SEPARATION AND DETERMINATION OF SEVERAL CHLORINATED PHENOXY-ACIDS BY GAS–LIQUID CHROMATOGRAPHY

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(Received June 10th, 1963)

Various chemical methods of analysis have been developed for chlorinated phenoxy-acids, such as 2,4-dichlorophenoxyacetic acid (2,4-D), involving the cleavage of the phenyl ether linkage with pyridine and subsequent colorimetric determination of the chlorinated phenol[1]. Recently 2,4-D and related compounds have been determined quantitatively by gas-liquid chromatography combined with a microcoulometric detector[2–5]. Although considerable work has been done on the quantitative determination of microamounts of single phenoxy-acids, no attempt was made to determine closely related phenoxy-acids from a mixture with sufficient sensitivity and specificity. For this reason two methods have now been developed for the simultaneous determination of three closely related phenoxy-acids combining gas–liquid chromatography of the methyl esters with spectrophotometry or radioactive counting technique.

EXPERIMENTAL

Gas–liquid chromatography

The phenoxy-acids were separated by gas–liquid chromatography as their respective methyl esters. To a 1-ml solution in benzene containing 10 mg each of 2,4-D, 2,4,5-T, and PCPA*** was added 1 ml of diazomethane solution in diethyl ether prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis.) as described previously[6,7]. Carbon-14 methyl esters of the phenoxy-acids were prepared from ^{14}C-Diazald (New England Nuclear Corp., Boston 18, Mass.). Aliquots of freshly prepared ester solutions were chromatographed on an Aerograph Model A-90C instrument, using a 6-ft. long, $^1/_4$ in. O.D. copper tubing, packed with 20% (w/w) Dow-11 high-vacuum silicone grease on 30/60-mesh Chromosorb P at a column temperature of 210° and a He gas flow of 60 ml per min. Fractions were manually collected at the predetermined retention times of the respective methyl esters using a 1.0 × 14.0 cm glass tube fitted with a 7/15 S/T outer joint and containing a small wad of glass wool saturated with the appropriate solvent as specified below[8].

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* Parts of this paper were taken from the M.S. thesis of DAVID L. GUTNICK, University of California, Davis, Calif., 1963.

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*** Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid; PCPA = 4-chlorophenoxyacetic acid.

Spectrophotometric analysis

This method is based on the qualitative test for esters as the red Fe (III) complex of the hydroxamates[9].

(*a*) *Preparation of reagents.* About 3 g of reagent grade hydroxylamine hydrochloride (Eastman Chemicals Co.) was placed in a test tube, and 15 ml of absolute ethanol added. The solution was allowed to boil on a steam bath for 10 min. About 6.8 g of KOH was dissolved in 20 ml absolute ethanol in a 100-ml Erlenmeyer flask and boiled on a steam bath for 10 min.

(*b*) *Colorimetric determination.* One hundred μg of the methyl esters was dissolved in 1 ml of absolute ethanol in a test tube. Gentle heating was applied to bring the material into solution. Four-tenths milliliter of the alcoholic hydroxylamine hydrochloride solution was pipetted into the test tube followed by about 0.9 ml of the KOH solution or until the pH was greater than 10. The mixture was boiled on a steam bath for 1 min with stirring, and 2.5 ml of 1 *N* HCl was added until the pH was less than 1. The reaction mixture was cooled in an ice bath, and 1 ml of 1% (w/v) aqueous ferric chloride added. The test tube was shaken for 10 sec, the solution was transferred to a 10-cm path length spectrophotometric cell, and the absorbance at 500 mμ was measured with a Beckman DU spectrophotometer using a reagent blank as reference. The total volume of the final solution was 5.8 ml.

(*c*) *Preparation of the standard curve.* A stock solution of each of the methyl esters was prepared by adding 50 mg of each of the methyl esters to a 50-ml volumetric flask and diluting to volume with absolute ethanol. Aliquots of 100, 50, 30 and 20 μl (100, 50, 30 and 20 μg, respectively) were pipetted into test tubes and the volume of each was made up to 1 ml with absolute ethanol. The colorimetric reactions were carried out as above, and the absorbancies were measured at 500 mμ immediately and plotted against micrograms PCPA, 2,4-D, and 2,4,5-T.

(*d*) *Gas–liquid chromatography followed by colorimetric analysis.* In order to determine the percentage recovery of the methyl esters of the three phenoxy acids from the gas chromatographic column, 100 μg of each of the methyl esters was injected into the gas chromatograph and collected manually over glass wool saturated with absolute ethanol. The condensed material was eluted with 1 ml of absolute ethanol into test tubes and the colorimetric analysis was performed as above. The absorbance at 500 mμ was compared with the absorbance for 100 μg from the standard curve and the percentage recovery calculated.

Analysis of radioactive derivative

An alternate method of analysis was the separation of ^{14}C-methyl esters of the phenoxy-acids by gas–liquid chromatography and the radioassay of the collected fractions by liquid-scintillation spectrometry.

(*a*) *Preparation of stock solution of ^{14}C-methyl esters.* 1 ml containing 1 mg of each of the phenoxy-acids was pipetted into a graduated centrifuge tube and 1 ml of ethereal diazomethane-^{14}C was added. A freshly prepared solution of ^{14}C-diazomethane had a specific activity of about $1.75 \cdot 10^5$ d.p.m. per ml. The solution was gently swirled for 5 min at room temperature and evaporated under an air stream to a volume of 1 ml. The ^{14}C-methyl esters thus prepared were stored in the refrigerator until further use.

(*b*) *Counting technique.* A Tri-Carb semi-automatic liquid scintillation spectrometer (Model 314-E, Packard Instruments, Inc.) was used to count the radioactive

samples. A non-aqueous counting solution was prepared by dissolving 4 g 2,5-diphenyloxazole (PPO, Eastman Chemicals Co.) and 50 mg 2,2-phenylene-bis-(5-phenyloxazole) (POPOP, Arapahoe Chemical Corp.) in 1 l redistilled toluene. The solutions were counted in 20 ml potassium-free counting vials fitted with disposable screw caps. The spectrometer was operated at a tap setting of 2–425 (825 V).

(*c*) *Calibration curve.* Aliquots of the ^{14}C-methyl esters (5-30 μg) were separated by gas–liquid chromatography, and each ester was collected for a period of 3 min, 1 min before and after its respective retention time. The collector contained glass wool soaked in liquid phosphor counting solution (see above). The condensed methyl esters were washed from the collector into a counting vial with 10 ml additional liquid phosphor solution, and the total volume was made up to about 15 ml with additional phosphor solution. One ml of benzene alone was treated in the same manner as the ester solution and served as a blank. For quantities of esters that were too low to give sufficient response by thermal conductivity (5, 10 μg respectively) 100 μg of the non-radioactive compounds were mixed with the radioactive material as internal standards. Calibration curves were prepared by plotting counts per minute against μg phenoxy-acid. A calibration curve was prepared for each new batch of diazomethane.

RESULTS AND DISCUSSION

Gas–liquid chromatography

The separation of the methyl esters of PCPA, 2,4-D, and 2,4,5-T is shown in Fig. 1. The retention times under the stated conditions (see EXPERIMENTAL and caption under Fig. 1)

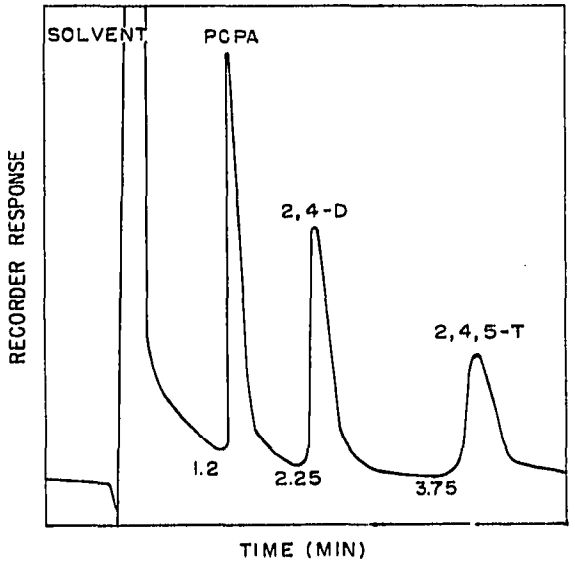

Fig. 1. Gas chromatography of methyl esters of PCPA, 2,4-D and 2,4,5-T on a Dow 11 high vacuum silicone column (20% on 30–60 mesh chromosorb) at 210°.

were 1.25, 2.25, and 3.75 min for PCPA, 2,4-D, and 2,4,5-T, respectively. The response of the thermal conductivity detector (hot-wire katharometer) of the Aerograph 90C instrument was directly proportional to the amount of esters chromatographed over the range from 25 to 100 μg. The esters of 2,4-D and PCPA had about the same thermal response, while the detectability for 2,4,5-T was reduced by about 30%.

Yield of esterification

The yield of esterification was determined by microcoulometric analysis[10], and the results are found in Table I. The percent recovery of the esterification process was determined for four different samples of diazomethane and the results represent aver-

TABLE I

YIELD OF ESTERIFICATION AS DETERMINED BY MICROCOULOMETRIC METHODS[10], *

Compound	*Peak area (sq. in.)*	*Wt. of ester recovered (μg**)*	*Wt. of acid (μg)*	*% Recovery*
2,4,5-T	1.70	3.06	2.89	96.3
2,4-D	1.29	3.03	2.84	94.7
PCPA	0.81	3.20	2.96	98.7

* Average of two independent experiments in which 3 μg-quantities were esterified.
** Micrograms chlorinated phenoxy acid =

$$\frac{\binom{\text{peak}}{\text{area}} \times \binom{\text{recorder}}{\text{sensitivity}} \times 35.5\,\frac{\text{g}}{\text{equiv.}} \times 60\,\frac{\text{sec}}{\text{min}} \times 10^{6}\,\frac{\mu\text{g}}{\text{g}} \times 10^{-3}\,\frac{\text{V}}{\text{mV}} \times 10^{2}}{\binom{\text{recorder input}}{\text{resistance, }\Omega} \times (\%\text{ chlorine in pesticide}) \times 96{,}500\,\frac{\text{C}}{\text{equiv.}}}$$

Recorder sensitivity = 1.09 mV./in.
Recorder input resistance = 64 Ω.

ages of these four separate analyses. As may be seen, 3 μg of each ester was recovered almost quantitatively but lower yields might have been caused by volatilization of the methyl esters during evaporation.

VORBECK *et al.*[11] have reported on a comparison of methylation techniques. In addition to diazomethane, they employed methanol–hydrochloric acid with microsublimation, methanol– hydrochloric acid on ion exchange resin, and methanol–boron-trifluoride. Diazomethanolysis was found to be the most efficient technique in the preparation of the methyl esters of low molecular weight fatty acids, and for this reason it was chosen as the methylating agent in these studies.

The use of microcoulometry in determining the yield of esterification had the advantage of providing a true electrochemical measurement of the amount of chloride in the sample. The quantitative application of thermal conductivity is by necessity a relative process. However, the combination of gas–liquid chromatography and microcoulometry[10] permits the effluent fractions to be combusted to hydrochloric acid which in turn is titrated with Ag^+ ions.

Stability of esters during gas–liquid chromatography

Fig. 2 illustrates infrared spectra of 2,4-D methyl ester before and after gas–liquid chromatography. The primary bands in the infrared spectra are: aliphatic C–H = 3.4 μ, C = O ester = 5.7 μ, C_6H_5–O–C = 8.26 and 9.25 μ. Although it is difficult to deduce the exact structure from the infrared data, it would seem apparent from the data that

no structural changes in the molecule took place as a result of gas–liquid chromatography. Similar results were observed with the methyl esters of PCPA and 2,4,5-T. Extreme care was taken to omit contamination such as moisture, which might have interfered with the spectra.

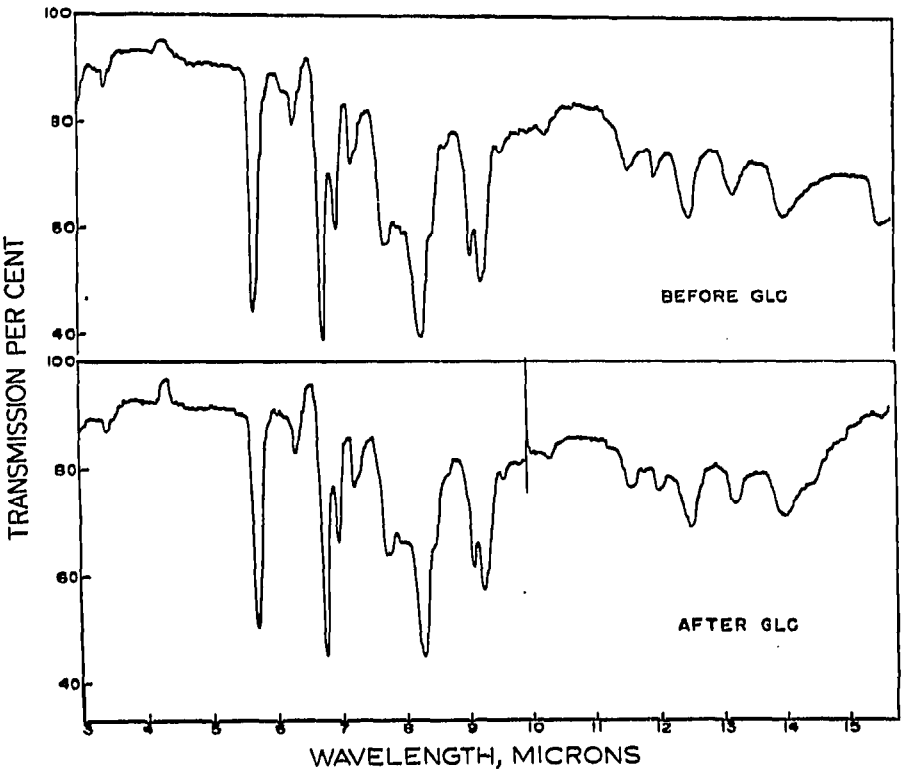

Fig. 2. Infra-red absorption spectra of 2,4-D methyl ester before and after gas–liquid chromatography.

Colorimetric analysis

The technique represents a modification of a qualitative determination of esters[9]. It was necessary, however, to use 10-cm path length cells for the spectrophotometric analysis owing to the low absorbance of the color. The minimum quantity, when

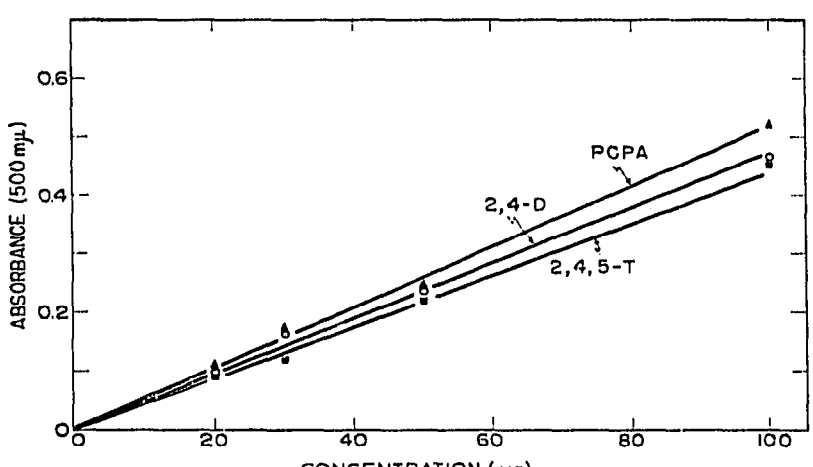

Fig. 3. Calibration curves of phenoxy-acids as hydroxamates.

analyzed in a 1-cm cell was about 200 μg of phenoxy-acid, while with a 10-cm long optical path, the least detectable amount was 20 μg.

The pH requirements for the stability of the color as determined by HESTRIN[12] were followed, but the color remained stable for only 4 min before degrading rapidly, making this method less desirable than the radiotracer technique discussed below.

The linear relationship between absorbance of the color complex and the quantity of phenoxy-acid analyzed is illustrated in Fig. 3. Within the 20 to 100 μg range, the standard curve obeys Beer's Law. The sensitivity of this method compares with that of the gas chromatography–thermal conductivity analysis. Since the direct colorimetric technique is non-specific, it was examined in conjunction with gas chromatography as a method of separating the phenoxy-acids from other compounds containing carboxyl groups prior to the colorimetric analysis.

The data in Table II show the percentage recovery of 100 μg of each of the acids from a mixture of PCPA, 2,4,-D, and 2,4,5-T. Recovery of each component by itself

TABLE II

RECOVERY OF PCPA, 2,4-D, AND 2,4,5-T FROM A MIXTURE OF THE THREE BY COMBINATION GAS–LIQUID CHROMATOGRAPHY AND COLORIMETRY*

Compound	Absorbance		Quantity recovered (μg)	
	1	2	1	2
PCPA	0.463	0.497	89.2	97.6
2,4-D	0.455	0.429	93.2	88.0
2,4,5-T	0.427	0.389	95.2	86.4
Average % recovery = 91.6% ± 4.5%.				

* A mixture of each of the three esters (100 μg of each) was injected into the gas chromatographic column and the fractions were collected at the respective retention times. The colorimetric technique was then applied to the analysis of each sample.

and from a mixture was over 90%. In all cases, the color was measured in the spectrophotometer within 4 min after development. The precision of the method as calculated by the standard deviation was ± 3.1%.

Radioactive analysis

Calibration curve. Data illustrating the linearity of the radioisotope assay are presented in Fig. 4. It was found that the background varies both with the type of compound and with the quantity of acid analyzed. A small amount of radioactivity remained on the column after each methyl ester was collected. Before each compound was chromatographed, therefore, the collector was placed on the exit port for 5 min, the effluent stream was collected, eluted from the collector, and a background count was taken. This background count ranging from 100–300 c.p.m. was subtracted from the total count. The standard deviation of each net count represented the error in the counting instrument and is the square root of the count[13]. It may be seen from Fig. 4 that with the radioactive-derivative method an ultimate sensitivity of 0.5 μg or less of each ester may be achieved; 5 μg of the esters of the three phenoxy-acids studied gave counts ranging from 1957 to 2683 c.p.m. at 57% counting efficiency of the liquid scintillation spectrometer.

Mixtures of the three ^{14}C-methyl esters were chromatographed and each respective fraction was collected and counted. It was found that quantitative recoveries could be made as shown in Table III, and the same standard curves plotted as for single com-

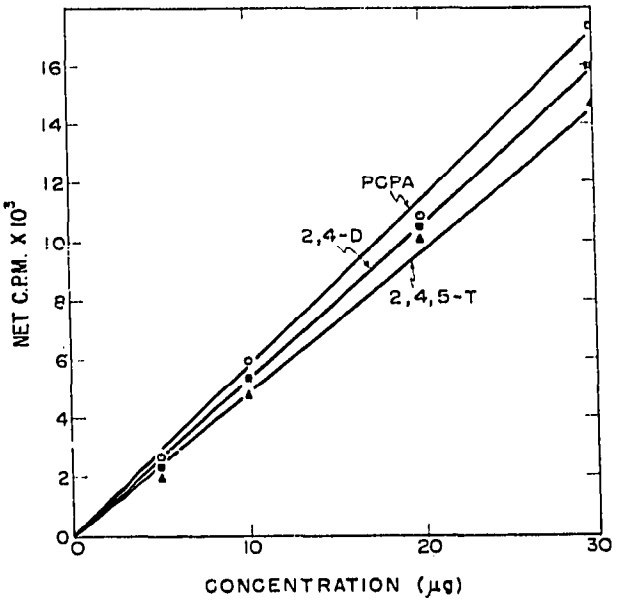

Fig. 4. Calibration curve for determination of phenoxy-acids by combination of gas–liquid chromatography and radioisotope technique.

ponents (Fig. 4). Overall comparison of the analytical techniques described led to the conclusion that the radioisotope analysis was more sensitive than either the colorimetric or thermal conductivity methods.

TABLE III

RECOVERY OF DIFFERENT QUANTITIES OF PHENOXY-ACIDS FROM A MIXTURE BY RADIOISOTOPE TECHNIQUE

	Fraction	*% Recovery*	*% Standard deviation*	*Number of determinations*
1	(PCPA)	91.6	8.9	15
2	(2,4-D)	95.3	6.2	16
3	(2,4,5-T)	96.7	6.8	16

ACKNOWLEDGEMENTS

The helpful advice of DRS. B. S. LUH AND R. A. BERNHARD, Department of Food Science and Technology, Univ. of Calif., Davis is gratefully acknowledged. One of us (DAVID L. GUTNICK) also acknowledges the financial assistance of the California Olive Advisory Board which made this study possible.

SUMMARY

Two procedures for the quantitative determination of micro-amounts of 2,4-dichloro-, 2,4,5-trichloro-, and 4-chlorophenoxyacetic acids are described. The first procedure is

based on the separation of the methyl esters by gas–liquid chromatography and the colorimetric determination of collected fractions as the hydroxymate–Fe (III) complex. The second procedure involves the formation of the ^{14}C-methyl esters, their separation by gas–liquid chromatography and the radioassay of collected fractions by liquid-scintillation spectrometry.

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