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Standard Practice for Conducting Subacute Dietary Toxicity Tests with Avian Species[1]

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1. Scope

1.1 This practice describes a procedure for determining the subacute dietary toxicity of a test substance administered to birds in their daily diet. The LC_{50} value time to mortality and slope of the dose response curve may also be derived.

1.2 This practice is applicable to substances that can be mixed uniformly into the diet.

1.3 This practice is intended primarily to be used with the young of the following species: northern bobwhite (*Colinus virginianus*), Japanese quail (*Coturnix japonica*), mallard (*Anas platyrhynchos*), and ring-necked pheasant (*Phasianus colchicus*). Other species or age groups, for example, with wild-trapped birds, may be used with appropriate husbandry modifications to the practice.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific precautionary statements see Section 6.

2. Referenced Documents

2.1 *ASTM Standards:*

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[2]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *LC_{50}*—the statistically derived estimate of the concentration of a test substance in the diet that would be expected to cause 50 % mortality to the test population under the specified test conditions.

3.1.2 *concentration*—the weight of test substance per unit weight of diet.

3.1.3 *substance or test substance*—the element, chemical compound, formulation, known mixture, or material mixed in diets and fed to birds for the purpose of determining an LC_{50}.

3.1.4 *negative control*—a group of birds maintained under conditions identical to the test birds except for the absence of the test substance in their diet.

3.1.5 *positive control*—a group of birds maintained under conditions identical to the test birds except for the replacement of the test substance in the diet with a substance known to elicit a consistent toxic response.

3.2 *Units and Symbols:*

3.2.1 Refer to Practice E 380.

4. Summary of Practice

4.1 This practice describes how to determine the subacute dietary toxicity of a test substance when administered to birds in their daily diet. The median lethal concentration (LC_{50}) in the diet is a measure of a specific toxic effect (that is, lethality). The LC_{50} has been used as a comparable index of toxicity. However, other expressions of toxicity also may be appropriate.

4.2 Groups of birds of the same species are fed diets containing a test substance or mixture of substances at selected concentrations for 5 days. This is followed by a minimum of 3 days (or for as long as the birds continue to exhibit toxic signs) on untreated food. The test substance is mixed into the diets, usually in a geometric series of concentrations.

4.3 General observations of the signs of toxicity and the acceptance of the test substance in the diet also must be reported.

4.4 Concurrent negative controls must be maintained throughout the test. A positive control also may be used.

5. Significance and Use

5.1 This practice provides a means of measuring the susceptibility of an avian species to a test substance in its diet under controlled conditions. The LC_{50} obtained in this test is a conditional measure of subacute toxicity because consumption is voluntary, and because the dietary route may introduce metabolic transformations of the test substance that might be absent in other exposure techniques.

5.2 Use of this practice contributes to the evaluation of the hazards of chemicals to birds because exposure is analogous to most field exposures, that is, through dietary intake.

5.3 The use of this practice allows for observation of signs of toxicity in addition to mortality.

5.4 The dose-response curve provides additional information about the response of birds to a test substance.

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[2] *Annual Book of ASTM Standards*, Vol 14.02.

5.5 This practice can be used to study the effects of test substances in combination in order to simulate situations where birds may be exposed to more than one substance simultaneously **(1).**[3]

5.6 This practice provides one basis for deciding whether additional toxicity testing should be conducted with birds.

6. Precautions

6.1 Contact with all test substances, solutions, and mixed diets should be minimized with appropriate protective clothing, gloves, eye protection, etc. The use of fume hoods and increased ventilation in test rooms is necessary when handling volatile substances. Mammalian toxicity and special handling procedures should be known before this practice is used.

6.2 Disposal of excess test substances, solutions, mixed diets, excreta, and treated birds should be done with consideration for health and environmental safety, and in accordance with all federal, state, and local regulations.

6.3 Cleaning and rinsing of glassware, feeders, and other equipment with volatile solvents should be performed only in well-ventilated areas.

6.4 Periodic medical examinations should be considered for all personnel caring for birds or handling test substances.

7. Facilities

7.1 Species requirements will vary, but pens and cages should include adequate room, clean food and water, heated areas for young birds, and protection from excessive disturbance. Space requirements have not been standardized for species normally used in this test. However, adherence to the general guidelines and principles found in the National Institutes of Health and the National Academy of Science publications **(2, 3, 4)** in addition to literature published on individual species should provide a basis for a humane approach to space requirements. Pens or cages must be placed so as to prevent cross-contamination **(5).**

7.2 Construction materials in contact with birds should not be toxic, nor be capable of adsorbing or absorbing test substances. Materials that can be dissolved by water or loosened by pecking should not be used. Stainless or galvanized steel, or materials coated with plastics are acceptable, but other construction materials may also be useful. Any material or pen shape is acceptable provided the birds are able to move about freely and that pens can be kept clean.

7.3 Ventilation, photoperiod, and relative humidity requirements vary little among test species, and these factors are particularly critical to the well-being of young birds. Relative humidity should be maintained at 45 to 70 %. Higher humidities may be appropriate for waterfowl. Photoperiod should be a minimum of 14 h of light. The amount and duration of heat for brooding is species specific **(6, 7).** A temperature gradient from approximately 38°C to approximately 22°C from an appropriate heat source should be established in brooders in order to allow the birds to seek a proper temperature. Ventilation should follow guidelines in *Guide for the Care and Use of Laboratory Animals.* Ventilation should be sufficient to supply 10 to 15 air changes per hour **(2).**

8. Diets

8.1 Dietary requirements vary according to the species and age of the test birds. Any unmedicated commercial diet that meets the minimum nutritional standards of the test species **(8)** is sufficient.

8.2 Contaminated feed may compromise a study **(9, 10, 11)**; therefore, feed should be analyzed periodically to identify background contaminants. Analysis may be especially important if the substance being tested is known or suspected of synergistic or antagonistic action with possible contaminants. Maximum allowable levels of heavy metals, pesticides, and other contaminants in feed have not been established.

8.3 Test diets should always be fresh and clean. The frequency that the diet is changed during a study is dependent upon the physical and chemical properties of the test substance, and the speed with which a test animal contaminates the feed with fecal matter or water, or both.

8.4 Test diets should be fed *ad libitum.*

8.5 Feed should not be used past its normal shelf life (usually 90 days).

8.6 Treated test diets should be stored so as to maintain the stability of the test substance in the diet.

9. Test Substance and Diet Preparation

9.1 Knowledge of the physical, chemical, and biological properties of the test substance is important in test diet preparation.

9.2 Test diets can be prepared by mixing the test substance directly into the feed or by dissolving or suspending the test substance in a solvent or carrier prior to mixing with the feed. The use of solvents or carriers may be necessary to achieve a uniform mix of the test substance in the feed.

9.3 The test substance is uniformly mixed into the diet. The physical and chemical properties of a test substance may cause variation in test diet concentrations and it is important to ensure that the test substance is available in the diet at the same concentration throughout the treatment period.

9.4 In addition to homogeneity and stability testing required by GLPs, it is recommended that concentrations of the test substance in the diet be confirmed by analysis at the beginning of the test.

10. Test Organisms

10.1 This practice is intended primarily to be used with the young of the following species: northern bobwhite (*Colinus virginianus*), Japanese quail (*Coturnix japonica*), mallard (*Anas platyrhynchos*), and ring-necked pheasant (*Phasianus colchicus*). Other species may be used, but changes in diet, caging, and other factors may be necessary **(12, 13)**.

10.2 If laboratory or commercially reared birds are used in this practice they must come from the same source, and be of the same age, because different strains or age cohorts can introduce variability into the test. These birds should be similar in appearance to a wild species. The parentage and dietary history of purchased birds should be known. If captured wild birds are used, they should come from the same source and be of similar maturity.

[3] The boldface numbers in parentheses refer to the list of references at the end of this practice.

10.3 Birds that are deformed, injured, emaciated, or phenotypically different from normal birds must not be used as test animals. The population of birds from which the test animals (treated and control) are selected shall be considered unsuitable for testing if mortality exceeds 5 % during the 3 days prior to testing.

10.4 The preferred age for Japanese quail and northern bobwhite is 14 days; for ring-necked pheasants, 10 days; and for mallards, 5 days **(17).** The preferred ages are based on the probability that test birds of these ages will not survive for 5 days without eating (see 12.1.4). Tests with younger or older birds also can be used to determine the LC_{50} **(14, 15, 16).** If data from one test are to be considered comparable with data from another test, the ages of birds between the two tests should deviate no more than one or two days.

10.5 Young birds of the species listed in 1.3 shall be conditioned to the test parameters of caging, food, water, and photoperiod from the time they hatch or are acquired until the initiation of the test. An acclimation period of at least 3 days is required (see 10.3). Older birds shall be conditioned for at least 7 days.

11. Procedure

11.1 *Range-Finding Test*:

11.1.1 To determine the test concentrations to be used in a definitive test, a range-finding test may be conducted for 5 days using three to five widely spaced concentrations.

11.1.2 One procedure is to use an initial concentration of at least 5000 ppm with two to four geometrically spaced lower concentrations. If there is no mortality at the 5000-ppm level, and test procedures and numbers of birds per concentration are the same as would be used in a definitive test, then the range-finding test may provide sufficient information to negate the need for a definitive test. If mortality does occur, then range-finding will suggest the approximate test concentrations to be used in a definitive test.

11.2 *Definitive Test*:

11.2.1 Individual test birds should be randomly assigned to groups and to control and test diet concentrations. Assignment to groups and initial weighing of the test birds should be done at the same time to avoid needless handling stress.

11.2.2 Water, and treated or untreated diets, should be available *ad libitum*.

11.2.3 The experimental (test and control) diets are available for 5 days after which they are replaced with untreated feed. Birds are held for a minimum of 3 days following treatment. In some situations, it may be necessary to extend the observation period in order to investigate prolonged or delayed effects.

11.2.4 Body weight must be recorded at the initiation and conclusion of the treatment and observation phases. Feed consumption must be recorded for both the treatment and observation phases; it is recommended that consumption during the treatment phase be recorded separately for the first two days and the last three days. Additional information may be gathered by measuring feed consumption daily. If the study continues beyond 8 days, body weight and feed consumption should be recorded weekly. Mortality, behavioral abnormalities, and other signs of toxicity should be recorded each day during the test.

11.2.5 Photoperiod during the test should be the same as during the conditioning period.

11.2.6 A minimum of 10 birds for each test concentration constitutes a treatment group, but groups may be subdivided into replicates with a minimum of five birds per replicate. The test concentrations should be geometrically spaced so as to result in 10 to 90 % mortality. Acceptable test results should have one concentration that kills more than 0 % but less than 50 % and one that kills more than 50 % but less than 100 %. These results usually can be obtained with four to six treatment levels. If it is necessary to extrapolate above or below the LC_{50} then three or more concentrations having partial mortality are desirable. However, test substances having steep dose response curves may make it difficult to obtain such results. Depending upon the characteristics and intended use of the test substance, fewer treatment levels with partial mortality may be acceptable.

11.2.7 Concurrent negative control groups are required. Natural mortality and genetic variability of the bird strain will determine the number of control birds. The number of birds in each control group pen should equal the number of birds in each treatment group pen. A minimum of 20 control birds is required. If the minimum number of negative control birds is used, they will be divided among at least four replicates. If more negative control birds are used, they should be divided among at least three replicates. If any of the species listed in 1.3 are used, any control mortality greater than 10 % is unacceptable and the test should be repeated. When variability and natural mortality are not adequately known for a given species or strain, additional control birds should be used, and the number of negative controls should be equal to the total number of treated birds. The LC_{50} value then may be adjusted for control mortality by using Abbott's formula **(20)**.

$$A = \frac{E - C}{100\ \% - C} \times 100$$

where:

A = corrected percent mortality,
E = percent mortality in an experimental group, and
C = percent mortality in controls.

Solvents, suspending agents, or other carriers added to the test diets must be added to the control diet at the same time and at the maximum concentration used in the test diets.

11.2.8 Positive controls can be useful in indicating differences in the toxic response of a given strain of birds or to compare differences in test results from different strains or laboratories. Whenever a source or strain of birds is changed or if there is reason to suspect that the response of a given strain has changed, then the use of a positive control is advisable. For laboratories maintaining their own colony, an occasional (twice per year) use of positive controls should help detect changes in the strain or in laboratory procedures. An ideal substance for positive controls should yield consistent results and have a mode of action similar to that of the test substance with which it is compared. At this time, dieldrin is the standard choice when a positive control is used, although dicrotophos has been used when testing suspected cholinesterase inhibitors. The

positive control is conducted under conditions identical to those used for test substances.

12. Limitations and Interpretations

12.1 The test described in this practice is designed, as an initial screening test, to determine the subacute dietary toxicity of a test substance to birds. Limitations and other considerations of this procedure are necessary to place this test in perspective.

12.1.1 The dietary route of exposure is important for wild birds. However, wild birds may be exposed to toxic substances by routes other than dietary, and other test methods may be necessary to evaluate potential hazards.

12.1.2 This test is not a chronic test and it is not designed to measure long-term effects of test substances on birds.

12.1.3 The toxic effects of a test substance in one species are not necessarily representative of the effects in another species. However, the use of several species of birds will establish general toxicological trends. Routine testing on more than a few species may be impractical.

12.1.4 Feed consumption is an important part of the interpretation of test results.

12.1.4.1 Decreased feed consumption due to the toxicity of the test substance is a normal phenomenon.

12.1.4.2 Sensory perception of the test substance may cause birds to refuse to eat the treated feed. Starvation, and not the test substance, may then be the primary cause of mortality. Refusal to eat contaminated food in a natural situation may cause birds to switch to noncontaminated food. Thus, experimental results may not always be representative of a field situation.

12.1.4.3 When the test substance is judged to cause feed avoidance, special studies to examine acceptance should be considered.

12.2 The LC_{50} computed using this practice is a measure of the toxicity of the test substance to birds under the conditions of the test. The methodology simulates a route of exposure birds might receive in the wild, however, the results of this test cannot always be predictive of potential adverse effects a test substance may present to birds in the wild. Knowledge of the physical, chemical, biological, and other factors contributing to the presence of a test substance in the environment is necessary to assess potential adverse effects to birds.

13. Quality Assurance

13.1 In order to ensure the quality and reliability of data developed using this practice, good laboratory practices should be followed **(18, 19, 20).**

14. Report

14.1 The report should include the following:

14.1.1 Name of the investigator, laboratory, laboratory address, location of raw data, and date when the test was started and finished.

14.1.2 Description of the species tested, including scientific name, source, age of the birds at the beginning of the test, and weights of birds at the beginning and end of the exposure and postexposure periods. If individual bird weights are measured, the extremes, mean, and a measure of variance should be included.

14.1.3 Description of the housing conditions (including test cages, room and brooder temperatures, light cycle, and humidity, if measured).

14.1.4 A description of the feed including proximate analysis, concentrations of contaminants and detection levels (if measured), name and source of feed. Any medication added to feed should be identified and its use justified.

14.1.5 A detailed description, to the extent known, of the test substance including its chemical name, structure, formulation, purity, source, batch, lot number, and physical appearance.

14.1.6 The dietary concentrations, respondents per concentration, number of birds and replicates per concentration, and the name of any substances used as a positive control; feed consumption, body weight, and signs of toxicity; the calculated LC_{50} value, 95 % confidence limits, slope of the dose-response curve, and the name and reference of the statistical method used (acceptable statistical methods for calculating the LC_{50} may be found in the following references: **21, 22, 23, 24, 25, and 26);** the highest treatment level at which no signs of toxicity are observed; anything unusual about the test, any deviations from recommended procedures; and other relevant information.

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