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.

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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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of injury were observed, the possibility that the peak in question was not 2,4-D also was considered. Concurrent observations in the field and greenhouse gave further supporting evidence. Herbicide-free brome grass and timothy, sprayed with deionized water so as to simulate rainfall and sampled at varying intervals after treatment, exhibited the same "2,4-D" peaks, though they represented concentrations approximately 90% that of similar peaks from samples which had received 2,4-DB. Beta-oxidation of 2,4-DB in treated samples, and the resulting production of limited quantities of 2,4-D, could not be completely discounted. However, these observations did indicate that plants produce electron-capturing materials with identical retention times to 2,4-D, and that the quantity of actual "2,4-D" detected might be in doubt. Concentrations of these compounds varied with stage of plant development, climatic variables affecting rate of plant growth, and levels of herbicide in plants. Thus, it became necessary to identify the interfering compounds and to find separation methods that would eliminate the errors in analysis for herbicides and facilitate identification of metabolites. This report is concerned primarily with the identification of interfering substances.

Procedures and Results

We attempted to find a gas chromatographic system that would separate the unknown from 2,4-D by using an Aerograph 600 gas chromatograph equipped with a concentric tube, electron-capture detector, and a 152-cm. by 0.3-cm. diameter coiled glass column. Hexamethyldisilizane(HMDS) - treated 60- to 80-mesh Chromosorb-W was used as support material. Chromatograph parameters were varied over the full range that would allow 2,4-D methyl ester to be detected quantitatively. Column temperatures were varied from 180° to 220° C. and injector temperatures were varied from 180° to 250° C. N_2 flow rate was varied from 25 to 40 ml. per minute. The following stationary phases—Dow 11 stopcock grease, DC200 silicone oil, Versamide 900, and SE30 silicone gum—tested at 5 and 10 weight % levels in relation to support material, were not capable of separating 2,4-D and the unknown compounds. These phases are commonly used in pesticide analysis.

Two columns that gave adequate separation were found: 10% ethyl acetate–fractionated Dow 11 which was baked for 5 days at 250° C. and purged with N_2 at 10 ml. per minute, and a relatively new column material, 5% FFAP (a modified Carbowax) (*13*). Both columns were operated under the same conditions: oven temperature 210° C., injector 210° C., and N_2 flow 30 to 35 ml. per minute. Both columns were capable of separating the unknown material into two peaks. Compared with the methyl ester of 2,4-D with a relative retention volume of 1.00, the silicone column produced a major peak at 0.47 and a minor peak at 0.65, and the FFAP column produced a major peak at 0.36 and a minor peak at 0.44. The major peak is referred to hereafter as unknown I and the minor peak as unknown II.

A system of extraction and cleanup, developed previously for determination of 2,4-DB and 2,4-D in forages, had been used routinely in this work (*5*). By extending the system it was possible to isolate and concentrate the unknowns. Briefly, the method requires blanching and subsequent extraction of plant material with 2-propanol. Chlorophyll, 2,4-DB, 2,4-D, and other organic acids are partitioned from the extract into a petroleum ether (b.p. 30° to 60° C.) epiphase. Highly polar materials remain in a 2-propanol–0.03*N* HCl hypophase. Methylation of herbicide, necessary for chromatographic analysis, can normally be carried out in the petroleum ether with diazomethane. Excess diazomethane is driven off by heating briefly or by vacuum, depending on whether or not materials of interest are thermolabile. Further cleanup necessary for some plant materials can be achieved by extracting the petroleum ether phase with 5% $NaHCO_3$ before methylation. The herbicide acids can be methylated after acidifying the bicarbonate solution and extracting with diethyl ether–*n*-hexane, 50–50 (v./v.).

On the columns we used, attempts to produce the unknown peak without prior methylation failed. This suggested that —COOH functional groups or ring-associated —OH groups could be involved (*6*). The unknown material was not quantitatively recovered by extraction of petroleum ether solutions of plant pigments and acids with 5% $NaHCO_3$. This indicated that processes of partition other than salt formation were active and, thus, the presence of ring-associated —OH or similar groups rather than —COOH as a functional group was favored. Percentage recovery was determined by difference between peak heights of unknowns produced in petroleum ether solution prior to and after shaking out in 5% sodium bicarbonate solution. The bicarbonate solution, when acidified with concentrated HCl and extracted with diethyl ether, yielded a solution which absorbed light in the carotenoid region between 420 and 500 mμ. The ether solution was methylated and analysis of this ether extract by gas chromatography using the Dow 11 column described previously and a flame detector resulted in separation of two components, one with the retention time of unknown I and a smaller peak with the retention time of unknown II. When the electron-capture detector was used and an aliquot of the sample was run on the same column, two electron-capturing peaks with retention time identical to unknowns I and II were recorded. Electron capture–flame response ratios were calculated as 15.0 to 1 for unknown I and 16.9 to 1 for unknown II.

The unknown material possessed solvent-partitioning characteristics similar to 2,4-D. An acetone-H_2O hypophase quantitatively removed the electron-capturing unknowns present in a petroleum ether epiphase from either a brome grass or a timothy extract. Percentage recovery of the unknowns was determined as explained above. Visible spectra of this mixture were similar to those obtained from the bicarbonate separation noted previously. The visible spectra indicated that the unknowns were carotenoids, so the acetone-H_2O fraction was chromatographed on a magnesium oxide–Celite column by the methods of Strain (*10*). Two yellow components were separated, the major one with the relative retention volume of lutein, and the minor one with the relative retention volume of violaxanthin. Methylation did not change the relative retention volumes of these two pigments on the Strain column, but did allow movement of the pigments on the Dow 11 gas chromatograph column and their subsequent detection by flame and electron-capture detectors. On the Dow 11 column the methylated major component eluted at the retention volume of unknown I and the methylated minor component eluted at the retention volume of unknown II. Visible absorption spectra of solutions of the two pigments closely matched those reported by Strain for lutein and violaxanthin in ethanol, respectively.

We obtained further quantities of the two pigments (unknowns I and II) from young (8 to 15 cm. in height) herbicide-free brome grass by the extraction and separation methods described. Visible spectra were run on both pigments in *n*-hexane, chloroform, ethanol, and carbon disulfide. Bathochromic spectral shifts for each unknown matched those published (*2*) for lutein and violaxanthin. Chloroform solutions of each pigment reacted positively to the Carr-Price reagent (*8*) for carotenoids. A diethyl ether solution of the minor pigment with a few drops of concentrated HCl gave an intense blue color. Violaxanthin produces an intense blue color under these conditions, whereas other epoxy pigments with almost identical visible absorption spectra do not (*11*). On this basis we conclude that the unknown pigments are the methylated derivatives of lutein and violaxanthin, respectively.

Electron-capture–flame response ratios were obtained for purified samples of the methylated pigments and compared to electron-capture–flame response ratios presented above for both methylated unknown pigments obtained from brome grass prior to purification. Electron-capture–flame response ratios were

also obtained for crystalline β-carotene and 2,4-D methyl ester. These data were obtained from a Model 204 dual channel Aerograph gas chromatograph equipped with a 152-cm. by 0.3-cm. diameter glass column packed with 5% ethyl acetate fractionated Dow 11 liquid phase on 60- to 80-mesh HMDS-treated Chromosorb W. The temperatures used were: column 210° C., injector 260° C., detector 220° C. The N_2 flow rate was 35 ml. per minute. A split ratio of 1 to 1 was used to supply both detectors from a single sample injection. The following electron-capture–flame response ratios were produced: β-carotene 29.5 to 1, methylated lutein 15.0 to 1, unknown I 15.0 to 1, violaxanthin 16.9 to 1, unknown II 16.9 to 1, and 2,4-D methyl ester 12.4 to 1. It is apparent that all these pigments are strong electron-capturing agents.

Each pigment was recrystallized twice. Lutein was crystallized from light petroleum ether by adding methanol, and violaxanthin was crystallized from methanol by adding H_2O. Melting points were determined in a Nalge micromelting point apparatus. The melting points were 193° and 190° C., respectively, in agreement with those published (*3*) for the all trans forms of lutein and violaxanthin. They serve as concluding evidence that the "2,4-D" chromatographic peak obtained initially from brome grass and timothy extracts was a composite of the methylated derivatives of the all trans forms of lutein and violaxanthin, respectively.

Discussion

These studies indicate that care must be exercised in using chromatograph peaks alone for quantitative determination of 2,4-D in plants because plant pigments which are electron-capturing may have retention volumes similar to the herbicide. Caution is necessary, especially if the quantity of the interfering compound is affected by climatic variables, stage of plant growth, or the herbicide. The need for adequate control samples under these conditions is obvious. Unfortunately, if herbicide affects production and metabolism of interfering pigments, or if there is herbicide-climatic or herbicide-climatic–stage of growth interaction affecting pigment production, these controls may be difficult or impossible to obtain.

The literature indicates that a large number of carotenoid isomers can be formed by laboratory manipulations conducted between extraction and final analysis—evaporating a solvent from an extract, allowing an extract to stand for periods of time, dissolving a crystalline pigment in some solvents, exposing an extract to high temperatures, etc. Other investigators have found that boron trifluoride, commonly used for esterification of organic acids in methanol, ethanol, and other alcoholic solutions, forms complexes with α- and β-carotene which result in hydroxy derivatives similar to those reported herein (*2*).

Work in our laboratory indicates that pigments and many of their isomers respond to detection by flame and electron-capture techniques over the entire range of columns and chromatograph parameters reported in this paper. Those pigments and their isomers lacking ring-associated hydroxyl groups (α- and β-carotene) do not require methylation to be detected.

Isomerization is a problem complicating metabolic studies—for example, methylated lutein and violaxanthin, when chromatographed, yielded one peak each. However, if we collected the material under the peak and rechromatographed it, lutein gave a total of five peaks and violaxanthin a total of eight peaks. Infrared spectrums of both pigments before and after chromatographic analysis were different at 10.3 and 12.8 to 13.0 microns. These data indicated that cis-trans isomers were being formed after the material had passed the chromatograph detector and in all probability were forming in the collection ports. Further evidence of potential isomerization was obtained by heating methylated lutein and violaxanthin in flasks under N_2 and cooling. Chromatographic analysis of this methyl lutein and violaxanthin yielded five and eight peaks with retention volumes identical to those above.

It is highly significant that carotenoid pigments are found in animals, insects, soils, and sediments (*1*, *9*), as well as in plants. Only two which interfere with the determination of 2,4-D, lutein, and violaxanthin, have been reported herein. These natural carotenoids and their isomers may possibly interfere with both qualitative detection and quantitative determination of other pesticides in other media.

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