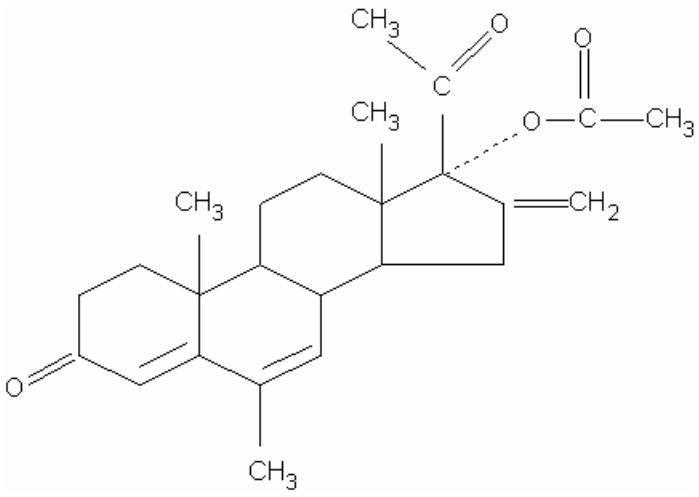
CLG-MGA4.04		Page 1 of 14
Title: Determination of Melengestrol Acetate		
Revision: 04	Replaces: CLG-MGA.03	Effective: December 12, 2007

Contents

A. INTRODUCTION

1. Theory and Structure

Melengestrol acetate (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, 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, and injected onto an HP 5 cross-linked PH ME siloxane 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 fat ≥ 10 ppb.

B. EQUIPMENT

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

1. Apparatus

a. Flask - boiling, round bottom, 250 mL Cat. No.10-101B, Fisher.

b. Funnel - separatory, Teflon stopcock, 500 mL.

c. Funnel -100 mL.

d. Rotary evaporator with control water bath (30-100 °C).

e. Pipettes - 0.5, 1, 2, 5, and 10 mL, Eppendorf .

f. Flask - volumetric (10, 25, 50, and 100 mL), Class A.

g. Reservoir - 24/40 male joint in the bottom - 500 mL, Cat. No. K422450-9007, Kontes.

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 37600, Thermolyne Corp.

q. Test tube - 15 mL.

r. Grinder - Robot Coupe - Model No. RSI 3Y-1.

s. Balance - Model No. PJ3000, Mettler.

2. Instrumentation

a. Gas chromatograph with Ni^{63} electron capture detector.

b. Capillary column - HP 5 cross-linked PH ME siloxane, 15M X 0.32, 0.25µm.

c. Liners – Agilent sp/less single taper glass wool Part No. 5062-358.

d. Ferrules – Agilent 0.5 mm ID Vespel/Graphite Part No. 5062-3514.

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, J.T Baker.

g. Isooctane – Spectrograde, Burdick and Jackson (B&J).

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):

      Add hexane to 2 liters of acetonitrile in 4 L bottle until about one centimeter of hexane is present above the surface of the hexane saturated acetonitrile. Shake.

   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:

   Melengestrol Acetate 250 mg Cat. No. 0215895283, MP Biomedicals.

2. Preparation of Standard Solutions

   a. Stock solution I (500 µg/mL):

      Dissolve an appropriate amount of MGA standard in 80:20 hexane:acetone to make 100 mL with a concentration of 500 µg/mL.

   b. Stock solution II (25 µg/mL):

      Dilute 5 mL of stock solution I to 100 mL with hexane or isooctane.

   c. Working Solution I (0.125 µg/mL):

      Dilute 0.5 mL of stock solution II to 100 mL in a volumetric flask with hexane or isooctane.

   d. Working solution II (0.250 µg/mL):

      Dilute 1.0 mL of stock solution II to 100 mL with hexane or isooctane.

   e. Working solution III (0.375 µg/mL):

      Dilute 1.5 mL of stock solution II to 100 mL with hexane or isooctane.

   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 (based on 12.5 g sample).

ii. Add approximately 1 mL of Working solution II (0.250 µg/mL) to auto-sampler vial. This is equivalent to 20 ppb (based on 12.5 g sample).

iii. Add approximately 1 mL of Working solution III (0.375 µg/mL) to auto-sampler vial. This is equivalent to 30 ppb (based on 12.5 g sample).

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.

E. SAMPLE PREPARATION

Grind 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 MGA in it.

ii. 1 recovery standard. Fortify at 20 ppb (D.2.f)

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 a 500 mL 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 and filter as in step g.

l. Remove the funnel, then stopper the separatory funnel with a Teflon or glass stopper, and shake vigorously for 1 minute. Wait for the acetonitrile-hexane to separate.

m. Collect acetonitrile (bottom layer) in a previously rinsed 250 mL round bottom flask or 250 mL Erlenmeyer flask.

n. Re-extract contents of the separatory funnel three times with 25 mL of the acetonitrile saturated with hexane as described in steps l - m. Add the extracts to the same 250 mL round bottom flask or 250 mL Erlenmeyer flask. Discard the hexane layer.

o. Evaporate to dryness on a rotary evaporator; water temperature at 90 $\pm$ 5 °C.

p. Add 20 mL hexane to the round bottom flask or Erlenmeyer flask and swirl.

2. Column Chromatography

a. Push a small wad of silanized glass wool into the column. Then pack the chromatography column (B.1.m.) with 10 -12 cm florisil and about 5 g anhydrous sodium sulfate. Pack the column by tapping gently.

b. Prewash 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.

c. Transfer the sample from the round bottom flask or Erlenmeyer flask to the chromatography column, rinsing the flask with two 20 mL portions of hexane.

d. Place the reservoirs (B.1.g) on the chromatography columns.

e. Wash the chromatography column with 400 mL of 95:5 hexane:acetone.

f. When the wash from step e has reached the top of the sulfate bed, close the stopcock. Discard the eluate.

g. Add 85 mL75:25 hexane/acetone to the chromatography column. Place a

500 mL round bottom flask or 250 mL Erlenmeyer flask under the column and collect the eluate at a rate of approximately 2 drops/second.

h. Evaporate the hexane:acetone to dryness on the rotary evaporator at 55 - 60 °C. The dried extract should show no discoloraton due to fat co-elution. Immediately add 10 mL hexane to the flask and swirl.

i. Transfer the sample to a 15 mL test tube. Evaporate hexane using N-evap at 55 - 60 °C under nitrogen to dryness and add 1.0 mL of iso-octane.

j. Vortex approximately 10 seconds.

k. The sample is ready for GC injection.

Note: The sample extract may be diluted if necessary.

3. Instrumental Settings and Conditions

The gas chromatograph is equipped with Ni^{63} electron capture detector and a capillary column - HP 5 cross-linked PH ME siloxane.

Note: System may be adjusted to insure optimum response.

a.	Oven temperature	240 °C (20 min)
b.	Detector temperature	350 °C
c.	Injection temperature	240 °C
d.	Injection mode	Set to inject 4 µL
e.	Make-up gas for ECD	Argon/methane or nitrogen
f.	Flow rate	30 - 35 mL/min argon/methane or 50-60 mL/min nitrogen.
g.	Carrier gas	Helium
h.	Flow rate	10 cc/min
i.	Volume injected	4 µL

4. Sample Chromatograms

See Section 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/area (y) for all standards. Do not force the curve through the origin.

Acceptable correlation coefficient (r) for standard curve: ≥ 0.995.

b. Compute ppb MGA in each sample using the formula:

PPB MGA = (y-b) (VSE) (DF) / (m) (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. Required Protective Equipment - Safety glasses, protective gloves, and lab coat.

2. Hazards

Reagents	*Hazard*	*Recommended Safe Procedures*
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.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
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 (B.1.m) 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

Analyte	*Analytical Range*	*Acceptable Recovery (%)*	*Acceptable Repeatability (CV)*
MGA	10 - 30 ppb	70 - 115	$\leq$ 20

Correlation coefficient (r) $\geq$ 0.995.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	12.5 ± 0.1 g
b.	Water bath temperature	55 - 60 °C, Water bath, step F. 1. e.
		90 ± 5 °C, Rotary evaporator, step F.1. o.
		55 - 60 °C, Rotary evaporator, step F.1.x
		55 - 60 °C, N-evap, step F.1. y.

3. Readiness to Perform

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.

Eight (8) 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
   a. System, minimum contents.
      i. Frequency: 1 per week per analyst when samples are 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 recovery fortified at 20 ppb.
   c. an external standard curve solutions of including 0, 10, 20, and 30 ppb.
   d. samples.

7. Sensitivity
   a. Minimum proficiency level (MPL): 10 ppb.

J. WORKSHEET

Following is an example of a worksheet.

MELENGESTROL ACETATE (MGA) DETERMINATIVE DATASHEET

Analyst: _____

Date Started: _____

Date Completed: _____

Set #: _____

Stopping Points

Step	Date	Time
1		From:_____ To:_____
2		From:_____ To:_____
3		From:_____ To:_____
4		From:_____ To:_____

Equipment	ID #	Temp, °C
Balance		-
Waterbath (55-60 °C)		
Rotovap I (90 ± 5 °C)		
Rotovap II (90 ± 5 °C)		
Rotovap I (55-60 °C)		
Rotovap II (55-60 °C)		
N-Evap (55-60 °C)	PR-EVAP-	
GC	GC #	-

Reagents	ID #
Fortification Standard	
80:20 Hexane/Acetone	
Hexanes	
Acetone	
Acetonitrile saturated with hexane	
Sodium Sulfate	
Florisil	
Iso-octane	

95:5 Hexanes/Acetone	
75:25 Hexanes/Acetone	
Working Solution I (0.125 ug/mL)	
Working Solution II (0.250 ug/mL)	
Working Solution III (0.375 ug/mL)	

Samples stored in _____

Sample Analysis Data

Sample No.	Internal Laboratory Numbe	Sample Wt.(12.50 ± 0.10 g)	MGA (ppb)*
0			
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Reviewed By Initials and Date _____

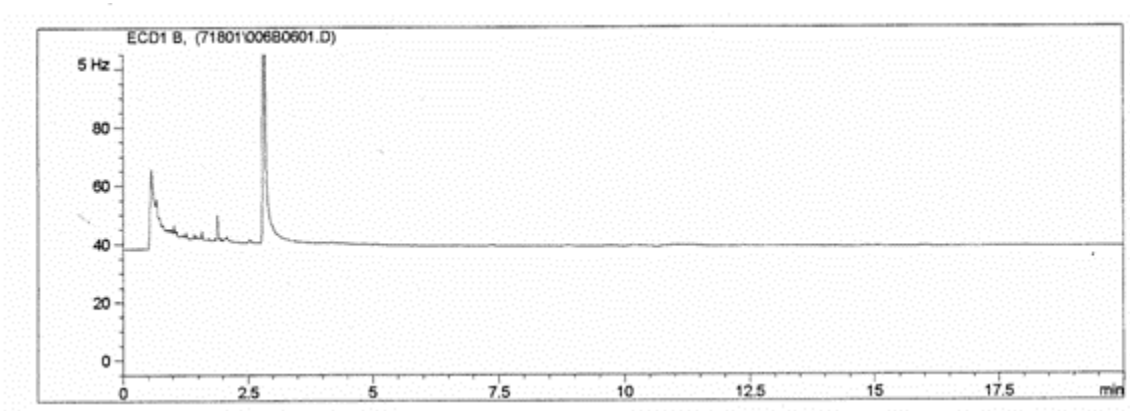
Supervisor Initials and Date _____

Approved/Date:

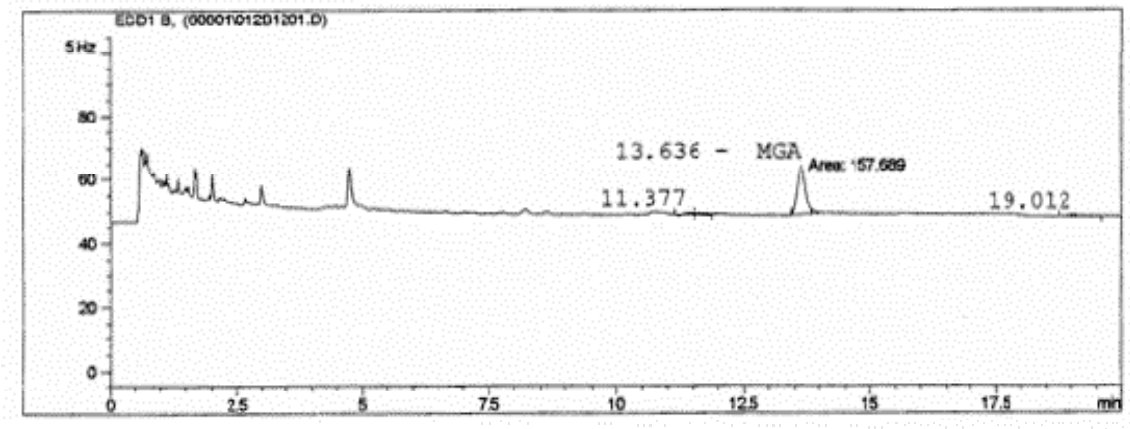
K. APPENDIX

1. Sample Chromatograms

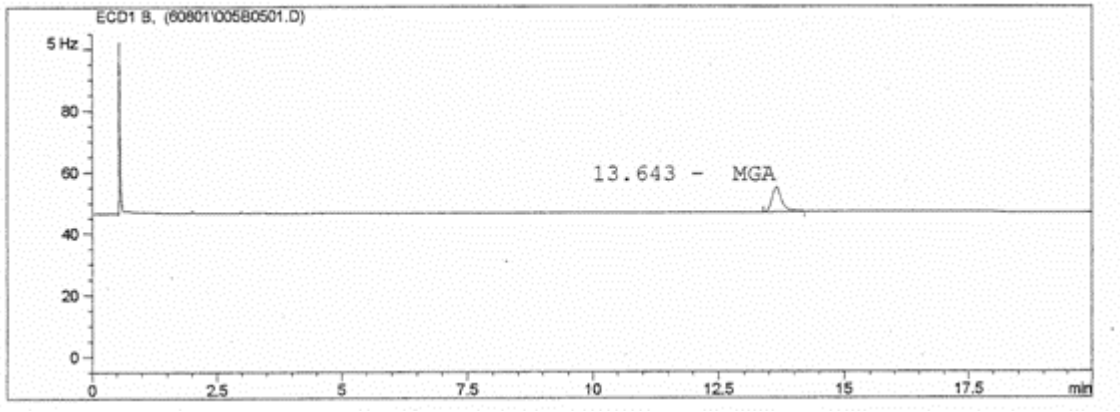
   a. Control Blank (beef fat)



   b. Recovery (15 ppb) in beef fat



c. External Standard (10 ppb)



2. Reference:

Krzeminski, Leo F.; Geng, Shu.; Cox, Byron L. Determination of Melengestrol Acetate in Bovine Tissue. *J. AOAC International.* **1976**, 59, 507-515.

L. APPROVALS AND AUTHORITIES

Approvals are on file.

Issuing Authority: Laboratory Quality Assurance Division.