(4), using 0.5 pound of heptachlor emulsion per acre, reported higher residues in milk of dairy cows grazing treated pastures as compared to those found in this study, but their experiment used a higher treatment level and was of short duration. The granular form of heptachlor resulted in lower levels of residue in butterfat as compared to the above reports where heptachlor in emulsion or Diesel oil was used.

Inspection of the curves after the animals were taken off the treated pasture (80 to 120 days after treatment) shows that heptachlor epoxide was still being excreted in the butterfat. This is probably due to the accumulation of the residue in the fatty tissues while animals were grazing the treated pasture, since the butterfat percentages for each cow were very comparable. The level of heptachlor epoxide excreted in the butterfat appears to be dependent upon the amount of insecticide or metabolite ingested and stored in the body fat and not the amount of butterfat produced by the dairy cow. A high intake of residue results in a high level excreted in the butterfat.

While on the treated pasture all animals were in good health and gained weight. The cows also showed good persistency in milk and butterfat production during the experiment.

Acknowledgment

Appreciation is extended to P. B. Polen and J. E. Buzzart, Velsicol Chemical Co., and to E. A. Cancienne and C. E. Smith, Louisiana Department of Agriculture Division of Entomology, for their cooperation and counsel in planning this project. The assistance of F. Ordoveza for chemical analyses and other phases of the project, and of B. R. Farthing, Louisiana Agricultural Experiment Station statistician, in the statistical analyses of the data is recognized.

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INSECTICIDE RESIDUES

Analytical Method for Determining 1,1-Dichloro-2,2-bis (*p*-ethylphenyl)ethane in Rat Fat and Cow's Milk

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Perthane, 1,1-dichloro-2,2-bis(*p*-ethylphenyl)ethane, was administered to rats under a 2-year feeding study for toxicological evaluation. A method was completed for analyzing the fatty tissue from rats and the analytical results have evinced well-defined patterns of absorption and storage. A procedure has also been developed for residual Perthane in cow's milk. These methods involve a general reaction based on dehydrohalogenation of Perthane, followed by reaction with strong sulfuric acid to produce the carbonium ion, which gives a color with maximum absorbance at 495 mμ. The complexities of handling milk and rat fat necessitated a modification of these methods to minimize background interference from extractives.

Perthane, which structurally is diethyldiphenyldichloroethane, has found wide acceptance for use on fruits and vegetables and in space sprays. Registration under the Miller Bill required a 2-year feeding study on rats for chronic toxicological evaluation. The analytical method developed involves a procedure reported by Miles and others (*4, 7, 8*) for determination of chlorinated 1,1-diphenylethane–type compound residues on agricultural crops. However, the complexities of handling rat fat necessitated a modification of these methods, to minimize background interference from extractives and allow accurate analysis at the sensitivity desired.

Procedure for Rat Fatty Tissue

The method, in general, involves the dehydrohalogenation of Perthane, followed by reaction with strong sulfuric acid to produce the colored carbonium ion complex with a maximum absorption at 495 mμ. In the procedure for rat fat, the tissues are macerated using sand, followed by extraction with diethyl ether added directly to the mortar. The ether extract is filtered and then evaporated off. The resulting fatty extractables plus any Perthane residue are picked up in petroleum ether and the Perthane is partitioned into acetonitrile and treated with adsorbent to remove substrate interferences. After removal of the solvent, dehydrohalogenation is carried out, followed by washing and evaporation of solvent and determination with 95% sulfuric acid.

Reagents. Perthane. Purified, technical material, recrystallized from methanol twice. Melting point 59–60° C.

Petroleum ether.

n-Hexane. Purified, technical grade, 95 mole % minimum passed through activated alumina. With a column

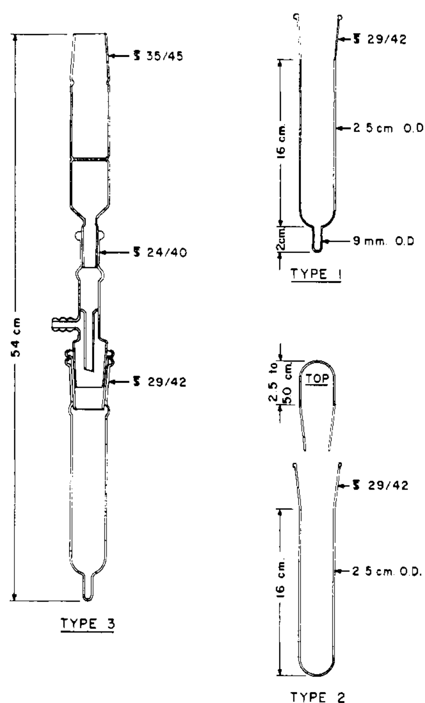

Figure 1. Apparatus

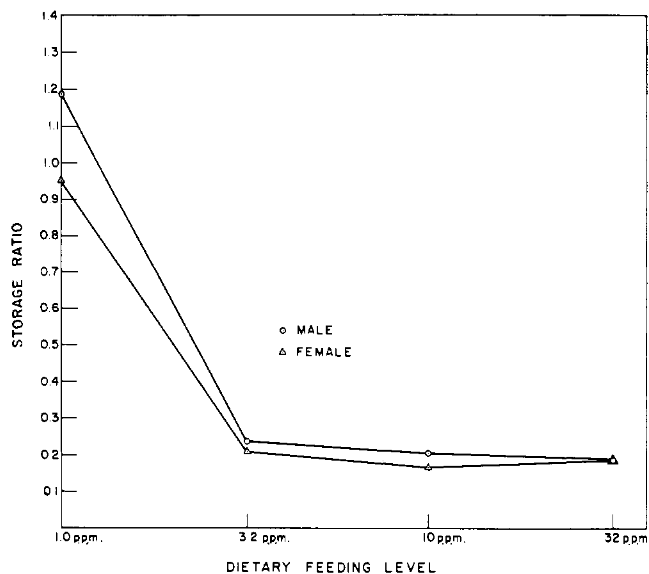

Figure 2. Relation of dietary level to storage in fat

1.5 inches in diameter, 1 pound of alumina will clean up 2 gallons of solvent.

Alumina, activated. Merck reagent grade.

Acetonitrile. Purified. technical grade, distilled, or reagent grade, passed through activated alumina (see *n*-hexane, purified).

Equilibrated solvents. Purified acetonitrile and petroleum ether saturated with each other.

Ottawa sand. Washed with hot water, then acetone, followed by diethyl ether. Dried.

Potassium hydroxide solution 1% in 2B ethyl alcohol. Prepared fresh daily. (Anhydrous ethyl alcohol may be substituted for 2B ethyl alcohol.)

Adsorbent mixture. 77 parts of sodium sulfate (anhydrous), 5 parts of Attasol, 5 parts of Filter Cel, and 2 parts of charcoal, activated (Nuchar). Mix well and dry for 24 hours at 110° C. Keep tightly stoppered until used.

Sulfuric acid, concentrated (96%).

Apparatus. Borosilicate glass tube, type 1 (Figure 1).

Borosilicate glass tube and top, type 2 (Figure 1).

Vacuum filter adapter (described in Figure 1). The fritted-glass filter is of medium porosity.

Receiver for vacuum filtration into vials (Figure 1). This is a 24/40 $ jointed tube approximately 7 cm. long below the joint with a side arm attached, type 3.

Spectrophotometer. Any instrument capable of performing well in the 500-mμ region.

Mortar and pestle.

Separatory funnels, 125-ml.

Filter paper, S & S Sharkskin.

Automatic 30-, 2-, and 5-ml. pipets facilitate the handling of large numbers of samples, but are not necessary. A vacuum source is necessary for filtration. In the studies conducted with this procedure, an aspirator vacuum has been found satisfactory.

Extraction. Weigh out approximately 3 grams of fatty tissue and macerate with sand in a mortar and pestle. After complete maceration, add 50 ml. of diethyl ether and grind for one-half minute. Allow the particulate matter to settle and decant the ether through a filter paper, collecting the filtrate in a tared beaker or bottle. Repeat this process twice, giving a total of 150 ml. of ether extractives in the beaker. Remove the solvent by placing the beaker on a steam bath and blowing a gentle stream of nitrogen into it. After the solvent is all removed, weigh the extractives.

Dissolve the fat in 15 ml. of petroleum ether (saturated with acetonitrile) and transfer to a 125-ml. separatory funnel. Rinse the beaker twice with 15-ml. portions of the same solvent and add these rinses to the separatory funnel. Extract with 20 ml. of acetonitrile (saturated with petroleum ether) by shaking vigorously for 2 minutes. Allow the phases to separate and draw the lower (acetonitrile) layer into another separatory funnel. Extract the petroleum ether with two additional 20-ml. portions of acetonitrile (saturated with petroleum ether) and combine these extracts. Discard the ether layers. For further removal of fat, shake the combined acetonitrile extracts with 15 ml. of petroleum ether. Drain the acetonitrile layer into a tube of type 2 (Figure 1). Add 4 grams of the adsorbent mixture, cap tightly, and shake for 2 minutes. Remove the adsorbent by passing the solution through a fritted-glass filter under vacuum into a receiver tube (type 1). Place the tube in a water bath at 55° C. and remove all but a few milliliters of the solvent with a gentle stream of nitrogen. When 1 or 2 ml. of the solvent remain, remove the tube from the water bath and blow off the remainder at room temperature with the nitrogen stream, just to dryness.

Dehydrochlorination. To the residue in the tube, add 15 ml. of 1% alcoholic potassium hydroxide and place the tube in a boiling water bath. Carry out the dehydrochlorination under a small stream of nitrogen. Keep the tube in the water bath exactly 20 minutes, then remove and cool to room temperature.

Separation. Add 30 ml. of *n*-hexane to the contents of the tube, then swirl to effect a clear solution, warming slightly, if necessary. Add 2 ml. of water (after tube has cooled to room temperature if it were heated), shake for 2 minutes, then withdraw the water layer by pipet. Repeat the water wash and withdraw the water. Remove a 25-ml. aliquot to a clean tube, type 2.

Color Development and Reading. Evaporate the 25-ml. aliquot almost to dryness under a gentle stream of nitrogen in a water bath at 55° C. Remove the final traces of solvent with nitrogen at room temperature while rotating the tube. Add 5 ml. of concentrated (96%) sulfuric acid and let stand for 15 minutes. Determine the absorbance at 495 mμ, using as a reference concentrated (96%) sulfuric acid.

Standard and Recovery Curves. Pipet aliquots of Perthane standard solutions (in acetonitrile solvent) containing 1 to 50 μg. into type 2 borosilicate glass tubes. Add acetonitrile to bring the total volume in the tube to 60 ml. Add 4 grams of adsorbent mixture and shake contents for 2 minutes. Filter

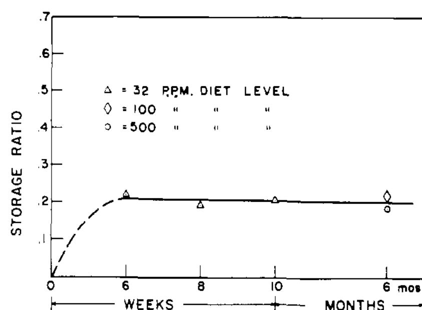

Figure 3. Relation of Perthane storage to duration of feeding

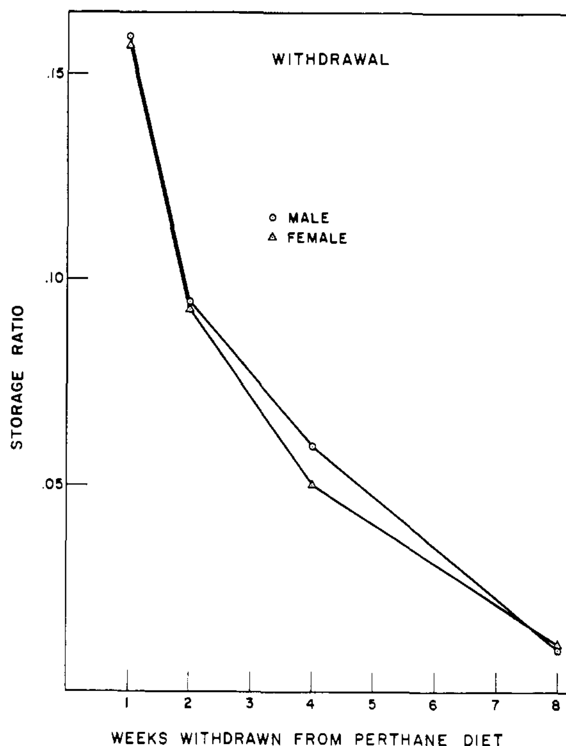

Figure 4. Relation of storage ratio to tissue depletion time

the solution through a fritted-glass filter funnel under vacuum into a receiver tube (type 1). Evaporate the acetonitrile solution almost to dryness in a water bath at 50° to 55° C. under a gentle stream of nitrogen. Remove the last bit of solvent at room temperature under nitrogen. From this point on, perform the dehydrohalogenation, separation, and color development steps exactly as for the rat fat method. Concentrations of 1 to 50 μg. of Perthane follow Beer's law. The average slope of the curve was found to be 0.015 (absorbance per μg.).

A recovery curve is developed by adding known concentrations of Perthane (1 to 50 μg.) to the fatty tissue before maceration in the mortar and pestles and then carrying through the procedure (Table II). Per cent recovery is based on values obtained with pure dehydrohalogenated Perthane undergoing the color development step only.

Discussion. Samples from the experimental feeding study consisted of 3 grams of rat fatty tissue. This presented a large potential source of interference. Small amounts of fat present during the color development step will char upon addition of concentrated acid. The amount of fat coming through the procedure is reduced to 0.2 gram by partitioning the petroleum ether extract with acetonitrile. Three acetonitrile extractions give a 95% recovery of Perthane. This cleanup is still not sufficient. Applying the follow-up steps normally carried out in the procedure for fruits and vegetables yielded high blank values. Various chromatographic procedures for further separation were tried. These adsorption techniques were carried out after the dehydrohalogenation step in a nonpolar solvent, *n*-hexane or petroleum ether. Low blanks can be obtained by performing the adsorption in a polar solvent, acetonitrile, prior to dehydrohalogenation using the adsorbent mixture given (*1*). This furnishes a good cleanup for the procedure up to the point of dehydrohalogenation.

However, the dehydrohalogenation itself, normally carried out with alcoholic KOH, produces an interfering brown color due to oxidation of trace amounts of fatty materials present. This seemingly necessitated an additional cleanup step. The disadvantage of losses through another cleanup step was eliminated by finding that a clean and colorless solution could be maintained during dehydrohalogenation by providing a blanket of nitrogen over the reacting mixture. Dehydrohalogenation produces a loss of 8.5%, while the loss through adsorption is 6.5%. These losses together with the extraction losses make the over-all recovery in the procedure around 70%.

Emphasis is placed on solvent purity. Constant low reagent blanks are not obtained until both the acetonitrile and *n*-hexane solvents are chromatographed through activated alumina. The petroleum ether and diethyl ether used for the initial extraction of the tissues should be of reagent purity.

Interferences. As pointed out by Miles (*7*), DDT, heptachlor, and methoxychlor do not interfere. The use of a rigorous dehydrohalogenation solution eliminates the interference of Rhothane (*8*).

Results. The fatty tissues were analyzed to determine the time necessary for storage levels to plateau, the storage *vs.* feeding levels, and time for the depletion of the insecticide from the fatty tissues upon withdrawal from a diet containing the insecticide (*5*). Results are presented as aids in demonstrating the validity of the method.

Perthane apparently has the properties necessary for storage in body fat, though at relatively low levels The dynamics of storage can be brought out by relating the storage ratio graphically to the dietary concentration. Figure 2 gives a graphical relationship for rats fed Perthane in their diet during an 8-week period at levels of 1, 3.2, 10, and 32 p.p.m. As the dietary level of Perthane decreases, a greater proportion of the ingested insecticide is stored. Both sexes follow the same pattern, the male having a slightly higher storage ratio.

Figure 3 indicates the proportion of administered Perthane stored over various time periods. Rats were fed at 32 p.p.m. for 6, 8, and 10 weeks. The plateau of the storage ratio is well defined. This same level resulted with animals fed 6 months on diets containing 100 and 500 p.p.m.

Figure 4 expresses the storage ratio *vs.* tissue depletion time. These data were obtained from animals fed at 32 p.p.m. for 10 weeks and then placed on withdrawal, with sacrificing at 1, 2, 4, and 8 weeks. Here again, male and female follow the same trend.

The data through this procedure, then, indicate Perthane following well defined relationships throughout the range employed for chemical detection. In this study, the sensitivity was about 0.3 p.p.m. About 100 samples were analyzed. A measure of reproducibility is indicated in replicate determinations (Table I).

Control samples and fortified con-

Table I. Results on Rats Fed 8 Weeks on Diet Containing 3.2 P.P.M. of Perthane

Type of Rat	Found, P.P.M.	
	1st detn.	2nd detn.
Control		
Male	1.43[a]	1.53[a]
Female	1.49[a]	1.45[a]
3.2 p.p.m. fed		
Male	0.67	0.80
Male	0.57	0.56
Male	0.67	0.76
Female	0.83	0.86
Female	0.67	0.56
Female	0.76	0.86

[a] Control values expressed in apparent p.p.m.

Table II. Recovery of Perthane in 3 Grams of Rat Fat

Fortification		Recovery	
μg.	P.p.m.	P.p.m.	%
5	1.6	1.03	69
10	3.3	2.34	71
20	6.6	4.62	70
30	9.9	7.23	73
40	13.2	9.25	70

trols were analyzed concurrently with each set of samples determined. Control samples gave absorbance readings of the order of 0.100 and averaged 1.4 ± 0.15 p.p.m. A standard curve was prepared by adding varying amounts of Perthane to the fatty tissue before maceration in the mortar and pestles. Per cent recovery (Table II) is based on values obtained with pure dehydrohalogenated Perthane undergoing the color development step only.

Perthane in Cow's Milk

Basically the same method is applied to residue analysis of cow's milk. However, the problem is again complicated by introduction of high interference values and several revisions are required. In the method finally adopted, Perthane is extracted from milk with an ether-acetonitrile solvent in a ratio of 1:1 to 1 volume of milk (*9*). The ether and acetonitrile are evaporated off, and the resulting fatty residue is picked up with *n*-hexane. Perthane is re-extracted with acetonitrile to separate it from the butter fats, and then put through the general procedure.

Reagents. The Perthane, *n*-hexane, acetonitrile, adsorbent mixture, alumina, potassium hydroxide solution, and sulfuric acid used for work with rat fat were used. Diethyl ether was reagent grade.

Special Apparatus. Borosilicate glass tubes (Figure 1). Vacuum filter adapter (Figure 1). Receiver for vacuum filtration (Figure 1).

Procedure. Place 100 ml. of milk in a 1-liter separatory funnel and add 100 ml. of acetonitrile and 100 ml. of diethyl ether. Shake the contents vigorously for 2 minutes and let stand for 20 minutes. (Three phases generally appear: an almost clear bottom, an emulsion-like center, and a relatively clear top.) Draw off and discard all but the top layer and a few milliliters of the emulsion layer. Shake the funnel again briefly to concentrate and settle the heavy layer and when the separation is complete, draw off the emulsion phase as clearly as possible and discard. Pour the remaining clear phase through the top of the funnel into a 500-ml. beaker, containing a few glass beads. Evaporate slowly under N_2 on a steam bath until only a fatty residue remains. Do not overheat or overdry. Pick up the residue with 15 ml. of *n*-hexane and transfer to a 125-ml. separatory funnel. Wash the beaker with 60 ml. of acetonitrile (saturated with *n*-hexane) and add the washings to the funnel containing the hexane wash. Shake the funnel and contents vigorously for one minute and let stand until separation is complete. Take off the acetonitrile into another 125-ml. separatory funnel. Wash the original beaker again with 15 ml. of hexane and add the washing to the funnel containing the acetonitrile. Shake 1 minute and let stand until the phases are separated. Take off the acetonitrile into a tube (type 1) and evaporate just to dryness at 55° C., using a gentle stream of nitrogen.

At this point carry out the dehydrohalogenation, separation, and water washing exactly as for the rat fat. Then to the 25-ml. aliquot removed after the second wash add 4 grams of the adsorbent mixture. Cap the tube tightly and shake for 2 minutes. Filter the solution through a medium-fritted-glass filter funnel under aspirator vacuum into a receiver tube (type 2).

For color development and reading, remove a 20-ml. aliquot to a test tube (type 2), and evaporate off solvent under a gentle stream of nitrogen in a water bath at 55° C. Remove the final traces of solvent at room temperature while rotating the tube under nitrogen. Add 5 ml. of 96% sulfuric acid and, after allowing to stand for 15 minutes, determine the absorbance at 495 mμ.

Standard and Recovery Curves. Pipet aliquots of Perthane standard solutions (in hexane solvent) containing 1 to 50 μg. into type 1 borosilicate glass tubes. Evaporate the hexane solution almost to dryness in a water bath at 50° to 55° C., under a gentle stream of nitrogen, the last bit of solvent being removed at room temperature under nitrogen. From this point on, perform the dehydrohalogenation, separation, adsorption, and color development exactly as for the milk procedure.

Develop a recovery curve by adding known concentrations of Perthane (1 to 50 μg.) to control milk prior to the acetonitrile-ether extraction and then carry through the procedure.

Discussion. Many methods for the initial extraction of whole milk were tried (*2*). As expected, refractory emulsions presented one of the major difficulties when liquid-liquid extractions involving such solvents as hexane, alcohol, and acetonitrile were tried alone. Some work was carried out using detergents for butterfat separation (*3*). However, this led to the problem of cleanup of traces of the detergent which came through and showed up in the final color development stage. Freeze-drying of milk followed by extraction with polar and nonpolar solvents yielded low recoveries (*6*). Early in the work, it was surprisingly noted that when milk was fortified with Perthane and then extracted promptly in a hexane system, Perthane recovery was complete. However, if the Perthane-milk mixture was allowed to stand a few days and then extracted, low recoveries resulted. This same behavior was also noted when employing a methanol-hexane extraction system, thus providing data which might erroneously indicate a low recovery with time due to decomposition of the insecticide. Stability of the insecticide is demonstrated by the method finally selected, employing acetonitrile and diethyl ether. This method has proved to be highly efficient in extracting Perthane-milk samples stored over long time periods and affords a quick and clean separation without refractory emulsion.

Table III. Per Cent Recovery of Perthane over Various Time Periods

Type of Fortification	Average Recovery on Duplicate Determinations			
	After prompt extraction	After 1-day storage	After 5-day storage	After 10-day storage
Perthane in methanol	83	84	86	80
Perthane in hexane	83	81	83	84

Table III shows the constancy of Perthane recovery from fortified milk stored under refrigeration over various time periods prior to extraction with acetonitrile-ether. Two types of fortification were employed: Perthane added to the milk from a hexane solution and Perthane added via methanol. Prompt extraction with *n*-hexane after fortifica-

Table IV. Recovery of Perthane from 100-Ml. Milk Samples

Type of Sample	Perthane Added		Perthane Recovered	
	μg.	P.p.m.	P.p.m.	%
Milk (control)	0	0	0.060[a]	..
	0	0	0.057[a]	..
	0	0	0.054[a]	..
Milk (fortified)	5	0.050	0.043	85
	10	0.100	0.083	83
	20	0.200	0.160	81
	40	0.400	0.340	86
Milk (fortified after stripping operation)	10	0.100	0.082	82
	20	0.200	0.170	86
	40	0.400	0.320	85
100 ml. H_2O	20	0.200	0.170	86
	20	0.200	0.160	83
	40	0.400	0.340	86

[a] Control values expressed in apparent p.p.m.

tion gave excellent recoveries when a hexane fortifying solution was used, but poor recoveries when Perthane was added in a methanol solvent. It is assumed that the Perthane was not carried first to the butterfat, as is probably the case in a hexane addition. This might indicate, then, since butterfat is always removed in good yield by the hexane extraction, that the Perthane is not carried by the butterfat exclusively but possibly is tied up in milk in other ways. Coinciding with this evidence is the previous observation of the inability of the *n*-hexane extraction to yield a suitable Perthane recovery on stored milk, even though the amount of fat extracted each time remains relatively constant. However, when the acetonitrile-ether method was used, recoveries proved constant over various time periods and the same values were obtained for both the methanol and hexane type fortification.

As in the rat fat procedure, it was necessary to incorporate an acetonitrile extraction prior to dehydrohalogenation to reduce the quantity of fatty residue. Four grams of butterfat when put through the acetonitrile-hexane extraction are reduced to about 0.2 gram. Most of the loss can be traced to the acetonitrile-hexane partition, where Perthane has a partition ratio of 3 to 1 for acetonitrile in this system. Only one extraction with the acetonitrile–diethyl ether is made on the raw milk, since re-extraction does not increase the recovery. When water is substituted for milk, the same per cent recoveries result.

In the rat fat procedure, when adsorption was carried out after the dehydrohalogenation step, high control values resulted; however, in the case with milk, it was necessary to adsorb after dehydrohalogenation in a nonpolar solvent, hexane. The adsorbent adopted was of the same type and formulation as that utilized in the rat fat work.

Results. Results of the chemical assay of 100-ml. samples of raw milk fortified with purified Perthane are shown in Table IV. Samples of untreated milk were fortified before and after the acetonitrile–ether stripping for an indication of losses due to this initial extraction. Both types of fortification exhibited approximately the same per cent recovery. This same recovery was realized when water was substituted as substrate in the procedure. Recoveries were based on determinations using pure Perthane through the basic procedure without substrate, thus reflecting the loss solely due to extraction.

As a further investigation for sensitivity, 200 ml. of milk were analyzed, with the same 85% average recovery. It was expected that higher blank values might develop. However, absorbance readings for the controls showed the same magnitude as obtained with 100-ml. samples and no complications were experienced with this larger quantity of milk.

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INSECTICIDE RESIDUES

Diazinon Residues in Treated Silage and Milk of Cows Fed Powdered Diazinon

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THIS STUDY is a part of a continuing program to find insecticides which may be used to control forage insects without imparting harmful residues to the forage. Harmful residues are those present in amounts which are toxic to livestock ingesting the forage or those which may cause meat or milk contamination when ingested by livestock. The present study concerns the stability of Diazinon in silage and the residues of Diazinon excreted in the milk of cows fed the insecticide in the diet. Diazinon [*O,O* - diethyl - *O* - (2 - isopropyl-4 - methyl - 6 - pyrimidinyl)phosphorothioate] was studied because of its effectiveness against a wide variety of insects. To find the persistence of Diazinon in silage a 22-day, small scale study designed to follow the rate of insecticidal breakdown was undertaken. The insecticide was also administered in various amounts in capsules to dairy cattle to find the maximum permissible dosage.

Experimental Procedure

Immature rye grass of 19.6% dry