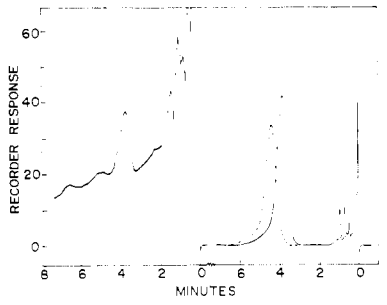


Figure 1. Chromatograms with electron-capture detection

— Compound 4072
– – – – Shell SD-8447
Right. 2-ng. amounts of pesticides injected in 5 μl. of benzene
Left. Raw benzene extract of corn (1.67 mg. equiv.) containing 0.833 ng. of Compound 4072 (0.50 p.p.m.).
Range setting 10^{-9} ampere, attenuation 3X with 10-mv. recorder

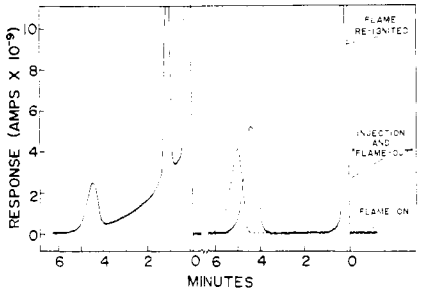


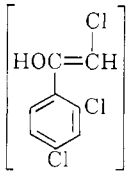
Figure 2. Chromatograms with flame-photometric detection

—— Compound 4072
– – – – Shell SD-8447
Right. 2-ng. amounts of pesticides injected in 5 μl. of benzene.
Left. Raw benzene extract of corn (16.67 mg. equiv.) containing 0.833 ng. of Compound 4072 (0.05 p.p.m.)

analysis could be further increased by injecting volumes larger than the 5 μl. used in this study and raising the column temperature to increase peak height.

In general, the flame-photometric detector was vastly superior to the electron-capture detector in terms of detector life, freedom from background interference, cleanup required, range of linearity, stability, and ease of operation. It would appear then that the flame-photometric detector is to be preferred if the application of either detector is possible and no specific interferences occur. One other advantage of the flame-photometric detector, which should prove of interest in the analysis of biological materials, is its insensitivity to water. The presence of water affects the electron-capture detector adversely.

The peak at 0.55 minute in the electron-capture runs of I appears to be the acid hydrolysis product (II) by its retention time and its p-value (*2,4*) in the hexane–acetonitrile system. The peak does not contain phosphorus or it would have appeared in the flame-photometric analysis. Since the structure of the hydrolysis product was not established, we prepared it by acid hydrolysis of I and found its melting point to be 54.5–55° C., which agreed with its reported value of 57° (*7*). The NMR spectrum, also in agreement with the structure of II, showed a singlet at δ4.70 (two methylene protons) and a complex multiplet at ca. δ7.50 (three aromatic protons). Its infrared spectrum showed a carbonyl absorption at 1706 cm^{-1}. [Subsequently, H. V. Claborn of this division informed us that he had identified II by comparing its infrared spectrum and R_f by his procedure (*6*) with the spectrum and R_f of a sample of II supplied by the General Chemical Co. His method of analysis of Compound 4072 cannot be used with the phosphorus detector.] The formation of II appears to take place by hydrolytic cleavage of I to give V, the enol form of II, which rearranges to give II.



V

In a similar manner Shell SD-8447 was hydrolyzed to give a compound melting at 64–65° C. Its NMR spectrum, in agreement with structure IV, showed a singlet at δ4.66 (two methylene protons) and two singlets at δ7.59 and 7.69 (one aromatic proton each; para protons do not couple). Its infrared spectrum showed carbonyl absorption at 1717 cm^{-1}. Since the compound could not be found in the literature, elemental analyses were run and found in agreement with IV:

ANALYSIS. Calculated for $C_8H_4OCl_4$: C, 37.2; H, 1.56; Cl, 54.9. Found: C, 37.19; H, 1.58; Cl, 53.78.

The carbon skeleton of IV was verified by carbon-skeleton chromatography (*1, 3*), a technique known to strip off chlorine and oxygen atoms; the expected product, ethylbenzene, was obtained. Compound IV and the impurity of Shell SD-8447 have the same retention time and the same p-value in the hexane–acetonitrile system (0.18) and therefore appear to be the same compound.

The extraction p-values (*4*) of Compound 4072 in the hexane–acetonitrile and hexane–dimethyl formamide systems are 0.058 and 0.026, and those of its hydrolysis product (IV) are 0.12 and 0.057, respectively. Extraction p-values of Shell SD-8447 and its hydrolysis product have been published (*4*).

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RESIDUE DETERMINATION

Residue Analysis of 4-Chlorophenoxyacetic Acid in Tomato Fruit

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THE ADVANTAGEOUS USE of 4-chlorophenoxyacetic acid (4-CPA) as a chemical to induce fruit set in tomatoes (*3*) has prompted the development of a sensitive analytical method for residue determinations of this plant growth hormone. Attempts to adapt the methods of Marquardt and Luce (*4, 5*) for determination of 2,4-dichlorophenoxyacetic acid (2,4-D) residues in grain and in sugar cane juice, and that of Erickson and Hield (*7*) for 2,4-D in citrus fruit proved unsatisfactory. A technique was developed whereby the 4-CPA is cleaved by pyridine hydrochloride to give 4-

A method for the determination of small residues of 4-chlorophenoxyacetic acid (4-CPA) in tomato fruits which had been treated with a 50-p.p.m. solution of this growth regulator shortly after the flower blossoms were open has been developed. The 4-CPA in the ether extract of raw tomatoes is cleaved by pyridine hydrochloride to give 4-chlorophenol which is purified by steam distillation. The 4-chlorophenol is brominated before acetylation, with final analysis by electron-capture gas chromatography as 4-chloro-2,6-dibromophenyl acetate with an over-all sensitivity of at least 0.001 p.p.m., or 0.5 μg. per 500-gram sample. Residues of 4-CPA at harvest time, 40 to 50 days after treatment, were negligible (below 0.001 p.p.m.).

chlorophenol (*5*); this compound is then steam distilled from the charred plant extracts and brominated to make it less soluble in water and a better electron capturer (*2*); the 4-chloro-2,6-dibromophenol is then extracted from the steam distillate, acetylated, and determined quantitatively as 4-chloro-2,6-dibromophenyl acetate using electron-capture gas chromatography. The reaction sequence for this procedure is shown in Figure 1.

Experimental

Apparatus and Equipment. Gas-liquid chromatography was accomplished using an Aerograph 600-C, equipped with a 5-foot, 1/8-inch o.d. copper column packed with 2% SF-96 on 30- to 60-mesh acid-washed Chromosorb W. The electron-capture detector was operated with a tritium source using nitrogen as a carrier gas at 30 ml. per minute with the column at 135° C.

Reagents. Acetone, ether, and benzene were all reagent grade (Baker Chemical Co.). Bromine–potassium bromide reagent was prepared according to the method of Shriner, Fuson, and Curtin (*6*). 4-Chlorophenoxyacetic acid was obtained from Distillations Products Corp. 2,6-Dibromo-4-chlorophenyl acetate (m.p. 81–83° C.) was prepared by brominating 4-chlorophenol and acetylating the 4-chloro-2,6-dibromophenol with acetic anhydride. Hexane used in the extraction procedure was purified by washing with concentrated H_2SO_4 two to three times, rinsing with water, drying, and distilling.

Residue Determination. Extraction. Five hundred grams of tomatoes were macerated in a 1-gallon Waring Blendor for 1 minute at high speed with 500 ml. of reagent grade acetone and 1 ml. of conc. HCl. The solids were filtered off using a 2-liter flask and a 15-cm. Büchner funnel. The solids collected were then returned to the blender, where 500 ml. of acetone were added, and macerated for 1 minute at high speed. The solids were filtered off using the same flask and then rinsed with acetone. After being rinsed, the solids were discarded and boiling chips added to the acetone extract. The acetone was completely boiled off on a steam bath and the remaining aqueous residue cooled to room temperature and extracted with one 125-ml. portion and two 100-ml. portions of ether. The aqueous portion was then discarded and the ether concentrated to a small volume for transfer to a 50-ml. Erlenmeyer flask.

Conversion to 4-Chloro-2,6-dibromophenyl Acetate. The ether extract was evaporated to dryness and 10 grams of pyridine hydrochloride were added. The flask was then placed in an air bath, covered with a watch glass, and heated to 200–210° C. for 6 hours. After being heated, the flask was cooled to allow addition of a small amount of distilled water from a 750-ml. portion. The contents were then transferred to a 1-liter round-bottomed flask, using small portions of distilled water to aid in the transfer. The last portion of water was warmed on the steam bath to transfer all of the insoluble material possible into the distillation flask. The remainder of the 750-ml. portion of distilled water was added to the round-bottomed flask, and 1 ml. of concentrated HCl and boiling chips were added. Distillation was continued until 500 ml. of water had been distilled into a 1-liter Erlenmeyer flask containing 5 ml. of 3*M* NaOH.

This basic distillate was extracted with one 125-ml. followed by two 100-ml. portions of ether. The ether extracts were discarded and the aqueous residue was acidified with 1 to 2 ml. of concentrated HCl. The bromination reagent was added dropwise until a pale yellow color was maintained in the acidified distillate. This was followed by addition of a small amount of sodium sulfite to remove excess bromine. The aqueous residue was again extracted with one 125-ml. and two 100-ml. portions of ether. These ether extracts were combined, concentrated to one half the volume, and the aqueous residue was discarded. The combined ether extracts were then extracted with 5 ml. of 3*M* NaOH solution. The basic extract was separated from the ether and 10 grams of ice and 0.5 ml. of acetic anhydride were added. The beaker in which this reaction was carried out was swirled until all the acetic anhydride had reacted. (If necessary, the beaker may be warmed on a steam bath for this reaction.) The reaction mixture was extracted with 25 ml. of redistilled ether, and the ether layer was separated.

The combined ether extracts again were extracted with 5 ml. of 3*M* NaOH and added to the aqueous portion of the first acetylation, and the mixture was reacetylated as described above. After the reacetylation, the reaction was extracted with a 25-ml. portion of redistilled ether and this extract combined with the first. The ether was evaporated to dryness and 1 ml. of benzene or 1 ml. of purified hexane added to the residue. One microliter of the resultant solution was injected with a syringe into the gas chromatograph for analysis. The sample analysis was preceded and followed by an injection of 1 μl. of 0.1 or 0.01 μg. per ml. of 4-chloro-2,6-dibromophenyl acetate standard for calibration of the peak area. Peak areas were determined using a planimeter, and by comparing peak areas of unknowns and standards, a

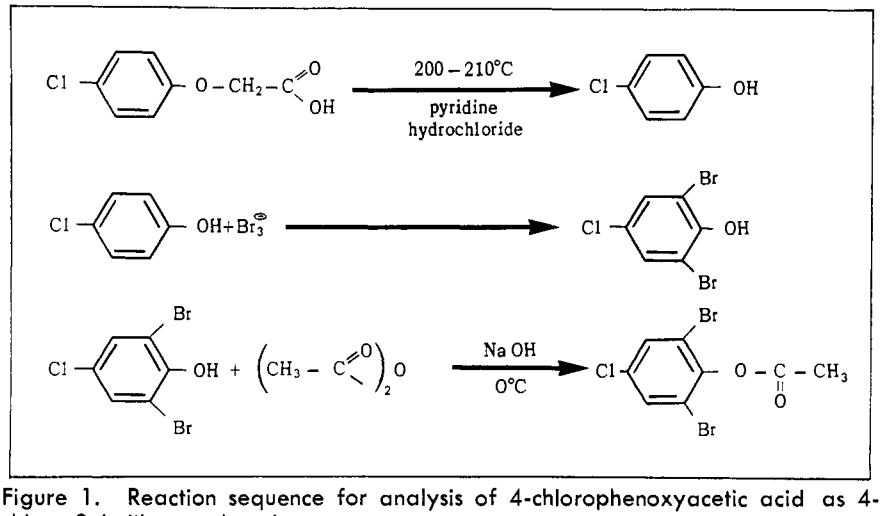

Figure 1. Reaction sequence for analysis of 4-chlorophenoxyacetic acid as 4-chloro-2,6-dibromophenyl acetate

quantitative estimate of 4-CPA in the tomato fruit sampled was calculated.

Results and Discussion

Cleanup and Analysis of 4-CPA. The determination of trace amounts of 4-CPA in tomato fruit posed a difficult problem. Attempts to apply the method of Marquardt and Luce (*4*) for 2,4-D in dried grain met with little success owing to very poor recoveries of added 4-CPA. Another method developed by Marquardt and Luce (*5*) for analysis of various phenoxy acid plant hormones in sugar cane juice was also tried. This method, which depended on the liberation and steam distillation of the corresponding phenol and colorimetric analysis after coupling with 4-amino antipyrine, was abandoned owing to the release of large amounts of phenol by the tomato fruit. An attempt was made to use the extraction procedure developed by Yip (*7*) for 2,4-D in spinach. However, when the solvent systems used for spinach were applied to tomato fruit, an almost intractable emulsion was obtained.

The method developed by Erickson and Hield (*1*) for 2,4-D in citrus fruit using the Dorman microcoulometric gas chromatograph as the method of quantitation was investigated. The extraction procedure seemed adequate for tomato fruit, but recovery of only 10 to 20% from samples spiked at the 0.1 p.p.m. level resulted from the gas chromatographic procedure. For this reason, and because of other difficulties in using the microcoulometric gas chromatograph, we decided to develop a method which could take advantage of the greater sensitivity and ease of operation of the electron capture gas chromatograph.

Since the methyl ester of 4-chlorophenoxyacetic acid is not a particularly good electron capturer, attempts were made to brominate the 4-CPA prior to methylation as suggested by Gutenmann and Lisk (*2*). However, trial brominations on pure 4-CPA resulted in a mixture of products which varied from one reaction to the next. Thus, it was decided to cleave the 4-CPA to 4-chlorophenol as was done by Marquardt and Luce (*5*) and then take advantage of the strong ortho-para activation by the phenolic hydroxyl group in the bromination step. Also, the steam distillation step thus added provided a simple and effective cleanup procedure. Bromination of pure 4-chlorophenol gave virtually a quantitative yield of 2,6-dibromo-4-chlorophenol.

Although 2,6-dibromo-4-chlorophenol could be gas chromatographed directly, it gave rather a broad trailing peak. Thus, attempts were made to make a less polar derivative which would give a sharper peak. Methylation to the corresponding anisole, using dimethyl sulfate, was attempted. However, by starting with pure materials, only about 50 to 60% yields of the 2,6-dibromo-4-chloroanisole were obtained. By using acetic anhydride, the 2,6-dibromo-4-chlorophenol could be acetylated with a yield of over 95%. This acetate gave a fairly sharp peak which was reasonably well separated from the other materials present in the analytical samples, as shown in Figure 2. The large peak which follows the 4-chloro-2,6-dibromophenyl acetate is probably 2,4,6-tribromophenyl acetate derived from natural phenol occurring in tomato fruit.

This method of analysis produced a technique for 4-CPA residues which was sensitive down to 0.001 p.p.m. with an average of approximately 50% recovery (Table I). A number of untreated tomato samples were spiked with 4-CPA at 0.0001, 0.01, and 1.0 p.p.m. levels. Some of these data are given in Table I which shows that 4-CPA recovered varied between 30 and 87% at the levels studied.

Table I. Recovery of 4-Chlorophenoxyacetic Acid from Tomato Fruit

4-CPA, P.P.M.	4-CPA in 500-Gram Sample, μg. Added		Recovered	Recovered, %
0	...		0.0000	
	...		0.0057	
	...		0.0055	
	...		0.0013	
		Av.	0.0031	
0.0001	0.05		0.027	53.7
	0.05		0.041	81.0
	0.05		0.020	40.0
	0.05		0.017	33.7
		Av.	0.026	52.0
0.01	5		1.63	32.6
	5		3.62	72.4
	5		3.72	74.3
	5		1.59	31.8
		Av.	2.64	52.8
1.0	500		282	56.5
	500		240	48.1
	500		435	87.0
	500		360	72.0
		Av.	329.3	65.9

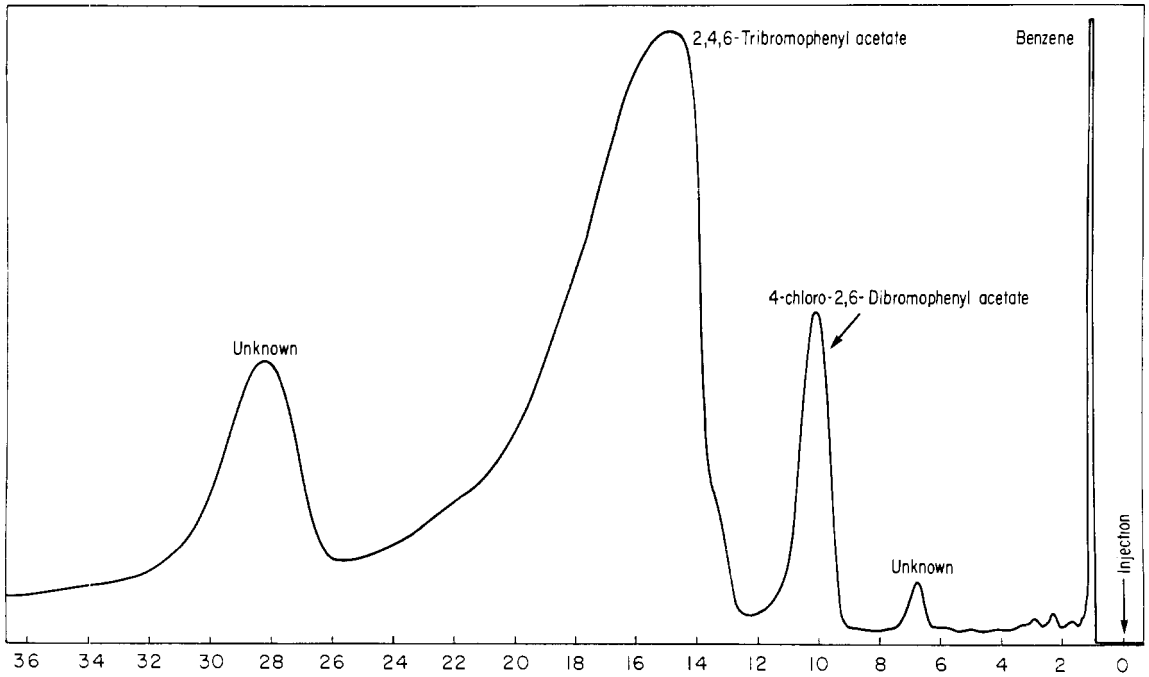

Figure 2. Chromatogram used in determination of residue in 500 grams of mature green tomatoes (*cf.* sample 15, Table II) which had undergone field treatment with 50-p.p.m. solution of 4-CPA

Final concentrated residue was dissolved in benzene and 1 μl. of this was injected as indicated. Abscissa in minutes

Table II. 4-Chlorophenoxyacetic Acid Residues in Tomato Fruit

Sample Number	Stage of Maturity at Harvest	4-CPA, P.P.M. Treatment	4-CPA, P.P.M. Found[a]
10	Breaker	None—Check	0.0000
14	Mature green	50	0.0008
15	Mature green	50	0.0009
19	Breaker	50	0.0009
21	Light pink	50	0.0007
23	Mature green	None—Check	0.0000
35	Light pink	None—Check	0.0000
51	Mature green	50	0.0001
57	Light pink	50	0.0008
58	Mature green	50	0.0019
65	Mature green	None—Check	0.0000
80	Table ripe	None—Check	0.0000
120	Mature green	50	0.0002
123	Mature green	None—Check	0.0000
124	Mature green	50	0.0004
129	Mature green	50	0.0003
182	Light pink	50	0.0010
188	Light pink	50	0.0002
212	Light pink	50	0.0012
218	Table ripe	50	0.0002
361	Table ripe	50	0.0003
371	Table ripe	None—Check	0.0000
		Average of treated samples	0.0007 ± 0.0005

[a] P.p.m. reported here have been adjusted assuming a 50% recovery of 4-CPA for the method described.

Residues in Tomatoes. Tomato blossoms were sprayed with a 50-p.p.m. aqueous solution of 4-CPA. These sprays were applied with a hand atomizer as a directed spray to the blossoms at the time the blossoms were open. This procedure was repeated at five weekly intervals so that new blossoms which opened each week would receive a 4-CPA treatment. Individual blossoms would receive only one of the five treatments. Fruits were harvested 40 to 50 days after 4-CPA treatment at various stages of maturity ranging from mature green (no color) to ripe (full red color).

Data from a portion of these analyses are given in Table II. While no samples were taken prior to the mature green stage, the data show that no detectable residues (above 0.001 p.p.m.) were found at the earliest harvest maturity.

Acknowledgment

Financial support of the San Diego County hormone tomato growers for the electron-capture gas chromatograph is gratefully acknowledged.

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ANALYTICAL INTERFERENCE

Carotenoid Pigments in Plants. Major Interfering Substances in Determining 2,4-D, a Metabolite of 2,4-DB

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Methylated derivatives of two natural plant pigments, the all trans isomer of lutein (3,3′-dihydroxy-α-carotene) and violaxanthin (3,3′-dihydroxy-5,6,5′,6′-diepoxy-β-carotene), isolated from brome grass (*Bromus inermis* Leyss.) and timothy (*Phleum pratense* L.), have retention characteristics identical to the methyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) on several types of chromatographic columns and ranges of column parameters that are commonly used in determining this herbicide.

IN RECENT studies of the metabolism and degradation of herbicides in forage plants, brome grass and timothy were treated with 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) and subsequently analyzed for herbicide by electron-capture and flame ionization gas chromatography. Not only could 2,4-DB be detected for a considerable time after treatment, but a chromatograph peak also was found with the same retention time and peak character as 2,4-dichlorophenoxyacetic acid (2,4-D). The peak height and area appeared to be dependent upon the concentration of 2,4-DB. Over a period of 30 days, it disappeared in a manner similar to 2,4-DB.

The peak in question was assumed to be caused by 2,4-D. This compound has been postulated to arise from beta-oxidation of 2,4-DB (*12*), and production of it from 2,4-DB in legume-grass mixtures has been reported by others (*4*). The relatively high levels of 2,4-D were thought to arise from an enzyme block allowing a pile up of herbicide.

Control samples yielded small peaks at the retention time for 2,4-D. These peaks were taken to be the "ghosts" referred to in chromatograph literature (*7*), and analytical data were corrected for them on this basis. However, the very high concentration of "2,4-D" that was found in 2,4-DB–treated plants (over 100 p.p.m.) should have resulted in visible herbicide effects even with the tolerant grass species under investigation. Since no symptoms

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