Determination of 2,4-D Residues in Animal Tissues

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A gas chromatographic method for determining 2,4-D (2,4-dichlorophenoxyacetic acid) in the body tissues of sheep is described. After extraction and cleanup the herbicide is hydrolyzed to 2,4-dichlorophenol and further cleanup is accomplished with steam distillation. Average recoveries of 82% were obtained from tissues spiked with known amounts of herbicide. The method will detect residues as small as 0.05 p.p.m.

For a number of years, 2,4-D (2,4-dichlorophenoxyacetic acid) and its salts and esters have been used widely for the control of many undesirable plants, including some which may be ingested by livestock in hay and silage, or while grazing. Previous work has shown that 2,4-D is excreted quantitatively by the sheep (*3*) and steer (*5*) following a single oral dose. The question of residues, which may be deposited in the body tissues following repeated oral doses, remains unanswered. Several analytical procedures have been published for 2,4-D residues in crops (*1*, *4*, *6*, *7*), milk (*2*), and animal excreta (*3*, *5*) but none have been published for animal tissues. This paper presents a procedure whereby 2,4-D residues in animal tissues may be determined.

Materials and Methods

Reagents. Phosphate buffer. 6.044 grams of KH_2PO_4 + 14.06 grams of Na_2HPO_4 per liter of redistilled water; pH 6.5.

Papain N. F., S. B. Penick & Co., New York, N. Y.

Solvents. Ethanol, petroleum ether, chloroform, diethyl ether, and petroleum ether–chloroform (3 to 1) washing solvent. Redistill before use (except ethanol).

Pyridine hydrochloride, K & K Laboratories. Wash with diethyl ether until crystals are white, then allow to stand in open air for 1 hour.

2,4-Dichlorophenoxyacetic acid, Dow Chemical Co., recrystallized from 95% ethanol.

2,4-Dichlorophenol, Eastman Chemical Co., purified by sublimation.

Fisher high temperature bath wax.

Apparatus. Waring Blendor, 500-ml. capacity with screw-on lids.

Water bath, Chicago Surgical and Electric Co., Catalog No. 13100 or comparable.

Soxhlet extractors, 500-ml. capacity.

High temperature oil bath, capable of maintaining 208° ± 2° C.

Steam distilling apparatus, Scientific Glass Apparatus Co., JM-4250

Gas chromatograph with electron-capture detector, Micro-Tek 2500R or equivalent.

Gas Chromatography. Prepare the analytical column as follows: Weigh 9.9 grams of Chromport XXX (60- to 80-mesh) into a 250-ml. beaker containing 0.1 gram of HI-EFF (HI-EFF-1a liquid phase—diethylene glycol adipate polyester, commercially available from Applied Science Laboratories, Inc., State College, Pa.) plus 0.1 ml. of 85% H_3PO_4 dissolved in 75 ml. of diethyl ether. Stir gently for about 5 minutes, then pour the contents of the beaker into a large flat dish and allow the solvent to evaporate with warm air and gentle stirring. Dry overnight in oven at about 180° C.

Fill a 4-mm. i.d. × 3-foot stainless steel column with the coated support, using vibration to settle. Plug both ends with glass wool. Condition the column for 48 hours at 200° C. with a stream of nitrogen (15 cc. per minute). (At an operating temperature of 175° C., the column showed no signs of deterioration after several weeks of constant use.)

For determination of 2,4-dichlorophenol, use the previously described column and, as carrier gas, prepurified grade nitrogen (The Matheson Co., LaPorte, Tex.) at 25 p.s.i. with column flow rate of 85 cc. per minute and detector scavenger flow of 15 cc. per minute. Use operating temperatures for the injector, column, outlet, and detector of 285°, 175°, 150°, and 150° C., respectively. Set the detector at 20 volts d.c. and the electrometer attenuation at 200X or 400X as required by the sample.

Prepare a series of standard solutions of 2,4-dichlorophenol in petroleum ether ranging in concentration from 0.02 to 0.10 μg. per ml. Inject 5-μl. portions into the gas chromatograph and compare the peaks with those resulting from tissue extracts. Peaks heights are directly proportional to 2,4-dichlorophenol concentration if the volume of solvent per injection is constant (Figure 1).

Sample Preparation. Weigh 25 grams of tissue into a Waring Blendor, add 100 ml. of boiling 95% ethanol, homogenize for 2 minutes, then vacuum-filter through Whatman No. 1 filter paper. Return the residue to the Blendor and repeat the ethanol homogenization twice more. Following the third filtration, transfer the residue into a Soxhlet thimble, add the ethanol extract to a 500-ml. round-bottomed boiling flask, and join it to the extractor. Allow the Soxhlet extraction to

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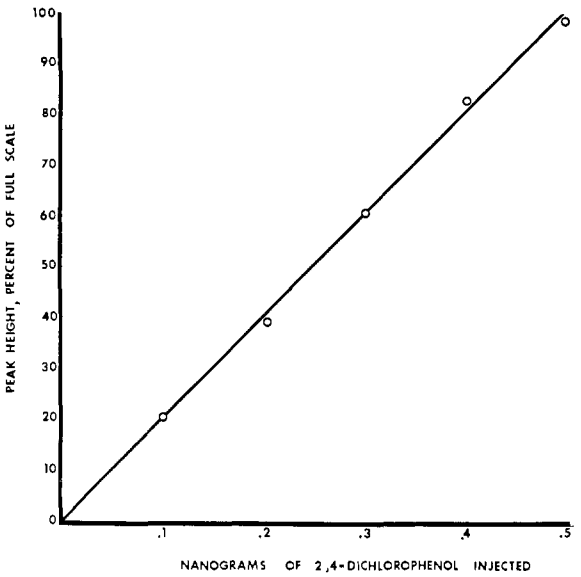

Figure 1. Standard concentration curve of 2,4-dichlorophenol by electron-capture gas chromatography at attenuation of $10^2 \times 2$

proceed for 1 hour with sufficient heat so that about eight flushings occur. Discard the thimble and its contents and evaporate the ethanol extract to dryness over a water bath with an air stream. For fat samples, chill the alcohol extract in a dry ice–ethanol bath, filter, and discard the solidified fat prior to evaporation.

Dissolve the dry residue in 100 ml. of the phosphate buffer (pH 6.5), add 1 gram of papain (0.5 gram for fat samples) and a few grains of NaCN, and digest at 37° C. overnight (16 hours). Remove from the water bath, add 11 grams of NaOH, and allow the samples to stand for about 30 minutes until a curd-like layer forms. Transfer the solution into a 500-ml. separatory funnel and wash it with two 50-ml. portions of petroleum ether, then with two 100-ml. portions of petroleum ether–chloroform (3 to 1), followed by four 50-ml. petroleum ether washings. Extract the combined washings with 25 ml. of 10% NaOH, then discard the organic layer. Acidify the aqueous phase to pH 1 with concentrated HCl (*Caution: HCN fumes*) slowly and allow to cool. Extract the 2,4-D from the acidified solution with five 50-ml. portions of diethyl ether. Dry the ether by passing it through a Na_2SO_4 column into a 500-ml. Erlenmeyer flask. Evaporate the ether down to about 5 cc. over a water bath with a gentle stream of air. Transfer the ether concentrate to a 50-ml. Erlenmeyer flask with a ground-glass fitting. Wash the 500-ml. Erlenmeyer flask with several small portions of diethyl ether and evaporate the entire ether extract to dryness. Add 5 grams of ether-washed pyridine hydrochloride, attach a Snyder column to the flask, and place in an oil bath at 208° ± 2° C. for 1 hour to hydrolyze the 2,4-D to 2,4-dichlorophenol. (The hydrolysis was 98% efficient.) Remove the flask from the oil bath and allow it to cool. Dissolve the contents of the flask with 6.5 ml. of 20% NaOH and transfer the solution to the steam distillation apparatus.

Wash the Synder column, the flask, and the mouth of the still with a total of 14 ml. of distilled water. Add 5 ml. of 12*N* H_2SO_4 and steam-distill the 2,4-dichlorophenol into a 250-ml. separatory funnel containing 10 ml. of 5% NaOH at a rate of about 5 ml. per minute. After a total of 60 ml. has distilled, remove the separatory funnel and discard the solution remaining in the still. Wash the distillate twice with 25-ml. portions of petroleum ether. Acidify the aqueous phase and extract the 2,4-dichlorophenol three times with 50-ml. portions of petroleum ether. Concentrate the extract to 1 ml. over warm water with a very gentle air stream. (2,4-Dichlorophenol is very volatile and care must be taken to avoid loss from overheating during concentration.) Evaporate the last 2 ml. with a gentle air stream and no heat. Inject 5 μl. of the concentrate into the gas chromatograph for analysis.

Results and Discussion

Several solvents, including benzene, petrolem ether, acetone, diethyl ether, sulfuric acid–benzene, chloroform, carbon tetrachloride, 0.05*N* NH_4OH, and 95% ethanol were tested for extraction efficiency. Tissue was spiked with a known amount of 2,4-D-^{14}C and the recovery determined radiometrically after homogenation with three 100-ml. portions of the various solvents. Extraction with hot 95% ethanol proved to be, by far, the most efficient of the solvent systems tested with consistent recoveries of above 96%.

Following the initial extraction, enzymatic hydrolysis of the extracted solids was necessary to release the 2,4-D, which is apparently bound to tissue protein, before further cleanup steps could be carried out quantitatively. Burchfield and Storrs (*2*) discovered that protein hydrolysis with pepsin was necessary when extracting 2,4-D from milk.

Several gas chromatographic columns were tested before one was found which would give a sharp peak, the required sensitivity, and adequate separation from tissue impurities. Chromport XXX, 60- to 80-mesh,

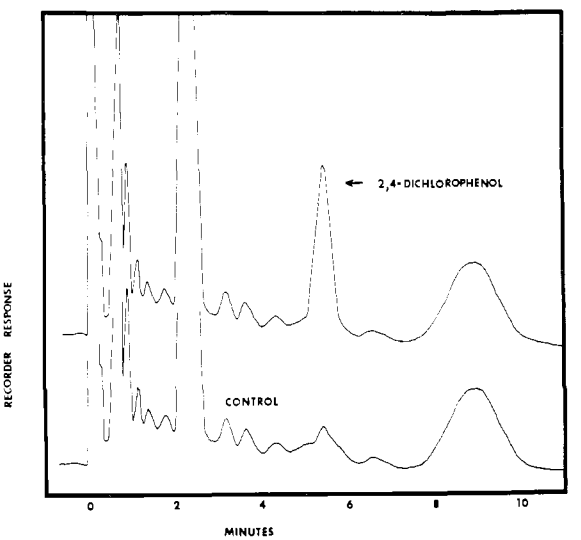

Figure 2. Gas chromatogram of extract of sheep muscle to which 2,4-D was added and control

Table I. Recovery of 2,4-D Residues from Tissues

Tissue	Per Cent Recovery			
	0.05 p.p.m.	0.1 p.p.m.	0.5 p.p.m.	1 p.p.m.
Muscle	84	80[a]	80	89
Liver	91	77[b]	86	81
Kidney	68	77[b]	90	82
Renal fat	92	91[b]	81	78
Body fat	90	84[b]	86	92

[a] Average of two samples.
[b] Average of three samples.

Table II. Value of Interference Peak in Various Tissue from Sheep Not Exposed to 2,4-D

Tissue	Apparent 2,4-Dichlorophenol, P.P.M.		
Muscle	0.015,	0.015,	0.012
Liver	0.015,	0.012,	0.010
Kidney	0.070,	0.005,	0.012
Renal fat	0.015,	0.007,	0.003
Body fat	0.005,	0.006,	0.004

coated with 5% SE-30 gave some separation at 120° C. but the 2,4-dichlorophenol peak appeared on the side of a very large interference peak in some tissues and the sensitivity was thus decreased. Apiezon L (10 and 20%) was tried but there was considerable tailing, and at the required temperature, the retention time was too short for good resolution from tissue impurities. Chromport XXX coated with 1% HI-EFF showed considerable tailing and very poor recovery of dichlorophenol from the column. However, with the addition of H_3PO_4, the peak geometry improved sufficiently to allow peak heights to be used as a quantitative measure and sensitivity was greatly increased and stabilized. Dichlorophenol peaked at approximately 5.4 minutes; however, approximately 40 minutes are required between injections of tissue extracts to allow the recorder to return to the base line (Figure 2).

The efficiency of the over-all procedure was determined by comparing the peak heights of standards with those from spiked samples (Table I). Unspiked samples were processed identically and were used as controls. An impurity which had identical retention times as 2,4-dichlorophenol with both SE-30 and HI-EFF/H_3PO_4 was present in all control samples (Table II). Of the tissues analyzed, kidney samples had the greatest concentration of the impurity and fat samples had the least. Recoveries were determined by comparing the difference between the spiked sample and the control with the standard.

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