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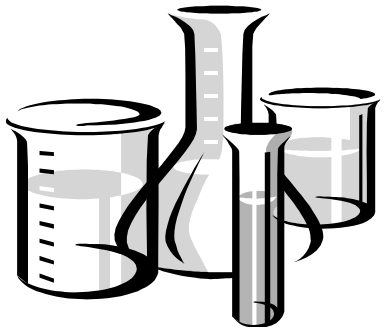
Ecological Effects Test Guidelines

OCSPP 850.3020: Honey Bee Acute Contact Toxicity Test



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to http://www.epa.gov/ocspp and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.3020: Honey bee acute toxicity.

(a) **Scope—**

(1) **Applicability**. This guideline is intended to be used to help develop data to submit to EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background.** The source materials used in developing this harmonized test guideline include the OPP 141-1 Honey Bee Acute Contact LD_{50} (Pesticide Assessment Guidelines Subdivision L); the Honey Bee—Acute Contact LD_{50} Standard Evaluation Procedure; and OECD 214, Honeybees, Acute Contact Toxicity Test.

(b) **Purpose**. This guideline is intended for use in developing data on the acute contact toxicity to honey bees of chemical substances and mixtures ("test chemicals" or "test substances") subject to environmental effects test regulations. The Agency will use data from this test in assessing the acute hazard a test substance may present to bees. While the study is specifically designed to allow calculation of the LC50, the study can be used to obtain information regarding sublethal effects which are used in Agency evaluations.

(c) **Definitions.** The definitions in OCSPP 850.3000 apply to this guideline. In addition, the more specific definitions in this paragraph also apply:

Acute contact toxicity is the adverse effects occurring within a maximum period of 96 hours of a topical application of a single dose of test substance.

Dose is the amount of test substance applied. Dose is expressed as a mass, microgram (µg) of test substance per bee (µg/bee).

Frank sublethal effects refers to overt or frank toxicological effects and include, but are not limited to lethargy, abnormal behavior, or ataxia. Less significant sublethal effects such as excessive grooming are not considered frank toxicological effects.

LD_{50} is the empirically derived dose of the test substance that is expected to result in mortality of 50 percent (50%) of a population of bees which is treated with a single contact dose level under the conditions of the test.

Mortality means an animal is recorded as dead when it is completely immobile.

(d) **General considerations—**

(1) **Summary of test.** The honey bee acute contact LD_{50} test, using the honey bee (*Apis mellifera*), is an acute, one-time dose laboratory study designed to determine the quantity of test substance that causes 50% mortality in a test population of bees. Test bees may be obtained directly from hives or from frames kept in an incubator. Test bees are immobilized and randomly assigned to the various dosage levels and controls. Test substance is administered as a single topical dose, either via microapplicator (topical drop) or via whole body exposure to impregnated dust. Bees are closely monitored within the first 4 hours (h) after treatment, and then observed for mortality and signs of intoxication at 24 and 48 h, with extended observations to 96 h as indicated in paragraph

(e)(2) of this guideline. The mortality pattern is examined and subjected to the appropriate statistical analysis to derive the LD_{50}, slope, and confidence limits. Results are expressed as LD_{50} in micrograms (μg) of test substance per bee. The complete mortality pattern, along with signs of intoxication, should be reported.

(2) **General test guidance.** The general guidance in OCSPP 850.3000 applies to this guideline except as specifically noted herein.

(3) **Range finding test.** If the approximate toxicity of the test substance is unknown, a range-finding test may be conducted to determine the dosage levels of the test substance to be used in the definitive test. Refer to paragraph (d)(4) of this guideline for details on dosage levels for definitive tests. If a test substance is expected to be of low toxicity, it may be useful to first conduct a limit test at 25 μg per bee under paragraph (d)(5) of this guideline. If mortality occurs at this level, then further range-finding at lower levels should be performed. The results of the range-finding test may then be used to establish the definitive test dosage levels. Results of range-finding tests should be reported along with the results of the definitive test, if range-finding tests are conducted.

(4) **Definitive test**. The goal of the definitive test is to determine the dose-response curve for honey bee mortality after 48 h acute contact, and to establish the median lethal dose (LD_{50}) (and its 95 percent (95%) confidence limits), as well as the slope of the dose-response curve, its associated standard error and 95% confidence limits. For this determination, a minimum of five dosage levels of the test substance should be used. A summary of test conditions is provided in Table 1 in paragraph (g) of this guideline, and validity elements for an acceptable definitive test are listed in Table 2 in paragraph (h) of this guideline.

(5) **Limit test.** For test substances expected to have relatively low toxicity, in place of a definitive test a limit test may be conducted with a single contact dose level, the limit dose, plus a control group. In these situations, it is only necessary to ascertain that the 48-hr LD_{50} is above the limit dose (*i.e.*, 48-h LD_{50} > limit dose). In a honey bee acute contact limit test at least 25 bees are exposed to the "limit dose" with the same number of bees in appropriate controls. For most pesticides the limit dose is 25 μg of active ingredient per bee (25 μg a.i./bee). If the expected environmental contact residue level exceeds 25 μg a.i./bee the test should be conducted at a higher dose which for pesticides is equivalent to twenty times the maximum expected dermal contact environmental concentration (see paragraph (e)(4)(ii)(B) of this guideline). Except for the number of treatment groups, an acceptable limit test follows the same test procedures, is the same duration and has the same number of controls as the definitive test (see Table 1 of this guideline). Signs of intoxication, if any, should be reported. At test termination if two or more mortalities occur among the 25 bees tested at the limit dose then a definitive dose-response test should be conducted. If frank sublethal effects are observed at the limit dose beyond the allowable mortality or observation of a sublethal effect(s) (*e.g.*, one bee out of 25 either died or exhibited sublethal effects), then a full definitive test may be necessary. For pesticides, if frank sublethal effects are observed in more than one bee and the limit dose tested was: 1) less than twenty times the maximum expected EEC, then a full definitive study is necessary; or 2) was at least twenty times the maximum EEC, but there

is other evidence or data that indicate a risk to terrestrial invertebrates, *e.g.*, pesticide use incident data, then a full definitive test is necessary.

(e) **Test standards—**

(1) **Test substance**. The substance to be tested should be technical grade unless the test is designed to test a specific formulation, mixture or end-use product. For pesticides, if more than one active ingredient constitutes a technical product, then the technical grade of each active ingredient should be tested separately, in addition to the combination, if applicable. OCSPP 850.3000 lists the type of information that should be known about the test substance before testing, and discusses methods for preparation of test substances.

(2) **Test duration.** The test consists of the administration of the test substance followed by an observation period of 48 h. 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%.

(3) **Test organism—**

(i) **Species.** Honey bee, *Apis mellifera*, is the test species.

(ii) **Source.** Bees may be obtained from on-site colonies or from a commercial apiary. All control and treatment bees used in a test should 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.

(iii) **Age.** The test is conducted using young adult worker bees that are of a similar age and feeding status.

(iv) **Acclimation.** No acclimation period is usually necessary.

(v) **Health status.** Bees used in the test should be in apparent good health. Only bees from apparently disease-free colonies should be used, and they should be kept in conditions conforming to proper cultural practices. Bees treated with chemical substances, such as antibiotics, anti-varroa, *etc.*, should not be used for toxicity tests for four weeks from the time of the end of the last treatment.

(vi) **Care and handling.** During holding and testing, bees should be shielded from excessive activity or other disturbance. Bees should be handled only as much as is necessary to conform to test procedures.

(vii) **Diet and feeding.** A 50% weight/volume (w/v) solution of sugar/water (500 grams/liter) should be provided *ad libitum* throughout the holding and test periods. Purified or distilled water should be used for the sugar solution.

(4) **Administration of test substance.** On the day of test initiation or the evening before, young bees should be 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 in holding cages. Exposure to CO_2 and N_2 gases should be kept to the minimum amount which immobilizes the bees. Dead bees should be rejected and replaced by healthy bees before starting the test. To initiate the test, bees in the holding cages are again immobilized, and distributed into treatment groups of at least 25 bees. A single dose is applied to the dorsal side of the thorax of each bee via microapplicator. Alternatively, test bees may be treated via a dusting apparatus (see paragraph (j)(1) of this guideline).

(i) **Preparation of dosing mixture.**

(A) A solvent is generally used to administer the test substance. The solvent of choice is acetone, although other volatile organic solvents of low toxicity (*e.g.*, dimethylformamide, dimethylsulfoxide) have been used successfully in cases where acetone was not suitable. Maximum dosage volume should not exceed 5 microliters (μL) per bee, to allow for adequate volatilization of the solvent.

(B) If a dusting apparatus is used, for a satisfactory test a nontoxic dust diluent is necessary as a carrier.

(ii) **Treatment levels.**

(A) A minimum of five dosage levels of the test substance should be used in the definitive test. These dosage levels should be spaced geometrically in such a manner so that the entire dose-response curve is characterized (curve between LD_{05} and LD_{90}). The dosage levels should be spaced so that at least three of the doses result in partial mortalities, and that at least one of these three doses kills greater than 50%, and at least one kills less than 50% of the bees. For some test substances (*e.g.*, shallow slope), it may be necessary to use more than five dose levels to achieve these results.

(B) For a limit test, there is single contact dose, plus the appropriate control (see paragraph (d)(5) of this guideline). A limit dose of 25 μg/bee is used unless environmental residues are expected to result in a higher acute contact dose. To calculate the acute contact limit dose (mg a.i./bee) for spray applications of pesticides Equation 1 of this guideline can be used for pesticide uses with a single application and Equation 2 for a use with multiple applications per year. The dietary residue estimates are based on a nomogram that relates food item residues to pesticide application rate; the highest residue level expected is with the broadleaf plants/small insects category (nomogram value of 135, assuming a 1 lb a.i./acre application rate). The nomogram is based on an EPA database called UTAB (Uptake, Translocation, Accumulation, and Biotransformation), a compilation of actual measured pesticide residue values on plants (see references in paragraphs (j)(4) and (j)(5) of this guideline). If there are multiple uses this study is supporting for

registration, the limit dose for the study should be based on the one resulting in the highest dose. The average weight of one honeybee is assumed to be 0.128 grams.

$$Contact\ \ Limit\ \ Dose = 0.128(ApRate)(135) \qquad \textbf{Equation 1}$$

$$Contact\ \ Limit\ \ Dose = 0.128\left(\sum_{i=1}^{n}\left((ApRate)(135)\left(e^{-\left(\frac{0.6931}{halflife}\right)(n-1)(\text{int}\,erval)}\right)\right)\right) \qquad \textbf{Equation 2}$$

ApRate = maximum single application rate (in lb a.i./acre);

Halflife = the foliar halflife (default is 35 days);

Interval = the minimum application interval (in days);

i = application event from 1 to n; and

n = total number of applications.

(5) **Controls.**

(i) Two concurrent controls are included in the test: a negative control and a solvent (or vehicle) control. Control bees are from the same source as the test groups and are kept under the same environmental conditions as treated bees. The test procedures should be the same for control and treated bees, except that negative controls receive no treatment, and solvent (vehicle) controls are treated only with the solvent (vehicle). Solvent control bees should receive a volume of solvent equal to the largest volume administered to the test bees. The use of shared controls is acceptable for concurrent tests as long as the same solvent or vehicle is used for all the tests.

(ii) A test is not acceptable if more than 20% of the control bees die during the test period.

(iii) A concurrent positive control or reference test with a substance of known toxicity is not a condition for an acceptable test. However, a quarterly or semiannual test with a laboratory standard (reference toxicant) is recommended as a means of detecting possible interlaboratory or temporal variation. A laboratory standard is also recommended when there is any significant change in source of bees.

(6) **Number of test organisms and replicates.** In the definitive test, a minimum of 25 bees should be used for each dosage level and for each control. Bees at a treatment level may be divided into replicates if desired. Test organisms are impartially or randomly assigned to dose levels in such a manner that the test results show no significant bias from the distributions.

(7) **Facilities, apparatus and supplies.** Tests should be conducted indoors to control lighting and other environmental variables, with bees being maintained in small test chambers. Test chambers may be constructed of metal, plastic, wire mesh, or cardboard,

or a combination of these materials. Chambers should be constructed so that a vial containing sugar water may be attached.

(8) **Environmental conditions.** Environmental conditions during the test should be maintained as follows:

(i) **Temperature and humidity.** Temperature should be maintained between 25 and 35 degrees Celsius (^{o}C), with relative humidity between 50% and 80%.

(ii) **Lighting and photoperiod.** It is recommended that test bees be maintained in the dark except during dosing and observations.

(9) **Observations—**

(i) **Environmental conditions.** Temperature and relative humidity should be recorded throughout the test.

(ii) **Measures of effect.** Bees should be observed for mortality and any other adverse effects at approximately 4, 24, 48, and if applicable, 96 hours after dosing. All signs of intoxication, other abnormal behavior, and mortality should be recorded throughout the test period and reported by dosage level and by time of occurrence. Signs of intoxication are those behaviors apparently due to the test substance and may include a wide variety of behaviors, such as ataxia, lethargy, and hypersensitivity. All signs of intoxication and any other abnormal behavior that may or may not be attributed to the test substance should be reported. Dead bees should not be removed from the test chambers until the test is terminated.

(f) **Treatment of results—**

(1) **Descriptive summary statistics—**

(i) **Environmental conditions.** Data should be summarized in tabular form, showing the range of temperature and relative humidity during the test, the mean temperature and relative humidity and standard deviation.

(ii) **Mortality.** The number of initial bees at each treatment and control and the number of dead bees should be summarized in tabular form by observation time and treatment, and replicate if applicable.

(iii) **Appearance and behavior.** Number of bees with abnormal appearance or behavior symptoms should be summarized in tabular form by symptom, time of observation, and treatment, and replicate if applicable.

(2) **Percent—**

(i) **Mortality**. Calculate the percent of mortality at each treatment level and control by observation time.

(ii) **Appearance and behavior.** Calculate the percent effected at each treatment level and control by symptom, and observation time.

(3) **Limit test—**

(i) **LD_{50} value.** At test termination (48 hours), if one or fewer bees are dead at the limit dose, the acute contact LD_{50} is considered to be greater than the limit dose (*i.e.*, LD_{50} > limit dose). This is because the Binomial Theorem predicts that when 25 bees are tested, the probability of seeing $\leq$1 dead bee if the true 48-h LD_{50} is at or below the limit dose is $\leq$0.001. Conversely the probability of seeing 2 or more dead bees if the true 48-h LD_{50} is at or below the limit dose is $\geq$0.999. Therefore, if $\leq$1 mortality occurs among the 25 bees tested, the 48-h LD_{50} is reported as greater than the limit dose (*i.e.*, 48-h LD_{50} >25 µg/bee for pesticides).

(ii) **Proportion of mortality ($\hat{p}$).** Assuming mortality follows the binomial distribution, an estimate of the true proportion of mortality ($\hat{p}$) in the laboratory test population as well as confidence bounds on that estimate (see Table A4 of the reference in paragraph (j)(3) of this guideline) can be obtained. For small sample sizes the interval may be large. For example, for a limit test resulting in no mortalities in 25 bees ($\hat{p} = 0$), the 99% confidence interval on the estimate of $\hat{p}$ is (0.00, 0.19) and the 95% confidence interval is (0.00, 0.14). For a limit test resulting in one mortality in 25 bees ($\hat{p} = 0.04$), the 99% confidence interval on the estimate of $\hat{p}$ is (0.01, 0.26) and the 95% confidence interval is (0.00, 0.20). Using the 95% confidence interval as an example, the true (unknown) proportion of mortality will be covered by the calculated confidence interval in 95% of repeated trials. For assessing risks, the confidence in the estimated proportion impacted is considered in determining acute effects at environmental exposure doses. If the uncertainty in $\hat{p}$ is high at the limit dose, and the expected environmental exposure dose is close to the limit dose, risks to threatened and endangered species may not be able to be discounted.

(iii) **Multiple-dose testing.**

(A) At test termination if two or more mortalities occur among the 25 bees tested at the limit dose or there are clinical signs of toxicity, a definitive dose-response test should be conducted. If frank sublethal effects are observed at the limit dose beyond the allowable mortality or observation of a sublethal effect(s) (*e.g.*, more than one bee out of 25 either died or exhibited sublethal effects), then a full definitive test may be necessary. For pesticides, if frank sublethal effects are observed in more than one bee and the limit dose tested was: 1) less than twenty times the maximum expected EEC, then a full definitive study is necessary; or 2) was at least twenty times the maximum EEC, but there is other evidence or data that indicate a risk to terrestrial invertebrates, *e.g.*, pesticide use incident data, then a full definitive test is necessary.

(B) A multiple-dose definitive LD_{50} test may be waived if at test termination no more than 1 bee dies at the limit dose, and there are also no clinical signs of toxicity at the limit dose.

(4) **Multiple-dose definitive test—**

(i) **Dose-response curve, slope and LD_{50}.** Statistical procedures are employed to calculate the 48-h LD_{50} (standard error and 95% confidence limits) based upon mortality. If a dose-response model (*e.g.*, probit) was fit to the data to determine the LD_{50}, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded.

(ii) **NOEL.** While calculation of the NOEL and LOEL is usually not required for acute tests, reporting these values is useful when testing pesticide and industrial chemicals. For pesticides, the reporting of a NOEL value is useful when evaluating risk to Federally listed Endangered and Threatened species.

(iii) **Statistical methods.** Statistical procedures for modeling quantal data are available and should be used. Additional discussion about endpoints and statistical procedures is found in OCSPP 850.3000.

(g) **Tabular summary of test conditions.** Table 1 lists the important conditions that should prevail during the definitive test. Except for the dose levels, Table 1 also lists the important conditions that should prevail during a limit test. Meeting these conditions will greatly increase the likelihood that the completed test will be acceptable or valid.

Table 1.—Summary of Test Conditions for the Honey Bee Acute Contact Toxicity Test

Test type	Acute contact
Test duration	48 h (96 h, if mortality increases >10% between 24 and 48 h)
Temperature	25 - 35 ^{o}C
Relative humidity	50 – 80%
Lighting	Darkness, except during dosing and observations
Test chamber	Metal, plastic, wire mesh, or cardboard
Test solution application	Microapplicator or dusting apparatus
Age of test bees	Young adult worker bees of similar age and feeding status
Number of bees per chamber	Variable
Number of bees per treatment and control	25 (minimum)
Number of dose levels	Minimum of 5 in a geometric series, a negative control, and a solvent control (if solvent used)
Feeding	50% sugar/water (w/v) solution *ad libitum*
Measure of Effect or Measurement Endpoint	LD_{50} based upon mortality at 48 h (96 h, if applicable), sublethal effects

(h) **Test validity elements.** This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 2 occurred. This list should not be misconstrued as limiting the reason(s) that a test could be found unacceptable or invalid. However, except for the conditions listed in Table 2 and in OCSPP 850.3000, it is unlikely a study will be rejected when there are

slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected, the precision of the test is reduced, the power of a test to detect differences is reduced, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly from this guideline, the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 2.—Test Validity Elements for Honey Bee Acute Contact Toxicity Test

1. Test bees were not of similar age and feeding status.
2. More than 20% of the test bees in any control treatment were dead at the end of the test.
3. All bees in a test were not from the same source.
4. The negative (untreated) control [and solvent (or vehicle) control, when a solvent was used] was not included in the test.
5. Test organisms were not impartially or randomly assigned to test chambers.

(i) **Reporting**—

(1) **Background information.** Background information to be supplied in the report consists at a minimum of those background information items listed in paragraph (j)(1) of OCSPP 850.3000.

(2) **Guideline deviations.** Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test.

(3) **Test substance.**

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and concentration of active ingredient(s)).

(ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.

(iii) Methods of preparation of the test substance and the treatment doses used in the range-finding and definitive test, or limit test.

(iv) If a vehicle (*e.g.*, solvent) is used to prepare stock or test solutions provide: the name and source of the vehicle, the nominal concentration(s) of the test substance in the vehicle stock solutions, and the vehicle dose(s) used in the test.

(4) **Test organisms.**

(i) Scientific name, race, and source.

(ii) Culture method and conditions.

(iii) Health status of colonies used for collection of test bees (*e.g.*, any adult diseases, use and application date(s) of any prophylactic or preventative treatments).

(iv) Collection method and date of collection.

(v) Holding period.

(vi) Age at test initiation.

(5) **Test system and conditions.** Description of the test system and conditions used in the definitive or limit test, any preliminary range-finding tests, and any positive control tests.

(i) Description of housing conditions: type, size, and material of test cages.

(ii) Description of any feeding during the test (if applicable), including: method, type of food, source, amount given and frequency.

(iii) Number of bees per test cage.

(iv) Number of cages (replicates) per treatment, negative control, and solvent control.

(v) Methods used for test cage and treatment randomization as well as methods for impartial assignment of bees to test cages.

(vi) Method of test substance application including the body part and volume of test solution applied.

(vii) Test duration.

(viii) Methods and frequency of environmental monitoring performed during the definitive or limit study and any positive controls for test room temperature, humidity and lighting.

(ix) For the definitive, limit, or positive control test, all analytical procedures should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.

(6) **Results.**

(i) Environmental monitoring data results (test room temperature, humidity and lighting) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).

(ii) For preliminary range-finding tests, if conducted, the number of dead and intoxicated bees at each dose level and in the control(s).

(iii) For the definitive or limit test, the number of dead bees at each observation period at each dose level and control(s) (provide the raw data).

(iv) For the definitive or limit test, a description of signs of intoxication and other abnormal behavior, including time of onset, duration, severity, and number affected at each dose level and control(s) (provide the raw data).

(v) Graph the dose-response curves at the end of the test.

(vi) For the definitive study, the slope of the dose-response curve at the end of the test and its standard error and 95% confidence limits.

(vii) For the definitive, limit, and positive control studies provide LD_{50} values, with 95% confidence limits, at each recommended observation time.

(viii) Description of statistical method used, including software package, for determining LD_{50} values and the dose-response model parameters and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(j) References. The references in this paragraph should be consulted for additional background material on this test guideline.

(1) Atkins, E.L., Jr. *et al*., 1954. Equipment and technique used in laboratory evaluation of pesticide dusts in toxicological studies with honey bees. Journal of Economic Entomology 47: 965-969.

(2) Atkins, E.L. *et al.*, 1975. Toxicity of pesticides and other agricultural chemicals to honey bees: Laboratory studies. University of California, Division of Agricultural Sciences, Leaflet 2287, 38 pp.

(3) Conover, W. 1980. Practical Nonparametric Statistics, 2nd Edition. John Wiley & Sons, Inc., New York, NY. 493 pp.

(4) European and Mediterranean Plant Protection Organization (EPPO), 1992. Guideline on Test Methods for Evaluation of the Side-effects of Plant Protection Products on Honeybees (No.170). Bulletin OEPP/EPPO Bulletin 22: 203-215.

(5) Mayer, D. & C. Johansen. 1990. *Pollinator Protection: A Bee & Pesticide Handbook.*Wicwas Press. Cheshire, Conn. p. 161

(6) Organization for Economic Co-operation and Development (OECD), 1998. OECD Guidelines for the Testing of Chemicals, TG 214, Honeybees, Acute Contact Toxicity Test. Adopted 21 September, 1998.

(7) U.S. Environmental Protection Agency, 1982. Pesticide Assessment Guidelines Subdivision L Hazard Evaluation: Nontarget Insects. Office of Pesticides and Toxic Substances, Washington, D.C., EPA-540/9-82-019.

(8) U.S. Environmental Protection Agency, 1985. Hazard Evaluation Division Standard Evaluation Procedure, Honey Bee—Acute Contact LD_{50} Test. Office of Pesticides Programs, Washington, D.C., EPA-540/9-85-002.