Method for Determining Residues of Mestranol and Ethynylestradiol in Foliage, Soil, and Water Samples

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INTRODUCTION

Mestranol (17-α-ethynyl-3-methoxy-estra-1,3,5,(10)-trien-17-β-ol), a synthetic estrogen often tested as a chemosterilant for animal damage control, has also shown promise as a repellent for the protection of Douglas-fir (Pseudotsuga taxifolia) seeds from deer mice (Peromyscus maniculatus). Laboratory tests conducted by the U.S. Fish and Wildlife Service and others (CROUCH AND RADWAN 1971) have shown that deer mice develop an aversion to seeds treated with the chemical; however, because of its strong estrogenic effects on both animals and birds, mestranol baits may be a potential hazard to wildlife. To assess the environmental contamination resulting from application of mestranol-treated seeds, and to support its registration as a control agent, an analytical procedure was needed for measuring residue levels of mestranol. Although several assay procedures are available for quantitatively measuring mestranol in formulations, they are not applicable for determining low levels of the compound in environmental samples. We have developed an analytical procedure for determining residue levels of mestranol and also its 3-hydroxy homologue, ethynylestradiol (EED), in plant, soil, and water samples. The lower limits of detection, based on recovery studies, were 0.05 ppm for foliage samples, 0.1 ppm for soils, and 0.01 for water samples.

SUMMARY OF PROCEDURE

Samples are extracted in an acidic medium to free any conjugated EED then cleaned up by Florisil column chromatography. Water samples usually can be analyzed by gas-liquid chromatography (GLC) at this point. Further cleanup of vegetation and soil samples and separation of mestranol from EED are made on a gel permeation chromatographic column. The EED fraction from this gel separation is clean enough to analyze by GLC. The mestranol fraction is cleaned up further by a second gel separation and one or more additional Florisil separations. After GLC, the samples are analyzed by thin-layer chromatography (TLC) for qualitative and semi-quantitative confirmation.

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EXPERIMENTAL

Materials and Equipment

Reagents.--Reference standards of mestranol and EED were supplied by Syntex Corporation*. All organic solvents used were Burdick and Jackson "distilled in glass" grade. Carbon tetrachloride was redistilled before use, and the chloroform contained commercially added 1% ethanol as a preservative.

Kuderna-Danish (KD) evaporative concentrator.--The K-D unit (Kontes Glass Company) consists of a graduated 10-ml concentrator tube, a 250-ml flask, and a 3-ball Snyder column.

Florisil columns.--Florisil (Floridin Company), 60-100 mesh, is heated at 130° C for 24 hours, then deactivated with 2-3% water and allowed to stand for at least 24 hours. To check the activity, add 0.25 g of the Florisil to 0.5 ml of a chloroform solution containing 100 ppm mestranol and compare the concentration of mestranol in the liquid phase with that in the original solution by GLC analysis. At optimum activity, the ratio of the two concentrations (with Florisil/original) should be 0.30 ± 0.02.

For a 10-g column, add 10 g of Florisil to a 400 X 22 mm ID chromatographic column. Tap the column lightly so that the Florisil packs uniformly, and add 10 g of anhydrous sodium sulfate over the Florisil. For a 5-g column, similarly pack 5 g of Florisil into a 200 X 10 mm ID glass column with a 125-ml reservoir, and add 10 g of anhydrous sodium sulfate.

Gel permeation chromatographic column.--The gel column apparatus consists of a 250 X 14 mm ID glass column with a 250-ml reservoir at top and a glass wool plug and Teflon stopcock at bottom. The top of the reservoir is fitted with an 18/9 ball joint to facilitate connection to an air pressure source. The gel (Biobeads S-X2 Gel, 200-400 mesh; Bio-Rad Laboratories) is swelled overnight in carbon tetrachloride, then poured as a slurry through a funnel into the column and allowed to pack by gravity until the length of the gel bed is 23 cm. Solvent should always be maintained in the column bed. A glass wool plug, approximately 1 cm in length, is placed on top of the gel. At least 50 ml of solvent should be pumped through the column until the carbon tetrachloride does not contain any substance that will interfere in the gas chromatographic analysis when evaporated from 10 to 0.1 ml. A constant flow rate between 2-3 ml per minute is maintained by applying air pressure to the solvent head by means of an air pump or gas cylinder. The column should be flushed with at least 20 ml of solvent between runs.

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* Reference to trade names does not imply endorsement of commercial products by the U.S. Government.

Gas chromatograph.--We used a Beckman GC-5 gas chromatograph, equipped with a Model 567100 Electrometer, flame ionization detector, and a 4-foot X 1/4-inch OD aluminum column packed with 3% OV-17 on 80/100 mesh Gas Chrom Q. The electrometer was operated at a sensitivity of 4 X 10^{-11} A/mV. Flow-rates were about 60 cc/min for hydrogen, 300 cc/min for air, and 60 cc/min for the nitrogen carrier gas. Operating temperatures were 260° C for the column, 275° C for the inlet, and 290° C for the detector.

Sample Preparation

Vegetation samples.--Weigh 10 g of air-dried and homogeneously ground sample into a glass-stopper 300-ml flask and shake for 30 minutes with 100 ml of acetonitrile containing 5 ml of 1.2 N HCl. Decant and filter the extract through a #1 Whatman filter paper into another 300-ml flask. Repeat the extraction twice with 50 ml each of acetonitrile. Add a boiling stone (Teflon/Halon; Chemware) to the flask, connect a 3-ball Snyder column to the flask and a water-aspirator to the column, and evaporate under vacuum just to dryness on a steam bath.

Soil samples.--Weigh 10 g of air-dried and pulverized soil sample into a glass stoppered 300-ml flask and shake for 30 minutes with 50 ml each of 1.2 N HCl and acetone. Decant and filter through a #1 Whatman filter paper into another 300-ml flask. Rinse twice with 50 ml each of acetone. Connect a 3-ball Snyder column to the flask and place it on a steam bath to evaporate off the acetone. After cooling, add 10 ml of ethyl ether and 50 ml of chloroform, and shake for 2 minutes. Transfer the contents and two 50-ml of chloroform rinses to a 250-ml separatory funnel and shake for 5 minutes. Draw off the lower organic layer and pass it slowly through 10 g of granular anhydrous sodium sulfate into a 300-ml flask, then rinse the sodium sulfate with a 10 ml of chloroform and add the rinse to the sample. Add a Teflon boiling stone to the flask, attach a 3-ball Snyder column to the flask, and evaporate just to dryness on a steam bath.

Water samples.--Shake 200 ml of water sample with 10 ml of 1.2 N HCl, 10 ml of ethyl ether, and 50 ml of chloroform in a 500-ml separatory funnel for 10 minutes. Pass the lower layer through 10 g of sodium sulfate into a K-D unit and evaporate to about 5 ml on a steam bath. Place the 10-ml concentrator tube in a tube heater (60° C) and pass a gentle stream of air over the sample to concentrate it just to dryness.

Sample Cleanup and Analysis

10-g Florisil column.--Dissolve the sample extract from the vegetation or soil sample in 20 ml of chloroform, using an ultrasonic cleaner to facilitate solution. Add this and a 10-ml chloroform rinse to the dry chromatographic column and elute with an additional 110 ml of chloroform. Collect all the eluate in a K-D unit and evaporate it just to dryness, then proceed to the first 5-g Florisil column cleanup.

First 5-g Florisil column.--Dissolve the extract (from water samples) or the concentrate (from the 10-g Florisil cleanup of vegetation and soil samples) in 1 ml of chloroform and add this and a 1-ml chloroform rinse to the dry Florisil column, then elute with 68 ml of chloroform. Discard the first 10 ml of eluate and collect the remainder in a K-D unit and concentrate it to just dryness.

Proceed to the gel column for further cleanup of concentrates from vegetation and soil samples. Dissolve the concentrate from water samples in 100 µl of carbon tetrachloride and analyze for mestranol and EED by GLC. If the sample contains interfering material and further cleanup is needed, proceed to the gel column procedure.

Gel column.--Open the stopcock, apply air pressure to the reservoir and remove enough solvent until its surface is level with the top of the glass wool plug. Disconnect the air pressure line, close the stopcock and place a 10-ml K-D concentrator tube under the column. Dissolve the sample from the first 5-g Florisil cleanup in 1 ml of carbon tetrachloride, withdraw the sample solution with a 1-ml syringe and transfer to the gel column. Open the stopcock, apply air pressure and allow the sample to sink just to the top of the glass wool plug. Repeat the transfer with another 1 ml of carbon tetrachloride. Add 80 ml of carbon tetrachloride to the reservoir and collect six 10-ml fractions in K-D concentrator tubes. Fraction 4 contains mestranol, and fractions 5 and 6 contain EED. Concentrate fraction 4, and the composite of fractions 5 and 6, in K-D units (without Snyder columns) to about 3 ml on a steam bath, then evaporate to just dryness in the tube heater under a stream of air.

Fraction 4 from water samples, and the composite of fractions 5 and 6 from vegetation, soil, and water samples, are clean enough for GLC analysis. For fraction 4 from vegetation and soil samples, repeat the gel column cleanup and concentration procedure, retaining only fraction 4 for additional cleanup by Florisil chromatography.

Second 5-g Florisil column.--Add fraction 4 from the second gel cleanup to a 5-g Florisil column, elute with 30 ml of chloroform and collect only the second 10 ml of eluate. Evaporate this fraction to dryness and dissolve it in 100 µl of carbon tetrachloride for analysis by GLC. If early emerging materials interfere with the measurement of mestranol, keep repeating the second 5-g Florisil column cleanup step until they are removed.

GLC analysis.--Dissolve the sample extracts from the cleanup procedures in 100 µl of carbon tetrachloride so that the sample equivalence is 100 mg/µl for vegetation and soil samples and 2 ml/µl for water samples. The amount of sample injected for gas chromatographic analysis will depend on the concentration of mestranol or EED in the sample solution and the sensitivity desired. The retention time is about 9 minutes for mestranol and 11-1/2 minutes for EED (Fig. 3), but at least 40 minutes should be

allowed between injection of mestranol sample extracts so that late emerging substances will not interfere with subsequent injections. The EED fraction is relatively free of these late interfering substances.

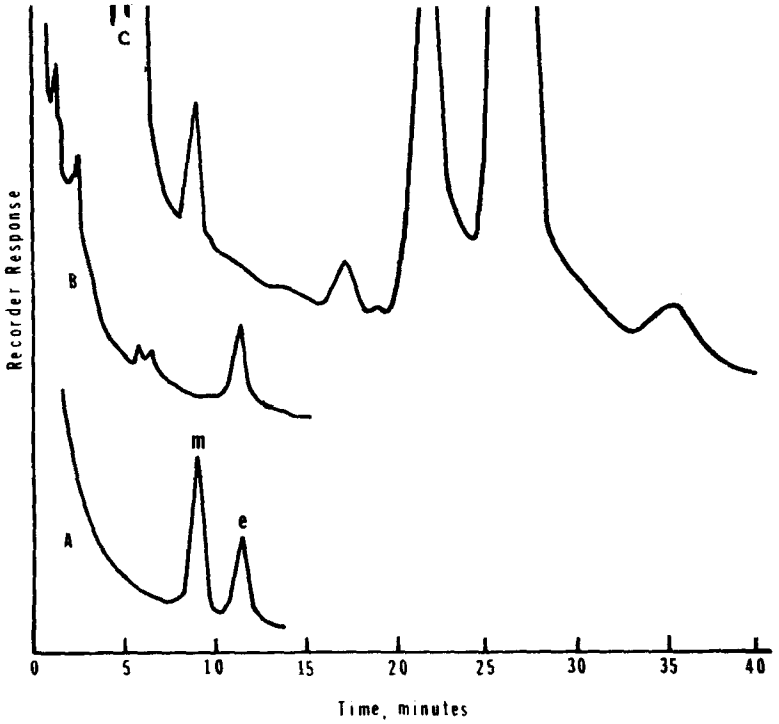

Fig. 1. Gas chromatographic analysis of extracts from cleanup of a grass foliage sample fortified with 0.1 ppm each of mestranol and EED:

A. Mixed reference, 2 µl containing 10 ppm each of mestranol (m) and EED (e).
B. Composite of fractions 5 and 6 from first gel cleanup, 4 µl (sample equivalence, 100 mg/µl).
C. Fraction 4 from second gel cleanup after two separations on second 5-g Florisil column, 4 µl (sample equivalence, 100 mg/µl).

TLC analysis.--Thin-layer chromatography is used for qualitative and semi-quantitative confirmation. Spot an appropriate amount of sample solution on a silica gel thin-layer sheet (Eastman Chromatogram, Type R 301R2) and develop with chloroform, allowing the solvent front to migrate about 10 cm. After drying, spray the TLC sheet with a 1:1 mixture of methanol and sulfuric acid and warm for 5 to 10 minutes at 60° C (APPLEGREN AND KARLSSON 1970). Mestranol (Rf = 0.75) and EED (Rf = 0.25) will appear as red spots under visible light and orange under ultraviolet light (254 nm). The lower limit of detection is about 100 ng for each compound.

RESULTS AND DISCUSSION

Recoveries for Cleanup Steps

The 10-g Florisil column is used initially to separate mestranol and EED from most of the co-extracted materials that are more polar. The first 5-g Florisil column is used to remove additional polar materials that were not removed in the 10-g column. Recoveries of both compounds added as standard solutions to the 10-g column were essentially quantitative (Table 1). On the 5-g column, mestranol and EED recoveries averaged 103% and 92%, respectively, in the eluate collected after the first 10 ml (Table 1).

TABLE 1.

Recoveries of Mestranol and EED Added as Standards to Florisil and Gel Permeation Chromatographic Columns.

Column	ml eluant	µg added	N	Compound	Eluate retained	% Recovery Mean	S.D.
Florisil							
10-g	140	10	6	Mestranol	All	100	3.0
				EED	All	100	3.6
1st 5-g	70	1	6	Mestranol	All but 1st 10 ml	103	2.5
				EED	All but 1st 10 ml	92	6.1
2nd 5-g	30	1	6	Mestranol	2nd 10 ml only	91	7.4
Gel	60	1	5	Mestranol	3rd 10 ml only	86	4.1
				EED	4th 10 ml only	77	5.8

On the second 5-g Florisil column, used as an additional cleanup step for mestranol only, recovery averaged only 91% because only the second 10-ml fraction is retained (Table 1). About 9% is sacrificed to exclude interfering substances, including chlorophyll and xanthophyll from plant samples, that appear after 20 ml of chloroform have eluted. For additional cleanup, repeated separations with the 5-g Florisil column were more effective than a single cleanup on a larger column.

Gel permeation chromatography is used to remove extractables of high molecular weight that are not easily removed on the Florisil column. The utility of gel permeation cleanup for residue analysis of biological samples has been described by STALLING et al. (1972). Substances of high molecular weight are eluted first, followed by mestranol and then EED. By beginning the mestranol fraction at 30 ml, some mestranol is lost in order to remove as

TABLE 2.

Recoveries of Mestranol and EED from Foliage, Soil, and Water Samples Spiked with Both Compounds.

Sample	Sample size	µg added	N	Compound	No. column separations: 1st 5-g Florisil	Gel	2nd 5-g Florisil	Recovery (X): Actual Mean	Actual S.D.	Corr.* Mean
Grass foliage	10 g	1.0	5	Mestranol	1	2	2	40	8.8	65
				EED	1	1	0	49	4.6	69
Mixed foliage	10 g	0.5	2	Mestranol	1	2	4	38	0	75
				EED	1	1	0	56	2.1	79
Soil A	10 g	1.0	2	Mestranol	1	2	2	26	3.5	42
				EED	1	1	0	13	1.4	18
Soil B	10 g	10.0	2	Mestranol	1	2	2	29	4.9	47
				EED	1	1	0	12	2.1	17
Soil C	10 g	10.0	2	Mestranol	1	2	2	30	2.8	49
				EED	1	1	0	34	0.7	48
Water	200 ml	2.0	5	Mestranol	1	0	0	99	7.2	99
				EED	1	0	0	73	7.1	79

* *Corrected for cleanup* losses on the basis of the following recoveries (Table 1): For mestranol, 100% for 1st 5-g Florisil, 86% for each gel, 91% for each 2nd 5-g Florisil; for EED, 92% for 1st 5-g Florisil, 77% for gel.

much of the interfering substances as possible. Some EED is sacrificed in the fourth 10-ml fraction to obtain maximum recovery of mestranol. Beginning the EED fraction at 40 ml also excludes remnants of interfering material that trail, producing a much cleaner fraction for GLC analysis. Recoveries from the gel column averaged 86% for mestranol and 77% for EED (Table 1).

Recoveries from Spiked Samples

Recoveries of mestranol and EED from spiked samples varied with sample type and with the number of steps required for cleanup. Recoveries from five replicate analyses of grass foliage samples spiked at 0.1 ppm averaged 40% for mestranol and 49% for EED, or 65% and 69% after correction for the losses due to the various cleanup steps (Table 2). Recoveries from a mixed foliage sample (field-collected grasses and forbs) spiked at 0.05 ppm averaged 38% for mestranol and 56% for EED, or 75% and 79% after correction.

Recoveries were lower in soil samples, averaging less than 50% even after correction (Table 2). This may have been due to degradation of the compounds by soil microorganisms (STURTEVANT 1971) or to chemical and physical interactions with the soil. Mestranol recoveries averaged 26% from soil A, a podzol type spiked at 0.1 ppm, and 29% and 30% from similar type soils B and C spiked at 1.0 ppm. Recoveries of EED were even lower, presumably because of its greater chemical reactivity due to the slightly acidic hydrogen in the 3-hydroxy position. The better recovery of EED from soil C than from A and B was probably due to relatively large amounts of less adsorbing vegetative debris in soil C.

Good recoveries were obtained from water samples. For a field-collected runoff sample spiked at 0.01 ppm, recoveries averaged 99% for mestranol and 73% for EED, or 99% and 79% after correction (Table 2). The lower EED recoveries in this case apparently reflect a less favorable partitioning of the compound into the chloroform phase.

REFERENCES

APPELGREN, LARS-ERIK, AND R. KARLSSON: Acta Pharmacol. et Toxicol. 29, 65 (1970).

CROUCH, G. L. AND M. A. RADWAN: USDA For. Ser. Res. Note PNW-170, 6 pp (1971).

STALLING, D. L., R. TINDLE, AND J. JOHNSON: J. Ass. Offic. Anal. Chem. 55, 32 (1972).

STURTEVANT, J.: Toxicol. Appl. Pharmacol. 19, 649 (1971).