Extraction and Location of Selective Chlorinated Hydrocarbon Residues in Alfafa Hay

by T. E. ARCHER and D. G. CROSBY
Agricultural Toxicology and Residue Research Laboratory, University of California, Davis California

Pesticides have become essential to the economical production of almost all animal feeds, including by-products of other industries, such as sugar beet pulp. Insects, plant diseases, nematodes, and weeds are combated with dozens of different chemicals. The chlorinated hydrocarbon class of insecticides has been predominantly responsible for feed contamination in the past, but almost any pesticide can cause residue problems.

Many commercial feeds contain DDT and related chemical residues in the low parts-per-million range. Pesticides pass rapidly from feeds into milk, eggs, and other animal products and, once established, cannot be quickly eliminated simply by withdrawing the bad feed.

The present investigations have the following objectives: (1) to study the characteristics and efficiencies of solvents and methods for the extraction of DDT and related chlorinated hydrocarbon residues from alfalfa hay; (2) to determine the nature of the residues present, whether they are chemically bound to the plant material or are on the surface as a loose deposit, or are in solution with the materials on the plant surface; (3) to identify the form of the chemical residues; and (4) to determine the sites of

Bulletin of Environmental Contamination & Toxicology,
Vol. 2, No. 4, 1967, published by Springer-Verlag New York Inc.

the residues (whether they are on the surfaces of the plant material, including the leaves and stems) and what amount is present.

Experimental

Detection and Determination of Pesticide Residues. The methods described in this section were used routinely. Exceptions are described later herein. Thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) procedures were used, either separately or in combination. The GLC method of Crosby and Archer (3) was used except that a 6-ft. x 1/8-inch stainless-steel column packed with 10% DC 200 on 100-120 mesh Gas Chrom Q was used occasionally instead of the one specified. Samples were analyzed with or without alkali treatment (dehydrohalogenation), and the results are reported on a dry-weight basis. Recovery trials gave essentially quantitative results.

TLC was employed for screening and in combination with GLC, as an analytical tool. Silica gel H absorbent, pentane solvent, and the silver nitrate:2-phenoxyethanol color test (7) were employed. For quantitative work, chromatogram areas (containing the unknowns) were quantitatively extracted from the silica gel with benzene or pentane after comparing parallel R_f values (0.41 DDE; 0.26 DDT; 0.14 DDD) with developed standard pesticide tracers. The solvent extracts were analyzed by GLC.

Hay samples were obtained from fields in Yolo County, California. The fields had not intentionally been treated with pesticides during the previous season, so the residues found represent accidental contamination in the fields. The hays used ranged from freshly baled (16.5% moisture) samples to those stored up to three years in the laboratory (8.0% moisture). Several were taken of each sampling. However, the hay examined in each particular experiment was subsampled carefully, chopped into approximately one-half inch pieces, pooled and thoroughly

mixed, subsampled, and analyzed prior to experimental treatment. All samples were analyzed for DDT [1, 1, 1-trichloro-2-2-bis(4'-chlorophenyl)ethane] isomers, DDE [1, 1-dichloro-2, 2-bis(4'-chlorophenyl)ethylene] isomers, and DDD [1, 1-dichloro-2, 2-bis(4'-chlorophenyl)ethane] isomers.

Results and Discussion

A. Solvent and Extraction Method Efficiencies. A series of experiments were conducted to determine the most efficient method and solvent for the extraction of DDT-RCH (DDT plus related chlorinated hydrocarbons) from alfalfa hay (Tables I, II, III). Subsamples from chopped, mixed, and composited hay were analyzed for DDT-RCH with six solvent systems and three extraction procedures: (1) 250 ml of solvent/15 grams hay (excess solvent) were refluxed for 1 hour and the extract and remaining plant solids were analyzed; (2) 650 ml/5 grams hay were refluxed in a Soxhlet apparatus for 32 hours, and the plant solids and extracts were analyzed; (3) 250 ml of solvent/15 grams hay were blended in a Waring blender for 5 minutes, and the extracts and plant solids were analyzed. The extracted plant solids remaining after the above treatment were refluxed for 30 minutes with ethanolic-KOH solution before GLC analysis. The advantage of using field-contaminated samples is that the pesticide is deposited in the plant material and not added by means of a solvent. If the plant material has little or no residue after repeated extractions, the extraction procedure is considered adequate.

Tables I, II, and III show that the solvents were similar in ability to extract DDT-RCH from hay (with pentane the poorest). Most efficient for removal of the residues was the Soxhlet method, followed by the reflux method, with the blending method the poorest. However, when the extracted plant solids were determined and the amount added to the previously extracted residues, all three methods appeared to be equivalent (Table III).

TABLE I

Comparison of Methods and Solvents
for the Extraction of DDT-RCH[1] from Alfalfa Hay

	DDT-RCH (ppb)[2]		
Solvents	Blending	Reflux	Soxhlet
Benzene	343	648	1058
Acetonitrile	271	528	1140
Pentane	300	460	729
Acetone	340	596	-
Ethanol	343	609	-
Isopropanol	290	433	847
Mean	315	536	944
Percent extracted	48%	75%	99%
Standard deviation	±29	±79	±164
Percent deviation	9.2%	14.7%	17.4%

[1] DDT plus related chlorinated hydrocarbons

[2] parts per billion

Refluxing hay for 1 hour with a suitable solvent recovered 75% of the total pesticide contaminants in the hay sample. However, blending for 5 minutes recovered only 48% in the solvent extract, while the Soxhlet extraction for 32 hours recovered 99% of the total residue. Warm and hot solvents were more efficient than cold solvent for extraction, which may partially account for the lower efficiency of the blending procedure. No difference in efficiency of extraction of DDT-RCH residues from

the alfalfa hay (Table III) could be shown when the total residue [that in the extraction solvent (Table I) plus that in the extracted plant material (Table II)] was compared with the mean value for the five other solvents (Student's t-distribution formula at a level of significance of 0.01). Also, no difference could be shown by statistical analysis for method efficiencies when the total residue values (Table III) were compared.

TABLE II

Residues from Previously Extracted Hay
Obtained by Ethanolic Alkali Extraction

	DDT-RCH (ppb)		
Original solvents	Blending	Reflux	Soxhlet
Benzene	394	160	9.4
Acetonitrile	363	228	2.4
Pentane	363	316	17.8
Acetone	338	133	-
Ethanol	294	133	-
Isopropanol	320	131	1.2
Mean	345	184	7.7
Percent of original	52%	25%	1%
Standard deviation	±32	±68	±6.6
Percent deviation	9.3%	36.9%	85.8%

TABLE III

Total Residues in Hay

(from Extraction Solvent plus Extracted Hay)

Extraction solvents	DDT-RCH (ppb)		
	Blending	Reflux	Soxhlet
Benzene	737	788	1067
Acetonitrile	634	756	1142
Pentane	663	776	749
Acetone	678	729	-
Ethanol	637	742	-
Isopropanol	610	564	859
Mean	660	716	957
Standard deviation	±41	±72	±159
Percent deviation	6.2%	10.1%	16.7%

B. Nature of the Residues. To determine whether the residues were in free or chemically bound form, solvent extraction was followed by repeated hydrolysis of the extracted plant material with ethanolic potassium hydroxide solution. The results are shown in Table IV. The hay was first extracted by three 1-hour benzene refluxings, which removed 96% of the total residue from the high-level hay and 82% of the total residue from the low-level hay. The remaining plant material was extracted 5 times with ethanolic alkali. The total residue found in the solvent plus the alkali extraction was 687 ppb for the high-level hay and 87 ppb for the low-level hay. Although the plant solids did not dissolve completely in the ethanolic alkali, after the first alkali

TABLE IV

Ethanolic-Alkali-Extractable Residues in Alfalfa Hay Previously Solvent-Extracted

	DDT-RCH (ppb)	
Method of extraction	High-residue hay	Low-residue hay
Benzene reflux (Three extractions; pooled)	659	71.5
Percent removed	96%	82%
Ethanolic alkali extraction of remainder		
Reflux #1	11.3	7.9
Reflux #2	7.5	3.8
Reflux #3	5.0	1.5
Reflux #4	3.4	1.0
Reflux #5	1.0	1.3
Total residue recovered	687	87

refluxing they progressively broke down and deteriorated. Most of the residues on the hay were removed by the solvent refluxing; those remaining were removed progressively by ethanolic alkali, with very little remaining after the fifth extraction. As shown in Table V, the subsample of hay composited as described above was hydrolyzed or extracted with ethanolic alkali directly without preliminary solvent treatment. The total DDT-RCH residue was 584 ppb for the high-level hay and 86 ppb for the low-level hay, respectively 85% and 99% of the residues shown in Table IV. Again,

the residues decreased progressively until only a small amount was found in the fifth extraction. The total residues recovered as presented in Tables IV and V showed a 10.3% deviation from the mean for the high-residue hay and a 0.6% deviation for the low-residue hay. The DDT-RCH residues in the plant material after solvent extraction probably represent those held by adsorption rather than by chemical combination with the plant material.

TABLE V

Residues in Alfalfa Hay by Direct Repeated Ethanolic Alkali Extractions

	DDT-RCH (ppb)	
Method of extraction	High-level hay	Low-level hay
Ethanolic alkali		
Reflux #1	451	47.0
Reflux #2	90	28.4
Reflux #3	23	4.8
Reflux #4	11	2.4
Reflux #5	9	3.6
Total residues recovered	584	86
Percent of total residue (See Table IV)	85%	99%

C. Nature of the Contaminants. All samples were analyzed for DDT, DDE, and DDD by TLC, GLC, or a combination of both. Residues (Table VI), expressed as the percentage of the total, ranged from 73.4 to 11.0% DDT, 84.7 to 17.7% DDE, and 8.9 to 4.3% DDD. These percentages represent not changes on the

plant material after sampling, but simply variations encountered due to sampling.

TABLE VI
Distribution (ppb) of DDT-RCH
in a High- and Low-Residue Alfalfa Hay

Sample	DDT	DDE	DDD	TOTAL
High-residue hay	469	113	57.0	639
Percent of total	73.4%	17.7%	8.9%	-
Low-residue hay				
Dehydrated meal	35.3	31.9	5.9	73.1
Percent of total	48.2%	43.6%	8.2%	-
Dehydrated pellets	9.5	73.5	3.7	86.7
Percent of total	11.0%	84.7%	4.3%	-

The values include all isomers for each chemical and represent principally the p, p'-isomers. No other chlorinated hydrocarbon residues were detected in the hay under the conditions of the experiments. The ratio of DDE to DDT was high in the pellets as compared to that in the dehydrated meal from which the pellets were prepared. Factors probably responsible for the degree of conversion of DDT to DDE were moisture, time, and temperature.

D. Site of the Contaminants. Tables VII through XI show the results of experiments to determine the site of the pesticide residues on hay samples. Table VII shows that the residue was not loosely distributed on the surface of the plant material as a thin film or deposited on the hay in dust or soil. The starting material contained 560 ppb DDT-RCH. The hay was put in a

TABLE VII

Removal of Residue from Alfalfa

by Various Water Washes

	DDT-RCH (ppb)	
	Cold-water wash	Hot-water wash
Content before treatment	560	560
Content after treatment	544	384
Percent loss due to treatment	2.86%	31.4%
DDT content of water rinse #1	46.3	8.8
DDT content of water rinse #2	5.5	5.0
DDT content of water rinse #3	10.5	2.6
Percent of total residue	108%	71.4%

large Erlenmeyer flask with 30 volumes of either cold or hot distilled water and shaken vigorously for 15 minutes on a rotary laboratory shaker. The water was filtered through a small loosely packed glass wool plug and stored. Washing was done three times; the glass wool plug was extracted with the first water washing three times with pentane, and the contents were pooled and analyzed. The second and third water washings were extracted in a similar manner without the glass wool plug. Only trace amounts of pesticide were found in the water washings, indicating that the residue was not loosely distributed on the plant material. This experiment was replicated twice with similar results. The difference between the 108% and the 71.4% figures in Table VII may be explained

by the volatility of DDT with water vapors. Acree et al. (1) observed that DDT is lost from various media as a result of the DDT-water codistillation phenomenon.

TABLE VIII

Residues (ppb) in Alfalfa Hay as Affected by Benzene Washing

	No dehydrohalogenation				Dehydrohalogenation
Method	DDT	DDE	DDD	Total	DDT-RCH
Before washing	331	42.6	21.0	395	363
Mean (ppb)				379	
Standard deviation (ppb) for unwashed dehydrohalogenated vs. non-dehydrohalogenated samples				±15.8	
Percent deviation				4.2%	
After various washings					
Wash #1	212.0	21.8	21.0	251.8	148.0
Wash #2	63.6	7.0	[a]	70.6	63.0
Wash #3	25.4	4.3	[a]	29.7	37.0
Wash #4	13.5	2.9	[a]	16.4	26.0
Wash #5	14.2	2.3	[a]	16.5	28.0
Wash #6	23.3	2.9	[a]	26.2	27.0
Wash #7	6.5	1.4	[a]	7.9	10.0
Total	359	42.6	21.0	419	339
Mean (ppb)				379	
Standard deviation (ppb) of total residue values in benzene washes by dehydrohalogenated vs. non-dehydrohalogenated samples				±40.0	
Percent deviation				11.8%	

[a] No detectable residue; <1 ppb

TABLE IX

Serial Extraction (ppb) of Hay Following Benzene Washing

Serial extraction solvent	DDT	DDE	DDD	TOTAL
Benzene reflux #1	27.7	4.4	ndr	32.1
#2	15.2	2.4	ndr	17.6
Acetonitrile reflux #1	8.1	ndr	ndr	8.1
#2	4.5	ndr	ndr	4.5
Acetone reflux #1	ndr	ndr	ndr	ndr
#2	ndr	ndr	ndr	ndr
Ethyl alcohol reflux #1	ndr	ndr	ndr	ndr
#2	ndr	ndr	ndr	ndr
Total	55.5	6.8	ndr	62.3

ndr, no detectable residue; <1 ppb

The experiment summarized in Tables VIII and IX was made to determine whether the pesticide residue on the plant surface was bound to the cuticle by solution in such materials as the plant waxes. The nature of plant cuticles and the residue problem have been discussed by several workers (2, 5). A 60-gram hay sample was rinsed for 3 minutes in 900 ml of benzene warmed to 80°C, removed, and drained dry. The hay was rinsed seven times (Table VIII). After solvent washing, the extracted plant material (Table IX) was subjected to a number of different solvent treatments in the ratio of 200 ml solvent/15 grams of extracted plant material. Two 1-hour refluxings for each solvent were performed with benzene, acetonitrile, acetone, and ethyl alcohol in that order as a serial solvent extraction on the previously washed plant material, and the results are shown in Table IX. The maximum residue found on the hay after the initial surface washings was

62.3 ppb, or 12.9% of the total residues (benzene washings plus the extracted plant material). The original hay and the surface washings were analyzed with and without ethanolic-potassium hydroxide treatment. The results are shown in Table VIII. Using the Student's t-distribution formula and at a level of significance of 0.01, no significant difference was found between the alkali and nonalkali treatment. The original residues present on the hay were essentially recovered in the benzene washes, indicating a concentration of the compounds in the cuticle.

Tables X and XI show the amount of contaminants in the leaves and stems. Statistical evaluation showed no significant differences exist between the alkali and nonalkali treatments. A series of 5 composited hay samples and 5 leaf and stem samples from the composited hay were also analyzed. The samples were found to have mean values of 41% leaves, 59% stems, and 8.4% moisture. All results were corrected to a dry-weight basis. As shown in Table X, the hay and its leaves and stems were refluxed with 200 ml of benzene/15 grams plant material for 1 hour, and the extract was stored for analysis. Refluxing was done three times, and the second and third extracts were pooled and stored for analysis. The extracted plant solids remaining were refluxed for 30 minutes with ethanolic alkali solution before GLC analysis. Three benzene extractions of the hay by refluxing resulted in recoveries in excess of 97%.

The leaves made up 82% of the total residue (923 ppb), and the stems 18% (198 ppb) (Table XI). If the residue in a theoretical composited sample of leaves and stems is collected, a residue of 1122 ppb is obtained. By direct analysis the mean of 5 field samples of hay containing both leaves and stems had a residue of 824 ppb. This residue represents 73.3% of the theoretical calculated value obtained by individual direct analysis of the stems and leaves. Experimental errors probably account for the 26% deviation from

TABLE X

Effect of Dehydrohalogenation on Residue Values for Alfalfa Hay

	Mean (ppb)	Standard deviations	Percent deviations
Field-cured composite sample (100%)			
No dehydrohalogenation			
DDT	519	±54	10.4%
DDE	96	± 2	2.1%
DDD	35	± 0	0 %
Total	650	±54	8.3%
Dehydrohalogenation			
Benzene extract	814	±99	12.2%
Extracted plant material	10	± 2	20.0%
Total	824	±99	12.0%
Field-cured leaf sample (41%)			
No dehydrohalogenation			
DDT	1399	± 5	0.4%
DDE	121	±18	14.8%
DDD	271	± 5	1.8%
Total	1791	±15	0.8%
Dehydrohalogenation			
Benzene extract	2239	±134	6.0%
Extracted plant material	15	± 2	13.3%
Total	2254	±135	6.0%
Field-cured stem sample (59%)			
No dehydrohalogenation			
DDT	219	±43	19.6%
DDE	21	± 7	33.3%
DDD	50	± 0	0 %
Total	290	±48	16.5%
Dehydrohalogenation			
Benzene extract	327	±17	5.2%
Extracted plant material	10	± 1	10.0%
Total	337	±14	4.2%

theoretical, because of the multiple operations involved during analysis of the individual components. However, it is evident that the greater percentage of pesticide contaminants in alfalfa hay is on the leaves.

TABLE XI

DDT-RCH Residues on a Dry-Weight Basis in Alfalfa Hay by Direct Analysis on Composite, Leaf, and Stem Samples, Compared with a Theoretical Composite Sample

Sample	Percent moisture	Percent leaves	Percent stems	DDT-RCH (ppb)
Field-cured composite	8.4	41%	59%	824
Field-cured leaves	8.4	100%	-	2254
Field-cured stems	8.4	-	100%	337
Theoretical composite based upon above data for leaves and stems	8.4	41%	59%	1122
Percentage of theoretical recovered by actual analysis				73.3%
Percentage of theoretical residue contributed by leaves				82.0%
Percentage of theoretical residue contributed by stems				18.0%

The basic information collected in the above data will contribute to future work in the study of the intentional removal of pesticide contaminants from animal feedstuffs.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Eugene Whitehead, James Stokes, and Dennis Garber.

References

1. F. ACREE, Jr., M. BERGER, and M.C. BOWMAN, J. Agr. Food Chem. 11, 278 (1963).
2. A.S. CRAFTS and C. L. FOY, Residue Reviews, p. 112 (1962), Academic Press, Inc., New York.
3. D.G. CROSBY and T.E. ARCHER, Bull. Environ. Contam. and Toxicol. 1, 16 (1966).
4. F.A. GUNTHER and R.C. BLINN, Analysis of Insecticides and Acaricides. Interscience, p. 208 (1955), New York.
5. H.F. LINSKENS, W. HEINEN, and A. L. STOFFERS, Residue Reviews, p. 136 (1965), Academic Press, New York.
6. LOUIS LYKKEN, Residue Reviews, p. 19 (1963), Academic Press, New York.
7. L.C. MITCHELL, J. Assoc. Offic. Agr. Chem. 41, 781 (1958).