# Utility of Bioassay in the Determination of Pesticide Residues

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A review of bioassay techniques for the quantitative determination of pesticide residues has been made. Several organisms are in use. Selection depends on the pesticide used, reproducibility, indication of the true quantity of toxicant present, cost, speed of assay, and other practical limitations. The types of exposure, responses measured, sensitivity, and complexity vary with different methods using the same organism and with different organisms. Though possibly less specific than chemical methods, bioassay offers the opportunity to determine the presence of toxic metabolites which may be bypassed by more specific methods.

Bioassay, in its broadest sense, may refer to any method in which some property of a material is measured in terms of a biological response. In the agricultural pesticides field, practically all chemical or biological control programs involving response of an organism to a chemical may be called bioassays. Hoskins (*51*) has divided bioassay into three groups: screening and formulation, resistance, and residue analysis. Bioassay will be defined here as the quantitative determination of a toxicant using biological material to measure the response as compared with a standard under standardized conditions.

Bioassay, often thought of as a new tool, has been used by pharmacologists since the turn of the century (*40*) to assay vitamins and drugs. In the pesticide field, it was used to evaluate rotenone residues and breakdown in the 1930's (*55*, *105*). Its use was revived by Laug (*60*) in 1946 for the determination of DDT residue in animal tissue using the house fly (*Musca domestica* L.). More recently, the discovery of many more highly toxic pesticides, and new regulations governing their use on or in edible commodities, have greatly stimulated the search for simple, sensitive, and specific methods to determine trace amounts of these chemicals. Chemical analysis has long been recognized as the standard method of determining pesticide residues. However, relatively few chemical methods are simple (*94*), or highly sensitive (*17*, *38*, *61*, *92*, *98*). Many are highly specific (*9*, *42*, *87*). If a material is new, no chemical method may be available, or if a method is otherwise satisfactory, adequate equipment may be lacking (*38*, *86*). For these reasons, many interested in pesticide residues have turned to bioassay.

The main problem with bioassay, as with chemical methods, is the difficulty of determining extremely small quantities of toxicants in the presence of large amounts of plant and animal tissue. The basic principles of quantitative determination used in analytical chemistry apply to bioassay. Gunther and Blinn (*45*) and Van Middelem (*99*) have reviewed these principles as they apply to pesticide residues.

## Suitable Organisms for Bioassay

Theoretically, any susceptible organism may be used to determine pesticide residue. However, the sensitivity required of the organism may range from 1.0 down to 0.1 p.p.m. or less of the pesticide. Relatively few organisms can meet this requirement. Further, the organism must be easy to rear or readily available in large numbers throughout the year. It must be easy to handle, easily produced to obtain a known age, stage, and uniform vigor, and should have a low natural mortality. The response curve should be relatively steep, reproducible, and a true measure of the toxicant. Other considerations include freedom from toxicity of plant extractives and other background interferences, space requirements, cost, speed of assay, versatility, and amount and type of help needed.

In the bioassay of pesticide residues, insects, microcrustaceans, fish, bacteria, yeast, fungi and other microorganisms, higher plants, and enzymes have been used.

**Housefly.** Houseflies were used by Laug (*60*) in 1946 and, since, they have been widely used to determine insecticide residues. Flies are easily reared in large numbers, are easy to handle, and have a relatively short life cycle. Although the males are usually more susceptible than the females (*60*), either or both sexes can be used. They are relatively unaffected by extractives or pesticides other than insecticides (*90*). Houseflies have been used to determine residues of at least 15 different insecticides (Table I), on or in 24 different fruits and vegetables (Table II), and in milk, cream, and several animal tissues (Table III).

**Mosquito Larvae.** Mosquito larvae have been demonstrated to be extremely sensitive to insecticides. Woke (*105*), Simmons (*87*), and Nolan and Wilcoxon (*71*) were among the first to use mosquito larvae for the determination of insecticide residues. Although *Aedes agypti* (L.) (*13–17*) have been used most frequently, *A. vexans*, *A. sticticus* (Meig.) (*14*), *Anopheles quadramaculatus* Say (*17*), *Culex quinquefasciatus* Say (*18*, *93*, *94*), and *C. fatigens* Wied. (*105*) have also been used. Mosquitoes are more difficult to handle and rear than houseflies, as most of them require a blood meal for egg production. They are subject to considerable variation unless special precautions are observed (*15*, *43*) and are sensitive to natural constituents extracted from plant or animal tissue (*90*). Although their extreme sensitivity is usually an advantage, they are susceptible to contamination and special care may be necessary to prevent loss of the colony. Mosquito larvae have been used on at least 18 different insecticides (Table I) for residues on or in 12 different fruits and vegetables (Table II), and in milk and animal tissues (Table III).

**Drosophila.** The use of adult fruit flies (*Drosophila melanogaster* Meig.) for determining insecticide residues was first reported by Pankaskie and Sun (*76*) in 1952. Both winged and vestigial forms have been used. *Drosophila* are more easily reared and handled in large numbers, require less rearing space, and have a shorter life cycle than houseflies or mosquitoes. Although they are about as sensitive as mosquitoes, the culture methods make them less subject to contamination. *Drosophila* have been used

**Table I. Insecticides and Some Bioassay Organisms Used in Residue Determination, with References**

| Insecticide | Housefly | Mosquito Larvae | Drosophila | Daphnids | Brine Shrimp |
|---|---|---|---|---|---|
| Allethrin | | (13) | | | |
| Aldrin | (9, 22, 56, 59–62, 91, 92) | (13, 14, 48, 93) | (26, 27, 33, 42, 76, 91) | (58) | |
| Gamma BHC (lindane) | (39, 51, 54, 59, 61, 99) | (14, 48, 93, 94) | (26, 73, 107) | (58, 101) | (69) |
| Chlordan | (59, 99) | (13, 48, 49, 93) | (26, 27, 107) | (58) | (69) |
| Demeton | | | (26) | | |
| DDT | (50, 59, 60, 62, 91, 99) | (13, 14, 87, 93, 94) | (26, 76, 91, 107) | (58, 101) | (69) |
| Dilan | | (13) | | | |
| Diazinon | (59) | (94) | | (58) | |
| Dieldrin | (9, 30, 59, 78, 89, 91, 92) | (13, 48) | (26, 27, 33, 42, 76, 91, 106) | (58) | |
| Endrin | (57, 59) | | (26) | | (69) |
| EPN | (38) | (93, 94) | | | |
| Heptachlor | (59) | (13, 48, 49) | (26, 107) | | |
| Isodrin | (59) | | (26) | | |
| Lead arsenate | | (47) | (106) | | |
| Malathion | (98) | (94) | (26, 106) | | |
| Methoxychlor | | (13, 14, 48, 94) | | | (69) |
| Parathion | (38, 50, 70, 71 98) | (70, 93, 94) | (26, 106) | (70, 101) | |
| Perthane | (59) | | | | |
| Schradan | | (13, 14) | (26) | | |
| Strobane | | (13) | | | |
| TDE | | (13, 48, 93, 94) | | | |
| TEPP | | (47) | | | |
| Toxaphene | (20, 59, 99) | (13, 48) | | | (69) |

**Table II. Plant Materials and Some Bioassay Organisms Used in Residue Determination, with References**

| Crop | Housefly | Mosquito Larvae | Drosophila | Daphnids |
|---|---|---|---|---|
| Alfalfa | (20–22) | | (76, 91) | |
| Apples | (59) | | (26, 27, 33, 76, 91) | |
| Beans | (59, 98) | | (26) | |
| Beets | (50) | (94) | (26) | |
| Broccoli | (57) | (94) | (26) | |
| Brussel sprouts | | | (26) | |
| Cabbage | (51) | (49) | (26) | |
| Carrots | (50, 59) | (49, 94) | (26, 42, 76, 91) | |
| Cauliflower | | | (26) | (58) |
| Celery | (98) | | (26) | |
| Clover | | (94) | | |
| Collards | (57) | | | |
| Corn | (50, 59) | (49) | (26) | |
| Cucumbers | (59) | | | |
| Kale | (57) | | | |
| Lettuce | | | (26) | |
| Onions | | (49, 94) | (26, 27, 76, 91) | |
| Peaches | (59, 98) | | | (58) |
| Peas | (59) | | (26, 27) | |
| Pears | (59) | | | |
| Peppers | (57) | | | |
| Potatoes | (50, 59) | (49, 94) | (26, 27, 73, 76, 91) | |
| Prunes | (59) | | | |
| Spinach | (59, 99) | (13) | (26) | |
| Squash | | | (26) | |
| Strawberries | | (94) | (26) | |
| Tomatoes | (50, 59) | | (26, 27) | |
| Turnips | | (49) | (76, 91) | |

**Table III. Animal Products—Tissues and Some Organisms Used in Residue Determination, with References**

| Product | Housefly | Mosquito Larvae | Drosophila |
|---|---|---|---|
| Milk | (22) | (17) | (91) |
| Cream | (19, 39) | | |
| Butterfat | | (17) | |
| Bile | (22) | | |
| Blood | (22) | | |
| Brain | (59) | | (91, 97) |
| Fat | | | |
| Renal | (78) | | (91) |
| Omental | (78) | | |
| Perirenal | (22, 59, 60) | | |
| Feces | (22) | | |
| Heart | (59) | | |
| Kidney | (22, 59, 60) | | (91, 97) |
| Liver | (22, 60) | | (91, 97) |
| Muscle | (22) | (17) | (91) |
| Chicken flesh | (52) | | |

on at least 14 different insecticides (Table I), for residues on or in 20 different fruits and vegetables (Table II), in milk and several animal tissues. *Drosophila* offer more versatility than most organisms. They may be exposed directly to macerated crops or whole soil without extraction, or to extract films (*26, 42, 76, 91, 106, 107*).

**Other Insects.** Several other insects have been used for determining insecticide residues, but on a much more limited basis than those discussed. Some of the insects used were the red flower beetle [*Tribolium castaneum* (Hbst.)] (*87, 101*), *Macrocentrus ancylivorus* Roh. (*34, 37, 79*), and Japanese beetle larvae (*Popillia japonica* Newm.) (*34–37*). Sun (*90*), in a search for sensitive insects for bioassay, studied the German cockroach [*Blatella germanica* (L.)], the confused flower beetle (*Tribolium confusum* Duv.), the red harvest ant [*Pogonomyrmex barbatus* (F. Smith)], and the large milkweed bug [*Oncopeltus fasciatus* (Dall.)]. Susceptibility of these insects to dieldrin descends in the order named. None was as sensitive as houseflies or *Drosophila*.

**Microcrustaceans.** Two *Daphnia* species have been used. *Daphnia magna* was reported by Anderson (*1*) in 1945 to be extremely sensitive to DDT. It is easily reared in large numbers and occupies little space for rearing (*2*). Sun (*90*) has suggested *Daphnia* as a possibility for bioassay of copper- and mercury-containing fungicides based on the high sensitivity, reported by Anderson (*3*), to salt solutions of cupric chloride, cupric ammonium chloride, silver nitrate, mercuric chloride, and cadmium chloride. *Daphnia pulex* (de Geer) has also been used for determination of residues of Diazinon in cauliflower, Isolan in peaches, parathion in cherries, and aldrin, BHC, chlordan, DDT, and dieldrin (*58*).

The fresh water shrimp (*Gammeus pulex* de Geer.) was used by Callaway, Dirnhuber, and Wilson (*18*) for determining quantitatively the presence of cholinesterase-inhibiting phosphates. The brine shrimp (*Artemia salina*), a salt water crustacean, has been found by Michael, Thompson, and Abramovitz (*69*) to be highly sensitive to insecticides. One of the advantages claimed for this organism is the reduction of rearing time to a minimum as the eggs may be purchased at most tropical fish stores. Variation in sensitivity has been reported.

**Fish.** Guppies (*Lebistes reticulatus* Peters) have been used by Pagan and Hageman (*75*) for detecting small quantities of DDT added to beans and tomatoes. Pagan (*74*) previously selected guppies because of their high sensitivity, availability in large numbers, uniformity of development, and ease of handling.

Goldfish (*Carassius auratus*) have been used on a limited basis to determine pesticide residues. Davidow and Saba-

tino (*24*) used them for making qualitative assays and Davidow and Schwartzman (*25*) used them for the identification and quantitative determination of endrin residues in kale, broccoli, and pepper extracts.

Top minnows (*Gambusia affinis*) have been used in the study of insecticide toxicity (*72*), but have escaped attention as a bioassay organism. Their response is reported to be more uniform than that of gold fish (*44*).

**Bacteria, Yeast, Fungi, and Other Microorganisms.** The fungi, *Glomerella cingulata* (Stoneman) and *Helminthosporium victoriae* Meehan and Murphy, were first used by Leben and Keitt (*63*) to determine small quantities of thiram. Richardson (*82*) also found *G. cingulata* sensitive to ziram and sodium dimethyl dithiocarbamate.

The yeast, *Saccharomyces pastorianus*, was used as a test organism by Prescott, Emerson, and Ford (*80*) to determine residues of the antibiotic fungicide cyclohexamide on cherries in the presence of dieldrin, methoxychlor, lead arsenate, basic copper, and glyodin without interference. Ferbam and captan were found to interfere. Residues as low as 0.04 p.p.m. on the fruit were detected with the yeast.

The bacterium, *Bacillus subtiles*, was found by Thornberry (*96*) to be more sensitive to copper 8-quinolate than to five other fungicides. Chloranil and dichlone completely inhibited growth at the concentrations used. The sensitivity for copper 8-quinolate may not be adequate for residue determinations.

Numerous bacteria, yeast, and protozoa were investigated by Angelotti *et al.* (*4*) for sensitivity to DDT, nabam, BHC, lindane, and parathion residues. They found *Micrococcus pyozenes* var. *epidermidis* to show a graded response to nabam. Ayers (*8*) investigated the effect of aldrin, dieldrin, chlordan, heptachlor, and lindane residues on the growth of several bacteria and fungi. Inhibition of growth of *Sarcina lutea* resulted from aldrin, heptachlor, and chlordan at levels of 1 p.p.m. or less.

**Plants.** Pinto beans (*Phaseolus vulgaris*) were used by Dimond and Waggoner (*28*) to determine quantitatively the mercury vapor arising from a phenyl mercury compound. The assay is based on the prevention of abscission in bean explants. The method required no special equipment, was simple, rapid, inexpensive, highly sensitive (60 to 900 $\gamma$ per cubic meter), and free from interferences which made other methods unsatisfactory. The method requires 4 days to complete.

Many plants are sensitive to herbicides and several quantitative methods for the study of toxicity have been reported. Little or nothing has been published on quantitative methods for detecting herbicide residues. A few methods have been used that may be adaptable to residue studies because of their high sensitivity to herbicides or growth regulators in use.

Weintraub *et al.* (*102*) used Black Valentine beans (*P. vulgaris*) in a curvature response of the decapitated bean epicotyl which they report as sensitive to 0.04 $\gamma$ of 2,4-D. The reported advantages of this method are relatively high sensitivity, precision, and simplicity. Another method using Black Valentine beans measures the repression of leaf growth quantitatively (*12*). Sensitivity reported was 0.005 to 0.05 $\gamma$ of 2,4-D per plant. Both of these methods use potted plants and have the disadvantage of requiring considerable space and facilities for growth and holding of the plants.

Ries (*83, 84*), studying the toxicity of isopropyl *N*-(3-chlorophenyl)carbamate (chloro-IPC), used the amount of root elongation of buckwheat (*Fagopyrum esculentum* or *F. saggittatum*), as a criterion. Buckwheat was selected because of its rapid germination and growth, sensitivity, and ease of measurement. Root extension was significantly inhibited by concentrations as low as 0.02 p.p.m.

Luckwell (*68*) developed a tomato ovary test for 2-napthoxyacetic acid. This method is not specific for auxins and may be valuable for fruit setting agents, although the sensitivity is somewhat low based on his study.

The best possibilities for bioassay of herbicide residues are probably those methods utilizing root growth or inhibition measurements, germination, or growth of the coleoptile or stem of sensitive plants. Leopold (*65*) on the basis of response to indole acetic acid has given the sensitivity range in micrograms for 10 readings as follows: pea root growth (*66*), 0.00001 to 0.1; root inhibition (7), 0.0001 to 1.0; *Avena* coleoptile curvature (*104*), 0.00026 to 0.0026; slit pea stem (*103*), 0.25 to 250.; pea and *Avena* growth tests (*11, 95*), 0.1 to 100; green bean stem (*102*), 0.001 to 10; and the leaf repression test to 2,4-D (*12*), 0.005 to 5.

**Enzymatic Methods.** These are also a type of bioassay. They have been discussed in detail by Giang (*41*) and will not be discussed here.

### Sensitivity

Sensitivity usually refers to the minimum amount of toxicant which can be detected in significant quantities. In bioassay, sensitivity varies with the organism, test method, type of exposure (*60*), length of exposure period (*54, 60*), size of exposure chamber (*47, 93*), crop or tissue used, presence of interfering substances, and toxicants. Within particular organisms such as insects, sensitivity may vary with sex (*33, 60*), age (*14, 33, 92*), stage (*2, 14, 70*), size (*52, 58, 60*), food (*14, 43*), temperature, and criterion of response. Comparison of the sensitivity of one organism with another or of bioassay with chemical methods is difficult unless it is determined on the same samples.

Sun and Pankaskie (*91*) compared the sensitivity of houseflies and *Drosophila* on extract films and concluded *Drosophila* to be four to nine times more susceptible to some insecticides than the housefly. They point out, however, that when *Drosophila* were used for detecting milk residues by the direct feeding method, they were far less sensitive than houseflies to aldrin, dieldrin, DDT, and the gamma isomer of BHC. From interpolation of data presented by Hartzell and Storrs (*48*), Sun and Pankaskie concluded *Drosophila* to be about as sensitive as mosquito larvae exposed in aqueous suspension for 20 hours (Table IV). However, if the photomigration technique (*14*) is used, mosquitoes are more sensitive than *Drosophila*. Hartzell and Storrs (*48*) point out in their early work with exposure of mosquito larvae in aqueous solutions for 20 hours that the range of sensitivity for most of the insecticides falls between 0.01 and 0.1

**Table IV. Comparison of Chemical and Bioassay Sensitivity**

| | | Bioassay, $\gamma$ | | | | |
|---|---|---|---|---|---|---|
| | | $LD_{50}$ | | | | $T_{50}$[a] |
| | Chemical[b] | Housefly,[c] | Drosophila[c] | | Mosquito,[d] | Mosquito, |
| Insecticide | Lower Limits | extract/ jar | Direct/ jar | Extract/ 15 ml. | death/ 15 ml. | opt. range/ 100 ml. |
| Aldrin | 1–2[e] | 1.2 | 2.0 | 0.25 | | 0.5–20 |
| Gamma BHC | 100[f] | 4.0 | | 0.45 | 1.5 | 1–20 |
| DDT | 10[f] | 60.0 | 100 | 10.0 | | 0.5–10 |
| | 10[g] | | | | | |
| Dieldrin | 1–2[e] | 0.75 | 2.0 | 0.2 | | |
| Parathion | 4[f] | | | | 0.045 | 2–20 |
| | 2[g] | 3.0/50 ml.[h] | | | 0.002/1.5 ml.[h] | |

[a] (*14*).
[b] (*44*) p. 89.
[c] (*91*).
[d] (*48*).
[e] Infrared.
[f] Ultraviolet.
[g] Colorimetric.
[h] (*70*).

p.p.m. in water solutions and, in processed food, from 0.5 to 5.0 p.p.m. Later Burchfield and Hartzell (*13*), using the photomigration technique, concluded that a 60-minute exposure period or less should indicate the absence of many insecticides on or in food in amounts greater than 0.1 p.p.m., and that some insecticide residues can be estimated with reasonable accuracy as low as 0.002 p.p.m.

Wasserburger (*101*) reports *Daphnia magna* as being more sensitive than *Musca* or *Drosophila* to DDT, BHC, and parathion. Kocher, Roth, and Trebaux (*58*) consider *Daphnia pulex* (de Geer) more sensitive than *D. magna* as an organism for insecticide assay. However, Newman (*70*) has reported the approximate quantities of parathion to produce $LD_{50}$ as follows: *D. pulex* and *G. pulex*, 5 γ; *Musca domestica* (Oral), 3 γ; *Aedes agypti* (3rd instar), 0.5 γ; and *A. agypti* (1st instar), 0.002 γ. Davidow and Schwartzman (*25*) obtained comparable sensitivity of houseflies and goldfish to endrin at 0.25 p.p.m. Odum and Summerford (*72*) found the minnow (*Gambusia affinis*) more susceptible than gold fish to DDT.

Leben and Keitt (*64*) used the fungus *G. cingulata* when thiram residues were in the range of 2.5 to 20 γ per ml. and *H. victoriae* for quantities larger than 20 γ per ml. Richardson (*82*), using *G. cingulata* and the same test method, reports a sensitivity range of 1.25 to 20 p.p.m. to thiram, 0.1 to 10 p.p.m. to sodium dimethyl dithiocarbamate, and 1.0 to 100 p.p.m. to ziram. Other methods using leaf disks (*63*) and seed (*5, 6*) were less sensitive.

Relatively little information is available comparing the sensitivity of bioassay and chemical methods. An attempt has been made to compare some of this information in Table IV. Values given for the chemical limits are based on the lower limits, while the bioassay data are based on $LD_{50}$ or $T_{50}$. Although $LD_{50}$ or $T_{50}$ is the most accurate figure, satisfactory reproducibility can be obtained at considerably lower limits. As an example, the optimum $T_{50}$ range is from 0.5 to 20 γ (Table IV) of lindane; yet Burchfield and Hartzell (*13*) state residues can be estimated as low as 0.2 γ. The $LD_{50}$ for aldrin to houseflies is 1.2 γ (Table IV) per jar; Sun (*90*) reports the sensitivity as 0.23 γ per jar. The addition of small sublethal doses of toxicant to a sample has been used to bring the mortality into the toxicity range to permit measurement of small sublethal quantities which otherwise might be overlooked (*26, 33, 92*).

When desirable, sensitivity can be further increased within a particular organism by taking advantage of the most susceptible species or strain, stage, size, or sex; by providing optimum temperature and light; by regulating the treatment chamber size for optimum results; by using the most sensitive criterion of response; and by increasing the length of exposure and holding periods. Also, improved sensitivity, as with chemical methods, can be obtained by removal of extractives and by concentrating the extract or using a larger volume of material for extraction. Thus, bioassay appears to approach the sensitivity of the chemical methods and in many cases is capable of detecting considerably smaller quantities than chemical methods.

### Agreement of Chemical and Bioassay Results

Numerous comparisons of bioassay and chemical results have been made. Some are presented in Tables V and VI. DDT, chlordan, and parathion (Table V) show generally good agreement. However, Laug (*60*) and Tressler and Jenkins (*98*) observed weathered DDT residues to be lower in bioassay than in chemical analysis. Possibly the difference was due to a nontoxic breakdown product of DDT causing a color reaction with the Schechter-Haller method (*85*).

Hartzell, Storrs, and Burchfield (*49*) reported chemical residues of heptachlor (Table VI) in seven crops to be approximately 2.5 times higher than those obtained with the mosquito photomigration technique. Part of this difference on further investigation was the result of masking by lipides. The remaining difference was probably due to background absorption caused by the pres-

**Table V. Comparison of Bioassay and Chemical Residues of Treated Materials**

| Insecticide Material | Bioassay | | Chemical | | References |
|---|---|---|---|---|---|
| | Organism | Residue | Method | Residue | |
| DDT | | | | | |
| Rat fat | Housefly | 2.98 | Colorimetric | 3.0 | (*60*) |
| Spinach | Housefly | 74.0 | Colorimetric | 71.0 | (*99*) |
| Spinach | Mosquito | 35.0 | Colorimetric | 45.0 | (*14*) |
| Peaches | Housefly | 8.3 | Colorimetric | 8.5 | (*99*) |
| Cabbage | *T. castaneum* | 2.0 | Colorimetric | 1.4 | (*100*) |
| Chlordan | | | | | |
| Potato | Mosquito | 0.047 | Colorimetric | 0.041 | (*49*) |
| Yams | Mosquito | 0.021 | Colorimetric | 0.040 | (*49*) |
| Cabbage | Mosquito | 0.020 | Colorimetric | 0.029 | (*49*) |
| Turnip | Mosquito | 0.026 | Colorimetric | 0.031 | (*49*) |
| Onion | Mosquito | 0.017 | Colorimetric | 0.020 | (*49*) |
| Corn | Mosquito | 0.035 | Colorimetric | 0.045 | (*49*) |
| Carrot | Mosquito | 0.036 | Colorimetric | 0.037 | (*49*) |
| Spinach | Housefly | 0.2 | Total chloride | 0.1 | (*99*) |
| Parathion | | | | | |
| Spinach | Housefly | 6.8 | Colorimetric | 6.8 | (*99*) |
| Spinach | Mosquito | 6.4 | Colorimetric | 6.0 | (*14*) |
| Potatoes | Mosquito | 0.15 | Colorimetric | 0.17 | (*88*) |
| Potato foliage | Mosquito | 0.064 | Colorimetric | 0.11 | (*88*) |
| Potato foliage | Mosquito | 0.20 | Colorimetric | 0.30 | (*71*) |
| Onion | Mosquito | 0.10 | Colorimetric | 0.11 | (*71*) |
| String beans | Mosquito | 0.83 | Colorimetric | 0.80 | (*71*) |

**Table VI. Comparison of Bioassay and Chemical Residues of Treated Materials**

| Insecticide Material | Bioassay | | Chemical | | References |
|---|---|---|---|---|---|
| | Organism | Residue | Method | Residue | |
| Aldrin | | | | | |
| Carrot | *Drosophila* | 0.298 | Colorimetric | 0.187[a] | (*42*) |
| | | | Colorimetric | 0.134[b] | (*42*) |
| | | | Total chloride | 0.297 | (*42*) |
| Beef fat | Housefly | 110.0 | Colorimetric | 18.5[a] | (*9*) |
| | | | Total chloride | 93.0 | (*9*) |
| Sheep fat | Housefly | 55.0 | Colorimetric | 7.5[a] | (*9*) |
| | | | Total chloride | 42.5 | (*9*) |
| Soil | Mosquito | 3.0 | Total chloride | 3.2 | (*93*) |
| Dieldrin | | | | | |
| Beef fat | Housefly | 86.0 | Colorimetric | 85.0 | (*9*) |
| Butter fat | Housefly | 95.0 | Colorimetric | 95.0 | (*9*) |
| Soil | Mosquito | 4.8 | Total chloride | 5.5 | (*93*) |
| Heptachlor | | | | | |
| Cabbage | Mosquito | 0.01 | Colorimetric | 0.012 | (*49*) |
| Onion | Mosquito | 0.006 | Colorimetric | 0.008 | (*49*) |
| Carrot | Mosquito | 0.008 | Colorimetric | 0.03 | (*49*) |
| Corn | Mosquito | 0.01 | Colorimetric | 0.016 | (*49*) |
| Turnip | Mosquito | 0.006 | Colorimetric | 0.022 | (*49*) |
| Potato | Mosquito | 0.008 | Colorimetric | 0.021 | (*49*) |
| Yams | Mosquito | 0.003 | Colorimetric | 0.026 | (*49*) |
| Clover | Mosquito | 0.3 | Total chloride | 0.3 | (*94*) |
| Soil | Mosquito | 2.2 | Total chloride | 2.5 | (*93*) |

[a] Colorimetric method for aldrin.
[b] Colorimetric method for dieldrin.

ence of impurities which resulted in high chemical residues.

Good agreement between *Drosophila* direct exposure assays and specific chemical methods was reported for several insecticides and crops by Dewey (*26*). However, bioassay residues of aldrin in carrots were consistently higher than chemical residues. Further investigation of this discrepancy by Glasser and associates (*42*) demonstrated the presence of a toxic metabolite which accounted for the difference in residues (Table VI).

Bann and coworkers (*9*) obtained much higher residues with bioassay than by colorimetric analysis. This led to the discovery that aldrin was changed to dieldrin in the animal body.

Generally the results obtained with chemical and bioassay methods have been in good agreement. Where differences occur, they may be due to such factors as the presence of metabolites, interfering materials, or removal of portions of the toxicants. These differences should be carefully investigated when they occur for the information about the toxicant or the methods in use.

### Bioassay Methods

The methods used in bioassay vary with the test organism, material in which the residue is located, and magnitude of the residue. As with chemical methods, control samples are always necessary, but larger quantities are needed for bioassay as control and test standards for a standard curve should be run simultaneously. The methods used can be divided into two broad groups depending on whether extraction of the residues is necessary or whether the test organism is exposed directly to the residue containing tissue.

**Direct Exposure Methods.** This method has been used primarily with *Drosophila melanogaster* (*26, 27, 33, 76, 91*), and on a limited scale with houseflies to determine residues in milk (*89*). The *Drosophila* method consists in the maceration of plant materials by chopping or grinding and then blending to obtain a fine puree-like consistency. Sample size and exposure methods vary, from 20-gram aliquots in 4-ounce jars (*91*), to 2-gram aliquots spread on a paper strip and placed in a test tube (*26, 27, 33, 42*). Direct exposure of soils has been with a 1 to 1 mixture of soil and canned pumpkin (*91*) or pureed applesauce (*26, 107*) to keep the flies alive and the soil suitable for exposure. Wylie (*106*) dampened 5 grams of soil with 2 ml. of corn sirup and spread it on the walls of a large vial.

In the housefly method (*89*), 20 grams of milk was pipetted on 1.2 grams of cotton in a half-pint jar and 100-day-old flies were introduced. This method is reported to produce more consistent results than a dry-film exposure of extracted milk residues.

Direct exposure methods have certain advantages over extraction methods. No extraction, nor extraction cleanup is necessary when the method can be used. The danger of poor extraction, poor recovery, or elimination of a toxicant in the cleanup procedure is not encountered. When *Drosophila* are used, the method has a high degree of sensitivity (Table IV), is inexpensive, and simple. Disadvantages are a lower sensitivity than with extraction methods (Table IV) and the possible toxicity or masking effect of some plant materials.

**Extraction Methods.** As with chemical methods, extraction procedures vary from one method or organism to another, with the tissues to be extracted and with the toxicant. The same principles apply to both. Some workers have used known amounts of toxicant added in a pure state to obtain a standard curve rather than to a sample of the same kind of material as that analyzed. They have tried to accomplish this by cleanup methods designed to eliminate all extractives except the toxicant (*29, 53*) or by compensating for the presence of extractives by adding oleic acid or an oil (*22, 54, 57, 60*). In either case, results would probably not be the same as by the addition of the same extractives to the standard. If additives or extractives are necessary (*92*), equal amounts should be added to both unknown and controls. The addition of compensating materials should be only on a "last resort" basis. Methods using extractives can be grouped together based on methods of exposure.

**Film Method.** This group includes those methods exposing the organism to a film of toxicant in solution or deposited as crystals. Insects such as the housefly and *Drosophila* are generally used. The type of film varies. Some use a "dry film" (*42, 90*), while others add small quantities of an oil-type material to each sample to reduce volatility and to improve distribution and pickup of the toxicant (*52, 60*). This latter procedure increases the difficulties of toxicity from extractives (*60, 61*) and from masking the toxicant (*57, 61*).

The film method has the advantage of being the most sensitive of those available for organisms such as the housefly and *Drosophila*. Its main disadvantage is the difficulty and time required in extraction and extraction cleanup, and the difficulty of obtaining satisfactory recovery of toxicants.

**Aqueous Solutions.** This method is used for the exposure of test organisms of aquatic habitat such as mosquito larvae, fish, and microcrustaceans. The method consists in suspension of the toxicant in water. As with other methods of exposure, this one varies in the size of container used, volume of water, and criterion of response. Hartzell (*47*) and Hartzell and Storrs (*48*) originally used 15 ml. of water suspension and death as an end point. Later Burchfield, Hilchey, and Storrs (*14*) and Burchfield and Storrs (*16*) used 100 ml. of water and the response of the larvae to light as an end point. Newman (*70*) was able to increase sensitivity by decreasing water volume from 50 to 1.5 ml. and using first instar larvae in place of third. Complete death or a behavior response may be used as an end point for fish and microcrustaceans.

This method has the advantage of having the whole organism constantly in contact with the medium. Some have claimed uniform administration of the toxicant, but settling of the suspended material may occur. High sensitivity is thought to be the result of circulation and absorption of the toxicant through the gills or equivalent organs. The disadvantages are characteristic of extraction methods. Further disadvantages may be the toxicity of the solvents used and possible sorption on the walls of the container.

**Oral Feeding.** This method has not been used extensively. Frawley, Laug, and Fitzhugh (*38*) evaporated an ether extract to dryness over sugar, which was then dissolved in water and fed to houseflies for 24 hours. The main advantage of this technique is that purification or cleanup of the extract is unnecessary. However, it may be less sensitive than contact assays, or require more time for response.

**Agar Plate Diffusion.** This method has been used more for exposure of bacteria, yeast, fungi, and other microorganisms to pesticides than the serial dilution or turbidimetric methods. It is dependent upon the diffusion of the fungicide from the treated paper disk (*96*), blotter (*64*), leaf (*63*), or seed (*5, 6, 64*) into the agar medium, which has been seeded with the appropriate organism. The quantity of toxicant is determined by measuring the circle around the disk in which the growth of the organism has been inhibited. The technique is highly sensitive. Usually ethyl alcohol or water solutions are made of the extract (*64, 96*) and volumes as small as 0.02 ml. can be used per disk (*46*). This method requires no special equipment other than that normally found in plant pathology laboratories. Once the method has been standardized, assays may be made without special technical training.

### Methods of Interpreting Data

The method of interpreting and calculating data will depend somewhat on the type of measurement or response selected, the organism used, the method

of exposure, the accuracy required, and the level of residue present. Biological variation is inherent in bioassay and may influence interpretation as well as requiring different methods of calculation. The design and statistical methods used in the bioassay of drugs and vitamins have been treated extensively by Bliss (*10*) and Finney (*32*). In pesticide assays, the methods most generally used have been of the dosage response or mortality type or of a time response type involving death or some other response.

Dosage mortality studies are those based on death or a response terminating in death of the organism. A dosage response curve from which unknowns can be determined can be obtained by adding known amounts of the same toxicant to the control samples. The data are plotted either as log-probits (*31*) or on log-probability paper to produce a straight line by visual inspection. Computation of the probit regression line (*31*, *32*) can be used for more accurate calculations, but frequently may not be necessary. Residues of the unknowns are read graphically from the standard curve (*26*, *42*, *47*, *48*, *52*, *70*, *71*, *93*, *94*). Standard curves must be constructed daily for each series of analysis (*92*). Unknowns should be read when the mortality is near the 50% area for maximum accuracy. If this is not possible, the mortality should at least fall within a range from 10 to 90% and preferably from 20 to 80%. Samples having mortality less than 10% or higher than 90% should be rerun with standard curves in the proper range (*31*).

A second method of greater precision may be used. Toxicant is added to both control and unknown and serially diluted providing two parallel regression lines. The residue present in the unknown is found by taking the difference in concentrations at the $LD_{50}$ point and multiplying by the dilution factor (*26*, *33*). Statistical treatment can be accomplished with probit analysis (*31*) or the short method of Litchfield and Wilcoxon (*67*).

Time response studies have been used most frequently with aqueous suspension methods. Time is plotted against death or other responses as in dosage mortality curves. $LD_{50}$ or $T_{50}$ is estimated by comparison with similar values obtained at known concentrations of the same toxicant. Burchfield and Hartzell (*13*) have interpolated results from plotting the logarithm of $T_{50}$ against the reciprocal of the concentration in parts per million. Michael, Thompson, and Abramovitz (*69*) used time-flotation graphs for brine shrimp from which the toxicant present was read directly by comparison with the standards.

In agar plate diffusion studies, standard curves having a straight line relationship over most of the curve can be obtained by plotting the logarithm of the concentration against either the square of the diameter of the inhibition zone, or against the area of the inhibition zone (*96*).

## Specificity of Bioassay

One of the main disadvantages claimed for bioassay has been the lack of specificity of the organism to a chemical. This necessitates a knowledge of the toxicant present prior to assay, or in the case of mixtures, a method of separation unless total toxicity will suffice. This difficulty is also common to chemical methods but usually of a lower magnitude. Some pesticide mixtures may be separated and determined by bioassay without serious difficulty. The principles applied may be physical, chemical, or biological as are also used in chemical methods.

The degree of susceptibility of a test organism may often help in separating mixtures or eliminating some materials because of the amount of toxicant required to cause response (*34*, *61*, *86*). With two insecticides present, Fleming, Coles, and Maines (*34*) were able to measure the quantities of each due to the difference in the speed of action and volatility of the toxicants present. Davidow and Sabatino (*24*) using characteristic time response patterns of goldfish, separated nine chlorinated insecticides.

In other cases, the reaction of the organism to toxicants may be such as to facilitate separation. *Aedas agypti* larvae treated with DDT contracted spasmodically on exposure to light, but apparently were unable to move away from the light (*16*). This is contrasted to a sluggish reaction of parathion in which they move away from the light at a reduced rate or even toward the light in more advanced stages. Difference in action time of houseflies has been suggested by Sun (*90*) to separate phosphate compounds from chlorinated compounds. He also suggests the possible use of cholinesterase inhibition for assaying phosphates in the presence of chlorinated materials, or the possibility of utilizing resistant strains for separating toxicants.

Chromatographic columns may be used to separate compounds which show different degrees of polarity as demonstrated by Erwin, Schiller, and Hoskins (*29*) for separating groups of insecticides. Mixed residues of closely related compounds such as aldrin, dieldrin, endrin or isodrin (*42*, *90*), or heptachlor and its epoxide (*23*, *81*) have been accomplished by using Attasol columns. Separation may occur inadvertently with a toxicant and its metabolite (*9*, *42*). As the metabolite may be more toxic than the original (*77*), care must be taken in the use of columns, either as a cleanup or separation procedure, that no toxicant remains in the column.

Others have separated or established the presence of a particular insecticide by taking advantage of differences in stability between compounds. Aldrin, dieldrin, and endrin can be refluxed with 2% potassium hydroxide without change, while DDT, BHC, methoxychlor, parathion, and malathion are degraded to relatively inactive compounds (*57*). Endrin is decomposed by strong acid treatment while DDT is stable (*57*). Sun (*90*) used varying concentrations of alcoholic potassium hydroxide for separating aldrin and dieldrin residues from other toxicants.

## Conclusions

Bioassay can be a useful tool for the determination and study of agricultural pesticide residues. It can be simple, swift, versatile, and highly sensitive to a wide range of toxicants. Generally little or no expensive equipment or highly trained help is required.

Bioassay may be used in the absence of suitable chemical methods or adequate equipment. It may be used to determine total toxicity, the presence of metabolites, or to speed residue determinations. Although used to best advantage when the toxicant is known, bioassay can be used in some instances to identify toxicants or to separate mixtures of pesticides.

The main disadvantage of bioassay appears to be the need in most cases of its use in conjunction with a chemical method for general acceptance of the data. Other disadvantages may be the complexity of rearing or assay methods necessary for a specific organism; the susceptibility to plant toxicity and extractives of a particular organism; and the possible lack of specificity.

Whether a bioassay can be used advantageously or not will depend on the particular type of problem, but often it will be found a useful tool in the determination of pesticide residues.

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**INSECTICIDE RESIDUES**

# Residues in Milk from Dairy Cattle Treated with Methoxychlor for Fly Control

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**Samples of milk from methoxychlor-treated cows on four New Jersey farms were analyzed to determine the insecticide residue present at various intervals after application. Results indicate that methoxychlor is present in minute, but detectable, amounts in the milk of treated cows and that the concentration diminishes rapidly with successive samplings after spraying or dusting.**

PRELIMINARY INVESTIGATIONS of milk samples from cows treated with methoxychlor spray formulations in 1955 indicated detectable amounts of methoxychlor residue. Therefore, residue analyses were conducted on milk samples taken in conjunction with tests made by Granett and Hansens in 1956 (*4, 5*), in which they set out to establish more firmly the finding that control of biting flies can result in a significant increase in milk production. Previous work on effect of methoxychlor residues in milk and on rate of excretion has been done (*7*). No attempt was made in this study to reproduce conditions of treatment used by other investigators.

### Procedure

Four farms in Salem County, N. J., were used in the experiment. On farms I and II, water emulsion sprays were applied once a week and, on farm III, twice a week. At each location, one third of the herd (group A) was treated with a 10% methoxychlor formulation diluted 1 to 19 parts of water; and one third (group B) with a formulation of 5% methoxychlor plus 50% butoxy polypropylene glycol (Crag Fly Repellent) diluted 1 to 9 parts of water. The methoxychlor was applied at the rate of approximately 1 quart of 0.5% solution per animal. Sprays were applied from a knapsack sprayer operated at 20 to 40 pounds pressure and with a nozzle 1 to 2 feet from the animal. The other third (group C) was untreated.

On farm IV, the cows were similarly grouped and 7.6 grams of 50% methoxychlor wettable powder per animal was applied as a dust to group A for 4 weeks. The above wettable powder of methoxychlor plus 10% butoxy polypropylene glycol was applied to group B and group C was left untreated. After a 2-week interval, groups A and B were treated with sprays of the wettable powders at a level of 1 quart of 0.5% methoxychlor per cow (8 pounds per 100 gallons of water). There were from four to six cows in each group on all four farms.

Milk samples were taken from each group before treatment and at intervals