SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 6
Effective Date: 26 June 2014
Protocol Title: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions
Study Sponsor: Bayer CropScience
Test Substance: Clothianidin
Study No.: 13798.4143

Amendment:

The protocol cover states:

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: Bayer CropScience

Address: 2 T.W. Alexander Dr., Research Triangle Park, NC 27709

Phone: 919-549- 2735

Study Monitor: Allen Olmstead, Ph.D. **E-mail:** allen.olmstead@bayer.com

Sponsor Protocol/Project No.: EBTIN114

Test Substance Name(s): Clothianidin

Purity: 98.6% **Batch or Lot #: AE1283742-01-10**

Analytical Standard:*

Purity:* % **Batch or Lot #:***

Sponsor Approval: **Date:**

TO BE COMPLETED BY SMITHERS VISCIENT LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Smithers Viscient, CRC, 2900 Quakenbush Rd., Snow Camp, NC 27349

Study Director: Jessica Louque Study No.: 13798.4143

Test Concentration: 10 µg/L, 20 µg/L, 40 µg/L, 80 µg/L, 160 µg/L,

Proposed Experimental Dates: (Start) June 2014 (Termination) Aug 2015

Study Director Signature **Study Initiation Date**

EPA-HQ-2016-007648_00001019

This should be changed to:

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: Bayer CropScience

Address: 2 T.W. Alexander Dr., Research Triangle Park, NC 27709

Phone: 919-549- 2735

Study Monitor: Allen Olmstead, Ph.D. **E-mail:** allen.olmstead@bayer.com

Sponsor Protocol/Project No.: EBTIN114

Test Substance Name(s): Clothianidin

Purity: 98.6% **Batch or Lot #: AE1283742-01-10**

Analytical Standard: K-785

Purity: 99.8% **Batch or Lot #: M00343**

Expiration Date: 30 Oct 2016

Sponsor Approval: **Date:**

TO BE COMPLETED BY SMITHERS VISCIENT LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Smithers Viscient, CRC, 2900 Quakenbush Rd., Snow Camp, NC 27349

Study Director: Jessica Louque Study No.: 13798.4143

Test Concentration: 10 µg/L, 20 µg/L, 40 µg/L, 80 µg/L, 160 µg/L,

Proposed Experimental Dates: (Start) June 2014 (Termination) Aug 2015

Study Director Signature **Study Initiation Date**

Justification:

The original analytical standard information was left intentionally blank and was to be added by amendment.

Approval Signatures:

04 NOV 2015

Jessica Louque
Smithers Viscient Study Director

Date

04 NOV 2015

Study Sponsor Representative

Date

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 5
Effective Date: 26 June 2014
Protocol Title: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions
Study Sponsor: Bayer CropScience
Test Substance: Clothianidin
Study No.: 13798.4143

Amendment:

Amendment 2 states:

The analytical method will be "An Analytical Method for the Determination of Residues of Clothianidin in Bee Relevant Matrices Using LC/MS/MS" **Bayer Method No. TI-006-A13-02**, by T. J. Gould.

This should be changed to:

The analytical method will be "An Analytical Method for the Determination of Residues of Clothianidin in Bee Relevant Matrices Using LC/MS/MS" **Bayer Method No. TI-006-A13-02**, by T. J. Gould. In addition to this method, capped honey and uncapped nectar may be analyzed for sugar content by using a refractometer.

Justification:

The sugar content was deemed a relevant data point to be added to the study.

Approval Signatures:

04 NOV 2015

Jessica Louque
Smithers Viscient Study Director

Date

04 NOV 2015

Study Sponsor Representative

Date

EPA-HQ-2016-007648_00001022

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 4
Effective Date: 26 June 2014
Protocol Title: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions
Study Sponsor: Bayer CropScience
Test Substance: Clothianidin
Study No.: 13798.4143

Amendment:

Protocol section 2.3 Study Locations states:

The field portion of the study will be conducted at 12 sites in the general vicinity of the test facility. Site locations will be at least 1 mile apart. Geographic coordinates and site location maps will be included in the raw data file and final report. Each study site will be characterized using the National Land Cover Database within a five mile radius.

This should be changed to:

The field portion of the study will be conducted at 12 sites in the general vicinity of the test facility. Site locations will be at least 1 mile apart. Geographic coordinates and site location maps will be included in the raw data file and final report. Each study site will be characterized using the National Land Cover Database within a one, three, and five mile radius, as well as Crop Land Data Layer within a one, three, and five mile radius.

Justification:

The additional database will offer a better characterization of sites.

Approval Signatures:

04 NOV 2015

Jessica Louque — Date
Smithers Viscient Study Director

04 NOV 2015

Study Sponsor Representative — Date

EPA-HQ-2016-007648_00001023

SMITHERS VISCIENT

PROTOCOL AMENDMENT

Amendment No.: 3
Effective Date: 26 June 2014
Protocol Title: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions
Study Sponsor: Bayer CropScience
Test Substance: Clothianidin
Study No.: 13798.4143

Amendment:

Protocol section 2.1 Test Substance states:

Active Ingredient: Clothianidin
Chemical Name:[C(E)]-N-[(2-Chloro-5-thiazolyl)methyl]-N'-methyl-N''-nitroguanidine
CAS No.: 210880-92-5
Batch Number: 0301200403
Purity: 99.8%
Certification Date: 10/30/06
Expiration Date: 10/30/16
Storage Conditions: Freezer condition when not in use

This should be changed to:

Active Ingredient: Clothianidin
Chemical Name:[C(E)]-N-[(2-Chloro-5-thiazolyl)methyl]-N'-methyl-N''-nitroguanidine
CAS No.: 210880-92-5
Batch Number: AE 1283742-01-10
Purity: 98.6%
Certification Date: 20 November 2013
Expiration Date: 20 February 2015
Storage Conditions: +10 to +30˚C

Justification:

The original section was errantly described with information from the Certificate of Analysis of the analytical standard instead of the test material.

Approval Signatures:

02 Nov 2015
Jessica Louque — Date
Smithers Viscient Study Director

04 Nov 2015
Study Sponsor Representative — Date

EPA-HQ-2016-007648_00001024

PROTOCOL AMENDMENT _X_ DEVIATION ___
AMENDMENT NUMBER 2

COMPOUND/PRODUCT: Clothianidin

STUDY TITLE: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions

STUDY NUMBER: 13798.4143

PERFORMING LABORATORIES:

Smithers Viscient, CRC
2900 Quakenbush Road
Snow Camp, NC 27349

Bayer CropScience
Product Safety Center
2 TW Alexander Drive
RTP, NC 27709

DEVIATION/AMENDMENT ASSOCIATED WITH:

- [ ] Field Project
- [ ] Processing Project
- [X] Analytical Project

DEVIATION/AMENDMENT RELATING TO:

- [ ] Facilities
- [ ] Samples
- [ ] Test Substance
- [ ] Support Areas
- [ ] Dosage and Preparation
- [ ] Test Cancellation
- [ ] Test System
- [X] Other: Personnel
- [ ] Application
- [X] Test Procedures
- [ ] Test Dates

EXPLANATION AND EFFECT ON STUDY:

1. The principal analytical investigator (PAI) is updated in this amendment. There is no adverse effect on the study.
2. The analytical method is added to the protocol with this amendment. There is no adverse effect on the study.

AMENDMENT:

1. The PAI is Chung Lam of Bayer CropScience.
2. The analytical method will be "*An Analytical Method for the Determination of Residues of Clothianidin in Bee Relevant Matrices Using LC/MS/MS*" **Bayer Method No. TI-006-A13-02**, by T. J. Gould.

APPROVED BY:

STUDY DIRECTOR — DATE: 15 Sep 2015

SPONSOR/MANAGEMENT/ — DATE: 02 OCT 2015

PROTOCOL AMENDMENT _X_ DEVIATION ___
AMENDMENT NUMBER _1_

COMPOUND/PRODUCT: Clothianidin

STUDY TITLE: Colony Feeding Study Evaluating the Chronic Effects of Clothianidin-Fortified Sugar Diet on Honey Bee (*Apis Mellifera*) Colony Health under Free Foraging Conditions

STUDY NUMBER: 13798.4143

PERFORMING LABORATORIES:

Smithers Viscient, CRC
2900 Quakenbush Road
Snow Camp, NC 27349

Bayer CropScience
Product Safety Center
2 TW Alexander Drive
RTP, NC 27709

DEVIATION/AMENDMENT ASSOCIATED WITH:

- [ ] Field Project
- [ ] Processing Project
- [X] Analytical Project

DEVIATION/AMENDMENT RELATING TO:

- [ ] Facilities
- [ ] Samples
- [ ] Test Substance
- [ ] Support Areas
- [ ] Dosage and Preparation
- [ ] Test Cancellation
- [ ] Test System
- [X] Other: Personnel
- [ ] Application
- [X] Test Procedures
- [ ] Test Dates

EXPLANATION AND EFFECT ON STUDY:

1. The principal analytical investigator (PAI) is identified in this amendment. There is no adverse effect on the study.

2. The method of analysis is identified in this amendment. There is no adverse effect on the study.

AMENDMENT:

1. The PAI is Thomas J. Gould of Bayer CropScience.

   The method of analysis is **Bayer Method No. TI-006-A13-01**, *An Analytical Method for the Determination of Residues of Clothianidin in Bee Relevant Matrices Using LC/MS/MS* by T. J. Gould, with method modifications as determined to be necessary by the PAI.

AMENDMENT HISTORY:

APPROVED BY:

7 JUL 2014
STUDY DIRECTOR DATE

02 OCT 2015
SPONSOR/~~MANAGEMENT/~~ DATE

FINAL STUDY PROTOCOL

Colony feeding study evaluating the chronic effects of clothianidin-fortified sugar diet on honey bee (*Apis mellifera*) colony health under free foraging conditions

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: Bayer CropScience

Address: 2 T.W. Alexander Dr., Research Triangle Park, NC 27709

Phone: 919-549- 2735

Study Monitor: Allen Olmstead, Ph.D. **E-mail:** allen.olmstead@bayer.com

Sponsor Protocol/Project No.: EBTIN114

Test Substance Name(s): Clothianidin

Purity: 98.6% **Batch or Lot #: AE1283742-01-10**

Analytical Standard:*

Purity:* % **Batch or Lot #:***

Sponsor Approval: Allen W. Olmstead **Date:** 6/26/14

TO BE COMPLETED BY SMITHERS VISCIENT LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Smithers Viscient, CRC, 2900 Quakenbush Rd., Snow Camp, NC 27349

Study Director: Jessica Louque Study No.: 13798.4143

Test Concentration: 10 µg/L, 20 µg/L, 40 µg/L, 80 µg/L, 160 µg/L,

Proposed Experimental Dates: (Start) June 2014 (Termination) Aug 2015

Study Director Signature

26 JUN 2014

Study Initiation Date

* - to be added by protocol amendment if necessary

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1.0 GENERAL PROVISIONS

1.1 Introduction

Clothianidin is a systemic insecticide in the neonicotinoid class of chemistry which acts on the central nervous system of insects as an agonist of the nicotinic acetylcholine receptor. Clothianidin is efficacious against a wide range of pests including aphids, leafhoppers, fleas, termites and wood boring insects.

1.2 Study Objective

This study is designed to determine potential long-term effects on the health of honey bee (*Apis mellifera* L.) colonies after dietary intake of clothianidin via fortified sugar solution. Results from this study will be used to establish a No Observed Adverse Effect Level (NOAEL) for use in tier II risk assessment of clothianidin for bees.

1.3 Justification of the Test System

Clothianidin is widely used in agricultural settings targeting crop-destructive insects. In such crops, residues may be present in the pollen and nectar that bees collect and use as food. In order to evaluate whether these potential dietary exposures pose a significant risk, data are needed that describe the relationship between food concentration and the magnitude of any resulting adverse effects. The proposed study area in central North Carolina (rural lands between Durham and Greensboro) is a patchwork of forested and open lands. There are some small tracts of crops such as tobacco, corn, and soybean; however, most land is non-intensively managed pasture and forest. Consequently, the potential for exposure of bees to pesticides is relatively low.

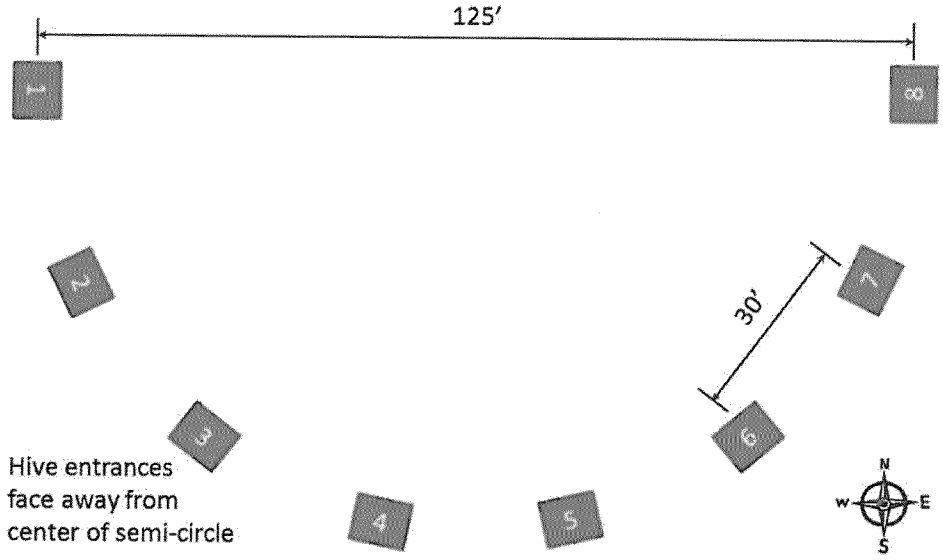
1.4 Experimental Design

In the pre-exposure portion of the study, approximately 160 colonies will be established from new packaged bees using new hive equipment in April 2014. Colonies will be supplementally fed sugar syrup in order to facilitate build-up of new comb and colony strength. In early May, Colony Condition Assessments (CCAs) will be conducted to select colonies meeting minimal criteria for colony strength and vitality. Each colony should consist of at least 3 frames of brood and 3 frames of stored food (pollen/honey mixture), and be queenright.

The exposure phase of the study will be conducted by feeding clothianidin-fortified artificial nectar diets to honey bee colonies in a rural field environment lacking extensive acreages of crops treated with pesticides and during a time of year when there is an anticipated dearth of available floral nectar. Colony development and health will be assessed via Colony Condition Assessments (CCA) which will quantify adult bee strength, food stores, and brood frame coverage. Samples of hive matrices will be collected from hives to monitor residues of the test material during the study.

Colonies selected for the study will be divided into 12 groups of 7 colonies based on total brood coverage as assessed during CCAs prior to exposure initiation. Each of these groups will be placed at different apiaries, which will be separated by at least 1 mile and equally separated from other known honey bee apiaries within the study region. Placement of the hives at an apiary will be in a half-circular pattern with hive

entrances facing outward from the center. This configuration is to minimize robbing and drift between colonies.



Within each apiary, the individual hives will be randomly assigned to treatment levels (control 1, control 2, and test substance concentrations 1 - 5, respectively). One additional hive will be used for monitoring purposes only. Sugar solution spiked with the test substance will be placed inside each treated hive twice a week for 6 weeks. Untreated sugar solution will be placed in each untreated (control) hive twice a week for 6 weeks. Hive health will be characterized via CCAs 8 times over the course of the study. Hive weights will be recorded continuously using dedicated digital scales for each hive.

Samples of adult honey bees will be collected from test hives to determine *Nosema* and *Varroa* levels the week before initiation of exposure, the week after cessation of exposure and after over-wintering. At pre-determined time points, samples of uncapped nectar stores, capped honey and bee bread will be taken and analyzed to determine residue levels of the test material in stored food from the colonies.

Monitoring hives will be placed at the study sites that have pollen traps affixed in order to characterize sources of available pollen forage and potential extraneous pesticide exposure. These monitoring hives will be chosen from those colonies remaining after treatment and control assignments. No CCA or other assessments of these hives will be done on the monitoring hives. These additional hives may be replaced if needed to maintain proper strength for pollen and nectar collection.

1.5 Control of Bias

Control of experimental bias will be achieved through the use of negative controls, including maintenance and sampling of the untreated hives, collection of representative samples from hives, use of validated analytical methods, analysis of untreated and blank samples within each analytical set, and analysis of laboratory-fortified samples within each set. Adherence to GLP standards, regulatory guidelines and standard operating procedures (SOPs), and associated hygiene procedures, will also control bias through standardization of experimental procedures and minimization of potential for contamination to the study test and reference substances or interfering compounds.

1.6 Good Laboratory Practice Standards Requirement

This study must meet all applicable GLP requirements as outlined in EPA FIFRA 40 CFR, Part 160. The Principal Investigators will submit a Statement of GLP Compliance to the Study Director noting any deviations from GLP requirements.

1.7 Standard Operating Procedures Requirements

The Study Director will ensure that appropriate SOPs are in place for activities which occur at their test site, such as equipment calibration, sampling methods, sample preparation, data generation and contamination precautions. SOPs must meet all applicable requirements of FIFRA GLPS, 40 CFR 160. These SOPs will be followed unless superseded by specific provisions of this protocol. All deviations from SOPs, except as required by this protocol, must be documented.

1.8 Quality Assurance

The Testing Facility QAU or designee will (i) review the protocol to ensure it provides for study conduct and collection of data consistent with the requirements of GLP, (ii) perform in-phase audits at field and analytical testing sites as appropriate to ensure the integrity of the study, and (iii) review the completed field notebook and analytical raw data to ensure all data required by the protocol were collected, that such data were properly recorded, that amendments and deviations were properly executed and filed, and that the raw data records and report accurately reflect the conduct of the study. The Study Director and Testing Facility Management will be provided with copies of all reports of QAU inspection findings and reports of actions taken in response.

2.0 FIELD PHASE

2.1 Test Substance

Active Ingredient: Clothianidin

Chemical Name:[C(E)]-N-[(2-Chloro-5-thiazolyl)methyl]-N'-methyl-N''-nitroguanidine

CAS No.: 210880-92-5

Batch Number: 0301200403

Purity: 99.8%

Certification Date: 10/30/06

Expiration Date: 10/30/16

Storage Conditions: Freezer condition when not in use

The test substance will be provided by the Sponsor. Upon arrival at the testing facility, the test substance will be received into the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody and use record established. The condition of the external packaging of the test substance will be recorded and any damage noted. The shipping packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor.

The test substance will be assigned a unique ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information will

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be provided by the Study Sponsor, if applicable: test substance lot or batch number, MSDS, safe handling procedures and a verified expiration or reanalysis date.

2.2 Disposition of Test Substance and Containers

Unused test substance and original containers will be retained at the Field Laboratory Facility until study completion and then returned to the Study Sponsor within 6 months unless other arrangements are made.

2.3 Study Locations

The field portion of the study will be conducted at 12 sites in the general vicinity of the test facility. Site locations will be at least 1 mile apart. Geographic coordinates and site location maps will be included in the raw data file and final report. Each study site will be characterized using the National Land Cover Database within a five mile radius.

2.4 Study Site History, Size and Layout

Hives will be placed in a rural area with minimal forage crops available during the exposure phase to reduce availability of alternative food sources for the honey bees.

The immediate area within a 500 ft radius around the test apiaries will not have been treated with any formulation containing the test substance or related material during 2013 through the experimental start date. Where applicable, crop history and recent pesticide-use data will be gathered and placed into the data file. The following parameters are required:

- Each site will contain one colony for each dietary concentration (treatment group) and two colonies for the untreated control group
- An additional monitoring hive will be maintained at each site for pollen and nectar collection
- The immediate area around the test apiaries within a 100 ft radius must be visibly free of significant bee forage for the duration of the exposure period
- Geographic coordinates of each study site will be recorded in the data file
- Hives must be separated by at least 30 feet (9.14 meters)

A map indicating the location of each colony will be placed in the raw data and provided in the final report. The history of the hive preparation and maintenance activities will be recorded in the data file.

A list will be developed of field crops and possible foraging sources or other sources of neonicotinoid exposure within a 3-km (1.86 miles) radius of each of the 12 sites.

2.5 Study Site Maintenance

Apiaries will be maintained as free of bee forage by mowing to the degree possible for the duration of the exposure period. Prior to and after the exposure period, bee forage may be available.

Maintenance chemicals which do not contain the test substance active ingredient, or a known bee-toxic ingredient, may be applied within the apiaries as necessary. No treatments will be made during the exposure period. Date, identity, rate and use method of maintenance chemicals, if any, will be recorded.

2.6 Honey Bee Colonies

The study will be comprised of six treatment groups: 1 untreated control group and 5 treated groups with 12 replications of each treated group and 24 replications of the untreated control group for a total of 84 colonies for biological assessments and residue sampling.

Colonies will be prepared by transferring packaged bees (3 lbs per hive, ~10,000 adults) onto new foundation in April. Colonies will be supplementally fed sucrose syrup in order to promote comb building until the exposure period. Honey bees (*Apis mellifera*) will be purchased from a commercial apiary and the identity of the source will be documented in the raw data and stated in the report. The bees will be typical of the bee stock used in commercial beekeeping operations. These are typically of the Italian race interbred with Carniolan or Russian strains. Approximately 160 colonies will be observed via CCA at the beginning of May to choose the 84 hives for the study (plus an additional 12 for monitoring hives). Colonies must be queen-right and will be chosen based on the strength of the total area of brood.

Before establishing colonies at the test sites, the colonies will be observed in a second CCA in late May. Colonies will be assigned to test sites in a stratified, random placement. The strongest 7 hives with respect to total brood frame coverage will be randomly assigned to each of the treatment groups within one apiary (test site), then the next 7 strongest hives will be randomly assigned to another apiary, and so on. Hives will be closed in the evening and transported to the test site the next morning. Placement of hives within the semi-circular arrangement at each apiary will be randomized.

Colonies will be allowed to acclimate until the third CCA, which should take place within one week of exposure initiation. Any hives damaged in transit will be replaced prior to exposure initiation. Damage may include the loss of a queen or a significant decrease in adult bees or larvae.

Hives will be labeled with the study number, treatment and replication information, as well as any other information to assure an unmistakable identification of each colony.

During the study, the colonies may be managed as typical for apicultural practice in this region, including application of antibiotic or miticide treatments, performance of beekeeper checks for swarm control and supplemental feeding during periods of dearth as required to maintain colony vitality. Any application of treatments or supplemental feeding will be made based on the assessments of control hives only and will only be performed after consultation with the study sponsor and the agencies (CDPR, EPA, PMRA). No supplemental feeding will be administered during the exposure period with only the test solutions being added to the colonies. All hives will be treated equally when such practices are employed.

2.7 Hive Components

Hive boxes will be standard 10-frame Langstroth type. As colonies increase in size, a second honey super with a queen excluder may be added to colonies. Determination

of the need for a honey super will be made by the study director for each individual hive. Bottom boards should be screen IPM boards.

A plastic-lined top feeder or a Boardman feeder will be used to administer the test substance during the exposure period. Control and treatment solutions will be sealed inside each hive and will not be accessible outside of the hive. Treatment and control solutions will not be exposed to sunlight except minimally during administering to the hive.

Robbing screens will be placed on hives before the exposure period to reduce incidental exposure from bees entering adjacent hives.

2.8 Monitoring Hives

An additional hive will be placed at each apiary for collection of pollen and nectar which will be analyzed for pesticide residues and pollen palynological identification. These monitoring hives will be chosen at random. No CCA or other assessments of these hives will be done on the monitoring hives. These additional hives may be replaced if needed to maintain proper strength for pollen and nectar collection. Any replacement of these monitoring hives should be documented and reported.

Pollen traps will be activated at the entrances of these monitoring hives at eight time points over the course of the study. These pollen traps will remain activated for approximately 24-48 hours. Uncapped nectar samples from monitoring hives will be collected at the same time as the pollen samples. Pollen collected from the traps will be used to identify local pollen sources. Pollen and uncapped nectar will be analyzed to assess pesticide exposure from outside sources.

Monitoring hives will also be utilized to characterize stability of the test material inside the hive. During the first and last week of the exposure, representative samples of the low, middle, and high test solutions will be placed in sealed containers (e.g., Falcon® tubes) and put inside monitoring hives. They will remain in the monitoring hives for the same period of time as that between application of test solutions to the treated hives (i.e., 3-4 days). These will be replicated three times each for both time points.

2.9 Environmental Monitoring

Rainfall data will be collected at the test facility and at the site furthest from the test facility, starting at the timing of the third CCA and continuing through the last CCA, encompassing the exposure period. A record of daily minimum and maximum air temperature from a weather station within 25 miles of the test sites, or from the data loggers associated with hive scales will also be recorded for the same duration.

Historical (10 year minimum) average precipitation and minimum/maximum air temperature data from the nearest weather station that provides such data will also be included in the raw data.

2.10 Treatments, Rate and Timing

Exposure to the test material will be done using spiked sucrose syrup (50% w/w) over a six week period. Twice a week, 2000 mL of freshly prepared spiked syrup will be provided to each colony for a total of 12 feedings (24 L in total). Any syrup

remaining from the previous feeding will be weighed and disposed of appropriately; however, all the sugar syrup is expected to be moved into the colony.

The feeding timing, syrup concentration and syrup volume are described below:

Treatment Group	Code	Feeding Timing	Amount a.i.	Syrup Volume
1 : UTC Sugar syrup	C1, C2	Twice a week	n/a	2000 mL
2 : Lowest Rate Sugar Syrup	T1	Twice a week	10 µg/L	2000 mL
3 : Low rate Sugar syrup	T2	Twice a week	20 µg/L	2000 mL
4 : moderate Sugar syrup	T3	Twice a week	40 µg/L	2000 mL
5: High rate Sugar syrup	T4	Twice a week	80 µg/L	2000 mL
6: Effect Rate Sugar syrup	T5	Twice a week	160 µg/L	2000 mL

Samples from freshly prepared control and treatment solutions will be collected in triplicate on week 1 and week 5 during the exposure to verify test concentrations.

2.11 Exposure via Feeding

Fresh treated sugar syrup will be delivered two times each week and be made accessible to bees inside a plastic-lined top feeder or Boardman feeder. Test material should be shielded from light. The previous feeding's syrup will be removed from the feeder and measured to calculate the consumed amount.

Two independent residue samples comprising at least 5 g each from the sugar syrup will be taken 1 week and 5 weeks after start of feeding (A: residue analysis and B: retained sample). Pre-feeding samples will be taken from the original diet before start of feeding. These samples will be kept separate from other samples during storage and shipping to avoid the potential for cross-contamination. Stability of the test material inside the hives will be evaluated at 1 week and 5 weeks after initiation of the exposure period.

Representative stored food samples (e.g., uncapped nectar and bee bread) will be collected for pesticide residue analysis (see section 2.14 Chronology list for timing). A sample for each matrix component comprising of at least 500 mg each will be taken from each hive if stores are present in sufficient quantities.

2.12 Data Collection

Data collected during this study is as follows:

Colony Condition Assessments (CCA)

CCAs will be done to determine potential colony-level effects by observing and recording the number of adult honey bees, honey, pollen, open brood and capped brood cells present in the hive. Swarm and supercedure cells will be counted for each frame. CCAs will be performed a total of 8 times during the study. Proposed weeks for CCAs are specified in 2.14 (Chronology List). The first and second CCAs will be used to choose the 84 colonies for the study. CCAs may be done either visually or through the use of digital photography. CCAs for any given time point must all be done with one method. For example, if the 5th CCA is assessed with an observer, then all colonies for that time point must be assessed in this manner.

For visual CCAs, trained observers will examine both sides of each frame and record the percent coverage of the frame for each component of the CCA. All the colonies within a given apiary will be assessed by the same observer so that any observer to observer variability is accounted for in the blocking factor of the statistical analyses. All observers for a study should perform a joint calibration exercise each year before study initiation. For this calibration exercise, each observer will perform CCAs on the same colonies. Digital photography of the frames from the same colonies will be performed at the same time in order to compare estimates of colony strength parameters between observers and the digital analysis.

For CCAs performed with digital photography, frame images will be taken in a controlled lighting photo box using an appropriate camera (e.g., Canon EOS D Mark III with 100 mm macro lens and lens hood). Images will be stored on a camera chip and a portable computer temporarily until a second digital storage file is created on one or more backup drives. The image assessments will be conducted using appropriate software (e.g., IndiCounter©, WSC Scientific GmbH, Heidelberg Germany). Queens must be located, captured and caged without harm before beginning CCAs with digital photography. Each frame of the colony must be photographed first for the adult bee coverage estimates. After these pictures, each frame must be completely removed of all adult bees by first shaking bees off and then brushing off any remaining bees. After all pictures have been taken, the queen will be released back into the hive.

During CCAs, each hive will be checked for abnormal behaviors of a significant numbers of bees. These behaviors include, but are not limited to, excessive crawling (numerous bees walking slowly, apparently unable/unwilling to fly) and disorientation (flying or walking erratically as if unable to determine direction). The presence of notable disease conditions such as foulbrood or chalkbrood will be recorded when observed.

Hive Weights

Weights of each hive will be recorded continuously by digital scales. Scales will record the weight and temperature at each hive on an hourly basis. Information regarding the scales will be documented in the study data.

Varroa and Nosema testing

Adult honey bees will be sampled at the third, fifth, and ninth CCA to assess *Varroa* and *Nosema* levels. No treatments for these pathogens will be administered during the exposure period.

Hive Matrices

Uncapped nectar, capped honey and pollen stores will be collected from control and treatment hives for clothianidin analysis at various time points (see study outline below). Samples will be collected, stored at ≤ -10 °C at the test facility, and then shipped frozen to the analytical lab for analysis.

A sample of nectar and bee bread will be taken at the second CCA from two hives at each apiary location prior to moving to the test locations to assess any pre-existing pesticide exposure. Samples will also be taken once during the exposure period, at the CCA just after the end of the exposure, at the CCA just before overwintering, and at the last CCA.

2.13 Sample Storage

Samples will be maintained frozen at ≤ -10 °C at the test facility until shipped under frozen conditions to the analytical lab. Daily minimum/maximum freezer temperatures are to be recorded for the duration of the storage period at the field facility.

2.14 Sample Shipment

Hive matrix samples will be shipped as soon as possible after collection. Samples will be shipped on dry ice directly to the selected analytical laboratory.

Packing and shipping procedures will be documented. Treated and untreated samples will be shipped in separate boxes, or triple bagged if shipped in the same box.

Chain of custody forms will accompany the samples during shipment. The forms will include an inventory identifying the samples in the shipment and will be signed and dated by the person responsible for shipping the samples. Upon shipment of the samples, the chain of custody forms should be faxed or e-mailed to the Study Director and the analytical laboratory. Copies of the chain of custody forms and bill of lading or tracking number will be maintained with the field data.

2.15 Chronology List

The following table outlines the order and potential weeks of actions performed during the test. Calendar weeks are designated by the date of the respective Monday.

Study Wk	Week (Mon)	Actions
-8	4/28	Install packages
-7	5/5	
-6	5/12	1st CCA
-5	5/19	
-4	5/26	
-3	6/2	2nd CCA Sample uncapped nectar and pollen stores for clothianidin residues in study hives
-2	6/9	Move hives to locations assigned based on 2nd CCA
-1	6/16	3rd CCA Replace any poorly performing hives Sample for Nosema/Varroa Sample trapped pollen and uncapped nectar from monitoring hives

0	6/23	Begin exposure
1	6/30	Sample treatment solutions for clothianidin analysis Assess clothianidin stability Sample trapped pollen and uncapped nectar from monitoring hives
2	7/7	
3	7/14	4th CCA Sample uncapped nectar for clothianidin residues in study hives Sample trapped pollen and uncapped nectar from monitoring hives
4	7/21	
5	7/28	Sample treatment solutions for clothianidin concentration analysis Assess clothianidin stability Sample trapped pollen and uncapped nectar from monitoring hives
6	8/4	End exposure
	8/6	5th CCA Sample for Nosema/Varroa Sample uncapped nectar and pollen stores for clothianidin residues in study hives
7	8/11	Sample trapped pollen and uncapped nectar from monitoring hives
8	8/18	
9	8/25	
10	9/1	Sample trapped pollen and uncapped nectar from monitoring hives
11	9/8	6th CCA
12	9/15	
13	9/22	Sample trapped pollen and uncapped nectar from monitoring hives
14	9/29	
15	10/6	
16	10/13	7th CCA Sample capped honey for clothianidin residues in study hives Sample trapped pollen and uncapped nectar from monitoring hives
~	~	
40	3/16	8th CCA
41	3/23	
42	3/30	
43	4/6	
44	4/13	9th CCA Sample for Nosema/Varroa Sample capped honey for clothianidin residues

This is a tentative schedule and is subject to change depending on weather conditions and other factors. If weather or other conditions prevent the opening of the hives to replace syrup for more than 4 hours from the normal time, subsequent treatments can either be moved to the later time, or the dosing may be done the next day with approval from the sponsor study monitor.

2.16 Statistical Methods

Statistical analysis is not required for the conduct of the field portion of this study. Any statistical analysis performed on data generated will be described in the Final Report.

2.17 Required Field Data

The data record supplied by the field facility will include the following:

1. The amount and lot/batch number of test substance received, the date received, carrier and bill of lading number or tracking number, the condition upon receipt, storage location, the minimum/maximum storage temperatures from receipt through final use, and test substance material balance (date, amount, purpose of withdrawal and final disposition)
2. A map of the general area, showing the test sites in relation to the nearest town, city or other geographic feature
3. A map of the test sites indicating their location and size, location of the hives, compass orientation, distance between hives, and distance from hives to a fixed point of reference (permanent marker) or GPS coordinates
4. A list of field crops or large floral sources in the surrounding area for approximately a 3 km radius from all field sites
5. Crop and pesticide-use history of apiary sites for the two years preceding study initiation
6. Detailed records describing cultural practices used during the trial, including use of maintenance chemicals used on hives or fields, and colony adjustments
7. Current year and historical weather data
8. Exposure data, including weights, equipment and calculations
9. Post-exposure syrup weights (how much consumed)
10. Detailed description of sampling techniques
11. Dates and times of sample collection and placement of samples into freezers
12. Identification of sample storage freezers and daily min/max sample storage temperatures from day of collection until shipment
13. Shipping information, including copies of the chain of custody and bill of lading
14. All study-specific correspondence and any other miscellaneous supporting raw data needed to reconstruct the trial

2.18 Field Report

The Principal Investigator is responsible only for submitting the completed raw data and associated raw data package to the Study Director. The Study Director is responsible for describing field procedures in the final report.

3.0 ANALYTICAL PHASE

Specific procedures for sample analysis will be added by amendment or appendix to this protocol.

4.0 PROTOCOL CHANGES – AMENDMENTS AND DEVIATIONS

The Study Director and the Sponsor Study Monitor must document all amendments to the approved protocol in writing. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any. If necessary, amendments other than the one providing the information required on

page one of this protocol, may initially be verbally authorized, followed by written documentation. In such cases, the effective date of the amendment will be the date of verbal authorization.

5.0 RECORDS TO BE MAINTAINED

At minimum, the following records will be maintained as described:

1. All original, study specific field raw data will be maintained at the Smithers Viscient Carolina Research Center until study completion. Copies of study specific field data should be maintained at least until safe receipt of original data.
2. The Analytical Laboratory will maintain all study specific analytical raw data until transfer to the Study Director.
3. At study completion, all records and raw data or true copies thereof, will be archived at Smithers Viscient Carolina Research Center. An archive to archive transfer will then be conducted to transfer the study package to the Sponsor facility, at the sponsor's request.
4. The Smithers Viscient Carolina Research Center shall maintain the original protocol, along with all amendments and deviations, until completion of the study. They will then be transferred to the Sponsor.
5. The Smithers Viscient Carolina Research Center shall maintain the original field, analytical and final reports (including any report amendments) until study completion. They will then be transferred to the Sponsor.
6. All correspondence that is necessary to reconstruct the study will be maintained by the parties originating and/or receiving such correspondence. All such correspondence in possession of Smithers Viscient Carolina Research Center shall be transferred to the Sponsor, if requested at the conclusion of the study.
7. Training and records of experience of field, laboratory and other personnel involved in the study shall be maintained by the Smithers Viscient Carolina Research Center as required by 40 CFR Part 160.

6.0 FINAL REPORT

A final report describing the conduct and results of the study will be prepared by the Study Director upon completion of a Sponsor-approved analytical report.

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