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Determination of Free and Hydrolyzable Residues of 2,4-Dichlorophenoxyacetic Acid and 2,4-Dichlorophenol in Potatoes

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Chemical methods were developed and used to provide residue and metabolism data in support of the registered use of 2,4-dichlorophenoxyacetic acid (2,4-D) on potatoes. The free and the free plus hydrolyzable forms of trace residues of both 2,4-D and 2,4-dichlorophenol (2,4-DCP) in potato tubers and vines were determined separately by the following methods: extraction of free residues with an acidified mixture of ethyl and petroleum ethers, or after hydrolysis, extraction of the free plus acid hydrolyzable forms with benzene; partitioning cleanup into alkali and then, after acidification, into ethyl ether; separation of 2,4-DCP from 2,4-D on an acidic alumina column; methylation of 2,4-D; quantification by GLC. Residues of 2,4-D averaged 0.11 part per million (ppm), whereas residues of 2,4-DCP were less than 10% those of 2,4-D. At least 18% of the total 2,4-D found was present in conjugated form. During simulated commercial storage, 2,4-D dissipated from whole potatoes with a half-life of about 12 weeks. Residues were not concentrated in the peel of potatoes, and baking had no influence on the residue present.

A major part of the mission of our laboratory involves the development of data to support the establishment of tolerances and registration of agricultural chemicals for use on minor crops. In 1971, the federal registration covering the use of 2,4-dichlorophenoxyacetic acid (2,4-D on potatoes was in danger of cancellation for lack of adequate data. This plant growth regulator has been used as a foliar spray on potatoes for many years to intensify the skin color of red varieties and to reduce the yield of oversize tubers while increasing the yield of medium, more desirable tubers of all table stock varieties (Nelson and Nylund, 1963; Wort, 1965; Hegazy et al., 1978). To support this use, data on the resulting residues of 2,4-D and one of its possible metabolites, 2,4-dichlorophenol (2,4-DCP), had been requested by the U.S. Environmental Protection Agency. This study was organized to provide the needed data. It required methodology capable of determining separately the free and the hydrolyzable residues of both 2,4-D and 2,4-DCP at low ppb (nanograms per gram) levels in potato tubers. High sensitivity was required because potatoes, susceptible to the herbicidal effects of 2,4-D, are treated with very low levels of commercial formulations.

Bevenue et al. (1963) reported that residue levels of 2,4-D in potatoes were very low using a method that involved the direct extraction of potatoes with an acidified organic solvent mixture. Using similar methodology, Nelson et al. (1971) reported that potato samples boiled in 6 N H_2SO_4 prior to extraction gave a 2–7-fold increase in the levels of 2,4-D found over those obtained by direct extraction with acidified benzene. Similar increases resulting from hydrolysis had been observed for residues of 2,4-D in beans (Thomas et al., 1963; Crosby, 1964) and in Forage (Yip and Ney, 1966). At the time this study began, Chow et al. (1971) reported that alkaline hydrolysis of wheat that had been treated with 2-methyl-4-chlorophenoxyacetic acid gave from 3 to 7 times higher residue levels than those found by direct extraction with acidified organic solvents. These studies demonstrated that hydrolysis plays an important role in the analysis of conjugated 2,4-D residues in plants. Since that time, numerous studies have shown that 2,4-D and other phenoxyalkanoic acids are metabolized extensively (Mumma and Hamilton, 1976; Feung et al., 1976, 1978; Bristol et al., 1977; Leng, 1977; Chkanikov et al., 1976, 1977; Lokke, 1975; Steen et al., 1974; Fleeker, 1973). However, no residue methods were available which satisfied the specific requirements of this study when it was undertaken.

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Salient features of the two methods described in this paper for separate determination of the free and the total (i.e., combined free plus acid hydrolyzable) residues of 2,4-D and 2,4-DCP in potatoes are as follows: extraction of free residues with an acidified mixture of ethyl and petroleum ethers, or extraction of the free plus hydroyzable residues liberated by reflux in 2 N H_2SO_4 with benzene; partitioning cleanup into 0.5 N NaOH and then, after acidification, into ethyl ether; separation of 2,4-D; quantification of the separate residues by GLC. The methods were validated by using radiotracer techniques. They were used to analyze field-treated potato samples and to conduct related residue studies.

EXPERIMENTAL SECTION

Reagents and Standards. All chemicals were ACS reagent grade except as noted. Ethyl ether and petroleum ether were distilled before use. Benzene was extracted with concentrated H_2SO_4 until colorless, washed with water, dried over calcium chloride, and distilled to remove interfering substances. Alumina, Woelm W-200 acid (ICN Pharmaceuticals, Inc., K and K Labs Division, Plainview, NY), was adjusted to activity II [4% water (w/w)] and stored in a glass-stoppered Erlenmeyer flask. Cleanup columns were prepared for each analysis by addition of 3.0 g of deactivated alumina to a 10 × 300 mm chromatographic tube (Corning Glass, Corning, NY) containing about 5 mL of ethyl ether. After the ethyl ether had been drained to the top of the alumina, the stopcock was closed.

Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co., Milwaukee, WI) according to instructions provided by the manufacturer. The ethereal reagent was redistilled and stored at −20 °C until used.

Commercial 2,4-D (Eastman Chemical Co., Rochester, NY) was recrystallized first from benzene and then from ethanol–water to provide a reference standard. A stock solution containing 1 mg of 2,4-D/mL in acetone (pesticide quality) was prepared.

Commerical 2,4-DCP (Eastman Chemical Co., Rochester, NY) was recrystallized from hexane. A stock solution containing 0.1 mg of 2,4-DCP/mL in acetone was prepared. A combined working standard solution for fortification experiments was prepared by dilution of combined aliquots of each stock solution with water to give a solution containing 10 μg/mL 2,4-D and 1 μg/mL 2,4-DCP. Additional working standards were made by serial dilution with water. Analytical-grade methyl 2,4-D (98%, Dow Chemical U.S.A., Midland, MI) was dissolved in benzene (0.1063 g/100.0 mL) to give a stock solution equivalent to 1 mg of 2,4-D/mL. Separate working standard solutions for GLC analysis were prepared by serial dilution of aliquots of the 2,4-DCP and methyl 2,4-D stock solutions with benzene to cover the range from 0.02 to 1 μg/mL phenol or ester.

[*acetic*-2-^{14}C]-2,4-D (4.2 mCi/mmol; of >98% radiochemical purity) and [*ring*-U-^{14}C]-2,4-D (1.0 mCi/mmol; of >98% radiochemical purity) were purchased from Mallinckrodt, St. Louis, MO. Stock and working standard solutions of [*acetic*-2-^{14}C]-2,4-D were prepared as described for the nonradioactive material; purity was checked by TLC analysis (Bristol et al., 1977) and, after derivatization with diazomethane of aliquots diluted in benzene, by GLC analysis. The [*ring*-U-^{14}C]-2,4-D was converted to the propylene glycol butyl ether (PGBE) ester and formulated as Esteron-99 by Dow Chemical U.S.A. (0.71 mCi/mmol as 2,4-D).

All standards were prepared and stored in volumetric flasks and culture tubes equipped with Teflon-lined screw caps (Corning Glass, Corning, NY).

GLC. A gas chromatograph (Barber Coleman Series 5000) was equipped with a Coulson conductivity detector (CCD, Tracor, Inc., Austin, TX) which was interchangeably connected by Teflon tubing to two glass columns (1.9 m × 4 mm i.d.) packed with either 10% OV-101 on Gas-Chrom Q (100–120 mesh) or 10% Silar-5 CP (80–100 mesh) on Gas-Chrom Q (both purchased from Applied Science Laboratories, State College, PA, and conditioned overnight with purge at 250 °C). On-column injections of 50 μL were made (CCD vent valve open), and the injection port was maintained at the same temperature as the column oven. Methyl 2,4-D was analyzed at 200 and 210 °C, whereas 2,4-DCP was analyzed at 150 and 180 °C, on the OV-101 and Silar-5 CP columns, respectively. These conditions gave retention times of 4 min or less. The detector transfer line, block heater, and furnace were heated at 235, 220, and 820 °C, respectively. Gas flow rates were as follows: carrier gas (prepurified, filtered He or N_2), 60 mL/min; CCD reaction gas (H_2), 80 mL/min; CCD vent gas (He), 60 mL/min. Peaks occurring in samples were quantified by interpolation of their peak heights between those of bracketing standards (within ±25%).

Radioassays. So that the radioactivity present in each liquid fraction could be assayed, the total volume of each fraction was measured, and duplicate 1.00-mL portions were withdrawn and added along with 3.0 mL of distilled water to 10.0 mL of Insta-gel (Packard Instrument Co., Downers Grove, IL). The resulting gel was shaken, cooled in the dark, and assayed at 15 °C by the automatic external standard channels ratio method using a liquid scintillation counter (Mark II, Nuclear Chicago Corp., Houston, TX). Solid samples of chopped and blended potato tuber or vine samples were lyophilized in 100.0-g batches, mixed, subsampled, and assayed after combustion to $^{14}CO_2$ in a Model 306 Tri-Carb sample oxidizer (Packard Instrument Co., Downers Grove, IL). All samples possessing activity above 1000 cpm were measured at 1% and those below at 3% standard error (95% confidence limit). A standard quench curve was prepared monthly from standards containing [^{14}C]benzoic acid. Different amounts of an equivolume quench mixture of acetone, carbon tetrachloride, and nitromethane were added in up to 1 mL of volume to cover the full range of efficiency (normally 20–96%).

Field Treatment, Sampling, and Storage. Replicate plots of Red Pontiac, Norland, and Red Norland potatoes were treated with a PGBE low-volatile ester formulation (as Dow Esteron-99 concentrate in 160 L of water/ha) corresponding to 0, 140, and 280 g of 2,4-D acid equiv (g of a.e.) per ha. One plot was treated with the [*ring*-U-^{14}C]-2,4-D formulation at 280 g of 2,4-D a.e/ha.

Applications were made with a bicycle-type sprayer equipped with adjustable boom and nozzles and operated at a constant pressure of 170 kPa (CO_2). Treated plots consisted of two adjacent 12-m rows which were separated from each other by three buffer plots (six rows) to prevent cross contamination. The adjusted spray of 2,4-D was directed onto the entire exposed plant.

Applications were made in two treatments. The first was made at the time of tuber initiation (determined by inspection of tubers), the second 2 weeks later. The plots were harvested 29 days after the second treatment.

Field samples consisted of at least 5 kg of tubers collected at random from the center 9 m of the 12-m plots. Each sample was collected in a double polyethylene bag, taken to the laboratory, and processed. Tubers were washed in water to remove surface soil before approximately 2.5-kg subsamples were cut into 10 × 10 mm

French fry type pieces and mixed thoroughly. From each subsample, 10 laboratory samples weighing 200.0 g each were double packaged in polyethylene bags, which were labeled, tied, and immediately frozen at −23 °C. They were stored at leass than −20 °C until analysis. The time from harvesting through freezing took less than 8 h.

In addition to the cut potato samples, 2.5-kg subsamples of washed whole potato tubers representing each treatment rate were place in storage at 3.3 °C to simulate the conditions of commercial storage. These were subsampled and analyzed periodically to check for dissipation of residues.

Hydrolyzable Residue Analysis (HRA) Procedure. Potato samples weighing 200 g were removed from frozen storage and allowed to thaw slightly at room temperature so that 100.0 g could be subsampled and placed directly into a quart Mason jar. After addition of 20 mL of 0.5 N NaOH solution and 80 mL of water, the jar was attached to a homogenizer (Lourdes Model MM-1A equipped with Mason jar adapter) and the contents were blended at 80% of full speed for 3 min. The macerated tissue was transferred with the aid of a powder funnel to a 1-L single-neck, round-bottom flask to which a 1-in., egg-shaped magnetic stir bar had been added. The jar and spindle were washed successively with 25 mL of water and 50 and 35 mL of 5 N H_2SO_4, all rinsings being transferred to the round-bottom flask to give a mixture 2 N in acid. The flask was fitted with a heating mantle situated over a magnetic stir plate (Thermolyne Type 1000, Sybron/Thermolyne, Dubuque, IA) and equipped with a water-cooled condenser. The contents were stirred and heated at reflux for 1 h. The mixture was monitored to assure that stirring occurred so as to avoid undesirable charring and caramel formation. Once relux had begun, stirring proceeded smoothly.

The acid hydrolysate was cooled in an ice bath. The pH of the mixture was adjusted to 10 or more by addition of 85 mL of 5 N NaOH through the top of the condenser. A Buchner funnel (126 mm) was fitted with glass-fiber filter paper (Whatman GF/B) covered with whatman No. 541 filter paper and supplied with a rubber adapter which fit onto a vacuum filter adapter (29/42, Kontes K-205000). The contents of the round-bottom flask were filtered with the aid of a slight water pump vacuum, and the yellow-brown filtrate was collected in a 500-mL separatory funnel. The round-bottom flask, filter cake, and apparatus were rinsed twice with 25-mL portions of water. The combined filtrate and washings could be stored overnight, if necessary.

Fifty grams of NaCl was dissolved in the alkaline filtrate, and the pH of the solution was adjusted to 3 or less by addition of about 20 mL of 5 N H_2SO_4 (pH checked with pHydrion paper). The acidified mixture was immediately extracted with three successive 50-mL portions of benzene, rocking and shaking gently for 1 min each time. After the mixture was allowed to stand for 5–10 min, the benzene layer of each extraction and any emulsion remaining were collected in a 500-mL centrifuge bottle. The combined extracts were centrifuged at 1400 rpm for 3 min, and the upper benzene layer was transferred to a 500-mL separatory funnel by decantation and/or with a transfer pipet. The centrifuge bottle and its aqueous contents (usually about 25 mL) were rinsed with two successive 25-mL portions of benzene, and the rinsings were decanted into the separatory funnel.

The combined benzene extracts and rinsings were extracted successively with 50-, 25-, and 25-mL portions of 0.5 N NaOH, and the alkaline extracts were combined in a 250-mL separatory funnel. Fifteen grams of NaCl and 25 mL of 5 N H_2SO_4 were added, and the acidified solution (pH 3 or less) was extracted with 12 mL of ethyl ether. The ethereal extract was applied to the alumina cleanup column by using a disposable pipet and allowed to run completely through. Care was taken to assure that none of the aqueous acid phase was transferred. The extraction was repeated 3 times, each extract being applied to the alumina column. Final traces of the ethyl ether eluate were blown through the column by application of a slight pressure of air.

The ethyl ether eluates (48 mL) containing 2,4-DCP were collected in a 25 × 200 mm culture tube. Five milliliters of 1 N NaOH was added, and the tube capped and agitated for 3 min. The separated ethereal layer was removed with a pipet and discarded, the last traces being removed under a stream of air. After addition of 1 g of NaCl, the alkaline extract was acidified to a pH less than 3 by addition of from 10 to 15 drops of concentrated H_2SO_4. Exactly 2.0 mL of benzene was added to the tube which was capped and agitated for 3 min. The separated benzene layer was either analyzed directly for residue of 2,4-DCP by GLC or a portion was transferred to a 13 × 100 mm culture tube which was capped and stored for later analysis.

So that the analysis of 2,4-D could be completed, the alumina column was eluted with 25 mL of 0.25 N aqueous $NaHCO_3$. The eluate, collected in a 25 × 150 mm culture tube, was acidified to pH 3 or less by the dropwise addition of about 12 drops of concentrated H_2SO_4, with careful intermittent mixing. Seven grams of NaCl and exactly 10 mL of ethyl ether were added to the tube which was then capped and agitated for 3 min. Exactly 5 mL of the ethereal extract was transferred to a 10-mL volumetric flask to which 2 mL of benzene had been added. The solution was concentrated to about 1.5 mL under a stream of air before 1–1.5 mL of diazomethane reagent was added. After 5 min, the solution was concentrated to about 1 mL under a stream of air to remove excess diazomethane and ethyl ether. The flask was diluted to volume with benzene, capped, and mixed, and its contents were either analyzed for methyl 2,4-D by GLC or stored for later analysis.

Free Residue Analysis (FRA) Procedure. A 100.0-g sample was weighed and placed in a quart Mason jar exactly as described for the HRA procedure. To it was added 50 g of NaCl, 200 mL of 3:1 (v/v) ethyl ether–petroleum ether (mixed ethers), and 10 mL of 5 N H_2SO_4. The contents of the jar were blended exactly as described for the HRA procedure. The spindle was rinsed with a stream of water, the rinsings were collected in the jar, and the contents were transferred to two paired 250-mL centrifuge bottles. The jar was rinsed twice with 5–10-mL portions of water, and the rinsings were used to equilibrate the weight of the centrifuge bottles. the bottles were stoppered with neoprene stoppers wrapped in aluminum foil and centrifuged at 1400 rpm for at least 3 min. The upper layer in each bottle was transferred with a pipet to a 500-mL separatory funnel. After addition of 10 g of NaCl and 20 mL of mixed ethers to each remaining aqueous layer, the centrifuge bottles were stoppered, agitated 3 min, and centrifuged as above. The upper layers were again transferred to the separatory funnel. The extraction was repeated with 20 mL of mixed ethers.

The combined mixed ethers were extracted successively with 50-, 25-, and 25-mL portions of 0.5 N NaOH solution. The alkaline extracts were collected in a 250-mL separatory funnel and further processed exactly as described in the HRA procedure.

Recovery Experiments. Along with every six treated potato samples, one control and one fortified control potato

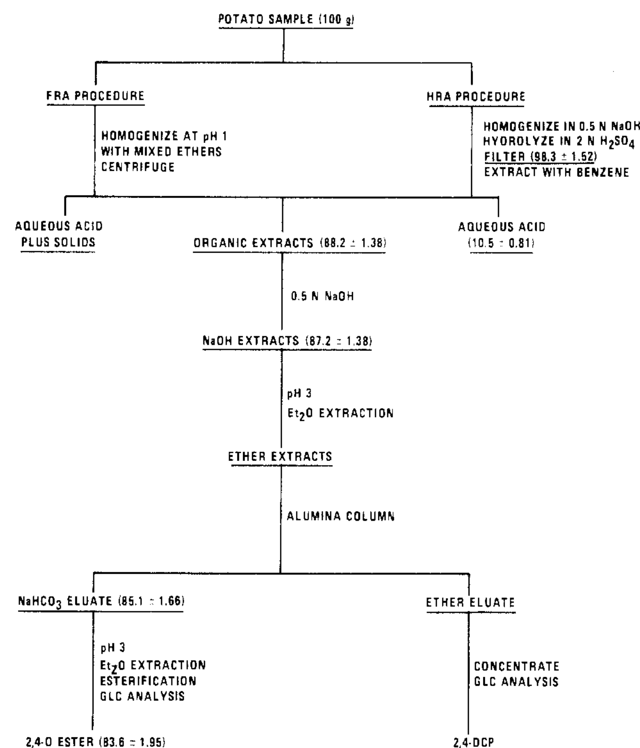

Figure 1. Outline of HRA and FRA procedures. The cumulative recoveries of [*acetic*-2-^{14}C]-2,4-D through the HRA procedure are given in parentheses as mean ± coefficient of variation ($N = 6$); all other fractions contained less than 1% of the radioactivity added.

sample were analyzed. The trace levels found in control samples were used to correct the recoveries of 2,4-D and 2,4-DCP in the corresponding fortified sample, when appropriate. Either a 1- or 2-mL aliquot of the appropriate aqueous combined working standard solution was pipetted directly onto the entire exposed surface of the 100.0-g control sample in the Mason jar.

For determination of the analytical behavior of 2,4-D through each step of the HRA procedure, 1-mL aliquots of an aqueous solution containing 23.65 μg/mL [*acetic*-2-^{14}C]-2,4-D (about 10^6 dpm/mL) were added to 100.0-g samples of potatoes. During analysis of each sample, duplicate 1.00-mL portions were removed from the alkaline filtrate before hydrolysis and from each organic and aqueous fraction resulting from a partition. The entire sample of alumina from the cleanup column was transferred under a slight pressure of air to a counting vial. All samples were analyzed as described under radioassays.

The identity of residues of 2,4-D and 2,4-DCP in two treated potato tuber samples was confirmed by combined GC–MS analysis (Model MAT CH5, Varian Aerograph, Palo Alto, CA).

RESULTS AND DISCUSSION

The analytical methods developed specifically for the low-level determination of free and hydrolyzable residues of 2,4-D and 2,4-DCP in potatoes are outlined in Figure 1. The FRA and HRA methods are applied separately to replicate samples and differ only in the manner in which residues are released from the plant tissue for extraction with organic solvent. The FRA method extracts free residues directly from macerated plant tissue by blending with acidified mixed ethers, a modification of the procedures of Yip and Ney (1966) and Nelson et al. (1971). The HRA method utilizes acid-catalyzed hydrolysis of the macerated tissue to convert conjugates (both soluble and insoluble or bound) to their free forms for extraction with

Table I. Effect of Reflux Period on Recovery of 2,4-D and 2,4-DCP from Potato Tuber Samples Using Acid Hydrolysis

length of reflux period, h	recovery, %, of added 2,4-D[a]	gross residue found, ppm[b]	
		2,4-D	2,4-DCP
1	86.9	0.11	0.0033
2	85.7	0.12	0.0042
4		0.12	0.0036
16	90.4	0.11	0.0010

[a] Reagent standard of 2,4-D added to control potato sample at 0.20 ppm. [b] Mean values of bioincurred residue levels found by duplicate or triplicate analysis of field-treated potato samples.

benzene. The subsequent steps of the two methods, involving cleanup and determination of the separated 2,4-D and 2,4-DCP residues, are identical to enhance comparison of the results obtained.

Good results have been obtained by using either acid (Crosby, 1964; Yip and Ney, 1966; Nelson et al., 1971; Shafik et al., 1971; Bjerke et al., 1972; Lokke, 1975) or alkaline (Crosby and Bowers, 1966; Chow et al., 1971; Jensen, 1973; Nony et al., 1976; Siltanen and Rosenberg, 1978; Cessna, 1980) conditions to hydrolyze conjugated residues of phenoxyalkanoic acids occurring in plant and animal tissues. This subject has been reviewed by Jensen and Glass (1981) and Lokke (1975).

Acid hydrolysis conditions were used for this study because the high starch content of potatoes presented a special problem. All attempts to work out conditions for alkaline hydrolysis of macerated potato tuber tissue, or for hydrolysis in acid at concentrations of less than 1 N, resulted in excessive foaming accompanied by the formation of intractable gel and caramel. Lokke (1975) encountered a similar problem when he tried to hydrolyze barley grain in alkali. Apparently, the rate of starch hydrolysis in alkaline or in dilute acid solution is slower than the competing rate of gel formation caused by heat. However, we found that the acid-catalyzed hydrolysis of potatoes proceeded smoothly in 1–5 N H_2SO_4, and 2 N acid was selected for detailed evaluation. Very high concentration of acid (>10 N) resulted in charring.

Once the optimum concentration of acid had been determined, the stabilities of 2,4-D and 2,4-DCP toward acid hydrolysis conditions were confirmed in two ways (Table I). First, control potato samples fortified with 2,4-D alone were subjected to hydrolysis for different lengths of time. The recovery of 2,4-D showed no significant decrease even after 16 h at reflux. Also, 2,4-D was not converted to 2,4-DCP since less than 0.1% of the amount of 2,4-D added was detected as 2,4-DCP. Yip and Ney (1966) found similar results using fortified forage samples hydrolyzed "for about 16 hours under highly acidic conditions". In a second test, replicate samples of potato tissue containing field-incurred residues were analyzed. Again, 2,4-D was found to be stable under the conditions of hydrolysis for up to 16 h; however, the residue level of 2,4-DCP decreased after more than 4 h. A 1-h reflux period was selected as optimum because longer reflux periods did not result in higher residue levels.

As a final check on the conditions for hydrolysis, duplicate potato samples that had already been hydrolyzed for 1 h in 2 N H_2SO_4 solution were subjected to an additional 1-h hydrolysis period in 0.1 N NaOH solution on a steam bath. No increase in residue level of 2,4-D over that found by use of the standard HRA method was observed. Thus, alkaline hydrolysis was no more effective than acid

Table II. Cumulative Percent Recovery of Field-Incurred ^{14}C Residue through the HRA Method[a,b]

HRA fraction[c]	tuber	vine
filtrate	98.7 ± 1.6	51.9 ± 11
organic extracts	42.4 ± 3.9	28.6 ± 3.3
aqueous acid	58.9 ± 7.5	21.8 ± 13
NaOH extracts	40.6 ± 5.2	11.6 ± 8.5
2,4-D ester fraction	34.6 ± 5.1	3.6 ± 15
2,4-DCP fraction	4.09 ± 23	5.9 ± 6.2

[a] Radiolabel applied as [*ring*-U-^{14}C]-2,4-D PGBE ester formulation (Esteron-99). [b] Values represent mean ± CV for triplicate analyses. Percentages calculated on the basis of total ^{14}C present before hydrolysis by combustion analysis. [c] Fraction as designated in Figure 1.

in releasing residues of 2,4-D for extraction. The accurate determination of any 2,4-DDP released by alkaline hydrolysis was precluded by the occurrence of large, interfering chromatographic peaks in the region of 2,4-DCP elution.

For determination of the extraction efficiency and partitioning behavior through the HRA method for all forms of 2,4-D and its transformation products present in potatoes, samples of potato tuber and vine that had been field treated with [*ring*-U-^{14}C]-2,4-D were analyzed after harvest. The total radioactivity present in each lyophilized sample was determined in triplicate (±1.5% CV) by combustion and liquid scintillation counting. When five different potato tuber samples were subjected to hydrolysis by using the HRA method, 99.4 ± 2.4% of the total radioactivity present was extracted into the aqueous filtrate (average of 155 700 dpm/100-g sample). HRA hydrolysis of three vine samples containing 363 500 dpm/100 g released only 51.9 ± 11% of the total radioactivity present.

These results indicate that while potato tuber contains no insoluble-conjugated (bound) residue of 2,4-D or 2,4-D transformation products, approximately 50% of the residue present in potato vine is bound (not freed by acid hydrolysis). Balba et al. (1979) have shown that phenolic or aromatic amine residues are incorporated into the insoluble lignin portions of plant cells, a possible explanation for the result observed for potato vine.

While the exact indentities of the solubilized transformation products of 2,4-D in potato plants were not determined, their behavior through the HRA method is shown in Table II. Comparison of the relatively large amount of radiolabel remaining in the aqueous acid fraction after extraction with benzene with the moderate amount present in the 2,4-D ester fraction indicates that field-incurred 2,4-D is transformed to polar products extensively in both the potato vine and tuber before harvest. When the residue level of 2,4-D present in the vine sample was quantified by both liquid scintillation counting and GLC analysis, the determinations were in excellent agreement, 0.0186 and 0.0185 ppm, respectively. These results indicate that the HRA method for 2,4-D residue is highly accurate and specific for 2,4-D in the presence of its transformation products.

The efficiency of each step of the HRA method was evaluated by fortifying potato tuber samples with [*acetic*-2-^{14}C]-2,4-D and is indicated by the recovery values given in Figure 1. Except for a 10% loss in the initial extraction with benzene, 2,4-D was recovered very efficiently. Attempts to substitute mixed ethers or chlorocarbon solvents for benzene resulted in the formation of emulsions that would not separate satisfactorily on standing or with centrifugation. Ethyl ether could be used in place of benzene only when the resulting emulsion was broken by centrifugation. However, because of the volumes involved, three ethyl ether extractions with centrifugation were found to be less convenient to perform than three extractions with benzene using separatory funnels. Meagher (1966) found the extraction of 2,4-D into hexane to be inefficient and compensated by using five successive extractions.

The transfer operations associated with the blending and hydrolysis steps of the HRA method had to be carried out in alkaline solution (pH 11) to minimize adsorptive losses of 2,4-D onto glassware and/or insoluble plant tissue. Initial attempts to carry out these operations all in 2 N H_2SO_4 gave 2,4-D recoveries that were low and quite variable. Recoveries of 2,4-DCP conducted simultaneously were not affected by pH. Ward and Getzen (1970) have shown that adsorption of 2,4-D to solid surfaces increases with lowering of pH, and this general subject has been reviewed (Adams, 1973; Hamaker and Thompson, 1972).

Attempts to derivatize 2,4-DCP using diazomethane or diazoethane were seen to result in incomplete and variable conversion when reaction mixtures were analyzed by using authentic reference standards of the derivatives. To circumvent this problem and to achieve both sensitive and reliable determination by GLC, we separated residues of 2,4-D and 2,4-DCP after extraction and cleanup. This was accomplished very conveniently by using an acidic alumina column procedure (Jensen, 1973; Bjerke et al., 1972) in which 2,4-D was retained by the column but 2,4-DCP was not. Concentration of the 2,4-DCP residue in the combined ethyl ether eluate from the alumina column (48 mL) was accomplished by extraction first into 5 mL of 1 N NaOH and then into 2 mL of benzene. This procedure avoided an adsorptive loss of 2,4-DCP encountered whenever the ethyl ether eluate was concentrated to dryness or to volumes less than 0.8 mL under a stream of dry nitrogen or air.

The FRA method of analysis relies on direct extraction of free residues into acidified mixed ethers. Since potato tubers are about 80% water, the aqueous phase of the FRA homogenization/extraction step (Figure 1) was 0.56 N in H_2SO_4. These conditions might hydrolyze very labile conjugates of 2,4-D or 2,4-DCP at room temperature, but stronger conditions were found to be needed to convert conjugated forms of 2,4-D to free form in grass samples containing field-incurred residues (Yip and Ney, 1966). Water-soluble 2,4-D conjugates extracted from wheat tissue culture cells were converted only slowly to the free acid under acidic extraction conditions (Bristol et al., 1977). However, when the residue levels found in potatoes by the FRA and HRA methods are compared, the possibility that some acid labile conjugates might be converted to free 2,4-D by the extraction conditions used in the FRA method should be kept in mind.

A fortified sample was included with each set of eight analyses performed. The recoveries of 2,4-D by using both the HRA and FRA methods are shown in Table III while corresponding recoveries for 2,4-DCP are shown in Table IV. No significant trends in recovery from samples fortified at different levels were observed by using either method, but recoveries of both 2,4-D and 2,4-DCP were about 10% higher by using the HRA method. While recoveries by using the FRA method were less than 80%, precison was good. Typical chromatograms of standards and of control and fortified samples analyzed by the HRA and FRA methods are presented in Figures 2 and 3. These methods are known to accurately recover 2,4-D and 2,4-DCP at the lowest fortification levels studied, and the chromatograms of control samples were still essentially free from interferences. The minimum amounts of 2,4-D and

Table III. Recoveries of 2,4-D from Fortified Potato Tuber Samples

fortification level, ppm	no. of samples analyzed	mean % recovery[a]
Hydrolyzable Residue Analysis		
0.20	4	89.2 ± 8.8
0.10	8	87.9 ± 6.8
0.05	4	89.5 ± 6.7
0.02	3	84.1 ± 7.2
0.20-0.02	19	87.9 ± 3.0
Free Residue Analysis		
0.20	3	75.2 ± 6.5
0.10	5	78.7 ± 2.6
0.05	3	83.6 ± 12.2
0.20-0.05	11	79.1 ± 2.9

[a] Mean ± 95% confidence limits for the mean.

Table IV. Recoveries of 2,4-DCP from Fortified Potato Tuber Samples

fortification level, ppm	no. of samples analyzed	mean % recovery[a]
Hydrolyzable Residue Analysis		
0.020	3	88.6 ± 27.1
0.010	6	78.6 ± 12.1
0.005	4	93.4 ± 9.4
0.002	3	80.5 ± 13.4
0.020-0.002	16	84.5 ± 5.7
Free Residue Analysis		
0.050	2	78.0 ± 47.0
0.020	3	71.8 ± 28.6
0.010	3	64.5 ± 9.7
0.005	4	76.8 ± 14.0
0.050-0.005	12	72.7 ± 4.8

[a] Mean ± 95% confidence limits for the mean.

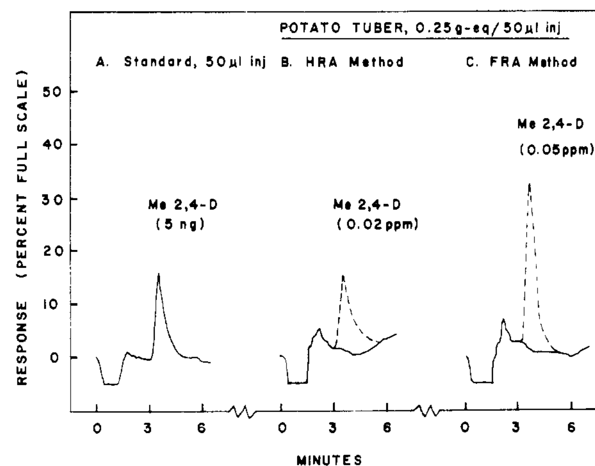

Figure 2. Gas chromatograms of the 2,4-D standard and control (—) and fortified (---) potato tissue.

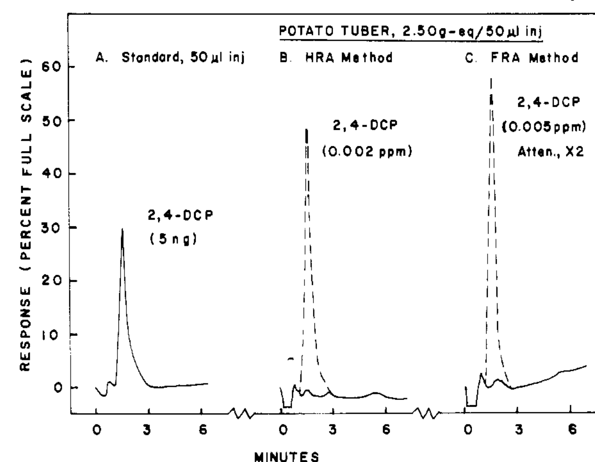

Figure 3. Gas chromatograms of 2,4-DCP standard and control (—) and fortified (---) potato tissue.

2,4-DCP detectable by the HRA method were 1 and 0.1 ppb, respectively, based on signals that were twice the background of control samples. Even at these levels, background interferences did not limit detection. However, lower detection limits were not required for this study, and the practical lower limit of reliable measurement was taken to be the lowest level at which successful recovery experiments were conducted (Tables III and IV).

At the outset of this study, GLC analyses were attempted with a ^{3}H-foil electron capture detector. Chromatograms of control samples were very complex and required at least 15 min for complete elution of each sample injected. This sample matrix problem was solved by using the very selective CCD, and since it accommodates 10-fold more sample volume, sensitivity was not sacrificed.

A summary of residue levels of 2,4-D and 2,4-DCP found in field treated potato tubers is presented in Table V. The residue levels of 2,4-DCP were very low in the 1972 samples, averaging less than 0.005 ppm. They could only be measured by using the HRA method. In each of the individual samples analyzed, 2,4-DCP was present at less than 10% of the residue level of 2,4-D. This phenol does not appear to be a significant metabolite of 2,4-D in potato tubers. Therefore, analyses conducted in subsequent years did not include the determination of 2,4-DCP residue. The residue level of 2,4-D found in each of the treated samples

Table V. Mean Corrected Residue Levels of 2,4-D and 2,4-DCP Found in Potato Tubers by Both the HRA and and FRA Procedures

year and variety[b]	treatment rate, g of a.e./ha[c]	mean 2,4-D found, ppm[a]		mean 2,4-DCP found, ppm[a]	
		HRA	FRA	HRA	FRA
1972, RP	0	<0.020	<0.050	<0.002	<0.005
	140	0.093 ± 0.011	0.070 ± 0.008	0.003 ± 0.0007	<0.005
	280	0.112 ± 0.017	0.100 ± 0.048	0.003 ± 0.0015	<0.005
1973, RP	0	<0.020		<0.002	
	140	0.124 ± 0.040		0.005 ± 0.0007	
1973, N	0	<0.020		<0.002	
	140	0.099 ± 0.027		0.0072 ± 0.0035	
1973, RN	0	<0.020		<0.002	
	140	0.104 ± 0.025		0.0088 ± 0.0038	

[a] Mean ± 95% confidence limits for the mean. In 1972, 10 replicate plot samples for each rate were analyzed, while in 1973, 4 replicate plot samples for each rate for each of three varieties were analyzed. [b] RP, Red Pontiac; N, Norland; RN, Red Norland. [c] Grams of 2,4-D acid equivalent per hectare.

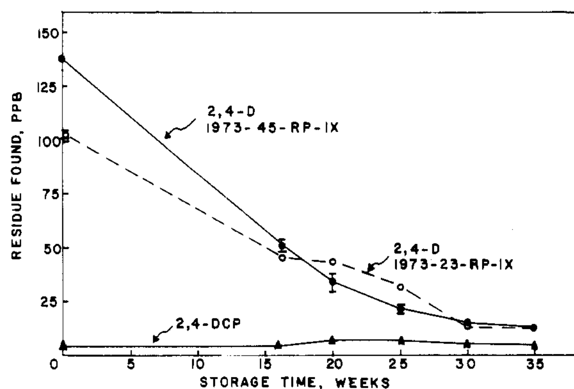

Figure 4. Dissipation of 2,4-D and 2,4-DCP residues from Red Pontiac potatoes treated with 140 g of 2,4-D a.e./ha and stored whole at 3.3 °C. Each point represents the mean ± CV of triplicate analyses; where not indicated, variation was too small to be plotted and the two samples gave essentially identical values for 2,4-DCP.

was low, the highest being 0.15 ppm in a sample analyzed by using the HRA method. The mean residue level for the 32 treated samples was 0.11 ppm. These results are in close agreement with those obtained by Nelson et al. (1971) using acid hydrolysis. For the 1972 replicate samples analyzed by both the HRA and FRA methods, the mean free 2,4-D residue represented 82% of the total 2,4-D residue level found. This difference was statistically significant at the 0.05% level by using Duncan's Multiple Range test. Nelson et al. (1971) found evidence of larger amounts of conjugated 2,4-D in potatoes. The lower amount of conjugated 2,4-D found in this residue study might be due to either greater extraction efficiency for free 2,4-D or partial hydrolysis of very acid labile, conjugated forms of 2,4-D by the FRA method used.

The HRA method used to obtain residue data in this study was also employed to answer several related questions. A dissipation study simulating commercial storage conditions (3.3 °C) showed that residues of 2,4-D in potatoes decreased with a half-life of about 12 weeks (Figure 4). Residues of 2,4-D in laboratory (sliced) potato samples were stable when stored under frozen conditions, but those of 2,4-DCP were not (Bristol, 1976). A fractionation study showed that residues of 2,4-D and 2,4-DCP were not concentrated in the peel of the potato tuber. Baking did not reduce the residue levels present when a correction was made for the water lost during cooking. Further details of these studies as well as residue and dissipation data obtained for the 1974 and 1975 growing seasons are presented elsewhere (Bristol et al., 1981).

The HRA method developed for this study requires slightly more time to perform than the FRA method. When only one analysis is to be performed, the HRA method is preferred, not only because it reflects the total rather than only the free residue present but also because it gives higher recoveries in fortification experiments. When analysis of 2,4-DCP is not necessary, a much more rapid method for the analysis of 2,4-D can be performed by omitting the alumina column separation step. The 2,4-D present in the combined ethyl ether extracts can be partitioned directly into $NaHCO_3$ solution while the derivatization and GLC analysis steps remain unchanged. Any 2,4-DCP present in the extract is partially derivatized but does not interfere with analysis of methyl 2,4-D.

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A Simple and Rapid Colorimetric Method for Determination of Vicine and Convicine

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A simple and rapid colorimetric procedure for determination of vicine and convicine is described based on complex formation between their corresponding aglycons and titanium reagent. The inability of other compounds such as nucleosides and nucleotides to interfere with the titanium complex is a distinct advantage over the UV procedure for measurement of vicine and convicine in fababean products.

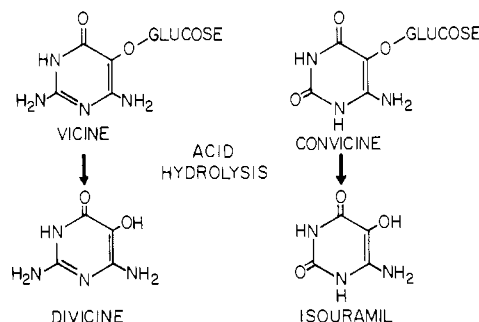
The aglycons of vicine and convicine have been implicated as the causative factors for favism in fababeans (Jamalian et al., 1977; Lin and Ling, 1962a,b; Mager et al., 1965). Two spectrophotometric methods for measuring vicine and convicine have been reported, including one based on the reduction of Folin–Ciocalteau phenol reagent (Higazi and Read, 1974) while the other method involved direct UV spectrophotometric scanning of a protein-free fababean extract (Collier, 1976). This paper reports a sensitive colorimetric procedure for measuring vicine and convicine based on complex formation of the corresponding aglycons with $TiCl_4$ (20% in concentrated HCl). This reagent has been developed in our laboratory to determine hydrogen peroxide (Gupta et al., 1977), lipid hydroperoxides (Eskin and Frenkel, 1976), phenolic compounds (Eskin et al., 1978), and sinapine (Ismail and Eskin, 1979) and as a chromogenic reagent for phenolic compounds (Eskin and Frenkel, 1978) and vicine and convicine (Hoehn et al., 1980) on thin-layer plates. While the reaction mechanism remains to be delineated, possible chelation via the oxygen or an oxidation–reduction type of reaction may be involved.

EXPERIMENTAL SECTION

Materials. Pure samples of vicine and convicine were provided by W. J. Pitz, Department of Crop Science, University of Saskatchewan, Canada, and Dr. R. R. Marquardt, Department of Animal Science, University of Manitoba, Canada. Titanium tetrachloride was purchased from British Drug Houses (Toronto, Canada). Fababeans (*Vicia faba* minor var. Diana) were supplied by the Department of Plant Science, University of Manitoba, Winnipeg, Canada. The beans were dehulled and then ground in a pinmill. The flour (30% protein) was air classified into a protein concentrate (70% protein) and starch fraction (7% protein) according to the method described by Vose et al. (1976).

Formation of Aglycons. Vicine and convicine do not react with the titanium reagent, but their corresponding aglycons, divicine and isouramil, form colored complexes due to the availability of the C-5 hydroxyl for interaction with the titanium salt. To establish the formation of aglycons, we hydrolyzed the glucosides in strong acid (Scheme I). So that the optimum conditions for aglycon formation were established, vicine (20 mg) was dissolved in 1.0 mL of concentrated HCl and heated at 60, 70, 80, and 90 °C over a time period of 0.5–5 min. Following each treatment the hydrolyzed solution was diluted to a final volume of 5 mL with concentrated HCl to give a concentration range of 0–1.0 mg/mL. To each solution was added 0.2 mL of $TiCl_4$ (20% in concentrated HCl) and mixed thoroughly on a vortex for a few seconds. The colored complexes were scanned between 400 and 700 nm in a Unicam SP800 spectrophotometer while individual absorbances were read at 480 nm by using a Unicam SP600 spectrophotometer against an equivalent blank.

Scheme I. Acidic Hydrolysis of Vicine and Convicine to Their Respective Aglycons, Divicine and Isouramil



Titanium Reagent Level and Absorbance of the Divicine–Titanium Complex. The relationship between titanium reagent levels and absorbance of the divicine–titanium complex was examined. Vicine (20 mg) was dissolved in 1 mL of concentrated HCl and hydrolyzed at 80 °C for 1.5 min. The hydrolyzed solution was further diluted with concentrated HCl to give solutions ranging from 0 to 0.5 mg/mL. Increasing amounts of titanium tetrachloride (0.05–3.2 mL) were added to the divicine solutions (5 mL), and absorbance at 480 nm was measured against an equivalent blank by using a Unicam SP600 spectrophotometer.

Preparation of Divicine and Isouramil Standards. Ten milligrams of pure vicine and convicine were each

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