chlorinated hydrocarbon insecticides had not been fully evaluated. Experiments were conducted to determine toxicity to quail and pheasants of aldrin, dieldrin, endrin, and strobane, and to determine effects of these compounds upon survival, growth, and reproduction.

Feeding of diets containing 0.02% DDT to breeding quail resulted in significant decreases in hatchability of eggs and in viability of chicks. Similar results were obtained by feeding 0.001% dieldrin, but effects upon reproduction of short-term feeding of aldrin and endrin could not be determined.

Aldrin, dieldrin, and endrin were lethal to both male and female quail when fed at levels of 0.0005% in the diets. Female pheasants appeared more resistant than males to the effects of these compounds.

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PESTICIDE RESIDUE ANALYSES

Preassay Purification of Tissue Extracts by Wax Column

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A reverse-phase partition column for removal of interfering extractives prior to bioassay or chemical determination of residual insecticides has been used with organic insecticides occurring as residues in plant and animal tissues. The column consists of finely ground alumina coated with a 2 to 1 mixture of petrolatum and low melting paraffin wax. The eluting liquid is 60% aqueous acetonitrile solution in most cases, but by successive use of 40, 60, and 75% solutions the toxicants may be separated into groups for easier identification. Recovery is satisfactory with most toxicants, and bioassay with houseflies is being used regularly with a wide range of produce.

THE FIRST STEPS in the determination of an organic insecticide or acaricide residue in a plant or animal product are subdivison of the material and extraction with an organic solvent. The resulting extract seldom contains the toxicant in a sufficiently pure state for analysis, but in addition to the residue in question, holds various fats, oils, waxes, and colored materials such as chlorophylls and carotenoids. These all interfere in varying degrees with such methods as colorimetric chemical determinations or bioassay involving response of a sensitive living organism. It is necessary therefore to purify the extract—i.e., in so far as practicable to separate the residual toxicant from the plant or animal extractives which have also passed into the organic solvent.

A number of purification methods have been advanced for cleaning up organic solvent extracts, but most of them are applicable to a limited number of toxicants. Sulfonation of the unsaturated fats, waxes, and colored materials, as in the Celite–sulfuric acid column (*7*) can be used only for acid-stable toxicants such as DDT. Saponification of the fats and removal as water-soluble soaps can be employed only with alkali-stable compounds such as aldrin or dieldrin. Adsorption columns with their highly specific characteristics must be developed individually for each toxicant and the ordinary partition columns of clays, cellulose, etc., work only with solutes that have considerable solubility in water. It seemed possible to develop a reverse-phase partition column that would work with extracts containing interfering extractives and any toxicants of low water solubility.

In reverse-phase partition columns, the stationary phase is of lower polarity than the moving one. Inclusion of water in the moving phase ensures this condition, but pairs of organic liquids of markedly different polarity and hence of low mutual solubility also give the same effect—e.g., hexane and acetonitrile. Such columns are suitable for holding back and separating compounds which are soluble in the typical organic solvents. At first sight, it might seem that such a column could be made without difficulty by treating some supporting solid with the chosen organic liquid and passing through any chosen water-solvent solution as developing liquid, but the situation is considerably more complicated.

The essential structure of a column having a relatively fixed polar phase—for example, a cellulose-water column—is that the water through hydrogen bonding forms a layer many molecules thick over all the surface of the substrate, which accordingly plays only a minor part in determining the distribution of solutes between this fixed aqueous phase and the external moving organic phase. The reverse of this situation would be a correspondingly thick layer of adsorbed organic liquid of low polarity held upon the supporting substrate. But such liquids usually are not capable of forming polymolecular layers, as they lack the necessary bonding atoms or groups. Ac-

cordingly, their capacity is small and the supporting solid contributes greatly to the distribution, and sometimes acts practically as an adsorption column instead of a partition column.

There appear to be no reports of reverse-phase partition columns used with insecticides. A number of applications of reverse-phase paper strips have been recorded—e.g., Winteringham (*7*) separated pyrethrins and cinerins on filter paper impregnated with Vaseline, using aqueous ethyl alcohol and ammonia as the moving phase. Metcalf and March (*4*) applied silicone (Dow 200, viscosity 550 centistokes) to filter paper strips and used an aqueous mixture of chloroform and ethyl alcohol to separate parathion, related phosphate esters, and degradation products. Such paper strips have very low capacity and are useless for removing the large amounts of extractives encountered with the volumes of extract needed for determination of toxicant residues. A reverse-phase column has been developed which has a much greater capacity to hold the interfering materials and at the same time passes the residual toxicants.

Choice of Column Materials and Eluents

Jones and Riddick (*3*) found that treatment of a hexane extract with acetonitrile resulted in considerable purification, because much of the extractives remained in the hexane, whereas the toxicants passed into the acetonitrile. This suggested use of a column having a hydrocarbon stationary phase and acetonitrile as eluent. Among the hydrocarbons tested were finely ground paraffin wax, finely divided mixtures of paraffin wax and petrolatum made by pouring hot acetone solutions into water, and paraffin wax plus petrolatum impregnated upon cotton fibers as in dental rolls, and upon cellulose powder, Filter-cel, sand, magnesium oxide, and alumina. The criteria for judging were ease of preparation, uniformity of impregnation, freedom from channeling, reproducibility of flow, and especially removal of the extractives and quantitative passage of toxicants. Chromatographic alumina coated with 15% by weight of a 2 to 1 petrolatum–paraffin wax mixture was chosen as the best column material. Granulated paraffin wax mixed with cellulose (1 to 1 by volume) plus starch (5% by volume) also gave satisfactory results with many extracts but was much less convenient to prepare and use.

The developing liquids tested, in addition to acetonitrile, were acetone, ethyl alcohol, pyridine, and acetic acid, all diluted with water to various extents to reduce solubility of the paraffin wax–petrolatum and of the various waxy extractives. Among three-component systems tried were pyridine-acetonitrile-water, acetic acid–acetonitrile-water, and acetic acid–pyridine-water. The more complex eluents had no advantages with the extracts tested over acetonitrile-water solutions and these were adopted as standard developing liquids. These eluents have the advantage that after strong dilution of the eluate with water, the contaminating toxicant may be extracted in any chosen solvent—e.g., petroleum ether if bioassay is to be used (Hoskins, Witt, and Erwin, *2*).

Special Materials and Reagents Used

Paraffin wax, melting point 160–165° F. (Standard Oil Co.).

White petrolatum, Protopet 1-S (I. Sonneborn Sons, New York).

Alumina, adsorption (for chromatographic analysis, 80–200 mesh, Fisher Scientific Co.)

Acetonitrile (Carbide & Carbon Chemicals Co.).

Petroleum ether, boiling range 30° to 60° C. (Merck & Co.).

Preparation of Column

To make the coated particles for the column, add 5 grams of paraffin wax (melting point 160–165° F.) and 10 grams of white petrolatum to 100 ml. of a 50:50 mixture of benzene and chloroform, warming, if necessary, to dissolve all the paraffin. Then add 100 grams of adsorption alumina (80 to 200 mesh) and stir while the solvents are evaporated on a steam bath. To remove the last traces of benzene, spread the coated powder on a flat surface and blow warm air over it—e.g., from a hair dryer. Removal is satisfactory if no odor of benzene is detectable upon opening a container in which a portion has been kept for a day. After passage through cheesecloth to remove lumps, the powder may be stored until used. Limited tests with other paraffin waxes and petrolatums gave satisfactory results, and the ratio may also be varied, but neither straight paraffin wax nor petrolatum equaled the mixture used.

The glass tube used to hold the column is of borosilicate glass 20 mm. in outside diameter and 26 cm. long, with 150-ml. reservoir at the top and a medium fine sintered-glass filter plate at the bottom. The plate leads into a drip tube surrounded by a 24/40 inner member of a ground-glass joint, in this respect differing from the column described by O'Donnell and others (*5*). During loading of the column the outer half of the ground joint is attached and connected to a vacuum while 35 grams of the coated powder are poured slowly into the tube, which is tapped to ensure uniformity of packing. This will give a column about 100 mm. high. Then the vacuum is relieved, a small plug of cotton is placed above the powder, and the column is rinsed with 50 ml. of the first eluent to be used, the flow being stopped just before all liquid enters the column.

Acetonitrile-water mixtures hold back nearly all the extractives from a wide variety of samples and quantitatively carry through many organic toxicants. By use of increasing concentrations of acetonitrile, the toxicants may be separated into groups.

Preparation of Extract

Residues of insecticides in watery tissues are very inefficiently extracted by water-insoluble solvents such as benzene, petroleum ether, or carbon tetrachloride. Driving off the moisture by heat often causes loss of toxicant. It is possible to dehydrate the samples with anhydrous sodium sulfate, but a simpler method is inclusion of enough water-miscible solvent to make a single liquid phase. Ethyl alcohol is very suitable for this purpose, as it is miscible with most other solvents, is a fairly good solvent itself for nearly all organic toxicants, reduces the tendency to form stable emulsions, and is easily removed by addition of excess water.

After many trials the following procedure has been developed. Following thorough subdivision of 1000 grams (or other convenient weight) of sample in a food chopper or grinder, the wet mass is quantitatively transferred to a gallon jar or other vessel which can be sealed tightly—1-gallon paint cans are very satisfactory. A volume of 1000 ml. of solvent and an equal amount of 95% ethyl alcohol are added and the whole is shaken or rolled mechanically for 1 hour. If the sample contains much water, two phases may result. This can be avoided by adding more alcohol or preferably by adding 100 to 200 grams of anhydrous sodium sulfate. The liquid phase is poured into a large separatory funnel, diluted with 2 liters of water, and shaken for 2 to 3 minutes. The aqueous phase which separates is withdrawn and the process is repeated. In the first rinse the aqueous phase is about 30% alcohol, in which solvents such as benzene or petroleum ether are soluble only to a small fraction of 1%. The various insecticides distribute themselves very strongly in favor of the conjugate water-insoluble phase, which contains only about 3% alcohol at most (*6*). This small amount is removed in the second water rinse. Hence 1 ml. of the remaining volume of solvent will represent 1 gram of sample (or another amount if a different ratio was taken), assuming only that there was uniform distribution of residual toxicant in the recovered and nonrecovered fractions of the solvent.

Fifty milliliters of extract, corresponding to 50 grams of sample, are put into a small beaker and the solvent is evaporated to approximately 5 ml. at as low a temperature as practicable in a warm air

stream. About 5 grams of fine white sand are added to the beaker and evaporation is continued with constant stirring to ensure that the plant or animal extractives present are left in a thin coat on the sand, thus facilitating solution of the insecticide in the next step. After the solvent has been removed, 5 ml. of acetonitrile are added to the beaker, which is placed on a warm plate. The mixture is stirred while it is being heated to the boiling point. Six milliliters of water are added to the hot solution, and after it has cooled the whole volume of approximately 10 ml. is added to the column. The beaker is rinsed with two 5-ml. portions of hot 40% acetonitrile and the washings are also put on the column.

It is convenient to apply about 2-pound air pressure to the column to hasten entry of the 40% acetonitrile solution of the extract. When the liquid level just reaches the top of the granular packing, the pressure is released and 150 ml. of developing eluent are added. The air pressure is restored and the eluate collected. Liquid should flow from the column at the rate of not more than 1 ml. per minute. The eluate is put into a 500-ml. separatory funnel and 200 ml. of water are added, plus two successive 100-ml. volumes of petroleum ether. After thorough shaking, the phases are allowed to separate, the petroleum ether phases are combined, and the aqueous one is discarded. Care should be taken to add enough water to give an aqueous phase containing less than 25% acetonitrile; otherwise all toxicant may not pass into the petroleum ether layer. All the organic toxicants listed below are completely extractable from 25% acetonitrile with petroleum ether in the above process. The petroleum ether is made to a convenient volume and aliquots are taken for bioassay.

The procedure for cleanup of extracts was developed primarily for use with the bioassay described by Hoskins, Witt, and Erwin (*2*) and the volume of petroleum ether solution needed for a test depends upon the toxicant concerned and its concentration. Essentially, the bioassay depends upon taking several amounts of toxicant, so that the resulting mortalities are distributed over a range, say from 10 to 90%. From a plot in probit–log volume units the LD_{50} volume is determined and equated to micrograms of toxicant needed to cause 50% mortality in a similar series of exposures to known amounts of the toxicant. For most precise work the toxicant should be added to extracts of uncontaminated tissue similar to that under investigation and carried through the column in the same manner as the unknown contaminant. From the known dilution of the petroleum ether solution, the parts per million of toxicant in the original sample may be calculated. It is, of course, possible to use some other bioassay or any suitable chemical method with the purified extract.

Table I. Purification of Extracts by Alumina-Wax Column

Solids in Solution Applied to Column, Gram	Solids in Eluate, Gram: 125 ml. eluate 1, 40 CH_3CN:60 H_2O	150 ml. eluate 2, 60 CH_3CN:40 H_2O	150 ml. eluate 3, 75 CH_3CN:25 H_2O
Fifty Grams of Spinach Extracted with Petroleum Ether in All Cases, except Blanks			
0.000 (blank)	0.001	0.003	0.006
0.141	0.002	0.004[a]	0.008[a]
0.142	...	0.003	...
0.143	...	...	0.009
0.141	0.003	0.004[a]	0.007[b]
0.143	...	0.004	...
0.145	...	...	0.008
Fifty Grams of Pears Extracted with Petroleum Ether in All Cases			
0.046	0.004	0.008[a]	0.008[b]
0.048	...	0.006	...
0.046	...	...	0.011

[a] 125 ml. of eluent I put through column prior to using eluent II.
[b] 125 ml. of eluent I and 150 ml. of eluent II put through column prior to using eluent III.

Table II. Toxicants Brought through Column by Three Eluents

Eluent I (40 CH_3CN:60 H_2O)	Eluent II (60 CH_3CN:40 H_2O)	Eluent III (75 CH_3CN:25 H_2O)
Rotenone	Dieldrin[a]	Aldrin
Malathion	DDT	Isodrin
Dilan	Chlordan	
Parathion	Heptachlor	
Lindane	Endrin	
Methoxychlor	Toxaphene	
Dieldrin[a]		

[a] Dieldrin is not completely eluted by 125 ml. of eluent I and therefore tends to divide between groups I and II if 40% acetonitrile is used as first eluent.

Use of Column

The data of Table I show the degree of purification secured with typical extracts of spinach and of pears, which are rather refractory representatives of vegetables and fruits. The material removed by the eluents from an unloaded column (first line), had the appearance of Vaseline. The material coming through the column from spinach samples had the consistency of a soft wax, while that from pears was hard. Both were colored green, especially that from spinach, as the relatively polar pigment molecules are not held by the petrolatum-paraffin. A considerable amount of pigment is precipitated when the warm 100% acetonitrile is diluted to 60% and is held in a thin layer at the very top of the column, especially in the layer of cotton. As may be expected, the degree of cleanup of extracts varies with the produce concerned. Spinach offers an example of relatively heavy weight of extractives because of the large area of leaf for a given weight, whereas pear has a much smaller surface-weight ratio. Table I shows that on the basis of original weight of extractives, spinach extract is easier to clean up than pear extract, but with all samples so far used the remaining extractives in the volume needed for the bioassay are too low to interfere.

An advantage of this procedure is that in addition to removing all but a small fraction of interfering extractives, possible contaminating toxicants may be separated into two or possibly three groups by use of a sequence of eluents containing increasing concentrations of acetonitrile. As bioassay in general does not identify toxicants, this separation aids greatly in determining what is present. The separation is fairly sharp when no extractives are in the solution and is less distinct as these increase in amount.

Table II gives the toxicants carried through the column under favorable conditions by the indicated eluents. The column used was either the granulated paraffin-cellulose-starch or the paraffin-Vaseline–coated alumina. In general, it is preferable to use 150 ml. of 60% acetonitrile followed by an equal volume of 75% acetonitrile as the routine eluents to determine presence or absence of a toxicant, as this will bring through all listed materials. If the toxicant is not known, a second portion of the extract may be treated with 100-ml. volumes of the three eluents in order to place it in one of the three groups. In case of heavy extractives, cleanup is effected by passage through the column with 75% acetonitrile; the eluate may be diluted with water, extracted in petroleum ether, evaporated, and put on the column in 40% acetonitrile by the process described. Then the column may be eluted successively with 100-ml. volumes of 40, 60, and 75% acetonitrile,

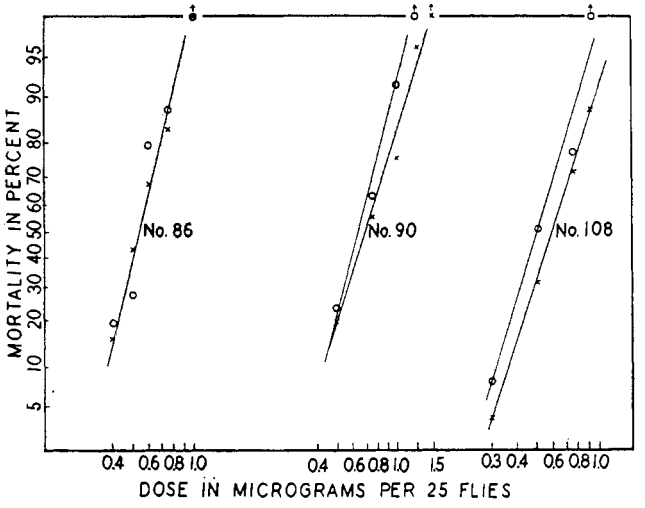

Figure 1. Mortality-dosage lines from three recovery tests

86. Dieldrin in extract from 50 grams of canned spinach
90. Heptachlor in extract from 50 grams of canned spinach
108. Lindane in olive oil
O Mortality from indicated amounts of toxicant in petroleum ether not put through column
X Mortality from indicated amounts added to extracts before passage through column
Arrows at top indicate that complete mortality resulted from corresponding doses

as desired. With some samples, inclusion of 10% alcohol in the 40% acetonitrile lessens the tendency to form stable emulsions.

An important criterion of the column is the quantitative recovery of toxicants, especially in presence of extractives from plant or animal tissues. The results of many tests with extracts from 50 to 60 grams of spinach, which are as difficult to handle as any so far encountered, may be summarized as follows: DDT, methoxychlor, lindane, chlordan, heptachlor, aldrin, toxaphene, parathion, and malathion have been recovered 90 to 100%; dieldrin, endrin, and isodrin 70 to 90%; and dilan 50 to 60%. Larger amounts of spinach result generally in lower recovery. On the other hand, extracts of 60 to 75 grams of green beans have always allowed over 90% recovery of any added toxicant. In the case of oils, such as olive or corn oil, a preliminary extraction with an equal volume of 100% acetonitrile leaves behind a major part of the waxes and other nonpolar components without serious loss of toxicant. This solution may then be diluted with water for passage through the column.

Three typical types of results are illustrated by the recovery runs shown in Figure 1. In each case the final purified petroleum ether extract used in the bioassay would have contained 1 γ of toxicant per milliliter if no loss occurred. The doses are plotted actually in terms of milliliters used for each point but expressed in the numerically equal hypothetical micrograms of toxicant. In no case was it possible to obtain a mortality-dosage curve with the original unpurified extract. In run 86 (dieldrin in spinach extract) the same line expresses the results for both the standard solution and the contaminated extract put through the column as described. Recovery was complete in this instance. In run 90 (heptachlor in spinach extract) the line for the contaminated extract has a somewhat lower slope than that for the pure toxicant solution. The most obvious explanation is that increasing amounts of unremoved extractives had correspondingly greater effect in preventing contact with the flies or in reducing penetration of the heptachlor. From the LD_{50}'s the recovery in this run was 94%. In run 108 (lindane in olive oil) the line for recovered lindane indicated lower mortality at all doses. The probable explanation is that in the course of the involved series of extractions with acetonitrile plus passage through the column, there was a more or less constant percentage loss of the toxicant, which would displace the line but not alter its slope. From LD_{50}'s the recovery even under such difficult circumstances was 85%. In testing action of the column only—i.e., by adding lindane to extracts—recovery of lindane was always at least 90%.

To date no product has been tried which could not be bioassayed successfully after passage of an extract through the column, provided that the toxicant can be determined in that manner. Not all toxicants have been tested in each product, as attention was given to those that are in practical use with the various products. In alphabetical order they are (R indicates raw and C canned): applesauce (C); green beans (C); kidney beans (R); lima beans (C); beef fat, heart, kidney, lean flesh, liver; carrots (R, C); sweet corn (C); cucumbers (R); olives and olive oil (C); peaches (C); pears (C); peas (C); sweet potatoes (R, C); white potatoes (R); prunes (C); sauerkraut (C); spinach (R, C); and tomatoes (R, C).

The physical nature of the alumina-wax particles of the column is not well understood. Attempts to coat thoroughly dry silica particles, sand, cotton fibers, and similar materials with the paraffin wax–petroleum mixture resulted in a heterogeneous product. Under the microscope the original solid appeared to be only irregularly coated and chunks of the wax mixture were scattered through the product. Even the 40% acetonitrile eluent brought through much wax, and when extracts were passed through such a column there was very unsatisfactory retention of extractives. The toxicants tended to be in the first eluent collected, just as in case of untreated alumina columns.

In contrast, coating the alumina particles gave a product which appeared entirely homogeneous and differed from the original alumina only in that some samples changed from a dull surface to a somewhat shiny one. The coated particles, made to consist of 100 parts by weight of alumina, 10 parts of petrolatum and 5 parts of paraffin, do not adhere upon contact but can be compacted by strong pressure. If the content of petrolatum is increased by a few per cent, the product becomes distinctly greasy and adheres readily. In the chosen composition the supercooled liquid nature of the coating is preserved, but packing is possible without consolidation into lumps.

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