Simultaneous Extraction and Detection of Residues of (2,4-Dichlorophenoxy)acetic Acid and Bromoxynil from Wheat

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An analytical method for the simultaneous gas chromatographic determination of residues of 2,4-D [(2,4-dichlorophenoxy)acetic acid] and bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) from wheat grain and green tissue has been developed by using an alkaline extraction procedure, derivatization with diazomethane, and electron-capture detection. The limits of detection for bromoxynil and 2,4-D as the corresponding methyl derivatives were 0.01 and 0.05 ppm, respectively. No significant differences in herbicide residues, determined at 1 day, 3 weeks, and 6 weeks after application, were observed whether the chemicals were applied singly or as a tank mixture. Amounts of residues of both chemicals from the mature grain and straw were less than their respective limits of detection.

Herbicide mixtures can be used to advantage where weed infestations in crops are composed of a range of weed species, all of which cannot by controlled by one herbicide. Thus, tank mixing permits control of a broad spectrum of weed species in a single field operation.

The tank mixture consisting of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and MCPA [[(4-chloro-*o*-tolyl)oxy]acetic acid] has been registered in Canada for several years for use as a postemergence treatment for the control of broad-leaved weeds in cereals. Early tolerance and efficacy studies (McConnell and Friesen, 1964; Hall and Friesen, 1965; Keys, 1965, 1966; Vanden Born and Schraa, 1965, 1966) have shown that a tank mixture of bromoxynil and 2,4-D [(2,4-dichlorophenoxy)acetic acid] could also be used successfully for broad-leaved weed control, with the advantage that some MCPA-resistant weed species, notably Russian thistle (*Salsola pestifer*), were controlled more effectively. However, this use has not been registered because the corresponding residue data were not available.

Studies by Chow et al. (1971) and Løkke (1975) have shown that residues of acidic herbicides, such as (chlorophenoxy)alkanoic acids, which form conjugates with plant substituents are most accurately determined when the extraction includes a hydrolytic step. In the present study, alkaline hydrolysis was used as a combined extraction/hydrolysis step to determine residues of bromoxynil and 2,4-D in wheat tissue, not only to generate residue data for use by the regulatory agencies for registration purposes but also to determine whether significant differences in herbicide residues were observed when the chemicals were applied singly or as a tank mix.

MATERIALS AND METHODS

Herbicide Treatments. Neepawa wheat was seeded into Regina heavy clay with a discer seeder on May 19, 1975, and immediately after seeding, 1.34 kg/ha triallate [*S*-(2,3,3-trichloroallyl) diisopropylthiocarbamate] was incorporated. Plots (1.8 × 12.2 m), separated by 0.5-m alleys, were staked out in a randomized complete block, and on June 17 the following herbicide treatments were applied when the wheat was in the three-leaf stage: 0.56 kg/ha 2,4-D octyl ester; 0.42 kg/ha bromoxynil octanoate; 0.56 kg/ha 2,4-D octyl ester plus 0.42 kg/ha bromoxynil octanoate as a tank mixture. All treatments were replicated 4 times and were applied in 45.5 L/ha by using a hand-held small plot sprayer operated by compressed air at 207 kPa.

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Sampling. Wheat plants, cut at the soil surface, were collected at random from each replicate plot until the sample size was collected. Green foliage samples were collected on June 18 (0.1 kg), July 9 (0.2 kg), and July 30 (0.4 kg). The mature wheat samples (0.4 kg) were collected on September 5 and then separated into grain and straw/chaff fractions by using a Almaco laboratory type small plant and heat thresher equipped with a rasping bar type cylinder (Allan Machine Co., Ames, IA). The replicate samples were not pooled.

All samples were frozen in sealed polyethylene bags immediately after harvest and stored in a freezer at −10 °C until extraction.

Chemicals. All solvents were pesticide grade (Caledon Laboratories Ltd., Georgetown, Ontario, Canada). The diethyl ester was stabilized with 0.25% ethanol.

Florisil, 60–80 mesh, was heated at 400 °C for 4 h and then deactivated by the addition of 5% water (Fisher Scientific Co.).

Sodium sulfate was heated at 600 °C for 48 h.

Caution: Diazomethane is very toxic and should be prepared and used in a well ventilated fume hood. Contact of ground glass apparatus with diazomethane, which is also explosive, should be avoided. *N*-Methyl-*N'*-nitro-*N*-nitrosoquanidine (MNNG), a precursor of diazomethane, is a cancer suspect agent and a very potent mutagen.

Bromoxynil methyl ether was prepared as follows. MNNG (1.32 g, 0.009 mol) was added to a 100-mL graduated cylinder which contained 10 mL of 6.0 N NaOH solution and 75 mL of diethyl ether. When the decomposition of MNNG was complete, the ethereal solution of diazomethane was slowly decanted into a 250-mL Erlenmeyer flask containing 2.0 g of bromoxynil (0.007 mol; Pfaltz & Bauer, Inc., Stamford, CT) in 25 mL of diethyl ether and the reaction mixture was allowed to stand at room temperature for 20 min. The reaction mixture was then placed in a water bath (35 °C) and the ether evaporated to dryness with a stream of N_2 to remove excess diazomethane. The residue was redissolved in diethyl ether (25 mL) and washed twice with 10 mL of 0.001 N NaOH solution and twice with 10 mL of water. The ether was removed by using a rotary evaporator and the residue recrystallized from ethanol/water by using decolorizing carbon to give 0.72 g of product, mp 115.3–116.3 °C [lit. mp 117 °C (Muggleton, 1979)].

Analytical grade 2,4-D and 2,4-D methyl ester were supplied by The Dow Chemical Co., Midland, MI, whereas the analytical grade bromoxynil was supplied by Allied Chemical Services Ltd., Calgary, Alberta, Canada.

Sample Extraction. (*a*) *Grain or Straw.* Ten grams of grain or straw, milled through a 1-mm screen, and 125 mL of 0.1 N NaOH solution were mechanically stirred for

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30 min at 80 °C (wax bath). The sample was cooled with stirring in a ice–water bath and then centrifuged at 13000 rpm for 10 min (straw samples, 5000 rpm for 10 min) by using 250-mL stainless steel centrifuge bottles. After the aqueous extract was decanted, 50 mL of 0.1 N NaOH solution was added to the plant residue, the centrifuge bottle was vigorously shaken, and the sample was centrifuged and decanted again as described above.

The decantates were combined, 50 mL of saturated NaCl solution was added, and the pH was adjusted to either 6.5 when only bromoxynil was present or 5.0 when 2,4-D was present with 4.0 N H_2SO_4. The sample was allowed to stand for 15 min and then was centrifuged for 10 min at 5000 rpm. The pH of the decantate was adjusted to either 2.5 when only bromoxynil was present or 1.0 when 2,4-D was present with 4.0 N H_2SO_4. The acidified decantate was extracted twice with 225 mL of diethyl ether. The ether extracts were combined, concentrated to approximately 150 mL by using a rotary evaporator, transferred to a 250-mL separatory funnel, and extracted 3 times with 25 mL of 4% $NaHCO_3$ solution. The $NaHCO_3$ extracts were combined, adjusted to pH 1.0 with 4.0 N H_2SO_4, and extracted 3 times with 25 mL of $CHCl_3$ in a 250-mL separatory funnel. The $CHCl_3$ extracts were combined and concentrated to approximately 10 mL by using a rotary evaporator. Hexane (25 mL) and a small amount of Celite (prewashed with $CHCl_3$) were added, and the mixture was filtered under suction through a glass fiber filter paper, followed by a 10-mL wash with 60:40 hexane/chloroform. The sample was then evaporated to approximately 2 mL by using a rotary evaporator.

(*b*) *Green Samples.* Fifty grams of green wheat tissue was cut into 1.3-cm lengths and blended in a 1-L stainless steel blender jar with 150 mL of 0.1 N NaOH solution at high speed for 5 min. The plant material was transferred to a 500-mL Erlenmeyer flask, including a 100-mL 0.1 N NaOH solution rinse of the blender jar, heated with stirring, and cooled as described above. The mixture was centrifuged for 10 min at 5000 rpm and decanted. An additional 100 mL of 0.1 N NaOH solution was added to the plant residue; the mixture was vigorously shaken and then centrifuged and decanted as before.

Saturated NaCl solution (100 mL) was added to the combined decantates and the pH adjusted as described above. The combined decantates were extracted with diethyl ether in the following manner to prevent emulsion formation. Half of the combined decantates was extracted with 225 mL of diethyl ether in a 500-mL separatory funnel. The aqueous layer was drained into a second 500-mL separatory funnel containing 225 mL of diethyl ether, the extraction was repeated, and the aqueous layer was discarded. The other half of the combined decantates was added to the first separatory funnel and extracted in the same manner. The ether extracts were combined, and the workup was continued as described above.

Diazomethane Derivatization. Diazomethane was prepared by the addition of 100 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Aldrich Chemical Co., Inc., Milwaukee, WI) to 10 mL of 6.0 N NaOH solution and 25 mL of diethyl ether in a 50-mL graduated cylinder.

The concentrated plant extract was transferred to an 18 × 150 mm test tube, and approximately 3 mL of diazomethane solution was added (using a flame-polished pipet). The test tube was immersed in a warm water bath (55 °C) and the excess diazomethane removed by boiling the ether just to dryness.

Florisil Column Cleanup. Florisil (10 mL) was added to 25 mL of hexane in a 17 mm i.d. × 300 mm column, topped with a layer of anhydrous sodium sulfate (10 mL), and the hexane was drained from the column. The methylated plant extract was then transferred to the column in 2–3 mL of hexane and washed onto the column with 2–3 mL of 0.5% acetone in hexane. The column was then eluted with 0.5% acetone in hexane, and the following fractions were collected: 25–90 mL for bromoxynil methyl ether, 50–180 mL for 2,4-D methyl ester, and 25–180 mL when both herbicides were present. The eluate was concentrated to approximately 5 mL by using a rotary evaporator and then taken to volume (10 mL) with hexane prior to quantitation of bromoxynil methyl ether and 2,4-D methyl ester by GLC.

Fortification. Recoveries of 2,4-D and bromoxynil were determined by the extraction of green wheat tissue and wheat grain fortified at 0.1 ppm. The green wheat tissue was fortified with either bromoxynil or 2,4-D as follows. Fifty grams of frozen untreated green wheat tissue was cut into 1.3-cm lengths and allowed to thaw in a 250-mL beaker. Bromoxynil or 2,4-D (5.0 mL, 1.0 ppm, in acetone) was added to the green tissue and the beaker sealed with parafilm to prevent the green tissue from drying. The wheat grain was fortified as follows. A 2.0-mL (0.5-ppm) amount of bromoxynil or 2,4-D in acetone was added to 10 g of milled (1-mm screen) untreated wheat grain in a 125-mL Erlenmeyer flask. All fortified samples were left in darkness at room temperature for 48 h prior to extraction.

Gas Chromatography. A Hewlett-Packard Model 5713A gas chromatograph equipped with a ^{63}Ni detector was used with a Honeywell Electronik 194 1-mV recorder. The 1.2 × 4 mm i.d. coiled glass column was packed with 100–200 mesh Ultra-Bond (RFR Corp., Hope, RI). The retention times for 2,4-D methyl ester and bromoxynil methyl ether were 6.0 and 4.3 min, respectively, with the following operating conditions: 95% argon/methane (carrier gas), 30 mL/min; injector and column, 140 °C; detector, 300 °C. Under these conditions with the attenuator set at ×16, 0.85 ng of 2,4-D methyl ester and 0.11 ng of bromoxynil methyl ether gave full-scale recorder deflections. Bromoxynil methyl ether gave a linear response over the range 0.04–4.0 ng, whereas the linear range for 2,4-D methyl ester was 0.04–2.0 ng.

Check on the Stability of Bromoxynil to the Extraction Conditions. Bromoxynil (1.0 μg in 1.0 mL of acetone) was added to 175 mL of 0.1 N NaOH in a 250-mL Erlenmeyer flask and mechanically stirred for 30 min at 80 °C (wax bath). The sample was cooled with stirring in a ice–water bath, 50 mL of saturated NaCl solution was added, and the pH was adjusted to 1.0 with 4.0 N H_2SO_4. The extraction, methylation, Florisil cleanup, and gas chromatographic analysis procedures were then performed as previously described.

The experiment was then repeated as described above except that the alkaline solution of bromoxynil was not heated and subsequently cooled prior to the addition of the saturated NaCl solution. Both of the above experiments were replicated 8 times.

RESULTS AND DISCUSSION

Phenoxyalkanoic acid residues are recovered most efficiently from plant tissues by an extraction procedure which effects hydrolysis of the tissue being analyzed (Chow et al., 1971; Løkke, 1975). Similar results have been observed for bromoxynil (Buckland et al., 1973). In the present study, the wheat tissue was hydrolyzed with 0.1 N NaOH solution. This alkaline concentration, reported by Chow et al. (1971) to give complete hydrolysis of MCPA conjugates extracted from wheat tissue, should not only

Table I. Residues[a] of Bromoxynil Found in Neepawa Wheat[b]

sampling date[c]	ppm, fresh wt basis, in plot no. A-2	B-2	C-2	D-2	av, ppm
June 18	57.0	53.0	50.0	66.0	56.5 ± 7.0
July 9	0.078	0.040	0.021	0.018	0.039 ± 0.027
July 30	<0.01	<0.01	<0.01	<0.01	<0.01
Sept 5					
straw	<0.01	<0.01	<0.01	<0.01	<0.01
grain	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Residue values are uncorrected for recoveries. [b] Treated with 0.42 kg/ha bromoxynil octanoate. [c] Moisture content of the wheat tissue: June 18, 86.0 ± 0.2%; July 9, 82.3 ± 0.2%; July 30, 57.1 ± 1.5%; Sept 5, 11.1 ± 0.4%.

Table II. Residues of Bromoxynil Found in Neepawa Wheat Treated with the 2,4-D/Bromoxynil Tank Mixture[a]

sampling date	ppm, fresh wt basis, in plot no. A-6	B-6	C-6	D-6	av, ppm
June 18	51.5	40.0	58.0	52.5	50.5 ± 7.6
July 9	0.014	0.015	0.012	0.013	0.014 ± 0.001
July 30	0.012	<0.01	<0.01	<0.01	<0.01
Sept 5					
straw	<0.01	<0.01	<0.01	<0.01	<0.01
grain	<0.01	<0.01	<0.01	<0.01	<0.01

[a] 0.42 kg/ha bromoxynil octanoate plus 0.56 kg/ha 2,4-D octyl ester. Residue values are uncorrected for recoveries.

effect hydrolysis of 2,4-D conjugates but also hydrolyze any 2,4-D octyl ester remaining in the plant tissue (Smith, 1972). Even less rigorous hydrolysis conditions (0.01 N NaOH) have been used for the analysis of 2,4-D residues from the pulp and peel of oranges (Moyer and McCornack, 1977). Although bromoxynil is susceptible to aqueous alkaline hydrolysis (Carpenter et al., 1964), no significant loss of bromoxynil was observed due to the alkaline extraction conditions employed in this study. The recoveries of bromoxynil from the stability experiments were 88.4 ± 2.0% with heating and 90.6 ± 3.1% without heating.

Recoveries of bromoxynil and 2,4-D as their respective methyl derivatives were determined from standard calibration curves constructed by plotting nanograms of methyl derivative against peak height. Four replicates of both green tissue (from the July 30 check samples) and mature grain fortified at 0.1 ppm with either 2,4-D or bromoxynil were analyzed. The recoveries of bromoxynil were 81.6 ± 8.5% from the green tissue and 74.6 ± 7.8% from the mature grain. The corresponding recoveries for 2,4-D were 80.4 ± 5.7 and 71.2 ± 7.4%.

Residue amounts of bromoxynil found in wheat tissue with time when applied alone or when tank mixed with 2,4-D are tabulated in Tables I and II, respectively. Because of the large residue amounts and the corresponding dilution factor, background interferences were negligible for the June 18 samples. The background interferences for the other three collection dates, based on analysis of the corresponding checks (Figure 1), were less than 0.005 ppm, and the limit of detection for bromoxynil was 0.01 ppm.

The corresponding residue amounts of 2,4-D are tabulated in Tables III and IV. Background interferences, negligible for both the June 18 and July 9 samples, were on the order of 0.03 ppm for the remaining samples (Figure 1), and the limit of detection for 2,4-D was 0.05 ppm.

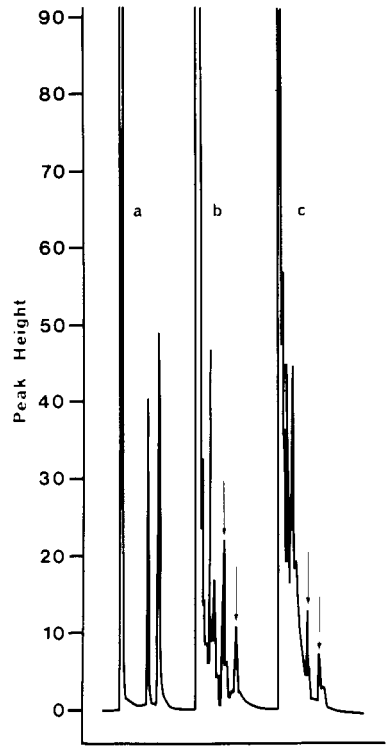

Figure 1. Chromatograph a, 0.04 ng of bromoxynil methyl ether plus 0.4 ng of 2,4-D methyl ester; chromatogram b, extract of July 30 wheat tissue from check plot; chromatogram c, extract of Sept 5 wheat grain from check plot.

Table III. Residues of 2,4-D Found in Neepawa Wheat[a]

sampling date	ppm, fresh wt basis, in plot no. A-4	B-4	C-4	D-4	av, ppm
June 18	136.4	165.2	151.9	168.1	155.4 ± 14.5
July 9	1.23	0.58	1.16	0.59	0.89 ± 0.35
July 30	0.08	0.08	0.10	0.10	0.09 ± 0.01
Sept 5					
straw	<0.05	<0.05	<0.05	<0.05	<0.05
grain	<0.05	<0.05	<0.05	<0.05	<0.05

[a] Treated with 0.56 kg/ha 2,4-D octyl ester. Residue values are uncorrected for recoveries.

Table IV. Residues of 2,4-D Found in Neepawa Wheat Treated with the 2,4-D/Bromoxynil Tank Mixture[a]

sampling date	ppm, fresh wt basis, in plot no. A-6	B-6	C-6	D-6	av, ppm
June 18	161.3	190.7	176.3	167.1	173.9 ± 12.8
July 9	0.80	1.13	0.33	0.26	0.63 ± 0.41
July 30	0.09	0.13	0.12	0.10	0.11 ± 0.02
Sept 5					
straw	<0.05	<0.05	<0.05	<0.05	<0.05
grain	<0.05	<0.05	<0.05	<0.05	<0.05

[a] 0.42 kg/ha bromoxynil octanoate plus 0.56 kg/ha 2,4-D octyl ester. Residue values are uncorrected for recoveries.

Although 2,4-D and bromoxynil were applied to the wheat in a ratio of 1.33:1, the observed initial residue amounts of 2,4-D and bromoxynil, whether applied singly or as tank mix, were roughly in a ratio of 3:1. Since formulations of bromoxynil octanoate are less volatile than those of 2,4-D octyl esters (Grover, 1979), the relatively

high initial residue amounts of 2,4-D may reflect either a more rapid uptake and/or metabolism of bromoxynil by wheat seedlings or a greater susceptibility of bromoxynil to photodecomposition.

Application of 2,4-D and bromoxynil to wheat seedlings as a tank mixture did not result in significant differences in residues when compared to the residues observed when the chemicals were applied singly.

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Determination of the Triglyceride Composition of Olive Oil by a Multistep Procedure

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Olive oil was analyzed by separating the total triglycerides into fractions, different in the number of double bonds per mole, by means of Ag^+ TLC. The fatty acid composition—overall and at the β position—was then determined both of total triglycerides and of single fractions; moreover, each fraction was oxidized and the products were separated by means of TLC into classes, each containing molecules having the same number of azelaic acid residues (A_3; A_2S; AS_2). The acids contained in each class were then quantitated by means of GLC. From the obtained data, the triglyceride composition of olive oil was determined (21 molecular species = 92.5% of total triglycerides), without the many assumptions usually conceived by other methods.

Many methods have been described to determine the glyceride composition of fats.

In some of these, the analytical procedure is carried out by working directly on the fat (Dutton and Cannon, 1956; Quimby et al., 1953; Riemenschneider, 1954; Scholfield and Dutton, 1958; Scholfield and Hicks, 1957) or on the oxidized products of unsaturated fatty acid components of the fat (Hilditch and Lea, 1927; Kartha, 1961; Youngs, 1961); other methods are based either on the ozonization of double bonds, followed by the catalytic reduction of ozonides and by their separation and TLC quantification (Privett and Blank, 1961, 1963), or on the action of pancreatic lipase in the 1–3 positions of the triglycerides (Vander Wal, 1960).

Otherwise, the component glycerides of some seed oils were separated by Ag^+ TLC on two chromatoplates and then quantitatively determined by GLC of the methyl esters of their fatty acid components, with methyl eptadecanoate as an internal standard (Gunstone and Padley, 1965); the results agree with those obtained by other methods, but the isomeric molecular species are not determinable.

Some GLC methods directly operating on triglycerides (Freyer et al., 1960; Huebner, 1961; Kuksis and McCarthy, 1962; McCarthy et al., 1962, Pelick et al., 1961) have been entirely successful, though they do not allow for the separation of unsaturated from saturate glycerides with the same carbon number.

This has been partly overcome with an oxidation process that converts the unsaturated glycerides into compounds having smaller carbon numbers; GLC analysis of these esterified compounds gives the distribution of the individual saturated fatty acids in the glycerol mojety, whereas the unsaturated fatty acids (palmitoleic, oleic, linoleic, and linolenic) are estimated together as azelaoglycerides (Youngs and Subbaram, 1964).

An improved method (Subbaram and Youngs, 1964) based on the fractionation of glycerides into groups dif-

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