The Determination of the Diallyl Derivatives of Diethylstilbestrol and Hexestrol in Feeds and Other Materials

J. B. HIMES[1] and L. D. METCALFE
Armour Industrial Chemical Co., McCook, Ill.
ROBERT HUBATA
Food Research Division, Armour & Co., Chicago, Ill.

A method of determining quantitatively the amounts of hexestrol, diethylstilbestrol, and their diallyl derivatives in animal feeds and other materials by solvent extraction and paper chromatography is described. This method provides an accurate means of analyzing animal feeds for these compounds even in the presence of phenolic compounds.

THE USE of 3,3′-diallyl- α,α′-diethyl-4.4′-stilbenediol (diallyl diethylstilbestrol, DAS) and 4,4′-[1,2-diethylethylene] bis(3-allylphenol) (diallylhexestrol, DAH) as animal feed additives has been reported (*5, 8–10*). As with all animal feed additives, analytical methods are necessary for proper control of the additive in the feed. It is also desirable to be able to determine the presence of the additive in animal tissues and other animal products (e.g., eggs, milk).

A number of analytical methods have been developed for α,α′-diethyl-4,4′-stilbenediol (diethylstilbestrol, DES) and 4,4′ - [1,2 - diethylethylene] - diphenol (hexestrol, HEX) in solutions and animal feeds (*1–4, 6, 9*). These are essentially extraction of the additive with a solvent, recovery and concentration of the additive from the extract, and finally colorimetric determination. Colors are developed with DES and HEX by nitrosation, reaction with vanadyl chloride, antimony pentachloride, and in the case of DES, exposure to ultraviolet light under acid conditions.

When DAS, DAH, DES, and HEX were extracted from plant materials, the naturally occurring plant phenols were carried through the analysis and interfered with the colorimetric methods. It was also desirable to separate DAS and DAH from DES and HEX in addition to separating them from interferences. A reverse-phase paper chromatographic method was found that would separate DAS and DAH from DES and HEX and also from the natural phenolic material occurring in plants.

The method first employs a continuous solvent extraction (*7*) of the sample. DAS and DAH are then separated from the solvent extract by washing with aqueous alkali. The aqueous solution is adjusted to pH 9 and then washed with chloroform to remove the DAS or DAH. The chloroform is evaporated to dryness, and the residue is dissolved in methanol. All or part of the methanol solution is spotted on a soybean oil–impregnated paper. The paper chromatogram is developed with 30% pyridine in water. The spots are developed by Folin's phenol reagent. Quantitative results are obtained by reading the density of the spots with a densitometer. DAS and DAH have approximately the same R_f values and are widely separated from DES and HEX. A spot containing less than 0.5 μg. of DAS or DAH could be detected.

[1] Present address, The Richardson Co., 2737 Lake St., Melrose Park, Ill.

Sample Preparation

Extraction. Samples of feeds are first ground to approximately 40-mesh. Milk, eggs, tissues, and liquids are weighed and then lyophilized and, if necessary, powdered. Animal feces are dried in a vacuum oven at 60° C. and then powdered. Usually 20-gram samples of feeds are weighed for extraction. Fifty-gram samples of the other materials mentioned are weighed, lyophilized, and the entire dried sample is extracted.

The weighed sample is mixed with 2 grams of fine asbestos fiber and placed in a folded filter paper (E & D No. 513). The paper is placed in a Butt funnel, and the contents are subjected to continuous extraction for 4 hours with a mixture of 7% ethanol in chloroform. The extract is then transferred to a 100-ml. volumetric flask—filtering, if necessary, through glass wool. Dilution to the mark is accomplished with additional extraction mixture.

Separation. A 25-ml. aliquot is transferred to a 150-ml. separatory funnel for isolation of DAS or DAH. One volume of extraction solvent mixture and 25 ml. of 1*N* aqueous sulfuric acid is added to the 25-ml. aliquot. The solvent fraction is washed 10 times using a rotary motion. The chloroform layer is transferred to another separatory funnel. Ten milliliters of chloroform is added to the acidic aqueous fraction and agitated with a rotary motion, as before. The chloroform fractions are combined; the aqueous layer is discarded. Ten milliliters of 1*N* aqueous sodium hydroxide is added and shaken with a rotary motion 10 times or longer if a persistent emulsion can be avoided. The chloroform fraction is transferred to another separatory funnel, and the extraction is repeated with sodium hydroxide three times. (With each succeeding extraction with the alkali, the number of rotary agitations can usually be increased without causing a persistent emulsion.) The solvent fraction is discarded, and the combined alkaline extracts are washed with 10-ml. portions of chloroform until the solvent washings are colorless. The chloroform is completely drained from the alkaline fraction. The alkaline fraction is transferred to a 100-ml. beaker, and the separatory funnel rinsed with 10 ml. of water. Phosphoric acid (2*N*) is added slowly with stirring until the pH at room temperature is 9.0 ± 0.1 (a pH meter is used). The alkaline fraction is returned to a clean separatory funnel, and the beaker rinsed with 2-ml. portions of water. Then the beaker is rinsed with 30 ml. of chloroform which is added to the alkaline fraction in the separatory funnel. The separatory funnel is shaken vigorously for 60 seconds, and the chloroform layer is drawn off into a clean separatory funnel. Extraction of the alkaline fraction is repeated twice with 30-ml. portions of chloroform. The alkaline fraction is discarded, and the combined chloroform extracts are washed with 50 ml. of water. The solvent layer is filtered into a beaker through a fritted-glass Gooch crucible of medium porosity containing a 1-inch layer of anhydrous sodium sulfate. The aqueous layer is extracted with two 10-ml. portions of chloroform, and these are used to rinse the crucible. The solvent is removed with a stream of filtered air and with enough heat from a hot plate to prevent condensation of water on the sides of the beaker. The beaker is rinsed with anhydrous methanol, and the rinsings are transferred to a 5-ml. centrifuge tube. The contents of the tube are concentrated to dryness in a

tream of filtered air and by holding the tube in a bath at 60° C. or cooler. The sample in the centrifuge tube is then diluted to the volume required for analysis.

Chromatography

Apparatus. CHROMATOGRAPHIC CHAMBER. A stainless steel pan, 9 × 13 × 2 inches, with a 1/4-inch flange is used as a tank for developing the chromatograms. A removable stainless steel rack (11 3/4 × 8 1/2 × 1 1/2 inches) is set inside to support a glass trough (1 1/2 × 8 inches) and six glass rods, parallel to the solvent trough, spaced 1 1/2 inches apart. The pan is covered with plate glass (10 × 14 × 1/4 inches) which has two 3/8-inch holes, each 1 1/2 inches from an end. Tygon sheeting (1/16 inch thick, 1 inch wide) is glued on the glass cover for a seal between pan flange and glass. (This chamber may be obtained from Labline, Inc., Chicago).

DENSITOMETER, Photovolt Model 525 and Varicord Variable Response Recorder.

Reagents. STANDARD SOLUTIONS OF HEX, DAH, DES, DAS. Prepare individual solutions of each by dissolving 2.0 mg. in 1.0 ml. of anhydrous methanol. One and 2-λ volumes of each solution are applied to each of two papers for chromatography.

PHENOL REAGENT OF FOLIN AND CIOCALTEAU. Prepare by adding 100 grams of sodium tungstate, 25 grams of sodium molybdate, 700 ml. of H_2O, 50 ml. of 85% H_3PO_4, and 100 ml. of concd. HCl to a 1500-ml. flask and refluxing gently for 10 hours. Then, add 150 grams of lithium sulfate, 50 ml. of H_2O, and a few drops of bromine water. Boil without a condenser for 15 minutes, then cool and dilute to 2 liters. Use one volume of this stock solution to two volumes of H_2O for dipping the chromatogram.

Paper Preparation. The paper is pretreated for chromatography before the samples are applied. Whatman No. 1 filter paper, 8 × 11 1/2 inches, is dipped in a 3% (v./v.) mixture of refined soyabean oil in anhydrous ethyl ether. The paper is dried in air overnight before being used for chromatography.

Chromatographic Procedure. The dried, concentrated extract is dissolved in sufficient anhydrous methanol so that the solution contains 1 to 5 mg. of DAS or DAH per ml. Sample volumes of 1 and 2 λ of the methanol solution are placed on the impregnated paper by means of a micro pipette so that the spots are 5/8-inch apart and 2 inches up from one of the 8-inch edges of the paper. Apply 1.0 and 2.0 λ of the appropriate known solution beside the unknown keeping the spots 5/8-inch apart. In this manner, three unknown samples and three known samples can be run on each chromatographic paper. To run duplicate determinations, the sample is spotted on two papers with the appropriate known solution. A 1 5/8-inch tab is folded at a right angle to the paper, 3/8 inch below the line of sample spots. The paper is placed in a chromatographic chamber containing 25 ml. of 30% pyridine in H_2O in the chamber bottom, so that the paper lies horizontally over the supporting glass rods with the folded-end tab extending down into the glass trough resting on the bottom of the chamber. Thirty milliliters of 30% pyridine in H_2O is added to the trough and a plate glass cover placed over the chamber. The chromatogram is allowed to develop at room temperature (24° C.) for 2 hours. The paper is then removed and hung in an exhaust hood to dry. The paper is dipped in Folin's phenol reagent, allowed to air dry at room temperature, redipped in 10% Na_2CO_3, air dried at room temperature, and then heated at 60° C. for 20 minutes.

Estimation of the Diphenylhexane Additives. The density of the DAH, HEX, DAS, or DES spots is read on a densitometer-recorder apparatus. The chromatogram is cut into strips and the spots on the strips are scanned in the densitometer. The concentration of HEX, DES, or the diallyl derivatives is calculated from the concentration of the known standard solution spotted on the paper, the area under the absorbance curve for the spot of the unknown sample, and the area under the absorbance curve for the spot of the known standard. Therefore,

$$\text{p.p.m. additive} = \frac{W_s}{A_s} \times \frac{A_u}{W_u}$$

where W_s = weight in μg. of known standard applied to paper
A_s = area under absorbance curve of standard
A_u = area under absorbance curve of unknown sample
W_u = weight in grams of unknown sample applied to paper

For duplicate determinations of feeds, samples are chromatographed along with the standards on the different papers; the results are calculated from each paper and then averaged.

Results and Discussion

In determining diallyl derivatives of HEX and DES in animal feeds, other phenol compounds present in the feeds will interfere with colorimetric analysis, ultraviolet absorption, and paper chromatography. When 30% pyridine in H_2O was used, the interfering compounds followed the front leaving the desired compounds behind. Table I lists the R_f values (average of three chromatograms of HEX, DES, DAS, and DAH.)

Table I. R_f Values

Hexestrol	0.54
Diallylhexestrol	0.13
Diethylstilbestrol	0.57
Diallyldiethylstilbestrol	0.15

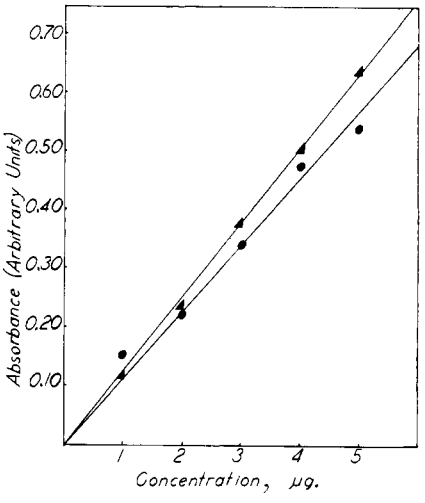

Figure 1. Concentration of DAS (▲) and DAH (●) versus absorbance

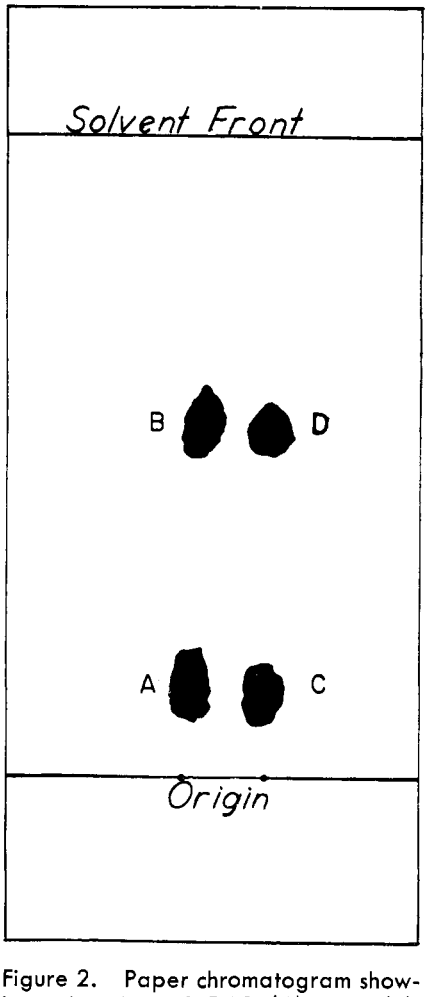

Figure 2. Paper chromatogram showing migration of DAS (A), DES (B), DAH (C), HEX (D)

Table II. Recovery of DAS and DAH from Feeds

Added (Mg./Lb.)	Recovered	Detns.
	Alfalfa	
2.5 (DAS)	2.1 ± 0.2	3
1.5 (DAH)	1.7 ± 0.2	2
2.5 (DAH)	2.5 ± 0.1	2
	Corn	
2.5 (DAS)	1.9 ± 0.1	2
40 (DAS)	40 ± 3.0	2
2.5 (DAH)	2.1 ± 0.1	2
	Soybean	
200 (DAS)	200 ± 4.0	9

Table III. Recovery of DAH and DAS in Milk and Eggs

Added, Mg.	Recovered	Detns.
	Milk, 50 ml.	
0.05 (DAS)	0.04 ± 0.01	4
0.25 (DAS)	0.25 ± 0.02	3
0.22 (DAH)	0.25 ± 0.02	3
	Eggs, 50 gm.	
0.35 (DAH)	0.35 ± 0.03	2
0.25 (DAH)	0.22 ± 0.02	2
0.35 (DAS)	0.33 ± 0.03	2

Samples of feed meal were prepared containing known amounts of DAS or DAH, and analyzed by extraction and paper chromatography. The results are tabulated in Table II.

In a further investigation, known amounts of DAS and DAH were added to known quantities of milk and eggs. Analyses of these products are listed in Table III.

Figure 1 shows curves for the concentrations of DAS and DAH on paper vs. the absorbance of the blue spots produced by the reaction of Folin's reagent with these compounds.

Figure 2 illustrates a paper chromatogram showing the migration of HEX, DAH, DES, and DAS.

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FEED ADDITIVES

Tissue Residues and Excretory Pathways of Orally Administered 2-C^{14}-Methimazole

NED S. RAUN[1] and WISE BURROUGHS

Iowa Agricultural Experiment Station, Iowa State University, Ames, Iowa

Methimazole residues were not detected (sensitivity 0.1 to 0.2 p.p.m.) in rat tissues 96 hours after daily administration of the isotopically labeled compound for periods ranging from 1 to 14 days. Excretory pathways appeared to be exclusively through the urine and feces. Approximately 80% of an oral dose was excreted in the urine within 24 hours, with the remaining 20% being excreted in the feces within 48 hours after administration. Total recovery ranged from 98 to 105%. No isotope activity was noted in expired carbon dioxide.

Methimazole (1-methyl-2-thiol imidazole) is a potent goitrogenic compound which, when properly fed to beef cattle, stimulates liveweight gains (*2*).

Chick bioassays employing a thyroidal iodine-131 uptake technique demonstrated the absence of methimazole residues in beef tissues of cattle previously fed this goitrogen (*5*). Ely *et al.* (*3*) working with a different goitrogen (thiouracil) found it to be both rapidly absorbed into the blood stream and essentially eliminated from the circulation of ruminants within a 24-hour period. Similarly in nonruminants, Paschkis *et al.* (*4*), Williams (*8*), and Williams and Chute (*9*) found thiouracil to be both rapidly absorbed from the gastrointestinal tract and rapidly excreted in the urine. The purpose of the present investigation was to characterize excretory patterns of orally administered 2-C^{14}-methimazole in the rat, and to study tissue residues following administration of this isotopically labeled goitrogen.

Experimental Procedure

Three trials were conducted with female rats to determine the excretory pathways of orally administered 2-C^{14}-methimazole. The isotope was administered via stomach tube at the rate of 0.5 mg. per pound of body weight (total dose approximately 1 μc.). After dosage, each rat was placed in a glass metabolism cage where feces, urine, and expired carbon dioxide were collected as outlined by Roth (*6*). Feces and urine samples were subjected to wet oxidation using a combination of a solid and liquid oxidant and an oxidation apparatus outlined by Aronoff (*1*). Feces or urine samples containing approximately 15 mg. of carbon were placed in the oxidation vessel to which 2 grams of solid reagent mixture of potassium iodate and potassium dichromate (10 to 1 ground together) and 10 ml. of a liquid reagent composed of 50 ml. of concentrated sulfuric acid, 50 ml. of sirupy phosphoric acid, and 1.5 grams of potassium iodate were added. The carbon dioxide produced was absorbed in a gas washer containing sodium hydroxide.

[1] Present address: Rockefeller Foundation, Mexico City, Mexico.