

2,4-DBCompounds Analyzed

2,4-DB	4-(2,4-dichlorophenoxy)butyric acid
2,4-D	2,4-dichlorophenoxyacetic acid
DCP	2,4-dichlorophenol

2,4-D and 2,4-DB Analysis

Overview of method. The 2,4-DB and 2,4-D were analyzed by an adaptation of the method by Yip (1971). The phenoxyacids are extracted from the tissue with acetonitrile-water. The acetonitrile is removed, the aqueous layer acidified and the phenoxyacids extracted into methylene chloride. The solvent is removed and the phenoxyacids converted to the methyl esters. The methyl esters are then purified with a Florisil column prior to analysis. Analysis is by gas chromatography with a Hall electrolytic conductivity detector.

Analytical Standards

The standards were obtained from the E.P.A. Standards Repository, Research Triangle Park, NC.

2,4-DB acid standard: Lot no. B02Y; purity: 99.66%
 2,4-DB methyl ester standard: Lot no. 948A;
 purity: not given

2,4-D acid standard: Lot no. 5397; purity 99.85%
 2,4-D methyl ester standard: Lot no. 092B; purity 99+%

All standards, when not in use, are stored at -20°C.

Standard Solutions The 2,4-DB acid stock solution was prepared in acetone at a concentration of 250 µg/mL. This solution was used to prepare 5 µg/mL and 1 µg/mL solutions in acetone. The 2,4-D acid stock solution was prepared at 500 µg/mL in acetone and then reduced to 5 µg/mL and 1 µg/mL concentrations, also with acetone.

Both methyl ester stock solutions were prepared in acetone at a concentration of 250 µg/mL. The concentrations were then reduced to 5 µg/mL with 2,2,4-trimethylpentane (TMP). A combined solution of the two esters was prepared at 1 µg/mL and this solution diluted to form the working standards. The working standards were at concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8 µg/mL. TMP was used as the solvent for all dilutions of the methyl esters.

All solutions, when not in use, are stored at 4°C.

Analysis of 2,4-DB and 2,4-D (Cont)**Storage Stability Samples**

No storage stability samples were prepared for this project (2,4-DB/Barley). Storage data were available from a previous 2,4-DB/Barley project.

Reagents

Acetone: Mallinckrodt, Nanograde
Acetonitrile: Mallinckrodt, ChromAR HPLC
Diethyl Ether: Baker Resi-Analyzed
Ethanol: 95% USP
Florisil: PR 60/100 activated 12 hr at 130°C
Hexane: Mallinckrodt, Nanograde
Hydrogen Chloride: Linde, technical grade
Methanol: Mallinckrodt, ChromAR HPLC
Methylene Chloride: Mallinckrodt, Nanograde
Sodium Chloride: Baker, Reagent
Sodium Hydroxide: Mallinckrodt, Analytical Reagent
Sodium Sulfate: Mallinckrodt, Analytical Reagent, Anhydrous
Sulfuric Acid: Mallinckrodt, Analytical Reagent
2,2,4-Trimethylpentane (TMP): Mallinckrodt, Nanograde

Equipment

Blender: Tekmar model SD45 with 45N head
Chromatography column: 1.5 cm X 30 cm with 250 mL reservoir
Coffee Mill: Girmi model MC18AS
Culture Tubes: 1.5 cm X 25 cm with Teflon lined screw caps
Paper Filters: Whatman #4, 7 cm
Wiley Mill: Intermediate model with 10 mesh screen

Extraction and Cleanup of 2,4-DB and 2,4-D

1. Cut the straw sample into 1 inch lengths and mix thoroughly. Grind the sample using a Wiley mill with a number 10 mesh screen. Grind the seed with a small coffee grinder to a fine consistency.
2. Place 20 g of ground seed or 10 g of ground straw (see addendum 1) in a 1000 mL tall glass jar. For recovery samples fortify at this point by adding the appropriate volume of the analytes in acetone to the ground tissue. After addition of the solution, mix, and after 10 minutes add 200 mL of acetonitrile-water (65:35, v/v). Blend for 2 minutes at moderate speed with the homogenizer.
3. Filter the homogenate by suction filtration through a 7-cm paper disk (Whatman No. 4). Do not wash the marc, but measure and record the volume of the filtrate.
4. Transfer the filtrate to a 500-mL round bottom flask. Add and dissolve 4 g of sodium chloride. Reduce the volume to 50-60 mL using a rotary evaporator with a water bath temperature of 40°C.
5. Transfer the concentrate to a 250-mL separatory funnel. Rinse the flask with 25 mL 3% sodium hydroxide solution; two 5-mL portions of distilled water and finally with a 50-mL portion of methylene chloride. Add all rinses to the funnel. In the case of the barley seed also add 20 mL of ethanol to the funnel.
6. Shake the mixture and allow the phases to separate. Discard the organic (methylene chloride) layer. Repeat the extraction with 50 mL methylene chloride and discard the organic layer.
7. Acidify the aqueous phase with 25 mL of 10% (v/v) sulfuric acid. Check with indicator paper to assure the pH is 1-2. Extract the solution with 50, 25, and 25-mL portions of methylene chloride. Drain each extract through a funnel with a plug of glass wool in the apex into a 250 mL round bottom flask. Rinse the glass wool with 5 mL of methylene chloride. Add 5 mL of TMP to the combined extracts and reduce the volume of the solution to approximately 1 mL using a rotary evaporator.

Analysis of 2,4-DB and 2,4-D (Cont)

8. Take up the concentrate with 2 mL of methanol and transfer to a culture tube. Rinse the flask with an additional 2 mL of methanol.

9. Add to the methanolic solution 1 mL of anhydrous 2N HCl in methanol. Seal the tube with a teflon lined cap and mix. Heat the culture tube in a steam bath for 10 minutes. After heating, cool the tube to room temperature.

10. Add to the tube 5 mL water and 5 mL TMP. Cap and shake for 1 minute. Allow the phases to separate.

11. Plug the bottom of a reservoir column with glass wool. Add 5.0 grams of fully active Florisil and overlay with 2 g of anhydrous sodium sulfate.

12. Transfer the TMP layer from step 10 to the column. Extract the aqueous layer with two additional 5-mL portions of TMP and transfer to the column. Allow the TMP extracts to pass through the column and discard the effluent.

13. Elute the phenoxyacid methyl esters from the column with 60 mL of diethyl ether-hexane (1:3, v/v) collecting the eluate in a 100-mL round bottom flask.

14. Add 5 mL TMP to the column eluate and reduce the volume to 1-2 mL on the rotary evaporator. Use a 40°C water bath.

15. Transfer the concentrate to a 10 mL volumetric flask rinsing the roundbottom with several 2-mL portions of TMP. Dilute to final volume of 10 mL with TMP. Store at 4°C until analysis.

16. Analyze by G.C. using a Hall electrolytic conductivity detector. Make successive dilutions, if necessary, with TMP in order to keep the analyte peaks within the range of the standards.

Analysis of 2,4-DB and 2,4-D (Cont)Chromatographic ConditionsGas Chromatograph

Instrument: Tracor model 550 gas chromatograph
with Hall electrolytic conductivity
detector, model 700A
Column: 6 ft. X 2mm OV-11 (10% on 80/100
Gas-Chrom Q)
Carrier: 20 mL/min Helium
Column Temp: 210°C; Inlet Temp: 220°C
Outlet Temp: 275°C; Transfer line: 280°C

Detector Parameters

Detector Temp: 870°C
Electrolyte: n-propanol (0.33 mL/min)
Hydrogen: 40 mL/min

Integrator Recorder Setting

Integrator: Shimadzu C-R1A
SLOPE: 350 MIN AR: 10
ATTEN: 0 SPEED: 10
METHOD: 1041 WIDTH: 5

Quantitation of 2,4-DB and 2,4-D

The analysis was done by comparing the response of the sample and that of standards giving a response immediately above and below that of the sample. Solution concentration was then determined by interpolation. Multiple injections of 5 μL were used and at least two values used to determine a mean value of response for the samples and methyl ester standards. The following illustrates the calculation method using barley straw (RRL-3009) treated with 3.0 lb 2,4-DB/A. The calculation is for 2,4-DB.

<u>Sample</u>	<u>Response</u>		
	<u>Run #1</u>	<u>Run #2</u>	<u>Mean</u>
2,4-DB Std (0.6 $\mu\text{g/mL}$)	357 ^a	367	362
2,4-DB Std (0.8 $\mu\text{g/mL}$)	527 ^b	509	518
RRL-3009	435 ^c	463	449

$$\frac{[\text{response of unknown}] - [\text{response of } 0.60 \mu\text{g/mL std}]}{[\text{response of } 0.8 \mu\text{g/mL std}] - [\text{response of } 0.6 \mu\text{g/mL std}]} = \frac{X - 0.6}{0.8 - 0.6}$$

$$\frac{449 - 362}{518 - 362} = \frac{X - 0.6}{0.8 - 0.6}$$

$$\frac{87}{156} = \frac{X - 0.6}{0.8 - 0.6}$$

$$\frac{(87)(0.2)}{156} + 0.6 = X = \mu\text{g/mL } 2,4\text{-DB methyl ester}$$

$$0.7115 \mu\text{g/mL} = X$$

$$(0.7115 \mu\text{g } 2,4\text{-DB methyl ester/mL})(25 \text{ mL})(20/1 \text{ dilution}) = 355.8 \mu\text{g methyl ester}$$

$$(355.8 \mu\text{g methyl ester}) \left(\frac{249.1 \text{ g } 2,4\text{-DB acid/mole}}{263.1 \text{ g } 2,4\text{-DB methyl ester/mole}} \right) = 336.8 \mu\text{g } 2,4\text{-DB Acid}$$

$$(336.8 \mu\text{g } 2,4\text{-DB Acid}) \left(\frac{200 \text{ mL solvent added}}{173 \text{ mL solvent recovered}} \right) = 389.4 \mu\text{g } 2,4\text{-DB acid corrected}$$

$$389.4 \mu\text{g } 2,4\text{-DB Acid}/10 \text{ g sample} = 38.9 \mu\text{g/g (ppm)}^{\text{d}}$$

^a Datum appears in figure 6A

^b Datum appears in figure 6B

^c Datum appears in figure 10 A

^d Result appears in table 4

Analysis of 2,4-DB and 2,4-D (Cont)Notes on Results

1. Standard response curves were obtained each day chromatographic analysis was done. Detector response varied day to day.

2. Both seed and straw from the controls contained 2,4-D and 2,4-DB and these background levels affected the quantitation limit. A level of five times background was used as the criteria for the detection and quantitation limit. The quantitation limit was 0.050 ppm in the seed and 1.0 ppm in the straw.

3. The storage stability data appear in the residue report from IR-4 project PR-2321, 2,4-DB/Barley (Wisconsin samples), prepared in January 1986. The method of analysis was the same method used for this analysis but with the following changes.

- a. In step no. 5 and 6 of this method (also no. 5 and 6 of 1986 report) 2-50 mL fractions of methylene chloride were used in the partition step instead of the 40 and 20 mL fractions used in the earlier analysis.
- b. In step no. 9 hydrogen chloride in methanol was used for methylation in this analysis with boron trifluoride in methanol having been used in the 1986 project.
- c. In steps no. 11-13 instead of the single Florisil cleanup column used in this analysis a Florisil column followed by a silica gel column cleanup was used.
- d. The analysis was done with gas chromatography using an electron capture detector in the earlier analysis and a Hall detector for this sample set.

Literature Cited

Yip, G., 1971. Improved method for determination of chlorophenoxy acid residues in total diet samples. J. Assoc. Off. Anal. Chem. 54:966-69.

Analysis of 2,4-dichlorophenol

Overview of the Method

Dichlorophenol (DCP) was analyzed by an adaptation of the method by Kan (1981). Ground tissue is steam distilled from an acid solution and DCP trapped in toluene. The toluene extract is analyzed directly by gas chromatography using a Hall electrolytic conductivity detector.

Analytical Standards

The dichlorophenol standard was obtained from the E.P.A. Standards Repository, Research Triangle Park, N.C. the standard was from Lot no. 1281 with a given purity of 99%. The standards, when not in use, are stored at -20°C .

The dichlorophenol standard stock solution was prepared at a concentration of $500\ \mu\text{g}/\text{mL}$ with acetone as the solvent. This solution was then diluted with acetone to a concentration of $5\ \mu\text{g}/\text{mL}$ and then to $1\ \mu\text{g}/\text{mL}$. These last two solutions were spiking solutions used to validate the method.

Analytical standards were prepared by dilution of the $500\ \mu\text{g}/\text{mL}$ solution with toluene to a concentration of $5\ \mu\text{g}/\text{mL}$ and then to $1\ \mu\text{g}/\text{mL}$. The $1\ \mu\text{g}/\text{mL}$ solution was then used to prepare the working standards with the concentrations of 0.10, 0.20, 0.40, 0.60, and 0.80 $\mu\text{g}/\text{mL}$. All solutions, when not in use, were stored at 4°C .

Storage Stability Samples

No storage stability samples were prepared for this project (2,4-DB/Barley). Storage data were available from a previous 2,4-DB/Barley project.

Analysis of 2,4-dichlorophenol (Cont)

Reagents

Acetone: Mallinckrodt, Nanograde
Phosphoric acid: Fisher, Certified Reagent
Toluene: Mallinckrodt, Nanograde

Equipment

Liquid-liquid extraction apparatus (Kan, et.al., and Figure 1).

Extraction of 2,4-dichlorophenol

1. Cut the straw sample into 1 inch lengths and mix thoroughly. Grind a subsample with a Wiley mill using a 10 mesh screen. Weigh out a sample with a weight equivalent to a 10 gram dry sample (See addendum number 1). Grind the seed with a small coffee grinder to a fine consistency. Place 20 g of the tissue in a 2000-mL round bottom flask. For recovery experiments spike at this point. Add the spiking solution to the ground tissue in the flask, mix and allow to stand for 10 minutes to allow the solvent (acetone) to evaporate.

2. Add to the flask: distilled water, 300 mL to the seed sample and 500 mL to the straw sample; 25 mL 85% phosphoric acid; 7-8 mL toluene and an egg shaped stir bar.

3. Assemble the extraction apparatus (Figure 1), and carry out reflux with stirring. Start timing when drops begin to fall from the condensor. Reflux 2 hours.

4. After reflux, allow the apparatus to cool for about 20 minutes. Remove the condensor from the extractor. Drain the water and collect the toluene layer. Rinse the extractor with a 2-mL portion of toluene and combine toluene fractions. Dilute to a final volume of 10.0 mL with toluene.

5. Determine the DCP concentration in the solution by directly analyzing with gas chromatography using a Hall electrolytic conductivity detector.

Analysis of 2,4-dichlorophenol (Cont)Chromatographic ConditionsGas Chromatograph

Instrument: Tracor model 550 gas chromatograph
with Hall electrolytic conductivity
detector, Model 700A
Column: 6 ft. X 2mm OV-11 (10% on 80/100 Gas
Chrom Q)
Carrier: 20 mL/min He
Column Temp: 130°C
Inlet Temp: 220°C
Outlet Temp: 275°C
Transfer Line: 270°C

Detector Parameters

Detector Temp: 870°C
Electrolyte: n-propanol (0.33 mL/min)
Hydrogen: 40 mL/min

Integrator Recorder Settings

Instrument:	Shimadzu model C-R1A		
SLOPE:	350	MIN AR:	10
ATTEN:	0	SPEED:	10
METHOD:	1041	WIDTH:	5

Quantitation of DCP

Quantitation was done by comparing the response of the sample and that of standards giving a response immediately above and below that of the sample. Solution concentration was then determined by interpolation. At least two values were used to determine a mean response for the samples and the DCP standards. The injection volume was 5 μ L.

The following is an example calculation used to convert the G.C. detector response to concentration of DCP. The extract is from straw from plants treated with 3.0 lb. 2,4-DB/A (RRL-3009).

<u>Sample</u>	<u>Response</u>		
	<u>Run #1</u>	<u>Run #2</u>	<u>Mean</u>
DCP Std (0.20 μ g/mL)	241 ^a	235	238
DCP Std (0.40 μ g/mL)	549 ^b	560	554
RRL-3009	559 ^c	529	544

$$\frac{[\text{response of unknown}] - [\text{response of } 0.20 \mu\text{g/mL std}]}{[\text{response of } 0.4 \mu\text{g/mL std}] - [\text{response of } 0.2 \mu\text{g/mL std}]} = X - 0.2$$

$$\frac{544 - 238}{554 - 238} = \frac{X - 0.2}{0.4 - 0.2}$$

$$\frac{306}{316} = \frac{X - 0.2}{0.4 - 0.2}$$

$$\frac{(306)(0.2)}{316} + 0.2 = X \mu\text{g/mL } 2,4\text{-DCP}$$

$$0.3937 \mu\text{g/mL} = X$$

$$(0.3937 \mu\text{g/mL})(10 \text{ mL})(4/1 \text{ dilution}) = 15.75 \mu\text{g DCP}$$

$$(15.75 \mu\text{g DCP})/10 \text{ g sample} = 1.58 \mu\text{g/g (ppm)}^d$$

- a Datum appears in figure 11 A
 b Datum appears in figure 11 B
 c Datum appears in figure 15
 c Result appears in table 6

Analysis of 2,4-dichlorophenol (Cont)

Notes on Results

1. Standard curves were prepared each day chromatographic analysis was done. The detector response varied day to day.

2. The control straw contained detectable levels of DCP. The quantitation limit for straw was about five times the endogenous level of DCP and was 0.20 ppm. The quantitation limit for seed was the lowest spike level for reproducible recovery and was 0.050 ppm.

Literature Cited

Kan, G.Y.P., F.T.S. Mah, N.L. Wade and M.L. Bothwell. 1981. Determination of 2,4-D butoxyethyl ester and its degradation products 2,4-D and 2,4-dichlorophenol in sediment. J. Assoc. Offic. Anal. Chem. 64:1305-8

Addendum 1Moisture Determination of Barley Straw Samples and Correction Factors

Three 2.00 g subsamples of each straw sample were weighed and dried in a forced air oven for 1 hour at 90°C. The samples were cooled and reweighed. The moisture level was determined and a correction applied to the damp sample when extracting.

The above drying conditions were determined to be sufficient to obtain a dry ground straw sample. Drying times longer than 60 minutes did not reduce the sample weight further.

The following table contains the resulting dry weights obtained for each of the triplicate samples.

Sample (No.)	Dry weights from 2.0 g subsamples (grams)			Moisture (%)	Correction factor
3003	1.402	1.405	1.408	29.75	1.42
3004	1.542	1.545	1.546	22.8	1.30
3005	1.672	1.63	1.627	18.6	1.23
3006	1.409	1.421	1.397	29.55	1.42
3007	1.454	1.458	1.457	27.2	1.37
3008	1.546	1.546	1.549	22.65	1.29
3009	1.39	1.396	1.398	30.25	1.43
3010	1.612	1.614	1.616	19.3	1.24
3011	1.534	1.516	1.526	23.75	1.31

Sample Calculation

The following is a sample calculation for the moisture correction for straw sample RRL-3003.

2.0 g damp sample - 0.598 g moisture loss = 1.402 g dry straw

mean of three triplicate measurements = 1.405 g

$(2.0 \text{ g wet straw}) / (1.405 \text{ g dry straw}) = 1.42$

$(1.42)(10.0 \text{ g dry straw desired}) = 14.2 \text{ g wet straw needed to equal } 10.0 \text{ g dry straw}$