dicofol (Kelthane), malathion, maleic hydrazide, methoxychlor, methyl parathion, naled, ovex, parathion, phorate, phosphamidon, schradan, carbaryl, tetradifon, endosulfan, toxaphene, carbophenothion, and 2,4-D.

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Received for review November 5, 1965. Accepted April 25, 1966. Division of Agricultural and Food Chemistry, Pesticides Subdivision, ACS, Atlantic City, N. J., September 1962. A partial description of the method has been published (8).

PLANT REGULATOR DETERMINATION

Determination of 2,4-Dichlorophenoxyacetic Acid and 2-(2,4,5-Trichlorophenoxy)propionic Acid in Citrus by Electron Capture Gas Chromatography

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Very dilute sprays (20 p.p.m.) of 2,4-dichlorophenoxyacetic acid and 2-(2,4,5-trichlorophenoxy)propionic acid will prevent preharvest fruit drop in citrus. A gas chromatographic electron capture method is described in which various forms of 2,4-D and 2,4,5-TP are isolated as the free acid and converted to 2-butoxyethyl esters for analysis. These esters are readily resolved from interfering citrus materials. Data show that 0.125 μg. can be determined in 500 grams of citrus peel (0.00025 p.p.m.) with 89 to 93% recovery and good reproducibility. The method demonstrates an important means of studying the metabolism of very low concentrations of these growth regulators in citrus.

WARM, dry, fall and winter weather, in Florida, can bring about preharvest fruit drop resulting in losses of 50 to 75% of the midseason Pineapple orange crop. Dilute sprays (20 p.p.m.) of 2,4 - dichlorophenoxyacetic acid (2,4-D) and 2-(2,4,5-trichlorophenoxy)-propionic acid (2,4,5-TP) inhibit preharvest abscission of oranges.

Isopropyl 2,4-D has been investigated in California and Florida for control of fruit drop (*2, 5, 7*), and 2,4-D is now registered for this use.

In Florida, 2,4,5-TP as the propylene glycol butyl ether ester (Kuron, Dow Chemical Co.) (*3*) and as the triethanolamine salt (*6*) has been demonstrated to be effective for fruit drop control of citrus. 2,4,5-TP is of importance because it is as effective as 2,4-D and causes less foliage damage to the spring flush of growth; however, 2,4,5-TP is not registered. Therefore, a sensitive and accurate method was needed for determining the relative residues of 2,4-D and 2,4,5-TP in citrus occurring under Florida climatic conditions.

Erickson and Hield (*1*) reported 2,4-D residues of 0.1 p.p.m. of free acid in whole oranges within one day after spraying. This approached the lower limit of measurement by their microcoulometric gas chromatographic method.

Since the electron capture detector is reported to be 1000 times more sensitive to many halogenated compounds, use of this type of detection should provide more definitive information concerning growth regulator residue concentrations below 0.1 p.p.m.

Cleanup procedures for citrus present special problems when the electron capture detector is used because of the wide variety of chemical entities in the oils and waxlike materials. If not removed, these rapidly foul the electron capture detector, greatly lowering sensitivity.

Many substances remaining in citrus even after cleanup have retention times very similar to those of 2,4-D and 2,4,5-TP methyl esters most commonly used in gas chromatography (*1, 4*). Presence of these interfering substances makes accurate quantitative measurements difficult or impossible because they produce peaks which are ill-defined, frequently unresolved from the solvent peak (Figure 1). Another ester was sought that would have a more desirable retention time. Yip (*8*) studied six commercial herbicide esters using microcoulometric gas chromatography and reported that the butoxyethyl ester of 2,4-D had the longest retention time. Therefore, this ester was selected for investigation.

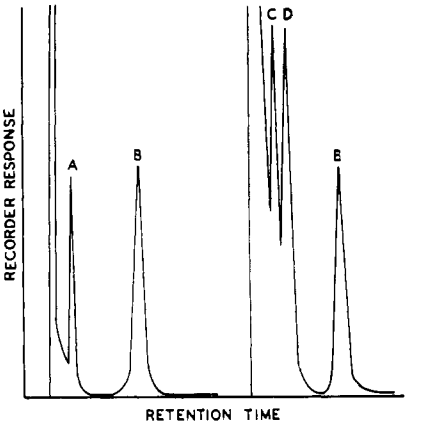

Figure 1. Relative retention times of 2,4-D methyl ester (Peak A) and 2,4-D butoxyethyl ester (Peak B)

In the tracing on right, butoxyethyl ester peak (B) is shown in the presence of citrus extractives. C and D are peaks from citrus. The appearance of peak B following the solvent peak represents a time lapse of 3 minutes

Experimental

2-Butoxyethanol—e.g., butyl Cellosolve—in the presence of dry HCl rapidly esterified the growth regulator acids. Butoxyethyl ester peaks appear after interfering citrus peaks on the chromatograph recording (Figure 1). Furthermore, 2-butoxyethanol has excellent solvent properties for citrus extractives and excess quantities of the reagent can readily be removed from the reaction mixture.

Reagents

Hexane. Petroleum naphtha, glass distilled over sodium.

Acetone. Technical grade, glass distilled.

Chloroform. Technical grade, glass distilled over calcium hydroxide. Alcohol added to 0.5% concentration.

Ether. Reagent, glass distilled over $FeSO_4$–H_2SO_4 immediately before use.

2-Butoxyethanol (butyl Cellosolve). Glass distilled, collecting fraction boiling at 170° C.

Butoxyethanol Reagent. 2-Butoxyethanol cooled to 0° C. and saturated with HCl gas. This is stable indefinitely in glass stoppered bottles stored in the refrigerator.

Florisil (activated at 1250° F.) 60 to 100 mesh.

Procedure

The extraction procedure is designed to divide the residues into three fractions as outlined in Figure 2. (In each fraction, the growth regulator is ultimately determined as the free acid.)

Oranges are juiced by hand with an electric reamer. Five-hundred-gram samples of peel in polyethylene bags are stored in the deep freeze at −40° C. prior to analysis. The peel is thawed in 750 ml. of hot acetone, and the mixture is blended in a gallon-sized Waring Blendor for 1 minute. The slurry is filtered by vacuum through a hardened filter paper and the residue resuspended in 600 ml. of acetone, boiled briefly, and again filtered. (Whole fruit may be treated in a similar manner.) The acetone is removed from the combined extracts in a rotary flash evaporator at a water bath temperature of not more than 30° C. with the condenser bath at −10° C.; this process takes about ½ hour.

Ten milliliters of 2N H_2SO_4 are added, and the mixture is extracted in a separatory funnel with 100 ml. of hexane, then six additional times with 50-ml. portions. The aqueous phase (A) is saved for determination of conjugated (bound) forms of the growth regulators.

The combined hexane extracts are extracted gently with 100 ml. of 0.2M K_2HPO_4, then twice more with 50-ml. portions, partitioning the free acid into the aqueous phase. The hexane phase (A) is saved for determination of esters. The aqueous K_2HPO_4 phase containing the free acid is washed once with 25 ml. of chloroform, which is discarded, then acidified with 25 ml. of 2N H_2SO_4 and extracted once with 25 ml. of chloroform, then twice more with 10-ml. portions. The combined chloroform extracts are evaporated to dryness in 50-ml. Erlenmeyer flasks on the steam bath. The flask must be removed the instant the chloroform is evaporated or acid residue will be lost. Preferably, the last traces of chloroform are removed by a current of air at room temperature. At this step, the residue can be held for a few weeks without loss.

Esters in the hexane phase (A) are determined by first removing the hexane by flash evaporation. The residue is refluxed in 100 ml. of 0.5N KOH for 1 hour for 2,4-D ester and 4 hours for 2,4,5-TP ester. The mixture is acidified with 100 ml. of 0.7N H_2SO_4, and the free growth regulator acid produced by hydrolysis is partitioned into hexane and determined in the regular manner.

The bound acid fraction in aqueous extract (A) is made to 0.5N KOH and hydrolyzed like the esters, but 15 minutes and 2 hours refluxing time are used for 2,4-D and 2,4,5-TP, respectively. Following hydrolysis, the free acids are partitioned into hexane and determined in the regular manner.

Esterification Procedure

One milliliter of 2-butoxyethanol saturated with HCl is added to the chloroform extract residue, while the flask is rotated to wet all of the residue. The esterification is completed by heating on the steam bath for 5 minutes. The mixture is cooled, 50 ml. of hexane is added, and the solution is transferred to a separatory funnel. The flask is rinsed twice with 25 ml. of 0.2M K_2HPO_4, and the washings are added to the separatory funnel. Following extraction, the aqueous phase is discarded. The hexane is extracted six additional times with 50-ml. portions of 0.2M K_2HPO_4, then several times with water. The water washes eliminate a broad peak appearing several minutes after the butoxyethyl ester peak. The hexane fraction is then transferred to a 20-mm. chromatograph tube containing 5 inches of Florisil, covered with 1 inch of Na_2SO_4, prewashed with 50 ml. of hexane. An additional wash of 50 ml. of hexane is passed through the column. Esters are eluted with 100 ml. of freshly distilled ethyl ether. Many orange extractives, including all of the waxlike materials and chlorophyll residues are retained in the column.

The ether eluate is concentrated by flash evaporation, and the residue is taken up in hexane—10 to 50 ml., depending on the amount of residue present. Five to 10 µl. are injected into the gas chromatograph for analysis.

All gas chromatography should be done on the day of esterification. Peak heights are markedly lower after 24 hours. Probably absorption on the glassware and hydrolysis occur causing low results.

Gas Chromatography

In these investigations, an Aerograph Model A600 gas chromatograph, equipped with a Wilkens concentric tube electron capture tritium detector operating at 90 volts, with a 5-foot × ⅛-inch glass column packed with 5% QF-1 on HMDS Chromosorb-W, 60 to 80 mesh, was used. Column temperature was 200° ± 0.2° C. Injector block was 240° C. Nitrogen carrier gas flow rate was 80 ml. per minute. A typical standard curve for 2,4-D and 2,4,5-TP 2-butoxyethyl ester is illustrated in Figure 3. The data are plotted on a log-log scale. From the standard curves, it was calculated that the electron capture cell is 7.0 times more sensitive to the 2,4,5-TP ester than to the 2,4-D ester.

2,4-D and 2,4,5-TP esters are not resolved by gas chromatography under the conditions specified; however, subsequent work has indicated that they can be resolved on 7-foot columns packed with 5% QF-1 or 5% DC-11 on HMDS Chromosorb-W.

Discussion of Method

Hexane is not as efficient as ether as an extractant for the free acids, but is more selective and is less prone to producing emulsions. The number of extractions necessary was previously determined using milligram quantities of growth regulators, following the course of extraction by ultraviolet spectrophotometry. The pH 6.8 buffer used by Erickson and Hield (*7*) will not extract free acid from hexane. Therefore, a more alkaline solution must be used. Two-tenths molar K_2HPO_4 solution has a suitable pH.

The effect of acetone remaining in the aqueous extract on the partition coef-

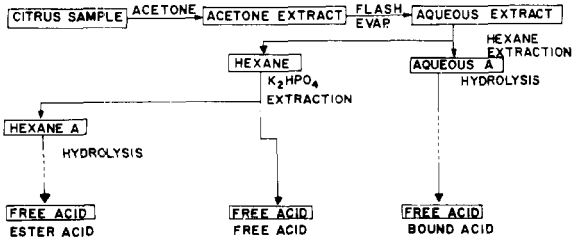

Figure 2. Schematic diagram indicating separation of residue fractions

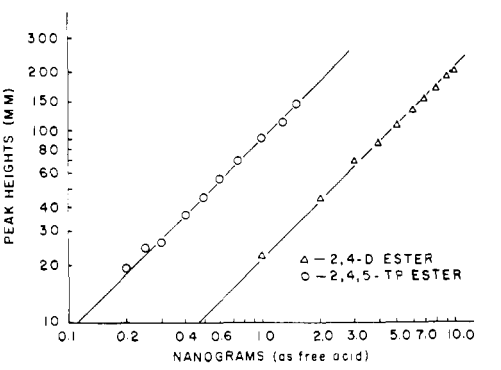

Figure 3. Standard curves for butoxyethyl esters of 2,4-D and 2,4,5-TP

Table I. Effect of HCl Concentration in 2-Butoxyethanol on Esterification of 2,4-D and 2,4,5-TP Acid

HCl Concn. in Butoxyethanol, M	Apparent Degree of Esterification, %	
	2,4-D	2,4,5-TP
0.0	Nil	Nil
0.1	56	40
0.2	60	56
0.4	70	60
1.0	75	80
2.0	82	90
3.0	96	94
4.0	100	100
5.8	100	100

ficient of free acids in the hexane-aqueous system was investigated by extracting 2,4-D and 2,4,5-TP from 400 ml. of aqueous solutions containing known amounts of acetone. It was found that acetone concentrations above 5% had a marked effect on extraction efficiency. Since acetone can be readily detected by odor at 1% concentration, any excessive amounts not removed can be detected readily.

Possibilities of the presence of bound growth regulators incorporated in protein were investigated as follows: The citrus pulp remaining after extraction with acetone was subjected to acid, alkaline, and enzymatic (papain) hydrolysis. No protein bound forms of the growth regulators were detected. The small amount of protein precipitate formed following removal of the acetone was similarly examined and found to be free of growth regulators.

Hydrolysis times for isopropyl 2,4-D and propylene glycol butyl ether ester of 2,4,5-TP were determined in both aqueous and methanolic 0.5*N* KOH. At reflux temperature, 1 hour was required for complete hydrolysis of 2,4-D ester and 4 hours for 2,4,5-TP ester, regardless of the solvent system. The author assumes that any new ester-like metabolites would also be hydrolyzed under these conditions. Optimum time for hydrolysis of the bound acid in 0.5*N* KOH at reflux temperature was measured also as time of maximum free acid liberation. This was 15 minutes for bound 2,4-D and 2 hours for bound 2,4,5-TP.

In studies of the esterification, peak heights obtained by gas chromatography were used as an index of the degree of esterification. Since the large excess of alcohol was expected to shift the equilibrium in favor of the esters with near quantitative yields, the highest peak height obtained for a given amount of growth regulator acid was estimated to represent 100% esterification.

Initially, equilibrium was not obtained within 5 minutes at room temperature; however, heating for 5 minutes on a steam bath yielded maximum peak heights. Longer heating times did not improve esterification. No precautions to exclude water from the reaction mixture were necessary. Since reproducible standard curves were consistently obtained, no further investigations were made on reaction time.

During heating, the HCl catalyst is rapidly evolved from 2-butoxyethanol. For this reason, various concentrations of HCl were investigated to determine if the HCl concentration was critical. The results shown in Table I indicate that the HCl concentration must be at least 4*M* to ensure optimum esterification. About 5.8*M* appears to be the saturation concentration of HCl in butoxyethanol at 0° C. Sulfuric acid was substituted in one experiment, and yields of butoxyethyl esters were very low.

A brief investigation was made to determine whether direct transesterification could be used to convert the hexane A residue esters to butoxyethyl esters avoiding the hydrolysis step. Under the present conditions of esterification, this reaction does not go to completion, probably because of the rapid loss of HCl from the alcohol.

Recovery Experiments

Recovery of Free Acid. Recovery of 2,4-D and 2,4,5-TP was determined by adding standard amounts of the growth-regulator acids in acetone to 500 ml. of water and to 500 grams of orange peel. In each case, the acid was extracted with hexane according to the procedure, except that no Florisil treatment was used in the recovery of the free acids from water.

Data indicate a quantitative recovery of 2,4-D acid from water and near quantitative recovery of 2,4,5-TP acid (Table II). The average recovery from peel with added 2,4-D acid was 89% and with 2,4,5-TP was 91%.

Recovery of Esters. Recovery of esters by the extraction procedure was determined by adding various amounts of standard solutions of isopropyl 2,4-D and propylene glycol butyl ether ester of 2,4,5-TP to both water and orange peel.

Table II. Recovery of 2,4-D Acid and 2,4,5-TP Acid from Water and from Orange Peel

Sample	P.P.M.	2,4-D Added, μg.	2,4-D Found, μg.	Recovery, %	2,4,5-TP Added, μg.	2,4,5-TP Found, μg.	Recovery, %
Water[a]	...	Nil	0.002	...	Nil	0.001	..
Water	...	0.50	0.50	100	0.10	0.090	90
Water	...	0.50	0.48	96	0.10	0.094	94
Water	...	0.50	0.50	100	0.10	0.094	94
Water	...	0.50	0.50	100	0.10	0.095	95
Water	...	0.50	0.50	100	0.10	0.095	95
Water	...	0.50	0.50	100	0.10	0.095	95
Peel[b]	Nil	Nil	0.03	...	Nil	0.01	..
Peel	0.00025	0.125	0.105	85	0.125	0.115	92
Peel	0.0005	0.250	0.220	88	0.250	0.230	92
Peel	0.0015	0.625	0.600	95	0.625	0.569	91
Peel	0.0025	1.25	1.10	87	1.25	1.15	92
Peel	0.005	2.50	2.25	90	2.50	2.30	92
Peel	0.010	5.00	4.50	90	5.00	4.45	89
Average recovery from peel				89			91

[a] Average of five control determinations.
[b] Average of five determinations on untreated peel.

Table III. Recovery of Isopropyl 2,4-D and Propylene Glycol Butyl Ether Ester of 2,4,5-TP from Water and Orange Peel

Sample	P.P.M.[a]	2,4-D Ester, μg.[a] Added	2,4-D Ester, μg.[a] Found	Recovery, %	2,4,5-TP Ester, μg.[a] Added	2,4,5-TP Ester, μg.[a] Found	Recovery, %
Water	0	...	0.002	..	0	Nil	..
Water	0.0002	0.10	0.088	98	0.10	0.091	91
Water	0.001	0.50	0.44	88	0.50	0.45	90
Water	0.01	5.0	4.50	90	5.0	4.46	90
Water	0.1	50.0	44.0	88	50.0	48.4	97
Water	0.2	100.0	91.0	91	100.0	91.0	91
Water	0.4	200.0	194.0	97	200.0	195.0	98
Peel	0	0	Nil	..	0	Nil	..
Peel	0.0002	0.10	0.090	90	0.10	0.088	88
Peel	0.0010	0.50	0.45	90	0.50	0.46	92
Peel	0.01	5.0	4.60	92	5.0	4.8	96
Peel	0.1	50.0	46.0	92	50.0	47.0	94
Peel	0.2	100.0	91.0	91	100.0	94	94
Peel	0.4	200.0	191.0	96	200.0	188	94
Average recovery from peel				91			93

[a] Calculated on free acid basis.

Esters were determined by the standard procedure; 91% of 2,4-D ester and 93% 2,4,5-TP ester were recovered from orange peel (Table III).

Free acid was also determined for each sample to determine whether hydrolysis of ester could have occurred in the procedure.

Initially, no free acid was detectable in the ester standard solutions. Approximately 0.3% of the 2,4-D ester added to the orange peel samples was hydrolyzed during extraction. No evidence for hydrolysis of 2,4,5-TP in the orange peel was found. Neither 2,4-D ester nor 2,4,5-TP ester was hydrolyzed during recovery from water. Consequently, no serious errors are introduced into the analysis through hydrolysis of the esters.

Residues in Citrus Sprayed under Grove Conditions

When citrus trees are sprayed with 20 p.p.m. (free acid basis) of these growth regulators, usual concentrations of 2,4-D and 2,4,5-TP found in the three fractions extracted from the peel were well below 0.1 p.p.m. During the first 2 weeks following spraying, the total residue in peel appears to be about 0.1 p.p.m. After this period, the residue slowly decreases.

No significant concentration of free acid has been found in the juice and no ester or bound fractions have been detected in this portion of the fruit.

These findings demonstrate the applicability of the method of analysis for determination of these growth regulators in sub-part per million concentrations for investigation of their fate when applied as physiological sprays to citrus.

Acknowledgment

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POLAROGRAPHIC DETERMINATION

Bipyridylium Herbicides. Polarography of 1,1′-Ethylene-2,2′-bipyridylium Dibromide

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Reduction of 1,1′-ethylene-2,2′-bipyridylium dibromide at the D.M.E. in 0.1M KCl gives two diffusion-controlled waves. The currents are linear functions of the concentration in the 1.00×10^{-5} to 9.80×10^{-3}M concentration range, and they vary directly as the square root of the mercury pressure. The $E_{1/2}$'s are independent of the pH within the 2.2 to 12.0 range. The reduction involves two steps of 1-electron transfer, and the reversibility of the first step is established. The lower limit of reproducible detection is approximately 0.5 μg. per ml. The method can be extended for the polarographic analysis of all *N*-substituted, 2,2′- and 4,4′-bipyridylium herbicides.

CURRENT interest in bipyridylium salts is the result of the discovery (*2*) of the herbicidal properties of 1,1′-ethylene-2,2′-bipyridylium dibromide (Diquat). Subsequent developments in this field are summarized by Boon (*1*).

The need for a sensitive analytical method for the determination of Diquat has arisen in connection with the estimation of residue levels in crops and food products. Some bipyridylium compounds undergo a reversible, 1-electron reduction to form free radicals. The reduction of 1,1′-dimethyl-, 1,1′-diethyl - , 1,1′ - betaine - , and 1,1′-dibenzyl - 4,4′ - bipyridylium cations by $CrCl_2$ in CH_3COOH, or by alkaline $Na_2S_2O_4$, has been reported (*10*, *11*). Elofson and Edsberg demonstrated (*5*) that 1,1′-dimethyl- and 1,1′ - dibenzyl-4,4′-bipyridylium dichloride are reduced at the D.M.E. Calderbank, Morgan, and Yuen (*3*) have proposed the spectrophotometric determination of the intensely colored free radical derived from Diquat by alkaline $Na_2S_2O_4$ reduction. However, Diquat is not stable in a strongly alkaline medium (*2*), and the formed free radicals, as other free radicals of bipyridylium origin, are only fleetingly stable under aerobic conditions even in the presence of a large excess of the reducing agent (*3*, *4*, *10*, *11*).

The suitability of the polarographic method of analysis was investigated then with special emphasis on anaerobic conditions and supporting electrolytes with favorable pH's, and the work was directed at finding the general conditions under which the method could be applied to other bipyridylium herbicides.

Experimental

Apparatus. A capillary (E. H. Sargent and Co.) was cut to 15.0-cm. length. Later, in order to renew the orifice, it was shortened to 13.7 cm. Estimation of the radius of the capillary orifice by the critical pressure and drop weight methods (*12*) yielded 32.8 and 32.2 microns, respectively. Direct microscopic measurement in polarized light gave 31.0 microns. A batch of mercury (Fisher, reagent) used throughout the experiments was vacuum-distilled three times in a nitrogen atmosphere to give a satisfactory foam test (*13*). The head of mercury was variable between 25.0 and 100.0 cm. An H-cell (*9*), maintained at 25.0° ± 0.1° C., was used with a S.C.E. For *iR* drop corrections the cell resistance was obtained from the shift of the apparent $E_{1/2}$ of Tl^+ with concentration.

The nitrogen gas used for deoxygenating the test solutions was freed from