Adopted:
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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Honeybees, Acute Oral Toxicity Test

INTRODUCTION

1. This Test Guideline is a laboratory test method, designed to assess the oral acute toxicity of pesticides and other chemicals, to adult worker honeybees. It is based principally on the European and Mediterranean Plant Protection Organization (EPPO) Guideline for evaluating side effects of plant protection products on honeybees (*Apis mellifera*) (1). The suggestions for improvements of the EPPO test, made by the International Commission for Plant-Bee Relationships (ICPBR) at its fifth International Symposium on the Hazards of Pesticides to Bees in Wageningen (the Netherlands) in 1993 have also been taken into account (2). Other existing guidelines have also been considered (3)(4)(5).

INITIAL CONSIDERATIONS

2. In the assessment and evaluation of toxic characteristics of substances, determination of acute oral toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute oral toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (6). Pesticides can be tested as active ingredients (a.i.) or as formulated products.

3. A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

4. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

5. Adult worker honeybees are exposed to a range of doses of the test substance dispersed in sucrose solution. The bees are then fed the same diet, free of the test substance. Mortality is recorded daily during at least 48 hours and compared with control values. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. ≤10%, it is appropriate to extend the duration of the test to a maximum of 96h. The results are analysed in order to calculate the LD_{50} at 24h and 48h (see Annex for definitions) and, in case the study is prolonged, at 72h and 96h.

VALIDITY OF THE TEST

6. For a test to be valid the following conditions apply:

- the average mortality for the total number of controls must not exceed 10 per cent at the end of the test.
- the LD_{50} of the toxic standard meets the specified range.

DESCRIPTION OF THE METHOD

Collection of bees

7. Young adult worker bees of the same race should be used, i.e. bees of similar age, feeding status, etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided as the bees have a changed physiology during this time. If tests must be conducted in early spring or late autumn, bees can be emerged in an incubator and reared for one week with "bee bread" (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

Test cages

8. Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic, disposable wooden cages, etc. Groups of ten bees per cage are preferred. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space.

Handling and feeding conditions

9. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50% w/v) is used as food. After given test doses, food should be provided *ad libitum***.** The feeding system should allow recording food intake for each cage (see paragraph 17). A glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter) can be used.

Preparation of bees

10. The collected bees are randomly allocated to test cages, which are randomly placed in the experimental room. The bees may be starved for up to 2 hours before the initiation of the test. It is recommended that the bees are deprived of food prior to treatment so that all bees are equal in terms of their gut contents at the start of the test. Moribund bees should be rejected and replaced by healthy bees before starting the test.

Preparation of doses

11. Where the test substance is a water miscible compound this may be dispersed directly in 50 per cent sucrose solution. For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). The concentration of the vehicle depends on the solubility of the test substance and it should be the same for all concentrations tested. However, a concentration of the vehicule of 1% is generally appropriate and should not be exceeded.

12. Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used: a solution in water, and a sucrose solution with the solvent/carrier at the concentration used in dosing solutions.

PROCEDURE

Test and control groups

13. The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95% confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2.2, and covering the range for LD_{50}, are required for the test. However, the dilution factor and the number of concentrations for dosage have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate concentrations for dosage.

14. A minimum of three replicate test groups, each of ten bees, should be dosed with each test concentration.

15. A minimum of three control batches, each of ten bees, should be run in addition to the test series. Control batches should also be included for the solvents/carriers used (see paragraph 12).

Toxic standard

16. A toxic standard should be included in the test series. At least three doses should be selected to cover the expected LD_{50} value. A minimum of three replicate cages, each containing ten bees, should be used with each test dose. The preferred toxic standard is dimethoate for which the reported oral LD_{50}-24h is in the range 0.10-0.35 µg a.i./bee (7). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

Exposure

Administration of doses

17. Each test group of bees should be provided with 100-200 µl of 50 per cent sucrose solution in water, containing the test substance at the appropriate concentration. A larger volume is required for products of low solubility, low toxicity or low concentration in the formulation, as higher proportions in the sucrose solution have to be used. The amount of treated diet consumed per group

should be monitored. Once consumed (usually within 3-4 hours), the feeder should be removed from the cage and replaced with one containing sucrose solution alone. The sucrose solutions are then provided *ad libitum*. For some compounds, at higher concentrations rejection of test dose may result in little or no food being consumed. After a maximum of 6 hours, unconsumed treated diet should be replaced with the sucrose solution alone. The amount of treated diet consumed should be assessed (e.g. measurement of volume/weight of treated diet remaining).

Test conditions

18. The bees should be held in the dark in an experimental room at a temperature of 25 ± 2 °C. The relative humidity, normally around 50-70 %, should be recorded throughout the test.

Duration

19. The duration of the test is 48 h after the test solution has been replaced with sucrose solution alone. If mortality continues to rise by more than 10 per cent after the first 24 h, the test duration should be extended to a maximum of 96 h provided that control mortality does not exceed 10 per cent.

Observations

20. Mortality is recorded at 4 h after start of the test and thereafter at 24h and 48h (i.e. after given dose). If a prolonged observation period is required, further assessments should be made at 24 hours intervals, up to a maximum of 96h, provided that the control mortality does not exceed 10 per cent.

21. The amount of diet consumed per group should be estimated. Comparison of the rates of consumption of treated and untreated diet within the given six hours can provide information about palatability of the treated diet.

22. All abnormal behavioural effects observed during the testing period should be recorded.

LIMIT TEST

23. In some cases (e.g. when a test substance is expected to be of low toxicity) a limit test may be performed, using 100 µg a.i./bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, the assessment of the amount of treated diet consumed, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see paragraph 22), these should be recorded.

DATA AND REPORTING

Data

24. Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (8)(9). Plot dose-response curves at each recommended observation time (i.e. 24h, 48h and, if relevant, 72h, 96h) and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95% confidence limits. Corrections for control mortality could be made using Abbott's correction (9)(10). Where treated diet is not completely consumed, the dose of test substance consumed per group should be determined. LD_{50} should be expressed in µg of test substance per bee.

Test report

25. The test report must include the following information:

Test substance:

- physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure);
- chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active ingredient(s)).

Test species:

- scientific name, race, approximate age (in weeks), collection method, date of collection;
- information on colonies used for collection of test bees, including health, any adult disease, any pre-treatment, etc.

Test conditions:

- temperature and relative humidity of experimental room;
- housing conditions including type, size and material of cages;
- methods of preparation of stock and test solutions (the solvent and its concentration must be given, when used);
- test design, e.g. number and test concentrations used, number of controls; for each test concentration and control, number of replicate cages and number of bees per cage;
- date of test.

Results:

- results of preliminary range-finding study if performed;
- raw data: mortality at each dose tested at each observation time;
- graph of the dose-response curves at the end of the test;
- LD_{50} values with 95% confidence limits, at each recommended observation time, for test substance and toxic standard;
- statistical procedures used for determining LD_{50};
- mortality in controls;
- other biological effects observed or measured e.g. abnormal behaviour of the bees (including rejection of the test dose), rate of consumption of diet in treated and untreated groups;
- any deviation from the guideline procedures and any other relevant information.

LITERATURE

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(5) US EPA (1995). Honey Bee Acute Contact Toxicity Test (OPPTS 850.3020). Ecological Effects Test Guidelines. EPA 712-C-95-147, Washington DC, United States of America.

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ANNEX

DEFINITIONS

Acute oral toxicity is the adverse effects occurring within a maximum period of 96h of an oral administration of a single dose of test substance.

Dose is the amount of test substance consumed. Dose is expressed as mass (µg) of test substance per test animal (µg/bee). The real dose for each bee cannot be calculated as the bees are fed collectively, but an average dose can be estimated (totally consumed test substance/number of test bees in one cage).

LD_{50} (median lethal dose) oral, is a statistically derived single dose of a substance that can cause death in 50 per cent of animals when administered by the oral route. The LD_{50} value is expressed in µg of test substance per bee. For pesticides, the test substance may be either an active ingredient (a.i.) or a formulated product containing one or more than one active ingredient.

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