Comparison of Free and Total Residues of (2,4-Dichlorophenoxy)acetic Acid and 2,4-Dichlorophenol in Millet Resulting from Postemergence and Preharvest Treatment

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Free and conjugated residues of (2,4-dichlorophenoxy)acetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP) resulting from postemergence and preharvest treatments of millet were determined in seed, straw, and forage samples. Hydrolyzable residues of 2,4-D in millet seed ranged from less than 0.020 ppm from postemergence to 0.29 ppm from preharvest treatment; residues of 2,4-DCP in seed were less than 0.020 ppm from postemergence to 0.031 ppm from preharvest treatment. Straw samples contained from 0.23 to 0.39 ppm of 2,4-D and 0.027 to 0.033 ppm of 2,4-DCP from postemergence treatment and from 13 to 26 ppm of 2,4-D and 0.24 to 0.40 ppm of 2,4-DCP from preharvest treatment. Only 15-19% of the 2,4-D and 2,4-DCP were recovered as free residues. From 70 to 85% of the 2,4-D and 2,4-DCP residues dissipated from millet forage within 4 weeks after postemergence treatment. Residues of 2,4-D and 2,4-DCP were found to be stable to frozen storage in glass containers for up to 1 year.

In order to establish a tolerance for the registered use of (2,4-dichlorophenoxy)acetic acid (2,4-D) on millet, the Environmental Protection Agency required residue data for 2,4-D and a metabolite, 2,4-dichlorophenol (2,4-DCP). Samples of Proso millet (*Panicum miliaceum* L.) were treated with the alkanol amine salt of 2,4-D at four application rates, and samples of the seed, straw, and forage were harvested at various time intervals after treatment. The project protocol required the analysis of total residues, including conjugated and free, of both 2,4-D and 2,4-DCP. This was accomplished by using the hydrolyzable residue analysis (HRA) and free residue analysis (FRA) procedures of Bristol et al. (1982) for the analysis of 2,4-D and 2,4-DCP in potatoes. Free residues, determined by the FRA procedure, are those removed by simple extraction of the plant tissue with acidified organic solvent. Total residues as determined by the HRA procedure include the free, unaltered forms of the compounds as well as residues that are conjugated to sugars and amino acids and are converted to free form by acid hydrolysis of the plant tissue. Residues of 2,4-D and 2,4-DCP were quantitated by gas chromatography using a halogen-specific detector.

EXPERIMENTAL SECTION

Reagents and Standards. All chemicals were ACS reagent grade except as noted. Acetone and hexane were pesticide quality. Ethyl ether was distilled before use. Benzene was extracted with concentrated H_2SO_4 until colorless, washed with water, dried over calcium chloride, and distilled to remove interfering substances. Celite-545 (J. T. Baker) was leached overnight in 0.5 N sodium hydroxide, filtered, and washed with distilled water. Alumina, Woelm W-200 acid (ICN Pharmaceuticals, Inc., K & K Laboratories Division, Plainview, NY), was adjusted to activity II (4% water, w/w) and stored in a glass-stoppered Erlenmeyer flask.

Diazomethane was prepared from N-methyl-N-nitroso-p-toluenesulfonamide (Diazald, Aldrich Chemical Co., Milwaukee, Wi) according to instructions provided by the manufacturer. The ethereal reagent was redistilled and stored at -20 °C until used.

Analytical-grade 2,4-D (99%, Dow Chemical U.S.A., Midland, MI) was dissolved in acetone to give a stock solution containing 1 mg of 2,4-D/mL. Commercial 2,4-DCP (Eastman Chemical Co., Rochester, NY) was recrystallized from hexane. A stock solution containing 0.1 mg of 2,4-DCP/mL in acetone was prepared.

A combined working standard solution for fortification experiments was prepared by dilution of combined aliquots of each stock solution with water to give a solution containing 10 μg of 2,4-D/mL and 1 μg of 2,4-DCP/mL. Additional fortification standards were made by serial dilution with water.

Analytical-grade methyl 2,4-D (98%, Dow Chemical U.S.A., Midland, MI) was dissolved in benzene (0.1063 g/100.0 mL) to give a stock solution equivalent to 1 mg of 2,4-D/mL. Separate working standard solutions for GLC analysis were prepared by serial dilution of aliquots of the 2,4-DCP and methyl 2,4-D stock solutions with benzene to cover the range from 0.02 to 1 μg/mL phenol or ester.

[*acetic*-2-^{14}C]-2,4-D (4.2 mCi/mmol, of 98% radiochemical purity) was purchased from Mallinckrodt, St. Louis, MO. Stock and working standards were prepared as described for the nonradioactive material.

All standards were prepared and stored in volumetric flasks and culture tubes equipped with Teflon-lined screw caps (Corning Glass, Corning, NY).

Gas Chromatography. The gas chromatograph (Barber Colman Series 5000) was equipped with a Coulson conductivity detector (CCD, Tracor, Inc., Austin, TX) and a glass column (1.9 m × 2 mm i.d.) packed with 10% OV-1 on 80-100-mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA). Injector and column temperatures were 180 °C for methyl 2,4-D and 130 °C for 2,4-DCP. Detector transfer line, block heater, and furnace temperatures were 220, 220, and 840 °C, respectively. Gas flow rates were as follows: carrier gas (prepurified, filtered He), 60 mL/min; CCD vent gas (He), 60 mL/min; CCD reaction gas (H_2), 80 mL/min. Sample peaks were quantitated by interpolation of their peak heights between those of bracketing standards (within ±25%).

Field Application, Sampling, and Storage. Samples of Proso millet were grown at the Rosemount Agricultural Experiment Station, Rosemount, MN, and were treated

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at two stages of growth: postemergence, when the plants were 8-in. tall; preharvest, 14 days later, when the plants were in the heading stage. Treatment was with a bicycle-wheel sprayer using a spray volume of 206 L/ha. Postemergence samples were treated with 0.84 and 1.68 kg of a.i./ha of 2,4-D alkanol amine (Formula 40, Dow Chemical Co., Midland, MI) and forage samples were harvested at 1, 2, 3, 4, and 5 weeks after treatment. Preharvest samples were treated with 1.12 and 2.24 kg of a.i./ha of 2,4-D amine and forage samples were harvested at 1, 2, and 3 weeks after treatment. Samples of grain and straw were taken 10 weeks after postemergence treatment and 8 weeks after preharvest treatment. Four replicates of an untreated control plot and of each treated plot were sampled. All samples were stored in glass jars at −20 °C until analysis.

Selected control samples of millet seed and forage were fortified with 2,4-D and 2,4-DCP and stored in glass jars at −20 °C along with samples containing bioincurred residues in order to evaluate the storage stability of these compounds.

Analytical Procedure. Details for both the FRA and HRA procedures for trace residues of 2,4-D and 2,4-DCP in potatoes have been published (Bristol et al., 1982). Some modifications were made to accommodate differences in sample matrix encountered with the millet samples. The ether–hexane extracts from the FRA procedure were extracted with 15-, 10-, and 5-mL portions of 0.5 N sodium hydroxide which were collected in a 50-mL centrifuge tube fitted with a Teflon-lined screw cap. After the addition of 8 g of sodium chloride and acidification with 20 drops of concentrated sulfuric acid, the solution was extracted with four 12-mL portions of ether. The emulsion which formed was broken by centrifuging at about 2000 rpm for 5-10 min. For samples analyzed by the FRA procedure, 35 mL of 0.25 N sodium hydroxide was substituted for 25 mL of 0.25 N sodium bicarbonate for the elution of 2,4-D from the alumina column. In the HRA procedure, filtration of the aqueous hydrolyzed solution was facilitated by layering a 1-cm pad of Celite-545 over a piece of glass fiber filter paper (Whatman, grade GF/B, Clifton, NJ). After acidification, the filtrate was extracted with four 50-mL portions of a 1:1 mixture of ethyl ether and hexane.

Recovery Experiments. One control and one fortified sample were analyzed along with each set of six treated samples. Fortifications were made by pipetting an appropriate aliquot of an aqueous fortification standard directly onto the sample immediately before blending. When trace levels of 2,4-D and 2,4-DCP occurred in the control sample, the amounts present were calculated and used to correct the levels found in the recovery samples. Recoveries of 2,4-D and 2,4-DCP were used to correct the values of residues found in treated samples.

For determination of the recovery of 2,4-D through each step of the FRA procedure, 1-mL aliquots of an aqueous solution containing 6.79 μg/mL [acetic-2-^{14}C]-2,4-D (about 271 000 dpm/mL) were added to 25.0-g samples of millet seed and forage. Duplicate 1.00-mL samples were taken at each step of the procedure, and radioassays were performed by using the procedure of Bristol et al. (1982).

RESULTS AND DISCUSSION

Both acid and alkaline hydrolysis procedures were tested for possible use in the analysis of the millet samples. Attempts to hydrolyze millet seed in hot alkaline solution resulted in excess foaming and in a gas chromatographic background that interfered with the 2,4-DCP analysis. Similar problems were encountered by Bristol et al. (1982),

Table I. Effect of Hydrolysis Reflux Period on Recovery of Metabolically Incorporated Residues from Millet Seed[a]

length of reflux period, h[b]	residue level found, ppb	
	2,4-D	2,4-DCP
not heated	0.070	0.002
0	0.220	0.013
$^1/_3$	0.240	0.023
$^2/_3$	0.240	0.028
1	0.300 ± 0.028[c]	0.033 ± 0.003[c]
2	0.340 ± 0.033[c]	0.034 ± 0.002[c]
4	0.310	0.027
16	0.320	0.031

[a] Single determinations except as noted. [b] Timing was begun just as the mixture reached the point of reflux. [c] Mean ± standard deviation for triplicate analyses.

Table II. Recoveries of 2,4-D and 2,4-DCP from Fortified Millet Using the HRA Procedure

sample type	2,4-D		2,4-DCP	
	fortification level, ppm	% recovery	fortification level, ppm	% recovery
seed	0.020-0.20	91 ± 5.4[a]	0.020	83 ± 14[b]
straw	0.020-2.0	90 ± 9.7[c]	0.020-0.20	97 ± 11[c]
forage	0.20-20	96 ± 16[d]	0.020-2.0	95 ± 12[d]

[a-d] Mean ± standard deviation for (a) six analyses, (b) seven analyses, (c) four analyses, and (d) nine analyses.

Chow et al. (1971), and Løkke (1975). Earlier tests of potatoes (Bristol et al., 1982) showed that acid hydrolysis was as efficient as alkaline hydrolysis in releasing bound residues of 2,4-D. Triplicate samples of 2,4-D-treated millet seed subjected to a 2-h acid hydrolysis contained 0.16 ± 0.007 ppm of 2,4-D. Three identical samples subjected to a 2-h acid hydrolysis followed by a 2-h reflux in alkali showed residues of 0.13 ± 0.002 ppm of 2,4-D. Since no additional 2,4-D was released by the alkaline hydrolysis, acid hydrolysis was used for the analysis of the millet samples.

For determination of the optimum hydrolysis time, a sample of millet seed containing biologically incorporated residues of 2,4-D and 2,4-DCP was subjected to acid reflux for periods of 0–16 h. The 0-h sample was heated just to the point of reflux and then cooled quickly on ice. Increasing residues of 2,4-D and 2,4-DCP were recovered as reflux time increased from 0 to 2 h and levels remained constant for up to 16 h (Table I). A hydrolysis period of 2 h was chosen since longer reflux periods did not result in greater residues of 2,4-D or 2,4-DCP.

Recoveries of 2,4-D and 2,4-DCP from untreated millet samples which were analyzed by the HRA procedure are presented in Table II. Samples were fortified at several levels in order to simulate the wide range of residues found in the treated samples. Overall recoveries for 2,4-D and 2,4-DCP from the millet samples were 92 ± 10% and 92 ± 12% (mean ± standard deviation), respectively.

The modified FRA procedure was evaluated by fortifying samples of millet seed and straw with ^{14}C-labeled 2,4-D (at 0.27 ppm) and assaying the radioactivity at each step of the procedure. The results in Table III indicate that the FRA procedure is very efficient in recovering free 2,4-D from the millet samples. Analysis of the samples by gas chromatography gave recoveries equivalent to those obtained by radioassay. Adequate recoveries of nonradiolabeled 2,4-D and 2,4-DCP from each type of millet sample were also obtained by using the FRA procedure (Table IV). Overall recoveries of 2,4-D and 2,4-DCP were 88 ± 8% and 92 ± 13%, respectively.

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Table III. Recovery of [*acetic*-2-^{14}C]-2,4-D from Millet Seed and Forage Samples at Successive Steps in the FRA Procedure[a]

FRA procedure step	successive % of initial ^{14}C label present	
	seed	forage
initial extraction	96 ± 0.14	99 ± 1.0
partition into NaOH	95 ± 2.4	99 ± 4.2
partition into ether	94 ± 0.11[b]	99 ± 1.4
elution from alumina	93 ± 1.1	96 ± 2.2
final esterified sample	90 ± 1.8	99 ± 1.2
2,4-D by GLC analysis	86 ± 5.0	97 ± 3.9

[a] Mean ± standard deviation for triplicate analyses except as noted. [b] Duplicate analyses.

Table IV. Recoveries of 2,4-D and 2,4-DCP from Fortified Millet Using the FRA Procedure[a]

sample type	2,4-D		2,4-DCP	
	fortification level, ppm	% recovery	fortification level, ppm	% recovery
seed	0.020	76	0.020	86
straw	0.20	90	0.020	105
forage	2.0	90	0.20	73
forage	20	97	2.0	103

[a] Single analysis.

Both 2,4-D and 2,4-DCP were found to be stable under conditions of frozen storage (Table V). Samples containing metabolically incorporated 2,4-D and 2,4-DCP showed no changes in residue levels for storage periods of up to 1 year. Similarly, recoveries of 2,4-D and 2,4-DCP from untreated millet samples remained constant for the same storage period. Biologically incorporated residues of 2,4-D and 2,4-DCP in potatoes were also found to be

stable during frozen storage (Bristol, 1976).

Residues of 2,4-D, as determined by either the HRA procedure (Table VI) or the FRA procedure (Table VII), were 72–91 times higher in straw than in seed. Levels of 2,4-DCP, which showed similar results, were less than 14% of the levels of 2,4-D. Chow et al. (1971) found no residues of 2,4,5-T or MCPA in the grain of treated wheat samples, indicating that the phenoxyalkanoic herbicides are not extensively stored in the seed. Differences in residue levels between the postemergence and preharvest treatments (Table VI) reflect both the shorter treatment-to-harvest interval for the preharvest samples and the differences in application rates of the 2,4-D amine.

Earlier studies (Chow et al., 1971; Crosby, 1964; Yip and Ney, 1966) indicated that significant amounts of phenoxyalkanoic acids occurring in plants are conjugated and may be converted to the free form by acid or alkaline hydrolysis. Conjugated residues of 2,4-D comprised 10–25% of the 2,4-D recovered from potato tubers (Bristol et al., 1982). Løkke (1975) found that 88–92% of the 2,4-D recovered from barley grain occurred as conjugated residues. Free residues of 2,4-D and 2,4-DCP (Table VII) were much lower than hydrolyzable residues (Table VI) in millet seed and straw with 81–85% of the recovered 2,4-D occurring as conjugated residues.

Samples of millet forage harvested 1–5 weeks after postemergence treatment and analyzed by the HRA procedure showed a rapid decline in residues of 2,4-D during the first 3 weeks after treatment (Figure 1). Residues of 2,4-DCP in millet forage averaged only 1–3% of the levels of 2,4-D and showed a similar decline for the same time period (Figure 2). Cessna (1980) found that residues of 2,4-D in wheat declined rapidly in the period 4–7 weeks after treatment.

Residue levels of 2,4-D and 2,4-DCP determined by the HRA procedure in millet forage from the preharvest

Table V. Stability of 2,4-D and 2,4-DCP Residues during Frozen Storage[a]

sample type	storage period, months	fortification level, ppm		% recovery[b]		residue level found, ppm[b]	
		2,4-D	2,4-DCP	2,4-D	2,4-DCP	2,4-D	2,4-DCP
forage	0	bioincorporated[c]				62 ± 5.4	0.77 ± 0.028
forage	3	bioincorporated				63 ± 1.1	0.76 ± 0.079
forage	6	bioincorporated				62 ± 0.42	0.78 ± 0.066
forage	12	bioincorporated				64 ± 1.0	0.73 ± 0.036
forage	0	0.20–20	0.020–2.0	96 ± 16	95 ± 12		
forage	3	1.0	0.1	94 ± 1.9	99 ± 2.6		
forage	6	1.0	0.1	86 ± 1.1	88 ± 7.2		
forage	12	1.0	0.1	110 ± 8.8	74 ± 1.0		
seed	0	0.020–0.20	0.020	91 ± 5.4	83 ± 14		
seed	3	0.20	0.020	97 ± 1.1	93 ± 20		
seed	6	0.20	0.020	88 ± 1.5	82 ± 3.7		
seed	12	0.20	0.020	100 ± 3.6			

[a] Analyzed by the HRA procedure. [b] Mean ± standard deviation for triplicate analyses. [c] Composite sample from plots treated with 2,4-D at 2.24 kg/ha.

Table VI. Residues of 2,4-D and 2,4-DCP in Millet Seed and Straw As Determined by the HRA Procedure

treatment stage	treatment rage, kg/ha	sample type	residue level found, ppm[a]	
			2,4-D	2,4-DCP
control	0	seed	<0.020	<0.020
postemergence	0.84	seed	<0.020	<0.020
postemergence	1.68	seed	<0.020	<0.020
preharvest	1.12	seed	0.16 ± 0.053	<0.020
preharvest	2.24	seed	0.29 ± 0.086	0.031 ± 0.007
control	0	straw	0.060 ± 0.024	<0.020
postemergence	0.84	straw	0.39 ± 0.29	0.027 ± 0.006
postemergence	1.68	straw	0.23 ± 0.16	0.033 ± 0.006
preharvest	1.12	straw	13 ± 1.9	0.24 ± 0.007
preharvest	2.24	straw	26 ± 5.7	0.40 ± 0.061

[a] Mean ± standard deviation for four replicate samples.

Table VII. Residues of 2,4-D and 2,4-DCP in Millet Seed and Straw As Determined by the FRA Procedure

treatment stage	treatment rate, kg/ha	sample type	residue level found, ppm	
			2,4-D	2,4-DCP
control	0	seed	<0.020[a]	<0.020[a]
preharvest	2.24	seed	0.055 ± 0.032[b]	<0.020[b]
control	0	straw	<0.020[a]	<0.020[a]
preharvest	2.24	straw	4.0 ± 1.3[b]	0.075 ± 0.046[b]

[a] Single analysis. [b] Mean ± standard deviation for four replicate samples.

Table VIII. Residues of 2,4-D and 2,4-DCP in Preharvest Treated Millet Forage Determined by the HRA Procedure

treatment rate, kg/ha	application to harvest interval, weeks	residue found, ppm[a]	
		2,4-D	2,4-DCP
1.12	1	21 ± 5.2	0.31 ± 0.074
1.12	2	11 ± 4.5	0.24 ± 0.087
1.12	3	14 ± 4.9	0.32 ± 0.11
2.24	1	58 ± 13	0.77 ± 0.19
2.24	2	36 ± 9.9	0.53 ± 0.13
2.24	3	32 ± 0.60	0.59 ± 0.013

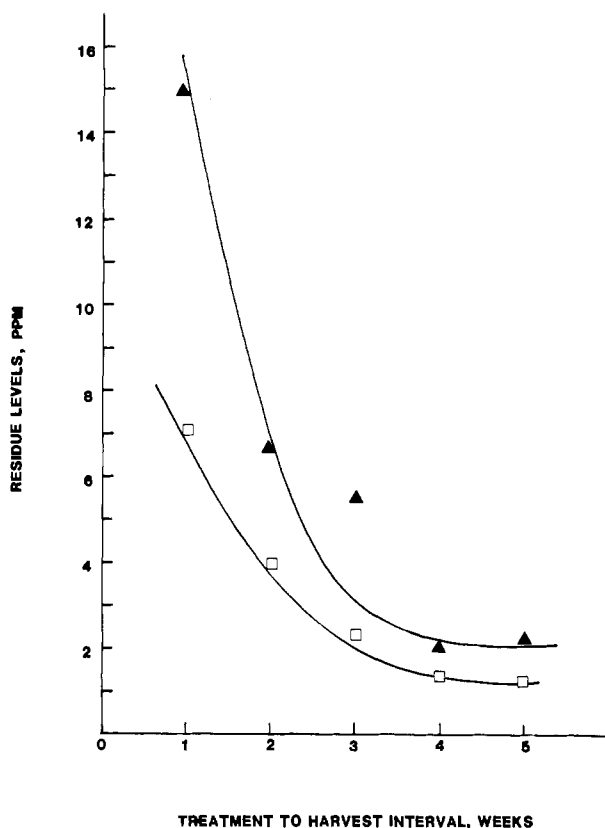[a] Mean ± standard deviation for four replicate samples.

Figure 1. Dissipation of 2,4-D in millet forage samples from plants treated postemergence at 0.84 (□) and 1.68 (▲) kg/ha active ingredient.

treatment were much higher than those in the postemergence treated samples (Table VIII). The higher residue levels reflect the higher treatment rates, the shorter application to harvest interval, and the stage of plant development for the preharvest samples. Although these samples appear to exhibit a decline in 2,4-D and 2,4-DCP
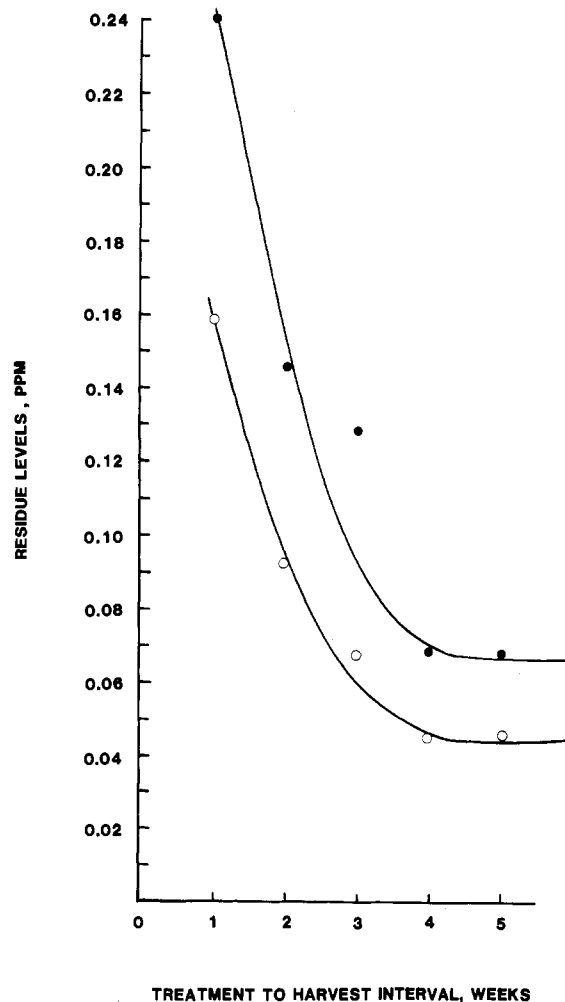

Figure 2. Dissipation of 2,4-DCP in millet forage samples from plants treated postemergence with 0.84 (O) and 1.68 (●) kg/ha active ingredient.

residues during the first 3 weeks after treatment, the variations in the replicate samples were too large to be statistically significant.

Registry No. 2,4-D, 94-75-7; 2,4-DCP, 120-83-2.

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