 2oz of the product per gallon of water (1. 56 % product V/V)

The two products exhibited 0 % bee mortality over a 48 hour test period and 0 % (1) and 8 %(2) bee mortality over a 96 hour test period, whereas the untreated control and solvent control exhibited 0% and 8% bee mortality over 96 hour test period, respectively. It can be concluded that no adverse effect of the test items to honey bees was found.

Aims and Objectives

A GLP laboratory study was required to assess the acute contact toxicity of two product to honey bees (*Apis mellifera*). The test generally followed the EPA (2012) test guidelines, however a single rate for each of the product was tested (as requested by the sponsor).
Experiments were conducted at i2LResearch Central Europe, a branch office of i2LResearch Ltd, in Ceske Budejovice, Czech Republic.

Methodology

Test system

The test was conducted using young adult worker honey bees (*Apis mellifera*) that were of a similar age and feeding status.
Bees were obtained from a commercial apiary in Ceske Budejovice, Czech Republic. During the holding and testing, bees were shielded from excessive activity or other disturbance. Bees were handled only as much as necessary to conform to test procedures. Honey comb and approximately 50% (w/w) solution of sucrose/water (500 grams/litre) was provided *ad libitum* throughout the holding and test periods.
The evening before test initiation, bees were randomly collected from the hive and placed into holding cages (45 cm x 45 cm x 65 cm) and kept at approximately 27 ºC and >70 % relative air humidity.

Test and reference items, product application

The test items were provided by the sponsor. Two concurrent controls were included in the test: a negative control and a solvent control. Distilled water was used as the solvent control.
Test items were transferred into the solvent shortly prior to application. The products were found not fully soluble in water, so the test volumes of the suspension were pipetted directly from the beaker placed on magnetic stirrer.
The test items were tested at one rate specified by the sponsor:

Organocide® 3-in-1 Garden Spray: 3oz of the product per gallon of water (2.34 % V/V)

Organocide™ 3-in-1 Garden Spray: 2oz of the product per gallon of water (1.56 % V/V)

Experimental design

To initiate the test, bees in the holding cages were immobilized by chilling, and distributed into treatment groups of at least 25 bees. A single dose of 4 µL was applied to the dorsal side of the thorax of each bee via an automated pipette. 25 bees were used for each product dose and for each control. After treatment, the bees were placed into test chambers similar to holding chambers (Figure 1)

Figure 1 The bees were kept in test chambers (approximately 45 cm x 45 cm x 65 cm) and provided with honeycomb and sucrose solution ad libitum.

Bees were observed for mortality and any other adverse effects at approximately 4, 24, 48 and 96 hours post-application. All signs of intoxication, other abnormal behaviour, and mortality were recorded throughout the test period. Knocked down and dead bees were pooled into one category.

Test conditions were maintained between 26 and 27.5 °C, with relative humidity between 55 and 87.5 %. Test bees were maintained in the dark except for during dosing and observations.

Results and Conclusions

Mortality data are displayed in Table 1. The two products tested exhibited 0 % bee mortality over 48 hours and 0 % (Organocide® 3-in-1 Garden Spray) and 8 % (Organocide™ 3-in-1 Garden Spray) bee mortality over the 96 hour test period whereas untreated control and solvent control exhibited 0% and 8% bee mortality over the 96 hour test period, respectively.

Table 1 Number of dead (D) and knocked down (KD) bees out of 25 worker bees per treatment exposed to the particular test treatments- untreated control, solvent control, Organocide® 3-in-1 Garden Spray or Organocide™ 3-in-1 Garden Spray

Time after dosing	Untreated control		Solvent control		OrganocideTM		Organocide®	
	KD	D	KD	D	KD	D	KD	D
4 hrs	0	0	0	0	0	0	0	0
24 hrs	0	0	0	0	0	0	0	0
48 hrs	0	0	0	0	0	0	0	0
96 hrs	0	0	1	1	2	0	0	0

Validity criteria

For the test to be considered valid according to the EPA (2012) test guidelines, mortality in the control treatment should not exceed 20% over a 48 h test period. The actual mortality was 0%, thus the test is considered valid.

Appendix I –References

EPA (2012) Ecological Effects Test Guidelines: OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test. United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention(7101), EPA 712-C-019

Appendix II – Study Protocol, protocol amendments and deviations

Study Protocol

Study Code: 15/141	Sponsor: Organic Laboratories, Inc.
Proposal approval date: 07.04.2015	Proposal approval method: email
Test system(s): *Apis mellifera*	Study Director: Pavel Foltan
Title of Study: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*)	
<u>Test substances</u> Test item(s): one product, two rates Control(s): negative control, solvent (or vehicle) control	Study start date: 08.04.2015 Experimental start date (Month/year): April 2015 Experimental end date (Month/year): June 2015 Study end date (Month/year): June 2015

GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*)

Testing organization	**i2LResearch Ltd** **Capital Business Park** **Wentloog** **Cardiff, CF3 2PX** **UK** **(1 copy)**
Testing facility	**i2LResearch Ltd** **Lipova 1789/9** **Ceske Budejovice, 37005** **Czech Republic** **(1 copy)**
Sponsor	**Rick Barr** **Organic Laboratories, Inc.** **2963 SE Dominica Terrace** **Stuart, FL 34997** **USA** **rick@organiclabs.com** **Phone Number 772-286-5581** **(1 copy)**
Submitted by:	**Dr Pavel Foltan**
Study Director:	**Dr Pavel Foltan** **Email: Pavel@i2lresearch.com** **Tel: +420604953278**
Proposal date:	**18th January 2015**

Aim

A GLP laboratory study is required to assess the acute contact toxicity of a product to honey bees (*Apis mellifera*). The generally follows the EPA (2012) test guidelines, however two rates are tested (as requested by the sponsor).

Experiments will be conducted at i2LResearch Central Europe, a branch office of i2LResearch Ltd, in Ceske Budejovice, Czech Republic.

GLP compliance

The study will be in compliance with the Czech GLP compliance programme (Title III of Act No. 350/2011 Coll. and Decree No. 163/2012 Coll. as amended), which is in accordance with the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (revised, 1997).

Test systems

The test will be conducted using young adult worker honey bees (*Apis mellifera*) that are of a similar age and feeding status.

Bees will be obtained from a commercial apiary. All control and treatment bees used in a test will be from the same source and race. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during this time. If tests have to be conducted during these times, bees can be emerged in an incubator and reared for one week with "bee bread" (pollen collected from the comb) and a sucrose solution. Bees used in the test should be in apparent good health. Only bees from apparently disease-free colonies will be used, and they will be kept in conditions conforming to proper cultural practices. Bees treated with chemical substances, such as antibiotics, anti-varroa, etc., will not be used. During holding and testing, bees will be shielded from excessive activity or other disturbance. Bees should be handled only as much as is necessary to conform to test procedures. Approximately 50% (w/w) solution of sucrose/water (500 grams/litre) will be provided ad libitum throughout the holding and test periods.

On the day of test initiation or the evening before, bees will be randomly collected from the incubator or directly from the hive, immobilized with cold temperatures, or carbon dioxide gas (CO_2) or nitrogen gas (N_2), and placed into holding cage. Dead or moribund bees should be rejected and replaced by healthy bees before starting the test.

Test Items.

The test item and its certificate of analysis will be provided by the sponsor. Two concurrent controls will be included in the test: a negative control and a
solvent (or vehicle) control. The particular solvent will be agreed with the sponsor.
Test item will be diluted in suitable solvent shortly prior to application and the solution will be thoroughly agitated to ensure its homogeneity. The test item will be tested at one rate specified by the sponsor.

Experimental design

To initiate the test, bees in the holding cages will be again immobilized, and distributed into treatment groups of at least 25 bees. A single dose will applied to the dorsal side of the thorax of each bee via microapplicator. A minimum of 25 bees will be used for each dosage level and for each control. After treatment, the bees will placed into test chambers constructed from wire and mesh (volume approximately 80,000 cm^3), separately for each treatment.
Bees will be observed for mortality and any other adverse effects at approximately 4, 24 and 48. If mortality increases by more than 10% between 24 and 48 h, the test duration should be extended up to a maximum of 96 h provided that control mortality does not exceed 20%. All signs of intoxication, other abnormal behaviour, and mortality will be recorded throughout the test period
Dead bees will not be removed from the test chambers until the test is terminated.

Temperature will be maintained between 25 and 35 °C, with relative humidity between 50% and 80%. Air temperature and humidity will be recorded during the course of the trial. If the ambient temperature or humidity falls outside of the required parameters for a short period of time, such as when assessments are made, this will not be considered to be a protocol deviation.

Test bees should be maintained in the dark except of during dosing and observations.

Validity criteria

For the test to be considered valid, mortality in the control treatment should not exceed 20%.

Statistical analyses

Results will be presented in a tabular format. Analyses will be performed by the Study Director and will depend on the outcome of the testing at the discretion of the Study Director. Statistical analyses performed will be fully documented in the report.

Protocol amendments and deviations

Any protocol amendments and/or deviations will be documented, fully justified and maintained with the protocol. All protocol amendments will be approved by the study director and sent to the sponsor.

Archiving records

The original raw data, final report and any amendments will be archived at i2LResearch Ltd for a period of ten years. The final report, including any protocol amendments or deviations, will be forwarded to the Sponsor. Any unused test substances will be either returned to the sponsor or disposed of with the sponsor's consent.

Proposed dates (month/year)

Proposed study start date: April 2015

Proposed study completion date: June 2015

Proposed experimental start date: April 2015

Proposed experimental completion date: June 2015

Please note that these are proposed dates, based on the date the proposal was submitted; for actual study dates please refer to the first page of the signed protocol.

References:

EPA (2012) Ecological Effects Test Guidelines: OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test. United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention(7101), EPA 712-C-019

The Sponsor must supply full safety information with any materials, e.g. material safety data sheets. Usage of all products received from the Sponsor will comply with i2LResearch Health and Safety requirements.

Protocol amendment no: 1

Study code: 15/141

Study Title: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*).

Description of amendment	1. Two instead of one dose rate will be tested. The required rates are 2oz per gallon of distilled water and 3oz per gallon of distilled water. *Notes to the units:* The US liquid gallon is equal to 3.785 litres. One american ounce [oz] = 29.6 ml. 2. The dosage volume applied to each bee will be 4µL.
Reason for amendment	Sponsor's request.
Impact on study	1. Extra 25 young adult bees in good health will be needed. 2. No impact.

Protocol deviation no: 1

Study code: 15/141

Study Title**: GLP laboratory study to determine acute contact toxicity of a product to honey bees (*Apis mellifera*).**

Description of deviation	Relative air humidity exceeded required parameters (50-80%)
Reason for deviation	Records of relative humidity have shown that the relative humidity was between 55 and 87.5%.
Impact on study	There was no negative impact on the study. Mortality in untreated group was 0% over 96 h test period.

Appendix III –GLP Certification