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Food Safety and Inspection Service, Office of Public Health and Science**

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Revision: .02	Replaces: CLG-MGA.01	Effective: 5-15-03

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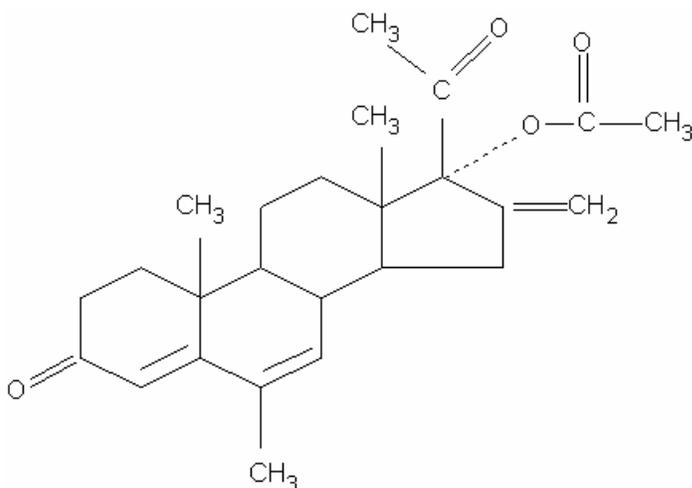
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A. INTRODUCTION

1. Theory and Structure

Melengestrol acetate (MGA) is a progestational agent added to the feed of heifers to suppress estrus and thereby achieve an increase in feed efficiency and the rate of weight gain. It is regulated as a suspect carcinogen in feedlot heifers.

MGA is extracted from bovine fatty tissue with hexane and partitioned into saturated acetonitrile. The acetonitrile mixed with hexane is evaporated to dryness. The residue is redissolved in hexane (while the flask is hot), placed on a Florisil column, washed with 95:5 hexane: acetone, and eluted with 75:25 hexane:acetone. The eluate is evaporated to dryness, re-dissolved in hexane (while the flask is hot), and injected onto an HP 5 cross-linked pH ME siloxane, 15M X 0.32, 0.25 μ m capillary column connected to a gas chromatograph equipped with an electron capture detector.



2. Applicability

This method is applicable to the analysis of MGA in bovine fatty tissue.

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B. EQUIPMENT

Note: Equivalent apparatus or instrumentation may be substituted for any of the following.

1. Apparatus

- a. Flask - boiling, round bottom, 250 mL.
- b. Funnel - separatory, Teflon stopcock, 500 mL
- c. Funnel -100 mL.
- d. Rotary evaporator with control water bath (30-100° C).
- e. Pipettes - volumetric (0.5, 1, 2, 5, and 10 mL).
- f. Flask - volumetric (10, 25, 50, and 100 mL).
- g. Reservoir - 24/40 male joint in the bottom - 500 mL, Kontes, K422450-9007.
- h. Stirring rods.
- i. Beakers - 250 mL.
- j. Glass or Teflon stoppers - sizes 9, 16, and 27.
- k. Water bath with temperature range 30-100° C.
- l. N-EVAP - Model 112 solvent evaporation system, Organomation Associates, Inc. P.O. Box 159, South Bend, MA 01549).
- m. Stopcock - Teflon, variable flow plug, #2 plug size 11/25 mm, Kontes, #821111.
- n. Chromatography columns 400 mm x 19 mm - fitted with medium porosity sintered glass disc, 24/40, Cat. No. K420550, Kontes size 224.
- o. Glass syringe - 10 µL.
- p. Vortex mixer - Thermolyne Maxi Mix model M-16715, Thermolyne Corp., Dubuque, Iowa.
- q. Test tube - 15 mL.
- r. Grinder - Robot Coupe - Model RSI 3Y-1.
- s. Balance - Mettler, Model MT-5.

2. Instrumentation

- a. Gas chromatograph with Ni⁶³ electron capture detector.
- b. Capillary column - HP 5 cross-linked pH ME siloxane, 15M X 0.32, 0.25µm.

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C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for the items below.

1. Reagents

- a. Acetone - HPLC grade, Burdick and Jackson (B&J).
- b. Hexane - Optima grade, Fisher Scientific.
- c. Acetonitrile - HPLC grade, B&J.
- d. Sodium sulfate - anhydrous granular.
- e. Florisil - 60-100 mesh, certified to have been activated at 660 - 675 °C, stored in glass bottle, and put in 130 °C oven for 48 hrs before use. Fisher F-100.
- f. Glass wool - silanized.
- g. Iso-octane - Spectrograde

2. Solutions

- a. 95:5 Hexane/Acetone
Add 95 mL hexane to a 100 mL graduated cylinder. Bring to volume with acetone. Mix.
- b. 80:20 Hexane/Acetone
Add 80 mL hexane to a 100 mL graduated cylinder. Bring to volume with acetone. Mix.
- c. Saturated acetonitrile (acetonitrile saturated with hexane)
- d. 75:25 Hexane/Acetone
Add 75 mL hexane to a 100 mL graduated cylinder. Bring to volume with acetone. Mix.

D. STANDARDS

Note: Equivalent solutions may be substituted for the items below.

1. Source:

The Upjohn Co. (melengestrol acetate, 99.5% pure).

2. Preparation of Standard Solutions

- a. Stock solution I (500 µg/mL):
Quantitatively weigh 50 mg MGA standard and transfer to 100 mL volumetric flask with 80:20 hexane:acetone; dissolve the MGA and dilute to volume with the same solvent mixture.

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- b. Stock solution II (25 µg/mL):
Dilute 5 mL of stock solution I to 100 mL with hexane.
- c. Working Solution I (0.125 µg/mL):
Dilute 0.5 mL of stock solution II to 100 mL in a volumetric flask with 80:20 hexane-acetone reagent.
- d. Working solution II (0.250 µg/mL):
Dilute 1.0 mL of stock solution II to 100 mL with hexane.
- e. Working solution III (0.375 µg/mL):
Dilute 1.5 mL of stock solution II to 100 mL with hexane.
- f. Recovery fortifications
Fortify 12.5 g sample according to the following table:

Volume of Working Solution I (mL)	Concentration of recovery (ppb)
1.0	10
2.0	20
3.0	30

Note: Other volumes may be necessary in order to assure that the samples will be bracketed by the standard curve.

- g. External curve preparation
 - i. Add approximately 1 mL of Working solution I (0.125 µg/mL) to auto-sampler vial. This is equivalent to 10 ppb.
 - ii. Add approximately 1 mL of Working solution II (0.250 µg/mL) to auto-sampler vial. This is equivalent to 20 ppb.
 - iii. Add approximately 1 mL of Working solution III (0.375 µg/mL) to auto-sampler vial. This is equivalent to 30 ppb.
3. Storage Conditions
All standards should be stored in tight sealed glass bottles at 2 - 8° C.
4. Stability
- a. Stock solution: one year.
 - b. Working solution/ External standards: three months.

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E. SAMPLE PREPARATION

Grind submitted samples of bovine fatty tissue in a meat grinder prior to analysis.

F. ANALYTICAL PROCEDURE

1. Sample Extraction

- a. Weigh 12.5 g of tissue into a 250-mL beaker.
- b. Weigh two 12.5 g of blank tissue. Use them as follows:
 - i. 1 control blank. A blank is a previously analyzed tissue that has no target compound in it.
 - ii. 1 recovery standard. Fortify at 20 ppb
 - iii. Prepare an external standard curve with levels of 10, 20, and 30 ppb.
- c. Add about 22 g anhydrous sodium sulfate to the beaker with the tissue. (The sodium sulfate added to the sample in the beaker removes the moisture in the tissue and eliminates emulsions.) Mix the sample with a glass stirring rod.
- d. Add 100 mL 80:20 hexane:acetone.
- e. Place sample in water bath at 55 - 60 °C for 30 minutes or until the fat is melted.
- f. Rinse a 100 mL funnel containing a small silanized glass wool plug and about 22 g anhydrous sodium sulfate (granular) with 20 mL acetone followed by 20 mL hexane, collecting rinse in the 500 mL separatory funnel. Remove the funnel, stopper the separatory funnel with the Teflon or glass stopper, and shake. Discard the rinse and allow the separatory funnel to drain dry. Replace the funnel and close the stopcock.
- g. Filter the hexane/fat mixture through the funnel into the separatory funnel.
- h. Wash the sides of the beaker with 20 mL of hexane and filter as in step g.
- i. Add 75 mL hexane to the beaker and agitate with a stirring rod. Filter into the separatory funnel as in step g.
- j. Add 25 mL acetonitrile saturated with hexane to the beaker and agitate and filter as in step g.
- k. Rinse funnel with 20 mL hexane.
- l. Remove funnel, stopper with the Teflon or glass stopper, and shake vigorously for 1 minute. Wait 15 - 20 minutes for the acetonitrile-hexane to separate.
- m. Collect acetonitrile (bottom layer) in a previously rinsed 250 mL round bottom flask.
- n. Add 25 mL acetonitrile saturated with hexane to the separatory funnel and shake as in step l.

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- o. Repeat step m.
- p. Repeat step n.
- q. Repeat step m.
- r. Repeat step n.
- s. Repeat step m.
- t. Discard the hexane layer.
- u. Evaporate to dryness on a rotary evaporator; water temperature at 90 ± 5 °C.
- v. Add 20 mL hexane to the hot round bottom flask and swirl.
- w. Pack the chromatography column with 10 -12 cm florisil and 5 g anhydrous sodium sulfate. Pack the column by tapping gently.
- x. Wash the column with 50 mL hexane, 50 mL acetone, and 50 mL hexane. Let each wash come to the top of the sulfate bed before adding to the next wash.
- y. Transfer the sample from the round bottom flask to the chromatography column, rinsing the flask with two 20 mL portions of hexane.
- z. Place the reservoirs on the chromatography columns.
- aa. Wash the chromatography column with 400 mL of 95:5 hexane:acetone.
- bb. When the wash from step aa has reached the top of the sulfate bed, close the stopcock.
- cc. Add 85 mL 75:25 hexane/acetone to the chromatography column. Place a 500 mL round bottom flask under the column and collect the eluate at a rate of approximately ≤ 2 drops/second.
- dd. Evaporate the hexane:acetone to dryness on the rotary evaporator at 55 - 60 °C. The dried extract should show no discoloration due to fat co-elution. Immediately add 10 mL hexane to the flask and swirl.
- ee. Transfer the sample to a 15 mL test tube. Evaporate hexane under nitrogen to dryness and add 1.0 mL of iso-octane.
- ff. Vortex approximately 10 seconds.
- gg. The sample is ready for GC injection.

Note: The sample extract may be diluted if necessary.

2. Instrumental Settings and Conditions

Note: System may be adjusted to insure optimum response.

- i. Oven temperature 240 °C (20 min)
- ii. Detector temperature 350 °C

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- | | | |
|------|-----------------------|--------------------|
| iii. | Injection temperature | 240 °C |
| iv. | Injection mode | Set to inject 5 µL |
| v. | Make-up gas for ECD | Argon/methane |
| vi. | Flow rate | 30 - 35 cc/min |
| vii. | Carrier gas | Helium |
| viii | Flow rate | 10 cc/min |
| ix. | Volume injected | 5 µL |

3. Sample Chromatograms
See Section I.K for chromatograms.

G. CALCULATIONS

1. Procedure
- a. Using linear regression, construct a standard curve by plotting Concentration in ng/mL (x), versus GC peak height (y) for all standards. Do not force the curve through the origin.
Acceptable correlation coefficient for standard curve: ≥ 0.9950 .
- b. Compute ppb MGA in each sample using the formula:

$$\text{PPB MGA} = (y-b) (\text{VSE}) (\text{DF}) / (m) (\text{WS})$$

Where:

- y = the observed peak height/area for the injected sample.
m, b = the slope and intercept of the standard curve calculated in step a.
VSE = Final volume of sample extract, in mL.
WS = Weight of sample matrix in grams.
DF = Any dilution factor (Volume of diluted aliquot / Volume of aliquot) that might be applied to the sample extract.

H. HAZARD ANALYSIS

1. Method Title - Determination of Melengestrol Acetate in Bovine Fat.

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2. Required Protective Equipment - Safety glasses, protective gloves, and lab coat.
3. Hazards

<i>Reagents</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Acetone Hexane Acetonitrile Iso-octane	Flammable. Avoid breathing vapors. May cause skin irritation.	Keep in well closed containers in a cool place and away from fire. Use it in well ventilated hood.

4. Disposal Procedures

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Organic Solvents	See above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Chromatography	See above	Columns can be left under the hood until dry; the packing can then be disposed of in the trash can.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Analyte</i>	<i>Analytical Range</i>	<i>Acceptable Recovery (%)</i>	<i>Acceptable Repeatability (CV)</i>
MGA	10 - 30 ppb	70 - 115	≤ 20

Regression coefficient (r) ≥ 0.995

The Measurement Uncertainty and Method Detection Limit should be recalculated yearly or whenever a change that affects method accuracy, precision, or sensitivity occurs.

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2. Critical Control Points and Specifications

	<i>Record</i>	<i>Acceptable Control</i>
a.	Sample weight	12.5 ± 0.1 g
b.	Water bath temperature	55 - 60 °C, N-evap, step F. 1. E 90 ± 5 °C, Rotary evaporator, step F.1. u 55 - 60 °C, N-evap, step F.1.dd

3. Readiness To Perform (FSIS Training Plan)

- a. Phase I: Standards- Standard curve on each of 3 consecutive days, which will include the following:
 - i. 0 ppb (0 µg/mL)
 - ii. 10 ppb (0.125 µg/mL)
 - iii. 20 ppb (0.25 µg/mL)
 - iv. 30 ppb (0.375 µg/mL)

- b. Phase II: Fortified samples- 3 duplicate curves fortified at 0, 10, 20, and 30 ppb over a period of 3 different days using bovine fatty tissue.
Note: Phase I and Phase II may be performed concurrently.

- c. Phase III Check samples for analyst accreditation.
15 bovine fat samples submitted by the supervisor or Quality Assurance Manager (QAM) fortified between 0 and 30 ppb.

- d. Acceptability criteria
Refer to section I.1 above.

4. Intralaboratory Check Samples

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- a. System, minimum contents.
 - i. Frequency: 1 per week per analyst as samples analyzed.
 - ii. Records are to be maintained.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses by that analyst.
 - ii. Take corrective action.
5. Sample Acceptability and Stability
- a. Matrix: Bovine fat (homogenized)
 - b. Sample receipt size, minimum: 50 g.
 - c. Condition upon receipt: Cold
 - d. Sample storage:
 - i. Time: 6 months
 - ii. Condition: Store in freezer
6. Sample Set
- The sample set should include:
- a. a tissue blank
 - b. a tissue blank fortified at 20 ppb
 - c. an external standard curve including 0, 10, 20, and 30 ppb
 - d. up to 18 samples
7. Sensitivity
- a. Lowest detectable level (LDL): 5 ppb.
 - b. Lowest reliable quantitation (LRQ): 10 ppb.
 - c. Minimum proficiency level (MPL): 10 ppb.
- J. WORKSHEET**
- An example of a worksheet on the following page can be removed for photocopying.

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MGA

Analyst: _____ Matrix: _____

Date started: _____ Date completed: _____

Standard Curve Data

Standard	ppb	Peak Height
1		
2		
3		
4		

Linear Regression

b =
m =
r =
s =

Sample Identification	Liquid/Liquid Extraction Emulsion (y/n)	Column Elution 2 drops/sec (y/n)	Sample Weight	Peak Height	Amount Found (ppb)

Remarks:

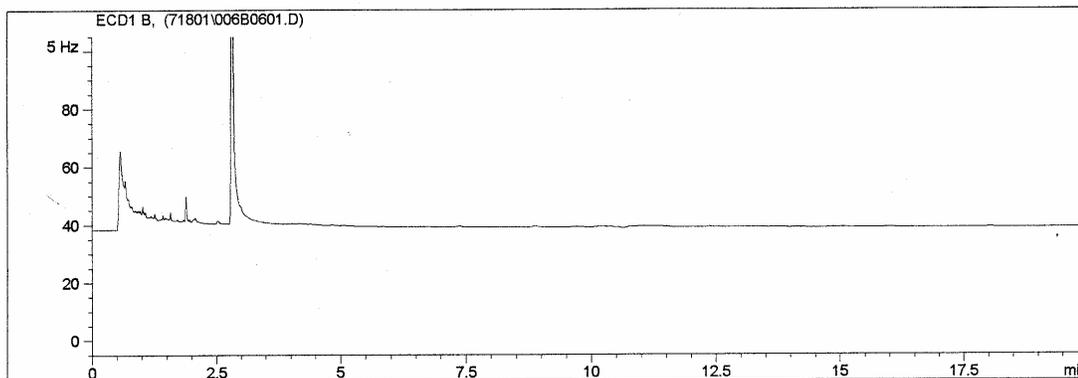
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K. CHROMATOGRAMS AND SPECTRA

1. Control Blank (beef fat)



=====
External Standard Report
=====

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 Multiplier : 1.0000
 Dilution : 1.0000

Signal 1: ECD1 B,

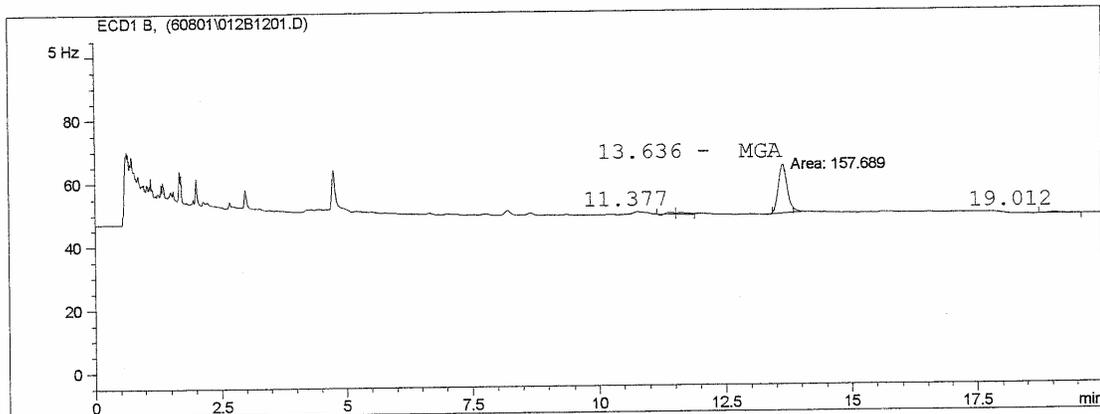
RetTime	Type	Area	Amt/Area	Amount	Grp	Name
[min]		[5 Hz*s]		[ng/mL]		
12.901	-	-	-	MGA		

Totals : 0.00000

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2. Recovery (15 ppb) in beef fat



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External Standard Report
=====

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 Dilution : 1.0000

Signal 1: ECD1 B,

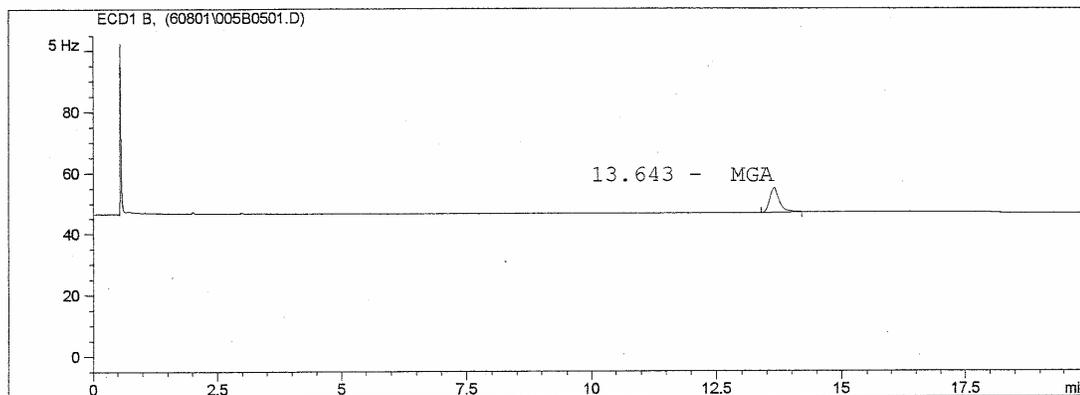
RetTime [min]	Type	Area [5 Hz*s]	Amt/Area [ng/mL]	Amount	Grp	Name
13.636	MM	157.68857	1.51529	238.94336	MGA	

Totals : 238.94336

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3. External Standard (10 ppb)



=====
External Standard Report
=====

Sorted By : Signal
Calib. Data Modified : Thursday, June 07, 2001 3:33:10 PM
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: ECD1 B,

RetTime	Type	Area	Amt/Area	Amount	Grp	Name
[min]		[5 Hz*s]		[ng/mL]		
13.643	PB	108.02644	1.53016	165.29748		MGA

Totals : 165.29748

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Approved By:

Date Approved:

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Terry Dutko	4-7-03
Jess Rajan	4-11-03
Charles Pixley	4-7-03
Phyllis Sparling	4-15-03