C.17. HONEYBEES - ACUTE CONTACT TOXICITY TEST

1. METHOD

This acute toxicity test method is a replicate of the OECD TG 214 (1998).

1.1 INTRODUCTION

This toxicity test is a laboratory method, designed to assess the acute contact toxicity of plant protection products and other chemicals to adult worker honeybees.

In the assessment and evaluation of toxic characteristics of substances, determination of acute contact toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute contact 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 (1). Pesticides can be tested as active substances (a.s.) or as formulated products.

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

1.2 DEFINITIONS

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

Dose: is the amount of test substance applied. Dose is expressed as mass (µg) of test substance per test animal (µg/bee).

LD_{50} (Median Lethal Dose) contact: is a statistically derived single dose of a substance that can cause death in 50 % of animals when administered by the contact. The LD_{50} value is given in µg of test substance per bee. For pesticides, the test substance may be either an active substance (a.s.) or a formulated product containing one or more than one active substance.

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

1.3 PRINCIPLE OF THE TEST METHOD

Adult worker honeybees (*Apis mellifera*) are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (dr oplets). The test duration is 48 h. If the mortality rate is increasing between 24 h and 48 h 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 96 h. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 24 h and 48 h, and in case the study is prolonged at 72 h and 96 h.

1.4 VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— the average mortality for the total numbers of controls must not exceed 10 % at the end of the test;

— the LD_{50} of the toxic standard meets the specified range.

1.5 DESCRIPTION OF THE TEST METHOD

1.5.1 Collection of bees

Young adult worker bees should be used, i.e. bees of the same age, feeding status, race 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 the time. If tests have to be conduced 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 products, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

1.5.2 Housing and feeding conditions

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. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space. Groups of ten bees per cage are preferred.

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. 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) should be used as food and provided *ad libitum* during the test time, using a bee feeder. This can be a glass tube (ca 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter).

1.5.3 Preparation of bees

The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and time of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

1.5.4 Preparation of doses

The test substance is to be applied as solution in a carrier, i.e. an organic solvent or a water solution with a wetting agent. As organic solvent, acetone is preferred but other organic solvents of low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a weak solution of a commercial wetting agent (e.g. Agral, Cittowett, Lubrol, Triton, Tween).

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, one treated with water, and one treated with the solvent/dispersant.

1.6 PROCEDURE

1.6.1 **Test and control groups**

The number of doses and replicates tested should meet the statistical requirements for determination 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 number of doses 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 doses.

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

A minimum of three control batches, each of ten bees, should be run in addition to the test series. If an organic solvent or a wetting agent is used three additional control batches of each ten bees for the solvent or the wetting agent have to be included.

1.6.2 **Toxic standard**

A toxic standard must 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 contact LD_{50}-24 h is in the range 0.10 - 0.30 µg a.s./bee (2). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

1.6.3 **Exposure**

1.6.3.1 *Administration of doses*

Anaesthetised bees are individually treated by topical application.The bees are randomly assigned to the different test doses and controls. A volume of 1 µl of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After application, the bees are allocated to test cages and supplied with sucrose solutions.

1.6.3.2 *Duration*

The duration of the test is preferably 48 hours. If mortality increases by more than 10 % between 24 h and 48 h, the test duration should be extended up to a maximum of 96 h provided that control mortality does not exceed 10 %.

1.6.4 **Observations**

Mortality is recorded at 4 h after dosing and thereafter at 24 h and 48 h. If a prolonged observation period is required, further assessments should be made, at 24 h intervals, to a maximum of 96 h, provided that the control mortality does not exceeding 10 %.

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

1.6.5 **Limit test**

In some cases (e.g. when a test substance is expected to be of low toxicity) limit test may be performed, using 100 µg a.s./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, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see section 1.6.4) these should be recorded.

2. DATA AND REPORTING

2.1 DATA

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) (3)(4). Plot dose-response curves at each recommended observation time (i.e. 24 h, 48 h and, if relevant, 72 h, 96 h) 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 (4)(5). LD_{50} should be expressed in µg of test substance per bee.

2.2 TEST REPORT

The test report must include the following information:

2.2.1 **Test substance:**

— physical nature and 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 substance(s)).

2.2.2 **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.

2.2.3 **Test conditions:**

— temperature and relative humidity of experimental room;

— housing conditions including type, size and material of cages;

— methods of administration of test substance, e.g. carrier solvent used, volume of test solution applied anaesthetics used;

— test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage;

— date of test.

2.2.4 **Results:**

— results of preliminary range-finding study if performed;

— raw data: mortality at each concentration 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 of the recommended observation times, for test substance and toxic standard;

— statistical procedures used for determining the LD_{50};

— mortality in controls;

— other biological effects observed or measured and any abnormal responses of the bees;

— any deviation from the test method procedures described here and any other relevant information.

3. **REFERENCES**

(1) EPPO/Council of Europe (1993). Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Products - Honeybees. EPPO bulletin, vol. 23, N.1, 151-165. March ,1993.

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(3) Litchfield, J.T. and Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, 99-113.

(4) Finney, D.J. (1971). Probit Analysis. 3rd ed., Cambridge, London and New-York.

(5) Abbott, W.S. (1925). A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol. 18, 265-267.