Determination of 4-(2,4-Dichlorophenoxy)-butyric Acid (2,4-DB) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Forage Plants

R. D. HAGIN and D. L. LINSCOTT

Crops Research Division, U. S. Department of Agriculture, Cornell University, Ithaca, N. Y.

A method for cleanup and analysis by gas liquid chromatography has been developed to investigate the disappearance of 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) and 2,4-dichlorophenoxyacetic acid (2,4-D) in forage plants. The method involves quick freezing and blanching of plant material, extraction with 2-propanol, separation of phenoxy acids from plant pigments using two-phase liquid separation, and analysis by electron capture gas chromatography. The dimethylamine salt of 2,4-DB was determined by gas chromatography after splitting off dimethylamine and methylating the acid radical. Herbicide recovery exceeds 96%.

The lack of suitable methods for isolation and determination of herbicides in plant materials limits progress in studies on herbicide metabolism and loss in plants (*4*). Although several methods now exist for determining 2,4-D (*2*, *3*, *5*) and 2,4-DB (*2*), they have limitations in forage plants and plants with very high concentrations of chlorophyll, waxes, sugars, and various other pigments. Detection of chlorinated phenoxy alkanoic herbicides is no longer a limiting factor with the advent of gas chromatographs and ultrasensitive electron capture detectors. However, satisfactory recovery of herbicide from forage plant material and presentation of herbicide quantitatively to the detector still remain as major problems.

Work in the authors' laboratory has dealt primarily with detecting herbicide after application to forage crops and its subsequent loss or degradation under climatic variables. This paper deals with a method of plant preparation and cleanup before analysis by electron capture gas chromatography. Attempts have been made to preserve forage and herbicide integrity as it exists at time of sampling from the field or greenhouse.

Apparatus

Lourdes Multi Mixer homogenizer with 400-ml. stainless steel cup.

Aerograph Hi-Fy gas chromatograph with a tritium electron capture detector and a Honeywell-Brown Electronik 1-mv. recorder.

Reagents

Diethyl ether, U.S.P., redistilled at 33–33.5° C.

Petroleum ether, 30° to 60° C. boiling range, analytical grade.

Isopropyl alcohol, analytical grade.

Acetone, analytical grade.

N-Methyl-*N*-nitroso-*p*-toluene sulfonamide (precursor for diazomethane), Eastman Organic Chemicals. Diazomethane prepared according to Arndt (*1*).

2,4-Dichlorophenoxyacetic acid, crystalline, Eastman Organic Chemicals.

4-(2,4-Dichlorophenoxy)butyric acid, crystalline, supplied by the American Chemical Co.

4-(2,4-Dichlorophenoxy)butyric acid, dimethylamine salt, commercial formulation (Butyrac 118), American Chemical Co.

Plant Material

Six forages were involved in this study: alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), red clover (*Trifolium pratense* L.), timothy (*Phleum pratense* L.), orchardgrass (*Dactylis glomerata* L.), and bromegrass (*Bromus inermis* L.). Forages used for laboratory research were grown in the field and greenhouse and harvested during a phase of rapid vegetative development prior to flowering.

Plant Preparation and Extraction

Sampling. Quick-freeze forages with dry ice immediately at sampling, seal in polyethylene bags, and store in a freezer at −10° C. until extraction.

Extraction. Chop frozen samples into 0.5-cm. lengths. Place 10 grams of the plant material in a 250-ml. beaker, pour 25 ml. of boiling water over the frozen plant sample, heat, and bring just to a boil. Remove from heat and swirl beaker contents to achieve adequate coverage. Cool and transfer quantitatively to a 400-ml. stainless steel blending cup. Rinse three times with 35 ml. of 2-propanol, adding each rinse to the blending cup. Blend with a Lourdes Multi Mixer for 5 minutes at 7000 r.p.m.

Transfer the homogenate to a Büchner funnel containing Whatman No. 42 filter paper, moistened with distilled water. Filter into a suction flask under reduced pressure. Rinse the plant residue from the blender's mixing head into the funnel, using 10 ml. of 2-propanol. Rinse the blending cup twice with 20 ml. of 2-propanol, adding each rinse to the Büchner funnel. Transfer the 2-propanol extract quantitatively to a 250-ml. volumetric flask and make to volume. This extract is equivalent to 1 gram of plant material (green weight) in 25 ml. of solution.

Cleanup and Analysis

Carry out all cleanup and methylation procedures in a fume hood.

Cleanup Procedure. Pipet 25 ml. of 2-propanol extract into a 60-ml. separatory funnel. (Use Teflon stopcocks or make certain that the glass stopcocks are completely free of grease. Traces of grease will interfere with herbicide determination.) Add 10 ml. of petroleum ether and shake for 30 seconds. Add 10 ml. of 0.03*N* HCl and shake for 30 seconds. Draw the lower layer into a second 60-ml. separatory funnel. Add 10 ml. of petroleum ether to this funnel and shake for 30 seconds. Draw the lower layer into a third 60-ml. separatory funnel and add 1 drop of concentrated HCl and 10 ml. (1 to 1, v./v.) of petroleum ether and diethyl ether. Shake 20 seconds and after separation discard the lower layer. Pool the three ether fractions in the first separatory funnel, rinsing each funnel with an additional 5 ml. of petroleum ether. Shake the combined petroleum ether fractions with 5 ml. of deionized water and after separation discard the lower layer.

Chromatographic Analysis. For 2,4-DB and 2,4-D as Acids. Transfer the petroleum ether fraction to a 50-ml. suction flask and rinse the separatory funnel several times with 1 to 2 ml. of petroleum ether. Add the rinse to the suction flask. Let the petroleum ether fraction react with excess diazomethane (approximately 20 mg. per sample) for 10 minutes. Shake occasionally. Evaporate excess diazomethane and reduce the petroleum ether volume to 1 ml., using a water bath and vacuum. Do not evaporate to dryness. (Evaporating organic extracts containing plant materials to dryness produces insoluble

residues which trap a portion of the herbicide.) Transfer the petroleum ether to a 10-ml. volumetric flask. Rinse the suction flask three times with 1 ml. of acetone, and add each rinse to the volumetric flask. Make solutions to volume with acetone and inject 1 μl. into a gas chromatograph.

FOR TOTAL 2,4-DB (including acids plus dimethylamine salts). Shake the petroleum ether collected in the previous step for at least 30 seconds with 10- and 5-ml. portions of 5% $NaHCO_3$. (An emulsion may be formed during the second shake-out. If so, break the emulsion by adding 1 ml. of 2-propanol and reshaking.) Pool the two bicarbonate fractions and rinse the petroleum ether with 3 to 5 ml. of distilled water. Add the water rinse to the bicarbonate and discard the petroleum ether. Acidify the bicarbonate fraction to pH 2 with concentrated HCl and shake out twice with 10 ml. of a 1 to 1 solution of diethyl ether and petroleum ether. Pool the ether fractions and wash with several milliliters of distilled water. Discard the water layer and methylate as above. Calculate the concentration of the dimethylamine salt of 2,4-DB as the difference between total herbicide found in this step and that found by direct methylation of the petroleum ether fraction.

If a gas chromatograph is not available, the following additional step may be taken to prepare samples for analysis by ultraviolet spectroscopy. After acidifying, shake out the bicarbonate fraction twice with diethyl ether. Reduce pooled diethyl ether fractions to 0.1 ml. under reduced pressure. Transfer the residue to a volumetric flask with 2-propanol and make to volume. Read at 286 mμ against a 2-propanol blank. Ultraviolet analysis will be much less sensitive and specific for herbicides than GLC analysis. Further, herbicide in red clover cannot be determined spectrophotometrically by this method without further cleanup.

Table I. Recovery of 2,4-DB and 2,4-D and Dimethylamine Salt of 2,4-DB from Forages after Cleanup and Analysis by Gas Chromatography

	2,4-DB			*2,4-D*			*2,4-DB Dimethylamine*		
	Added,	*Recovered*		*Added,*	*Recovered*		*Added,*	*Recovered*	
Forage	*p.p.m.*	*P.p.m.*	*%*	*p.p.m.*	*P.p.m.*	*%*	*p.p.m.*	*P.p.m.*	*%*
Alfalfa	10	10	100						
	22	23	104	24	25	104	60	59	98
	50	52	104	50	52	104	155	153	97
Birdsfoot trefoil	10	10	100						
	22	20	91	24	21	87	60	56	93
	50	49	98	50	51	102	155	148	95
Red clover	22	23	104	24	25	104	60	56	93
	50	50	100	50	44	88	155	150	97
Timothy	22	20	91	24	23	96	60	59	98
	50	50	100	50	49	98	155	150	97
Orchardgrass	22	21	96	24	24	100	60	63	105
	50	51	102	50	50	100	155	146	94
Bromegrass	22	22	100	24	25	104	60	52	87
	50	46	92	50	48	96	155	163	105

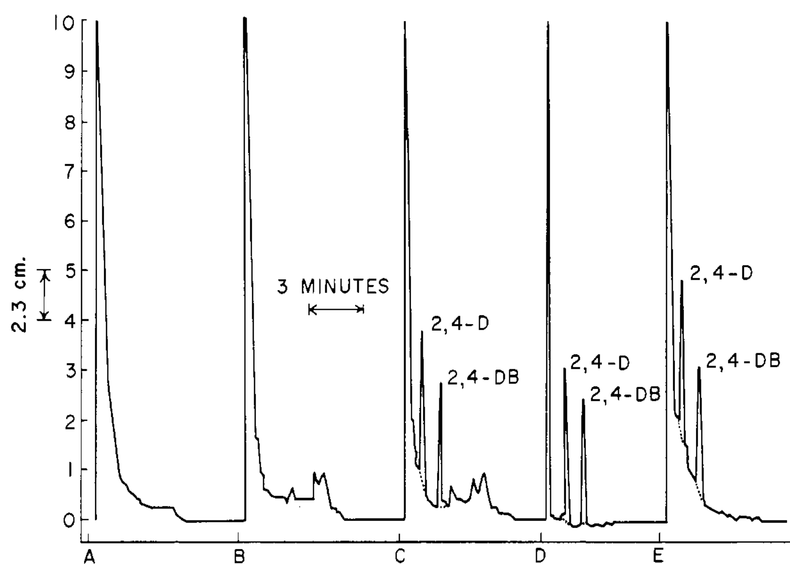

Figure 1. Chromatographic scans of cleanup fractions

A. Methylated diethyl ether phase after shaking out 2-propanol–water cleanup fraction (normally discarded)
B. Methylated petroleum ether phase containing plant constituents but no herbicide
C. Methylated petroleum ether phase containing plant constituents and 1 + 2 nanograms of 2,4-D and 2,4-DB, respectively
D. Standard solution containing 1 + 2 nanograms of 2,4-D methyl ester and 2,4-DB methyl ester, respectively
E. Methylated ether phase after $NaHCO_2$ shake-out containing remaining plant constituents and 1 + 2 nanograms of 2,4-D and 2,4-DB, respectively

Chromatograph Parameters

Column, 1/8- × 60-inch coiled borosilicate glass.

Column packing, 60- to 80-mesh, HMDS treated–Chromosorb W, coated with ethyl acetate fractionated Dow 11 silicone grease (10% by weight).

Oven and injector temperatures, 210° C.

Carrier gas, nitrogen, 40 ml. per minute.

Determination of Standard Curves and Per Cent Recovery

Reaction of varying concentrations of 2,4-DB and 2,4-D acids (up to 1500 p.p.m.) with excess diazomethane gave straight-line curves; therefore, for convenience, standard curves of 2,4-DB and 2,4-D were prepared by serial dilution of previously methylated acetone stock solutions. The acetone solutions contained 10 and 20 p.p.m. of 2,4-D methyl ester and 2,4-DB methyl ester, respectively. One microliter of solutions containing from 0 to 10 nanograms of 2,4-D methyl ester and 0 to 20 nanograms of 2,4-DB methyl ester were injected. Standard curves were constructed by plotting nanograms against peak height.

Recovery percentages were calculated from the ratio of herbicide detected to herbicide added to forage. As an example, for running 50 p.p.m. recoveries by gas chromatography, a 2-propanol solution containing 500 p.p.m. of 2,4-D plus 500 p.p.m. of 2,4-DB was prepared from stock solutions and 1 ml. of this solution was added to 10 grams of forage prior to blanching. The sample was then extracted with 2-propanol, cleaned up, methylated, and analyzed in the chromatograph.

Results

Typical chromatograph scans of the various cleanup fractions are shown in Figure 1. Scan *A* illustrates the complete removal of herbicide from plant material–acid-2-propanol into the petroleum ether phase, as evidenced by an absence of herbicide peaks. Some electron-capturing substances are contained in petroleum ether extracts (scan *B*) but do not seriously interfere

with the detection of 2,4-DB and 2,4-D. Retention times for the methyl esters of 2,4-DB and 2,4-D were 3.33 and 2.00 minutes, respectively. It is significant that passage of herbicide through the column was not affected by previous cleanup measures, as evidenced by identical retention times in scans *C*, *D*, and *E*.

Unknown plant constituents cause tailing at the origin and, as a result, herbicide peaks are imposed on a somewhat sloping base line. Manipulation of column constituents, gas flow, and column temperature would result in longer retention times and peaks originating from a horizontal base. However, analytic time per injection would be increased considerably. The authors have had no difficulty in obtaining good recovery data using the techniques described.

Recovery of 2,4-DB and 2,4-D acids in a range of 10 to 50 p.p.m. by the technique described varied from 87 to 105% for all six forages (Table I). The mean percentage recovery considering all forages is better than 96%. Herbicide recovery from an individual forage at a particular herbicide concentration varied by no more than ±4%. Recoveries are based on addition of a known quantity applied to forage before extraction and cleanup. All data represent means of three replications.

Previous unpublished research by the authors indicated that the dimethylamine salt of 2,4-DB as well as the acid was detectable for a considerable time after treatment with the commercial salt, especially at high herbicide concentrations. Isolation of herbicide amines and acids by paper chromatography and evolution of dimethylamine from plant extracts after KOH additions gave good evidence that both forms of the herbicide were present. Therefore, both the salt and acid should be considered in determination of herbicide. Recovery of the dimethylamine salt of 2,4-DB at 60 and 155 p.p.m. by this cleanup and analysis method for six forages ranged from 87 to 105% and averaged 96% (Table I). Recovery of the amine salt closely paralleled acid recovery.

The lower limit of detection for the tritium detector employed was 0.06 nanogram of 2,4-DB and 0.03 nanogram of 2,4-D. For routine operations the procedure is reproducible within 0.1 p.p.m. for 2,4-DB and 2,4-D.

Discussion

Several steps are critical for successful cleanup and analysis by the method described: inactivation of enzymes and denaturing of proteins, prevention of chlorophyll degradation, extraction of a broad range of organic plant constituents, and removal of highly polar compounds. Plants must be quick-frozen immediately after harvest to inactivate enzyme systems. Frozen plant material must be briefly immersed in boiling water or exposed to steam to denature proteins without fragmenting chlorophyll molecules. The last and most critical step in this method is the partitioning of chlorophylls and herbicide from highly polar compounds. This is done by two-phase separations involving acidified 2-propanol–water and petroleum ether. It is essential to remove the polar carotenoids, sugars, and leucoanthocyanins to enhance life of the chromatograph column and to prevent fouling of the detector. Failure to carry out these steps carefully will result in decreased recoveries and in some instances may preclude analysis.

Petroleum ether is not an ideal solvent for extracting organic acids in pure 2-propanol solutions and normally would not be used for this purpose. However, it does remove free herbicide acids quantitatively from a plant extract–acid–2-propanol solution. The authors had observed in previous research that chlorophenoxyalkanoic acids had a marked affinity for intact chlorophyll A. Chlorophyll A is highly soluble in petroleum ether, while the chlorophenoxyalkanoic acids are not. This procedure takes advantage of the herbicide-chlorophyll affinity by removing interfering polar constituents in the 2-propanol–dilute acid phase and quantitatively sweeping herbicide-chlorophylls into the petroleum ether phase. Diethyl ether should not be used as a substitute for petroleum ether for partitioning chlorophylls and herbicides from 2-propanol–water, because too many polar compounds will be carried over.

Chlorophylls and organic acids are partitioned into the petroleum ether phase. If the investigator is interested only in free herbicide acids or molecules of herbicide parentage that are readily converted to acids in the acid-alcohol shake-outs, methylation can be accomplished directly in petroleum ether. However, to analyze a mixture of herbicide amines and acids a $NaHCO_3$ treatment must be imposed. In the authors' experience dimethylamine salts of phenoxy herbicides in plant organic solvent extracts are not converted quantitatively to the corresponding acids by addition of acid. The pH must be elevated to at least 8.6 to cleave dimethylamine from the parent herbicide. Once dimethylamine is split off, the herbicide molecule can be easily converted to the acid and methylated.

The $NaHCO_3$ shake-out acts as an additional cleanup step to remove most chlorophylls, waxes, and other materials. Removal of these compounds prior to herbicide analysis will extend column life and lessen incidence of detector cleaning. However, as primary interfering substances have been removed previously by petroleum ether–acid–2-propanol partitioning, for free herbicide acid analysis it may be less troublesome to change a column more frequently than to proceed through the bicarbonate separation.

Birdsfoot trefoil and red clover extracts are the most difficult of the six forages to clean up for herbicide analysis by the methods described. More effort is required to obtain distinct phase separations. However, the usefulness of the procedure is not seriously affected. Probably the high saponin content of the two forages is a factor, particularly in birdsfoot trefoil.

The technique of cleanup and analysis as described is reasonably simple to perform and should offer a competent analyst little difficulty. Over 600 runs can be accomplished before a column change or detector cleaning is required. Approximately 75 samples per week can be analyzed from sample preparation through recording phases.

Literature Cited

(1) Arndt, F., *Org. Syn.* **15,** 3 (1935).
(2) Gutenmann, W. H., Lisk, D. J., J. Agr. Food Chem. **11,** 304 (1963).
(3) Freed, V. H., *Science* **107,** 98 (1948).
(4) Linscott, D. L., J. Agr. Food Chem. **12,** 7 (1964).
(5) Yip, G., *J. Assoc. Offic. Agr. Chemists* **45,** 369 (1962).

Received for review April 27, 1964. Accepted September 21, 1964. Cooperative investigations of the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and New York State College of Agriculture, Cornell University, Ithaca, N. Y. Agronomy Paper No. 647.