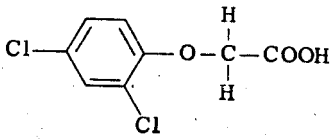
# 2,4-Dichlorophenoxyacetic Acid

R. P. Marquardt, H. P. Burchfield, E. E. Storrs, and Arthur Bevenue[1]



## I. GENERAL

### A. Empirical Formula

$C_8H_6O_3Cl_2$ (Mol. wt. 221.04).

### B. Alternate Name

2,4-D acid.

### C. Source of Analytical Standard

Bioproducts Center, The Dow Chemical Company, Midland, Michigan.

### D. Biological Properties

2,4-Dichlorophenoxyacetic acid and related compounds have such outstanding and unusual herbicidal properties that they have become some of the leading synthetic plant hormones. By selective action they are highly toxic to most broad-leaved plants and relatively nontoxic to monocotyledonous plants; thus they are frequently used as weed killers. These phenoxy acids are also important for such selective uses as the prevention of preharvest fruit drop, the production of seedless fruit, and the regulation of the growth of plants.

### E. History

Shortly after Pokorny (1941) reported the preparation of 2,4-dichlorophenoxyacetic acid, investigators in England and at the

[1] Sections I and I,A are by R. P. Marquardt; II,B by H. P. Burchfield and E. E. Storrs; and II,C by Arthur Bevenue.

Boyce Thompson Institute for Plant Research in the United States proved the growth-regulating properties of the aryloxyalkylcarboxylate series of compounds. In 1942, Zimmerman and Hitchcock first described the use of 2,4-dichlorophenoxyacetic acid as a plant growth regulator. Many of the discoveries which resulted from the research on these compounds during the war years were not published until after the war because of their military value.

Since the war, the manufacture of 2,4-dichlorophenoxyacetic acid and related compounds and their use as weed killers and plant growth regulators have grown tremendously. Other widely used members of this family of herbicides are 2,4,5-trichlorophenoxyacetic acid (2,4,5-T acid), 2-(2,4,5-trichlorophenoxy)propionic acid (Silvex), 2-methyl-4-chlorophenoxyacetic acid (MCP acid), 4-chlorophenoxyacetic acid, and 2,4-dichlorophenoxyethylsulfuric acid (Sesone). 2,4-Dichlorophenoxyacetic acid especially is a multimillion pound per year chemical in manufacture and use. The amine salts and esters are generally used to obtain desired solubilities and volatilities.

### F. Physical Properties

Melting point: 138°C (Pokorny, 1941); 139–140°C (uncorr.) (Synerholm and Zimmerman, 1945); 140–141°C (Zimmerman, 1943).

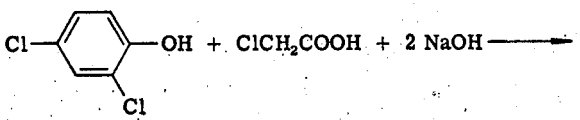
Solubilities (grams of 2,4-D acid/100 g solvent at 25°C). Data by Kaufman (1954):

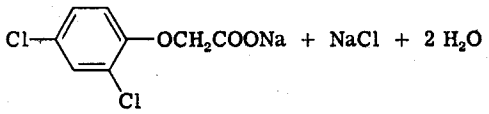
| | |
|---|---|
| Water | 0.089 |
| Ethanol | 129.9 |
| Ethyl ether | 24.34 |
| *n*-Heptane | 0.111 |
| Toluene | 0.67 |
| Xylene | 0.58 |

White, crystalline material, stable and nonhygroscopic.

### G. Preparation

2,4-Dichlorophenoxyacetic acid is usually prepared by reacting 2,4-dichlorophenol and monochloroacetic acid in aqueous sodium hydroxide:






The related compounds are prepared in a similar manner.

## II. RESIDUE ANALYSIS

### A. Colorimetric Analysis of 2,4-D Acid and Related Compounds

#### 1. Review of Methods

A bioassay method involving the use of plants has been reported (Swanson, 1946). Two methods measuring the ultraviolet absorbancy of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid have been used by Warshowsky and Schantz (1950) and by Gordon and Beroza (1952); however, these methods were not capable of detecting microgram quantities of the two acids. A sensitive colorimetric test for phenoxyacetic acids reported by Freed (1948) involves heating the phenoxyacetic acids with chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) in concentrated sulfuric acid. A quantitative adaptation of this test was used by Marquardt and Luce for determining 2,4-dichlorophenoxyacetic acid in milk (1951) and in grain and seed (1955); however, these two methods were limited to the determination of phenoxyacetic acids (phenoxypropionic acids do not give a color test with chromotropic acid) and occasionally gave high blanks when interfering materials were difficult to remove. Finally, a basic analytical scheme involving cleavage with pyridine hydrochloride of the ether linkage common to the phenoxy acids was developed by Marquardt and Luce (1961).

Pyridine hydrochloride is an acidic onium salt, especially in the fused state. Prey (1941) showed that the molten compound cleaves the phenyl ethers. It was found that with the same conditions pyridine hydrochloride cleaves the phenoxy acids. The phenol derivatives liberated by the cleavage are determined colorimetrically as measures of the original phenoxy acids.

#### 2. Determination of 2,4-Dichlorophenoxyacetic Acid in Sugar Cane Juice

a. *Principle*

The 2,4-D acid is extracted from a sample of sugar cane juice with chloroform. After suitable clean-up, cleavage of the ether linkage in 2,4-

dichlorophenoxyacetic acid with pyridine hydrochloride produces 2,4-dichlorophenol, which is determined colorimetrically as a measure of the original compound.

b. *Reagents*

*Filter-aid.* Hyflo Super-Cel, a Celite product, diatomaceous silica (Johns-Manville Co.).

*Phosphotungstic acid solution.* Dissolve 200 gm of phosphotungstic acid (approximately $P_2O_5 \cdot 24WO_3 \cdot xH_2O$) in water and dilute the solution to 500 ml. Disregard any insoluble material in the solution.

*Hydrochloric acid,* concentrated.

*Hydrochloric acid,* dilute. Dilute 60 ml of concentrated hydrochloric acid to 1 liter with water.

*Chloroform,* redistilled. Center 90% cut of technical grade chloroform.

*Pyridine hydrochloride,* practical grade.

*Ammonium hydroxide,* approximately 1 *N*. Dilute 70 ml of concentrated ammonium hydroxide (28%) to 1 liter with water.

*Skellysolve F.* A 35° to 60°C petroleum fraction (Skelly Oil Co.).

*Ammonium hydroxide,* approximately 0.05 *N*. Dilute 50 ml of 1 *N* ammonium hydroxide to 1 liter with water.

*Buffer solution.* Dissolve 300 gm of dibasic potassium hydroxide in water and dilute the solution to 1 liter. This solution should have a pH of 9.1 ± 0.1 (check with a pH meter). If necessary, adjust the pH by adding a small amount of monobasic potassium phosphate or tribasic potassium phosphate.

*4-Aminoantipyrine,* 1.0% solution. Dissolve 1.00 gm of 4-aminoantipyrine in water and dilute the solution to 100 ml. Store the reagent in an amber bottle in a dark place. Make a fresh solution weekly.

*Potassium ferricyanide,* 2.0% solution. Dissolve 2.00 gm of potassium ferricyanide in water and dilute the solution to 100 ml. Store the reagent in an amber bottle in a dark place. Make a fresh solution weekly.

*2,4-Dichlorophenoxyacetic acid* (2,4-D acid), commercial product assaying 99+%.

*Methanol,* A.C.S. grade.

c. *Apparatus*

*Evaporative concentrator,* Kuderna-Danish, with 500-ml upper flask and 25-ml lower flask (Fig. 1).

*Peanut oil bath.* Regulated to a temperature range of 207° to 210°C.

*Wire holder.* For suspending the 25-ml flask of the Kuderna-Danish evaporative concentrator in the peanut oil bath.

*Steam distillation apparatus* (Fig. 2).

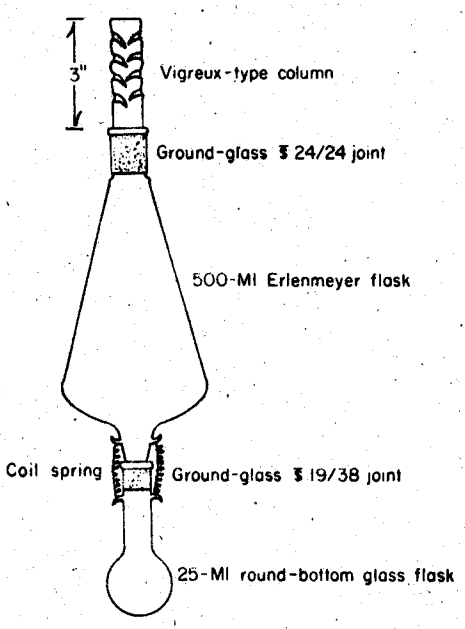



FIG. 1. Kuderna-Danish evaporative concentrator.

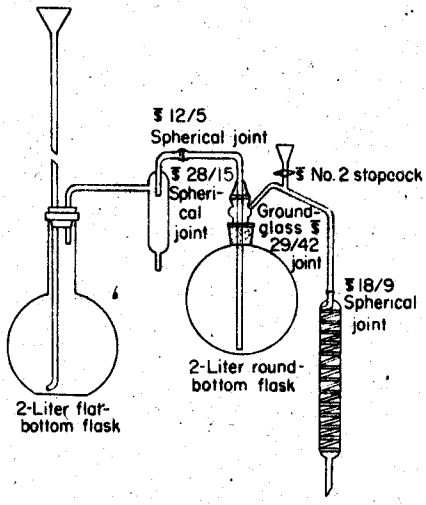



FIG. 2. Steam distillation apparatus.

*Coleman spectrophotometer.* Model 14, equipped with 4-cm absorption cells. Any photometer measuring light transmittance at 515 mμ should be suitable.

d. *Experimental Procedure*

i. *Sample Preparation and Procedure.*

Weigh 150 gm of sugar cane juice in a 250-ml beaker. Add 5.0 gm of filter-aid, 10 ml of phosphotungstic acid, and 10 ml of conc. HCl. Stir the mixture in the beaker occasionally for 15 minutes.

Filter the solids on a 7-cm Büchner funnel and wash with three 25-ml portions of dilute HCl. Discard the solids. Pour the combined solution of filtrate and washings into a 500-ml separatory funnel.

Place a few boiling chips in the Kuderna-Danish evaporative concentrator. Extract the 2,4-D acid from the solution with three 50-ml portions of chloroform. Combine the extract solutions in the concentrator and attach the Vigreux column.

Using a steam bath, evaporate the chloroform. When the liquid level is in the 25-ml flask of the concentrator, remove the Vigreux column and continue the evaporation. After all of the chloroform has evaporated, blow a gentle stream of air in the concentrator for 1 minute.

Disconnect the 25-ml flask from the concentrator and add 10 gm of pyridine hydrochloride to the residue. Using the wire holder, suspend the flask with contents in a peanut oil bath kept at a temperature of 207–210°C. After 10 minutes, swirl the contents of the flask to make a homogeneous solution and then keep the flask suspended in the oil bath for an additional 50 minutes. Cool the flask and contents to room temperature.

Dissolve the pyridine hydrochloride by filling the flask with water from a 150-ml portion of water. Add the aqueous solution to 150 ml of water and 10 ml of conc. HCl in the 2-liter round-bottomed flask of the steam distillation apparatus. Wash the 25-ml flask with the remainder of the 150-ml portion of water, adding the washings to the solution in the 2-liter flask. Steam-distill 500 ml of distillate into a 1-liter Erlenmeyer flask containing 25 ml of 1 *N* $NH_4OH$.

Pour the distillate into a 1-liter separatory funnel and wash it three times with 50-ml portions of Skellysolve. Discard the washings.

Acidify the washed distillate with 5 ml of conc. HCl. Extract the 2,4-dichlorophenol with three 50-ml portions of Skellysolve and combine the extract solutions in a 250-ml separatory funnel. Wash the solution with three 25-ml portions of water and discard the washings.

Extract the 2,4-dichlorophenol from the washed Skellysolve solution with one 10.0-ml and two 5.0-ml portions of 0.05 *N* $NH_4OH$ and combine the extract solutions in a 50-ml volumetric flask. Add 20.0 ml of buffer solution (pH 9.1) and 1.0 ml of 1.0% 4-aminoantipyrine solution and mix well. Add 1.0 ml of 2.0% potassium ferricyanide solution and

again mix well. After 1 minute, dilute to volume with water and mix well. Fill a 4-cm absorption cell with the solution. Three minutes after the addition of the potassium ferricyanide, determine the absorbance with a spectrophotometer at 515 mμ, using water as a reference liquid.

Determine the micrograms of 2,4-D acid represented by the absorbance by referring to the standard calibration curve. Subtract any apparent 2,4-D acid found in the control sugar cane juice and correct for the per cent recovery of 2,4-D acid obtained from the juice.

Calculate parts per million of 2,4-D acid, as based on the weight of the juice sample:

$$\text{p.p.m. of 2,4-D acid} = \frac{\text{micrograms in sample}}{150}$$

ii. *Preparation of Standard Calibration Curve.*

Prepare standard solution I as follows: Dissolve 0.100 gm of 2,4-D acid in about 50 ml of methanol and dilute the solution with methanol to 1 liter. Concentration: 100 μg of 2,4-D acid per milliliter.

Prepare standard solution II by diluting 15.0 ml of standard solution I to 200 ml with methanol. Concentration: 7.5 μg of 2,4-D acid per milliliter.

Prepare standard solution III by diluting 30 ml of standard solution I to 200 ml with methanol. Concentration: 15 μg of 2,4-D acid per milliliter.

Pipette 0, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 ml of standard solution II and 15.0 and 20.0 ml of standard solution III into respective 25-ml flasks of the Kuderna-Danish evaporative concentrator. Proceed with the known amount of 2,4-D acid in each flask as follows:

Add a few boiling chips and assemble the evaporative concentrator. Using a steam bath, evaporate the methanol. After all of the methanol has evaporated, blow a gentle stream of air in the concentrator for 1 minute.

Disconnect the 25-ml flask from the concentrator and add 10 gm of pyridine hydrochloride. Continue the determination as described in the procedure.

Prepare a standard calibration curve by plotting the data on graph paper. Beer's law is followed over the range from 0 to 300 μg of 2,4-D acid.

iii. *Recovery.*

Prepare standard solution A as follows: Dissolve 0.100 gm of 2,4-D acid in 25 ml of 1 *N* $NH_4OH$ and dilute the solution with water to 1 liter. Concentration: 100 μg of 2,4-D acid per milliliter.

Prepare standard solution B by diluting 15.0 ml of standard solution A with water to 200 ml. Concentration: 7.5 μg of 2,4-D acid per milliliter.

Prepare standard solution C by diluting 30.0 ml of standard solution A with water to 200 ml. Concentration: 15.0 μg of 2,4-D acid per milliliter.

Weigh 150 gm of nontreated sugar cane juice in a 250-ml beaker. Using standard solution B or C, add a known amount of 2,4-D acid in the same range as for the standard calibration curve.

Continue the determination as directed in the procedure.

Determine the micrograms of 2,4-D acid represented by the absorbance reading by referring to the standard calibration curve and subtract any apparent 2,4-D acid content found in the control juice. Calculate the per cent recovery obtained.

Recovery data obtained from known amounts of 2,4-D acid added to nontreated sugar cane juice are shown in Table I. Analysis of the nontreated juice itself showed no apparent 2,4-D acid content.

TABLE I
RECOVERY OF 2,4-D ACID FROM SUGAR CANE JUICE

| Added, p.p.m. | Found, p.p.m. | Recovery, % |
|---|---|---|
| 0.050 | 0.0450 | 90 |
| 0.050 | 0.0425 | 85 |
| 0.10 | 0.100 | 100 |
| 0.10 | 0.100 | 100 |
| 0.25 | 0.220 | 88 |
| 0.25 | 0.222 | 89 |
| 0.50 | 0.460 | 92 |
| 0.50 | 0.460 | 92 |
| 0.75 | 0.692 | 92 |
| 0.75 | 0.702 | 94 |
| 1.00 | 0.919 | 92 |
| 1.00 | 0.914 | 91 |
| 1.00 | 0.943 | 94 |
| 1.50 | 1.485 | 99 |
| 1.50 | 1.354 | 90 |
| 2.00 | 1.984 | 99 |
| 2.00 | 1.907 | 95 |

## 3. DETERMINATION OF PROPYLENE GLYCOL BUTYL ETHER ESTER OF 2-(2,4,5-TRICHLOROPHENOXY)PROPIONIC ACID IN SUGAR CANE JUICE

### a. *Principle*

The propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)-propionic acid[2] is extracted from a sample of sugar cane juice with carbon

[2] The commercial product is the active ingredient in Kuron (registered trademark of the Dow Chemical Co.) herbicide formulations.

tetrachloride. After suitable clean-up, cleavage of the ether linkage in the ester with pyridine hydrochloride produces 2,4,5-trichlorophenol, which is determined colorimetrically as a measure of the original compound.

b. *Reagents*

The following reagents are in addition to, or supplant reagents already listed:

*Methanol,* dilute. Mix equal volumes of methanol and water.

*Carbon tetrachloride,* redistilled. Center 90% cut of technical grade carbon tetrachloride.

*Buffer solution.* Dissolve 200.0 gm of dibasic potassium phosphate and 20.0 gm of monobasic potassium phosphate in water and dilute the solution to 1 liter. This solution should have a pH of $7.8 \pm 0.1$ (check with a pH meter). If necessary, adjust the pH by adding a small amount of dibasic potassium phosphate or monobasic potassium phosphate.

*4-Aminoantipyrine,* 0.3% solution. Dissolve 0.30 gm of 4-aminoantipyrine in water and dilute the solution to 100 ml. Store the reagent in an amber bottle in a dark place. Make a fresh solution weekly.

*Potassium ferricyanide,* 1.0% solution. Dissolve 1.0 gm of potassium ferricyanide in water and dilute the solution to 100 ml. Store the reagent in an amber bottle in a dark place. Make a fresh solution weekly.

*Propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid.* May be obtained from Bioproducts Center, The Dow Chemical Company, Midland, Michigan.

c. *Apparatus*

Same as already listed.

d. *Experimental Procedure*

i. *Sample Preparation and Procedure.*

Weigh 150 gm of sugar cane juice in a 400-ml beaker. Add 150 ml of methanol, 10 gm of filter-aid, and 10 ml of phosphotungstic acid solution. Stir the mixture in the beaker occasionally for 30 minutes.

Filter the solids on a 7-cm Büchner funnel and wash with three 25-ml portions of dilute methanol. Discard the solids. Pour the combined solution of filtrate and washings into a 1-liter separatory funnel. Add 150 ml of water and 10 ml of conc. HCl hydrochloric acid to the solution.

Place a few boiling chips in the Kuderna-Danish evaporative concentrator. Extract the propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid from the solution in the separatory funnel with three 50-ml portions of carbon tetrachloride. Combine the extract solutions in the concentrator and attach the Vigreux column.

Continue the determination as in the procedure for 2,4-D acid until ready to add the buffer solution.

Pipette 20.0 ml of buffer solution (pH 7.8) and 1.0 ml of 0.3% 4-aminoantipyrine solution into the 150-ml volumetric flask and mix the solution well. Add 1.0 ml of 1% potassium ferricyanide solution and again mix well. After 1 minute, dilute to volume with water and mix well. Fill a 4-cm absorption cell with the solution. Three minutes after the addition of the potassium ferricyanide, determine the absorbance with a spectrophotometer at 505 mμ, using water as a reference liquid.

Determine the micrograms of propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid represented by the absorbance reading by referring to a standard calibration curve. Subtract any apparent ester found in the sugar cane juice and correct for the percent recovery of the ester obtained from the juice. Calculate parts per million, as based on the weight of the juice sample.

ii. *Preparation of Standard Calibration Curve.*

Prepare standard solutions of propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid with methanol in the same way as described for 2,4-D acid.

Obtain the absorption data on known amounts of the ester in the same manner as described for 2,4-D acid but using the procedure for the ester.

Prepare a standard calibration curve by plotting the data on graph paper. Beer's law is followed in the range from 0 to 300 μg of the propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid.

iii. *Recovery of Propylene Glycol Butyl Ether Ester of 2-(2,4,5-Trichlorophenoxy)propionic Acid Added to Sugar Cane Juice.*

Weigh 150 gm of sugar cane juice in a 400-ml beaker. Using the standard methanol solutions already prepared, add a known amount of propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid in the same range as for the standard calibration curve.

Continue with the determination as described in the procedure for the ester.

Calculate the percent recovery in the same manner as with 2,4-D acid in sugar cane juice.

Recovery data obtained by the authors are shown in Table II. Analysis of nontreated juice showed an apparent ester content of 0.028 p.p.m.

TABLE II

RECOVERY OF PROPYLENE GLYCOL BUTYL ETHER ESTER OF 2-(2,4,5-TRICHLOROPHENOXY)PROPIONIC ACID FROM SUGAR CANE JUICE

| Added, p.p.m. | Found, p.p.m. | Recovery, % |
|---|---|---|
| 0.050 | 0.033 | 66 |
| 0.050 | 0.047 | 94 |
| 0.10 | 0.087 | 87 |
| 0.10 | 0.082 | 82 |
| 0.25 | 0.224 | 90 |
| 0.25 | 0.247 | 99 |
| 0.50 | 0.461 | 92 |
| 0.50 | 0.492 | 98 |
| 0.75 | 0.651 | 87 |
| 0.75 | 0.618 | 82 |
| 1.00 | 0.839 | 84 |
| 1.00 | 0.879 | 88 |
| 1.50 | 1.320 | 88 |
| 1.50 | 1.356 | 90 |
| 2.00 | 1.834 | 92 |
| 2.00 | 1.834 | 92 |

## 4. APPLICABILITY OF THE BASIC ANALYTICAL PROCEDURE TO DIFFERENT AGRICULTURAL PRODUCTS

The basic analytical scheme, in which the respective phenols produced by cleavage of the phenoxy acids with pyridine hydrochloride are determined colorimetrically as measures of the original herbicides, is generally applicable to all types of agricultural materials. Extraction and clean-up procedures will have to be modified, of course, and colorimetric or other methods adapted for the phenols involved.

## 5. DISCUSSION

Chloroform is used in the procedure for 2,4-dichlorophenoxyacetic acid because carbon tetrachloride, employed in the same manner, does not extract this acid quantitatively.

Carbon tetrachloride is used in the procedure for propylene glycol butyl ether ester of 2-(2,4,5-trichlorophenoxy)propionic acid because it quantitatively extracts 2-(2,4,5-trichlorophenoxy)propionic acid yet extracts less of other materials along with it. However, chloroform can be used if on occasion it should be the preferred solvent.

Since pyridine hydrochloride is very hygroscopic, do not expose it to the air more than necessary. However, avoid desiccating the com-

pound, for the small amount of water already present appears to promote proper cleavage of the phenoxy acids.

## B. Gas Chromatographic Analysis of 2,4-D in Milk Using Internal Standards

### 1. RECOMMENDED METHOD

#### a. *Principle*

In this method (Storrs and Burchfield, 1961) milk is acidified and hydrolyzed enzymatically with pepsin to degrade the proteins. The 2,4-D and lipoidal materials are then removed by liquid-liquid extraction with diethyl ether. The 2,4-D is separated from the lipids by partition between hexane and acetonitrile; the 2,4-D appears in the acetonitrile phase. The solvent is evaporated, and the residue is methylated with $BF_3$-methanol (Metcalfe and Schmitz, 1961) or diazomethane.

The residue obtained after methylation is then injected into a gas chromatograph equipped with a 6-foot silicone column operated at 201°C. The methyl ester is eluted in about 6 minutes. Known amounts of 4-chlorophenoxyacetic acid and 2-chloro-4-bromophenoxyacetic acid are added to the milk immediately before extraction to serve as internal standards (Storrs and Burchfield, 1962). This permits calculation of the amount of 2,4-D in the milk from peak-area ratios, even though all of the steps in preparing the sample for chromatography are not quantitative. The background from milk constituents, which are not completely removed during the clean-up step, is substantially eliminated by using a microcoulometric detector which is selectively sensitive to halogen (Coulson *et al.*, 1960) (see also Volume I, Chapter 9).

#### b. *Reagents*

4-Chlorophenoxyacetic acid used as an internal standard can be obtained from Eastman. 2-Chloro-4-bromophenoxyacetic acid is synthesized from 4-bromophenoxyacetic acid by chlorination with sodium hypochlorite using the general method of Hopkins and Chisholm (1946). $BF_3$-methanol reagent (Applied Science Laboratories, Inc.) is used to esterify the acids. Pepsin N.F. Powder (Merck and Co.) is used to hydrolyze the milk prior to extraction. The magnesium sulfate used to dry the ether extracts is washed with an ether solution of *p*-toluenesulfonic acid and then with dry ether until neutral. It is dried and stored in a tightly capped bottle until used.

c. *Apparatus*

Milk is hydrolyzed in 1-liter Erlenmeyer flasks on a Gyrotary shaker (New Brunswick Scientific Co.). All-glass liquid-liquid extractors equipped with individual Glas-Col heaters and powerstats are used for the extraction of milk. Five hundred-milliliter separatory funnels are used for partition of extracts between hexane and acetonitrile, and 200-ml Erlenmeyer flasks for evaporation of the solvents and for methylation of acids. Extractions of the esterified residues are carried out in 300-ml separatory funnels, and 200-ml Erlenmeyer flasks are then used for drying the ether extracts and evaporation of the solvent. A small tube drawn to a closed capillary tip is used for the final evaporation of the solvent and concentration of the residue.

A gas chromatograph equipped with a microcoulometric titration cell for the detection of halides (Dohrmann Instrument Co., Model G-100) is used for the separation and quantitative analysis of the esters of 2,4-D and the internal standards.

d. *Experimental Procedure*

i. *Sample Preparation.*

A sample of milk (350 ml) which has been frozen and then warmed to room temperature is placed in a 1-liter Erlenmeyer flask. 2-Chloro-4-bromophenoxyacetic acid (70 $\mu$g) and 4-chlorophenoxyacetic acid (35 $\mu$g) are added as internal standards. Concentrated HCl (4.5 ml) is added while swirling the flask, followed by 2 gm of pepsin. The flask is swirled until all of the pepsin dissolves. The mouth is then covered with a film of plastic such as Saran wrap, and the flask is placed on a rotary shaker adjusted to a slow speed for 15 hours at 40°C. After digestion is complete anhydrous sodium sulfate (50 gm) is added, and the sample is boiled for 2 minutes and cooled to room temperature. It is then extracted with diethyl ether in a liquid-liquid extractor for 20 hours. At the end of the extraction period, the ether layer is dried over magnesium sulfate (10–15 gm). The ether extract is then filtered through Whatman No. 2 filter paper and the solvent evaporated under a stream of clean, dry nitrogen with the flask warmed over a water bath at 65°C. The residue is taken up in four 25-ml portions of hexane and added to a 500-ml separatory funnel. The flask is then rinsed with four 25-ml portions of acetonitrile which is also added to the funnel. The milk extract is partitioned between the two layers by shaking vigorously. When the phases separate, the hexane is drawn off with an aspirator. The acetonitrile

solution is extracted with three 50-ml portions of hexane, and the upper layers are drawn off and discarded. The acetonitrile layer is shaken vigorously with a fourth portion of hexane (50 ml), and the lower layer transferred quantitatively to a 200-ml Erlenmeyer flask. The acetonitrile is evaporated slowly to complete dryness under a gentle stream of clean, dry nitrogen while warming the flask in a water bath at 85 to 90°C.

The flask is cooled to room temperature and a small amount of acetonitrile (1–2 ml) is added. The flask is swirled to dissolve the residue, diethyl ether (10 ml) is added and the solution filtered through Whatman No. 2 filter paper into a 125-ml Erlenmeyer flask. The flask is rinsed with three 25-ml portions of ether, and the combined filtrate and washings are evaporated in a water bath at 85 to 90°C.

The residue is methylated by adding the $BF_3$-methanol reagent (5 ml) and boiling for 4 minutes on a steam bath. The reaction mixture is quenched with distilled water (100 ml) and extracted with two successive portions of diethyl ether (100 ml, then 40 ml) in a separatory funnel. The ether extract is neutralized with $NaHCO_3$ and dried over magnesium sulfate. The dried extract is decanted quantitatively into another flask and evaporated slowly at room temperature to a small volume, then transferred quantitatively to a small tube with a capillary tip. The remaining ether is evaporated, and the residue (20–50 $\mu$l) contained in the capillary tip is ready for injection into the chromatograph.

ii. *Chromatography.*

A Dohrmann Instruments Co. chromatograph with the injection block at a temperature of 230°C and the column at a temperature of 201°C is used. The carrier gas is nitrogen with an inlet pressure of 20 psig and the outlet at atmospheric pressure. A 6-ft × ¼-in. O.D. aluminum column packed with 20% Dow-Corning High Vacuum silicone grease on acid-washed Chromosorb is used.

Oxygen is introduced into the combustion tube to burn organic compounds to $CO_2$ and $H_2O$, while halogen-containing compounds are burned to $CO_2$, $H_2O$, and HX. Some elemental halogen may be produced if insufficient hydrogen is present. Only one-half the detector response is obtained from elemental halogen as from HX, since hypohalide as well as halide are produced when the halogen reacts with water. To prevent this as much as possible, the oxygen is bubbled through water or 0.5 *M* ammonium hydroxide prior to entering the combustion tube to add a source of hydrogen to the gas stream.

A portion of the milk extract (5 to 20 $\mu$l) is injected into the chromatograph, and the effluent from the column is allowed to vent into the

atmosphere for 1 minute to elute volatile components. The vent valve is then closed and the sample passed through the combustion tube. Methyl 4-chlorophenoxyacetate is eluted first, followed by the methyl esters of 2,4-D and 2-chloro-4-bromophenoxyacetate. If 2,4,5-trichlorophenoxyacetic acid is present, the methyl ester is eluted after methyl 2-chloro-4-bromophenoxyacetate.

iii. *Interferences*

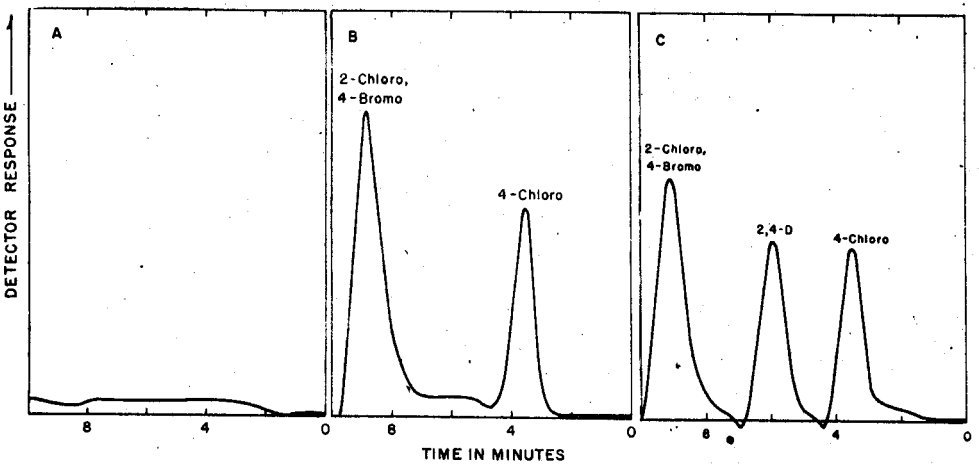



FIG. 3. Recovery of 2,4-D from milk using 4-chlorophenoxyacetic acid and 2-chloro-4-bromophenoxyacetic acid as internal standards. A, milk background without 2,4-D or standards. B, standards without 2,4-D added to milk. C, standards with 2,4-D added to milk.

The only interferences other than normal milk background (Fig. 3A) would be other halogen-containing compounds with the same retention volume as 2,4-D methyl ester.

iv. *Sensitivity.*

Using the procedure described above, as little as 0.01 p.p.m. of 2,4-D can be determined on chromatographing ¼ to ⅓ of the residue remaining on extraction of 350 ml of milk.

v. *Recovery and Calculations.*

Recovery need not be quantitative when an internal standard is added to the sample before extraction (Burchfield and Storrs, 1962). 2-Chloro-4-bromophenoxyacetic acid and 4-chlorophenoxyacetic acid are suitable standards for the analysis of 2,4-D, since the former compound emerges from the gas chromatograph just before 2,4-D and the latter

just after it. Moreover, these two compounds have chemical properties similar to those of 2,4-D and behave in a parallel manner during extraction, clean-up, and esterification. Thus, when the internal standards are added to milk free of 2,4-D, and the sample is processed and chromatographed, two peaks are obtained, one for each standard (Fig. 3B). However, when 2,4-D is present, a third peak appears (Fig. 3C). The amount of 2,4-D present in the milk can be calculated from the ratio of the area of this peak to that of either standard by the relation

$$p = (A/A_o)p_oR$$

Where $p$ is parts per million of 2,4-D, $p_o$ the parts per million of standard added to the milk, $A$ the area of the 2,4-D peak, $A_o$ the peak area of the standard, and $R$ an empirical correction factor determined by adding a fixed amount of standard and various amounts of 2,4-D to uncontaminated milk. Either standard can be used, but a separate value for $R$ must be determined for each.

The amount of 2,4-D in the milk can also be interpolated from a curve in which peak area ratios are plotted against micrograms of 2,4-D. The relationship obtained in the absence of milk is shown in Fig. 4.

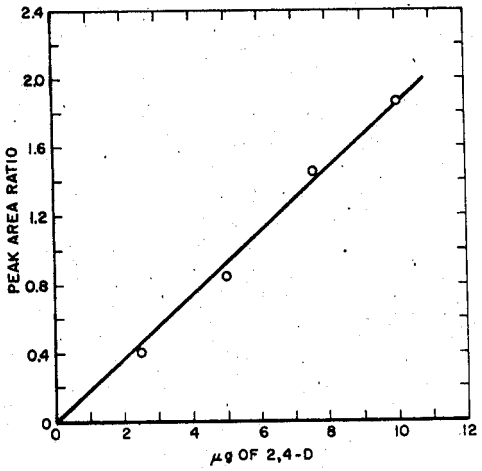



FIG. 4. Relation between micrograms of 2,4-D and peak area ratio using 2-chloro-4-bromophenoxyacetic acid as an internal standard in the absence of milk.

A linear relation is also obtained when 2,4-D and standard are extracted from milk in the range of 0 to 140 μg of 2,4-D in 350 ml of milk (Fig. 5).

The internal standard method is convenient, since it eliminates the need for quantitative recovery at each step and injection of the entire sample into the gas chromatograph. It also provides a safeguard against artifacts occurring during analysis, which might otherwise go undetected.

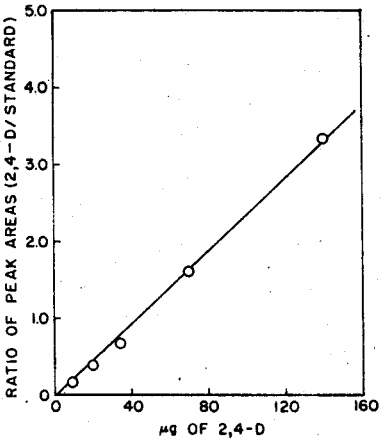



FIG. 5. Relation between micrograms of 2,4-D added to milk and peak area ratio using 70 μg of 2-chloro-4-bromophenoxyacetic acid per 350 ml of milk (0.2 p.p.m.) as an internal standard.

For example, analyses carried out on a series of putrified milk samples indicated that no 2,4-D was present. However, the standards could not be detected either, indicating that substances were present in the putrified milk which bound compounds of the 2,4-D type, and prevented their extraction. Without the standards, it would have been concluded, perhaps erroneously, that no 2,4-D was present. The use of internal standards also provides a convenient check on mechanical errors during routine analyses. If the peaks representing them are much smaller than usual, or are missing from the chromatogram entirely, it may be possible that manipulative errors were made.

### 2. APPLICABILITY TO DIFFERENT FOOD CROPS

This particular method was devised for the analysis of milk. However, there is no reason why it could not be applied to other dairy products and crops, with appropriate changes in methods used for extraction. However, a separate calibration curve should be run for each food product in which a constant amount of standard and variable amounts of 2,4-D are added and recovered. This involves no more work than demonstrating recovery by conventional analytical techniques, since in this case also, the validity of the method must be established for each food product.

### 3. DISCUSSION OF METHOD

It seems probable that many of the difficulties encountered in pesticide residue analysis could be reduced by use of the internal stand-

ard method coupled with gas chromatography. Ideally, the standard should have physicochemical properties identical to those of the pesticide except for a difference in retention volume great enough to permit good peak resolution.

The following criteria are suggested in selecting a standard: (1) The molecular structure and physicochemical properties of the standard should approach those of the pesticide as closely as possible. (2) The retention volumes of the two compounds should be as close as possible while still permitting baseline resolution. (3) The experimentally determined value $R$ should be as close to unity as possible. (4) The standard should not be a compound that is likely to be found as a major impurity in the pesticide. (5) The standard should not be another pesticide or have the same retention volume as known pesticides that might be carried through the analysis with it.

#### 4. Modification of Methods

Where laboratory safety is not a problem, the methyl esters can be obtained in higher yield and in a shorter time by substitution of diazomethane for $BF_3$-methanol. The residue after the final evaporation is taken up in ether containing 10% methanol (3 ml) and methylated by passing diazomethane through the solution for 20 minutes, as described in Burchfield and Storrs (1962). The solvent is evaporated and the residue is ready for injection into the gas chromatograph.

The amount of milk residue injected is quite large, so that the injection block becomes contaminated and the column overloaded with continued use. When the peaks become asymmetric, cleaning of the block and changing of the column is indicated. However, quantitative results, under otherwise unsatisfactory conditions, can be obtained when internal standards are used, since the ratios of peak areas remain constant even though their absolute magnitudes may vary from injection to injection. It is probable that column life could be prolonged by further clean-up of the milk extract by liquid-solid chromatography, but this has not been necessary in any of the work carried out to date.

### C. Gas Chromatographic Analysis of 2,4-D in Dry Crops

#### 1. Recommended Method

a. *Principle*

In this method (Bevenue *et al.*, 1962) crops of low moisture content are partially rehydrated with water followed by extraction with an acidified organic solvent mixture. After several base-acid extractions the

2,4-D is treated with diazomethane to convert it to its corresponding methyl ester. The methyl ester is determined with a microcoulometric gas chromatograph by measuring the areas of the peaks, recorded on a strip chart recorder calculating by means of an equation derived from Coulomb's law. Because of the specificity of the microcoulometric detector, only halide components will be recorded on the chromatogram. The principle of gas chromatography and microcoulometric detection is described in Volume I, Chapter 9.

b. *Reagents*

*Sulfuric acid solution,* 10% (v/v).
*Ethyl alcohol,* 95%.
*Ethyl ether.*
*Petroleum ether,* 30–60°C, redistilled.
*Benzene,* redistilled.
*Sodium bicarbonate,* 3% aqueous solution (w/v).
*Solvent mixture A:* 10 ml 10% sulfuric acid + 15 ml 95% ethyl alcohol + 25 ml petroleum ether + 75 ml ethyl ether.
*Solvent mixture B:* Ethyl ether-petroleum ether (30–60°C) v/v.
*Diazomethane.* A 100-ml distilling flask is fitted with a dropping funnel and an efficient condenser set downward for distillation. The condenser is connected to two receiving flasks in series, the second of which contains 20–30 ml of ether. The inlet tube of the second receiver is dipped below the surface of the ether and both receivers are kept cooled in an ice bath. Add 5 gm of KOH to the distillation flask and dissolve in 8 ml of water. Add 25 ml of 95% ethyl alcohol to the KOH solution. Heat the flask containing the alkali solution to 65°C. Add through the dropping funnel over a period of about 25 minutes a solution of 7.0 gm of "Diazald" (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., Milwaukee 10, Wis.) in about 130 ml of ethyl ether. The distillation rate should equal the rate of addition. When the dropping funnel is empty, slowly add 20 ml of ethyl ether and continue distillation until the condensate becomes colorless.

c. *Apparatus*

*Dohrmann microcoulometer gas chromatograph,* Model 100. Six-foot, ¼ inch O.D. aluminum or stainless steel spiral column containing 20% Dow-11 silicone grease on acid-washed Chromosorb P.
*Wiley mill,* equipped with a 2-mm screen.
*Shaker,* gyrotory (New Brunswick Scientific Co., New Brunswick, N. J.).
*Buchler,* batch model, evaporator.

d. *Experimental Procedure*

i. *Sample Preparation.*

Crop samples of low moisture content are ground in a Wiley mill to pass a 2-mm screen. Leafy or grasslike materials of higher moisture content are macerated by hand or in a food chopper. The meal or shredded material (25–50 gm) is transferred to a 500-ml Erlenmeyer flask, 25 ml of water are added and the contents mixed until the water is dispersed throughout the sample material. Solvent mixture A (150 ml) is added and the flask is agitated on a mechanical shaker for 1 hour. Grasslike materials will require 5 to 6-fold amounts of the solvent mixture A. Materials with high fat content may be preferentially mixed with the water and solvent mixture A in a Waring Blendor prior to the 1-hour shaking period.

The slurry is filtered through Whatman No. 1 filter paper on a Büchner funnel and the residue is washed twice with 20 to 25 ml aliquots of solvent mixture B. The filtrate and washings are combined. The filtrate is transferred to a separatory funnel, 75 ml of bicarbonate solution are added and the contents are carefully mixed. Two additional 50-ml amounts of bicarbonate solution are added, the flask is shaken vigorously, and the two phases are allowed to separate completely. The aqueous lower layer is retained. This solution is washed twice with 50-ml aliquots of petroleum ether and the ether phase discarded. Any flocculent precipitate present in the water phase may be removed by filtration through a plug of glass wool. The bicarbonate solution is acidified with 10% $H_2SO_4$ to about pH 3 and extracted with three 50-ml aliquots of ethyl ether. The ether layer is concentrated in a flash evaporator to about 10 ml and transferred to a 25-ml conical type centrifuge tube with benzene. One to two milliliters of diazomethane solution are added and the esterification is completed, usually within 10 minutes, as judged by the persistence of the yellow color due to excess diazomethane. The contents are transferred with benzene to a 6.5-ml calibrated hematocrit tube and, using a warm-air stream, the volume of the concentrate is reduced to a known amount (100–250 $\mu$l). Suitable aliquots of the sample are injected into the gas chromatograph apparatus.

ii. *Chromatography.*

Optimum chromatographic conditions are an injection block temperature of 250°C, column temperature 210°C, combustion zone temperature 825°C, and nitrogen and oxygen flow rates of 100 ml/minute.

The Dohrmann instrument is designed so that the chromatographic eluents are combusted to $CO_2$, $H_2O$, and halogen. A microcoulometer cell

connected to the combustion zone will determine the amount of halogen present by continuous automatic titration with internally generated silver ions. A permanent record of the results are produced on a strip chart recorder.

Sample sizes for injection into the gas chromatograph will vary from 10 $\mu$l to 50 $\mu$l (1–20 gm of original sample material) depending upon the amount of 2,4-D present in the sample. The amount of 2,4-D found in the sample is calculated by means of the following equation:

$$\text{Micrograms 2,4-D} = \frac{\text{(Peak area)(Recorder sensitivity)(35.5 gm/eq.)(60 sec/min)} \times (10^6)(10^{-3}V/\text{mv})(10^2)}{\text{(Sensitivity range, ohms)(\% Cl in 2,4-D)(96,500 coulombs/eq.)}}$$

Where the peak area is in square inches.

If the recorder sensitivity and chart speed each are unity, the above equation can be reduced to

$$\text{micrograms 2,4-D} = \frac{\text{(Area)(2210)}}{\text{(Ohms)(32.1\%)}}$$

For 1 to 5 $\mu$g of the 2,4-D methyl ester, optimum sensitivity is 64 ohms.

iii. *Interferences.*

Interferences will be limited to the possible presence of naturally occurring halides or halogen-containing pesticide contaminants having the same retention times as 2,4-D methyl ester.

iv. *Sensitivity.*

The lower limit of detection is 0.1 p.p.m. and may be as low as 0.05 p.p.m. if the background response is practically nil.

v. *Recovery.*

The average recovery from crop materials, such as lima beans, corn grain, corn and sorghum stovers, and walnuts will be about 80–85%. This can be determined by adding known amounts of 2,4-D to a plant material which is known to have no history of 2,4-D contamination. Gas chromatograph column efficiency for the methyl ester of 2,4-D was 95–105%.

## 2. DISCUSSION

With crops of low moisture content, it is important to rehydrate the sample material prior to extraction with the acidic organic solvent mixture; otherwise, 2,4-D recoveries will be very poor.

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