CLG-HRM.00		Page 1 of 16
Title: Determination of Multiple Hormones in Beef Muscle by LC/MS/MS		
Revision: Original	Replaces: NA	Effective: 09/15/2014

Contents

A. INTRODUCTION

1. Summary of Procedure

   This multi-hormone method uses acetonitrile extraction, followed by acidification and subsequent liquid-liquid partitioning with ethyl acetate. The extract is eluted through both a Bond Elut SAX column (anion exchange mechanism) followed by a Bond Elut C_{18} SPE column, filtered, and injected onto LC/MS/MS (APCI).

2. Applicability

   This method is suitable for the determination of melengestrol acetate (MGA), megestrol (MEG), hexestrol (HEX), and zeranol (ZER) in beef muscle at levels ≥ 1 ng/g (ppb).

   Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

   a. Micropipettors, adjustable - to deliver 100, 200, and 1000 µL, Rainin.

   b. Analytical balance - 0.1 mg sensitivity.

   c. Autosampler vials - 2 mL with accompanying clean 11 mm snap seal caps with pre-slit PTFE/Sil liner, catalog numbers N9303418 and N9303416, Perkin Elmer.

   d. Centrifuge - refrigerated, contains a swing-out rotor for 15 mL and 50 mL tubes and temperature control between 0 and 30˚C, catalog number 75-004-521, Thermo Scientific.

   e. Volumetric flasks - Clear-glass, 50,100, and 1000 mL, Class A, catalog numbers K92810W-50, 10-212-7A, and 13-756-687, Fisher Scientific.

   f. Polypropylene centrifuge tubes - disposable, 15 and 50 mL, catalog numbers14-959-49B and 14-432-22, Fisher Scientific.

   g. Disposable glass culture tubes - 12 x 75 mm, catalog number 14-962-10B, Fisher Scientific.

   h. Analog multi-tube vortexer - catalog number 58816-115, VWR.

   i. Solvent evaporator - with capability to hold 15 mL and 12 x 75 mm test tubes, Turbo Vap LV, catalog number C103198, Biotage.

   j. Solid phase extraction manifold - 12-port, catalog number 57160-U, Sigma-Aldrich.

k. SAX solid phase extraction column - 500 mg, 3 mL, Bond Elut catalog number 12102044, Agilent Technologies.

l. C_{18} solid phase extraction column - 500 mg, 3 mL, Bond Elut catalog number 12102028, Agilent Technologies.

m. Polypropylene syringes - 3 mL, luer lock, catalog number Z192104, Sigma-Aldrich.

n. Syringe filters - 13 mm, 0.2 µm, PTFE, Acrodisc CR catalog number 629-4423, KSE Scientific.

o. Ultrasonic cleaner - catalog number 97043-972, VWR.

p. Vortexer - Vortex-Genie 2, catalog number 58815-234, VWR.

q. Solid borosilicate glass beads - 4 mm, catalog number 13500-4, Kimball Chase.

2. Instrumentation

a. HPLC-QQQ system - Model G6410A, Agilent Technologies

b. Liquid chromatography column - Poroshell 120 EC-C18, 100 x 3.0 mm, 2.7 µm and C-18 guard column cartridge (C-18, 4 x 3 mm), catalog numbers 695975-302 and 823750-911, Agilent Technologies.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

a. Methanol, HPLC grade, Catalog Number AH230-4, Honeywell Burdick & Jackson

b. Acetonitrile, HPLC grade, Catalog Number AH015-4, Honeywell Burdick & Jackson.

c. Ethyl Acetate, HPLC grade, Catalog Number AH100-4, Honeywell Burdick & Jackson.

d. 1N Hydrochloric Acid, Catalog Number SA48-1, Fisher Scientific.

e. Nitrogen, 99.9% Purity (for sample evaporation)

2. Solutions

a. Methanol/Water - 50/50 v/v

Mix 500 mL of methanol with 500 mL of millipore water. Solution may be stored up to 1 year at room temperature.

b. Methanol/Water - 40/60 v/v

Mix 400 mL of methanol with 600 mL of millipore water. Solution may be stored up to 1 year at room temperature.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

a. Hexestrol (HEX) - Catalog Number H0200, Steraloids, Inc.

b. Megestrol (MEG) - Catalog Number P0952-000, Steraloids, Inc.

c. Melengestrol acetate (MGA) - Catalog Number P0951-030, Steraloid, Inc.

d. Zeranol (ZER) - Catalog Number Z0200-000, Steraloids, Inc.

Internal Standards (Optional)

e. Zeranol-d_4 -Catalog Number RIKILT-0006, Sigma-Aldrich RTC.

f. Melengestrol-d_3 -Catalog Number RIKILT-0014, Sigma-Aldrich RTC.

2. Preparation of Standard Solution(s)

a. Concentrated Individual Stock Solutions (~ 100 ng/µL):

Accurately weigh 10 ± 2 mg of each compound to the nearest 0.1 mg. Quantitatively transfer to a 100 mL volumetric flask. Bring to volume with methanol and mix well. Correct for purity if necessary and calculate final concentration of each in ng/µL.

b. Intermediate Stock Solution of Positive Hormones (2.00 ng/µL):

Calculate the volume of each positive hormone (MGA, and MEG) stock solution (D.2.a) necessary to prepare 100 mL of a 2.00 ng/µL solution (~ 2.0 mL). Using an automated micropipettor, accurately measure these volumes into a single 100 mL volumetric flask. Bring to volume with methanol and mix well.

c. Intermediate Stock Solution of Negative Hormones (2.00 ng/µL):

Calculate the volume of each negative hormone (HEX and ZER) stock solution (D.2.a) necessary to prepare 100 mL of a 2.00 ng/µL solution (~ 2.0 mL). Using an automated micropipettor, accurately measure these volumes into a single 100 mL volumetric flask. Bring to volume with methanol and mix well.

d. Working Stock Solution of All Hormones (1.00 ng/µL):

Accurately measure 25 mL of the Intermediate Stock Solution of Positive Hormones (D.2.b) and 25 mL of the Intermediate Stock Solution of Negative Hormones (D.2.c.) into a 50 mL volumetric flask to prepare a 1.00 ng/µL mixed solution. Alternatively, mix equal volumes of the two intermediate stock solutions.

e. Standard Solutions for Fortification of Samples and for Preparation of Calibration Standards:

Prepare standard solutions as described in Table 1 using volumetric flasks and automated micropipettors. Bring to volume with methanol.

Table 1. Preparation of Calibration and Fortification Standards

Standard Name	Stock	Volume (mL) of stock	Volume (mL) of methanol	Final Concentration (ng/µL)
AHORM #4	Working Stock (D.2.d)	0.800	9.200	0.0800
AHORM #3	Working Stock (D.2.d)	0.400	9.600	0.0400
AHORM #2	Working Stock (D.2.d)	0.200	9.800	0.0200
AHORM #1	Working Stock (D.2.d)	0.100	9.900	0.0100

Note: Internal standards are optional in this method. If internal standards are not used, appropriate volumes and chemicals must be adjusted as outlined in appropriate method steps below.

f. Internal Standard Stock Solutions (2 ng/µL):

Internal standard solutions are prepared following the manufacturer's instructions. Allow the ampoule to warm to room temperature and open it by carefully breaking the neck. Pipette 1.00 mL of ethanol into the ampoule. Seal the opening of the ampoule with parafilm and vortex for at least 1 minute. Sonicate the ampoule for at least 5 minutes, quantitatively transfer to a 50 mL volumetric flask with methanol, and dilute to volume. Store the solution at ≤ -10˚C. The internal standard solutions are stable for two years at ≤ -10˚C.

g. Working Mixed Internal Standard (IS) Solution (0.04 ng/µL):

Accurately measure 2.00 mL each of the stock solution (D.2.f) into a 100 mL volumetric flask. Bring to volume with methanol. Store the solution at ≤ -10˚C. The working mixed internal standard solution is stable for two years at ≤ -10˚C.

h. Working Standards for the Calibration Curve:

To prepare calibration standards, use automated micropipettors to pipette 100 µL of the standard listed in the second column of Table 2 into 2 mL autosampler vials. Add 50 µL of the working mixed internal standard (D.2.g) and 850 µL of 40/60 (v/v) methanol/water to each autosampler vial and vortex. The final volume is 1000 µL. After preparation, the calibration curve standards are stable for one year at ≤ -10˚C.

Table 2. Preparation of Standard Solutions for Calibration Curves

Calibration Standard	From Table 1 Pipette 100 µL of:	µL of 40/60(v/v) MeOH/H_2O	µL of IS *	IS ppb in 2g tissue *	Hormone ppb in 2g tissue	Final Conc in 1000 µL (ng/µL)
STD#1, 0.50 ppb	AHORM#1 (0.010 ng/µL)	850	50	1.00	0.500	0.00100
STD#2, 1.0 ppb	AHORM#2 (0.020 ng/µL)	850	50	1.00	1.00	0.00200
STD#3, 2.0 ppb	AHORM#3 (0.040 ng/µL)	850	50	1.00	2.00	0.00400
STD#4, 4.0 ppb	AHORM#4 (0.08 ng/µL)	850	50	1.00	5.00	0.00800

* - Addition of IS is optional.

Note: The volume of acetonitrile will be increased by 50 µL to maintain a total volume of 1 mL if no internal standard solution is added.

E. SAMPLE PREPARATION

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10˚C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Homogenize samples and store frozen (< -10˚C) prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. One negative control/blank (i.e. hormone-free) and one fortified control/recovery (2.0 ppb) must be included in the sample batch. See fortification table in appendix for complete details for preparation of fortified tissues.

      Note: The optional working internal standard solution (D.2.g.) is added to all samples at 1 ppb in 2 g tissue.

2. Extraction Procedure

   a. Weigh 2.0 ± 0.2 g of homogenized tissue into a 50 mL polypropylene centrifuge tube.

      Note: If using the optional internal standard, fortify all samples with the mixed internal std solution (D.2.g., 0.04 ng/µL) See fortification table in appendix for full details.

   b. Add 6 mL of ACN and 3 - 4 solid glass beads to each sample tube and briefly vortex or shake each sample individually until the tissue begins to disperse. Vortex for 5 min on multi-tube vortexer.

   c. Centrifuge at ~ 3000 g (3800 rpm) for 5 min at 4 ± 1˚C.

   d. Pour the ACN layer into a clean 15 mL polypropylene (PP) centrifuge tube.

   e. Add a second 6 mL aliquot of ACN to the tissue pellet (step F.2.a) and vortex a second time for 5 min on the multi-tube vortexer.

   f. Centrifuge at ~3000 g (3800 rpm) for 5 min at 4 ± 1˚C.

   g. Combine the ACN layers into the 15 mL PP centrifuge tube (tube 1) from step d.

      Stopping Point: The procedure may be stopped at this point. The samples can be stored overnight in a refrigerator.

   h. Evaporate the combined ACN layers to ~1 – 2 mL or until the sample volume remains unchanged using an evaporator set at 40 ˚C.

3. Liquid-Liquid Extraction

   a. Add 1 mL of 1N HCl solution to acidify the aqueous layer (F.2.h) to pH ~2.

   b. Partition each sample twice with 6 mL of ethyl acetate. During each partition, mix the samples by gently inverting the tubes 10 - 20 times.

   c. Centrifuge at ~3000g (3800 rpm) for 2 - 5 min at 4 ± 1˚C for phase separation. Combine the ethyl acetate layers into respective new 15 mL polypropylene centrifuge tubes, via plastic disposable pipettes.

   d. Evaporate the combined ethyl acetate layers to dryness (some residue water may remain) using an evaporator set at 40˚C.

e. Add 1 mL of methanol, vortex for 15 s, and sonicate for 2 min. Add 1 mL of H_2O and vortex for 15 seconds.

Note: This methanol wash of the sides of the tubes is essential to obtain a good recovery of analytes,

4. SAX Cleanup:

Condition each SAX (Bond Elut, 500 mg, 3 mL) column with 2 mL methanol followed by 2 mL 50% aqueous methanol.

a. Place a rack of new 15mL polypropylene centrifuge tubes into the vacuum manifold. Load the solutions from step F.3.e. onto the corresponding SAX columns. The eluent must drip at ~2 drops/second. Collect the eluate into respective 15 mL polypropylene centrifuge tube.

b. Add 2 mL of 50% aqueous methanol to each empty sample tube, vortex for 15 seconds, and sonicate for 2 min. Add this rinse to the corresponding SPE columns. Continue collecting this effluent.

c. Vacuum dry the SPE column for 2 min.

d. Take out the sample rack; add 4.5 mL of H_2O to each sample, and vortex for 15 seconds. The final volume should be ~9 mL.

5. C18 SPE Cleanup:

a. Condition each C_{18}SPE column with 3 mL methanol and 3 mL H_2O.

b. Load the solution from step F.4.d. onto a conditioned C_{18}SPE column and elute into a waste container positioned in the manifold. Let the solvent drip at ~1 drop/second.

c. Rinse each sample tube with 1 mL methanol, vortex for 15 seconds, and sonicate for 2 min. Add 2 mL of H_2O to each sample and vortex. Pour the rinse onto the SPE column and elute into a waste container positioned in the manifold.

d. Dry the column for ~1 min (longer if needed).

e. Place glass 12 x 75mm tubes into the vacuum manifold. Elute the analytes off the column with 5 mL ACN at a rate of ~2 drops/second. Dry the column for ~2 min.

f. Concentrate the eluate to dryness using a evaporator set at 40 ˚C.

g. Reconstitute the residue in 1000 µL of methanol/H_2O (40/60, v/v).

h. Vortex for 15 seconds and sonicate for 2 min.

i. Filter the solution with a BD syringe through an Acrodisc PTFE filter (13 mm, 0.2 µm) into a 2 mL autosampler vial.

j. Inject 20 µL onto LC-MS/MS.

Stopping Point: The samples can be stored at <-10˚C for one week, pending LC-MS/MS analysis.

6. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. HPLC Operating Parameters

i. LC System: Agilent 1200

ii. HPLC column: Poroshell 120 EC-C18, 100 x 3.0 mm, 2.7 µm

iii. Guard column: Same material as analytical column

iv. Oven temperature: 40°C

v. Mobile Phase: Isocratic Elution MeOH/H_2O/ACN (30/40/30)

vi. Flow: 0.400 mL/min

vii. Autosampler temperature: 10°C

viii. Injection volume: 20 µL

ix. Run time: 12.5 min

x. Pressure: ~ 150 - 200 bar

xi. Needle wash: Methanol

b. MS/MS Operating Procedures

i. MS/MS Instrument: Agilent 6410 Triple Quad LC/MS

ii. Source: APCI probe

iii. Gas Temperature: 325°C

iv. Vaporizer: 350°C

v. Gas Flow: 4 L/min

vi. Nebulizer: 20 psi

c. LC-MS/MS Transitions

The protonated molecules [M+H]+ are selected as the precursor ions for multiple reaction monitoring (MRM) in the positive ion mode, and the deprotonated [M-H]- molecules for MRM in the negative ion mode. See Appendix.

Table VI. MRM transitions for analysis of hormones in positive ion mode

Compound	Retention Time	Precursor Ion	Daughter Ions	Collision Energy	Fragmentor
Melengesterol Acetate	8.9	397.2	337 279*	20	135
Megestrol	5.2	343.2	325 267*	20	135

* - Quantitative ion

Table VII. MRM transitions for internal standards in the positive ion mode

Compound	Retention Time	Precursor Ion	Daughter Ions	Collision Energy	Fragmentor
Melengesterol d_3	5.1	358.1	282.4	20	100

Table VIII. MRM transitions for hormone analysis in the negative ion mode

Compound	Retention Time	Precursor Ion	Daughter Ions	Collision Energy	Fragmentor
Zeranol	3.0	321.2	303.2 277.2*	18 20	100
Hexestrol	4.1	269	119* 134	40 8	100

* - Quantitative ion

Table IX. MRM transitions for the internal standards in the negative ion mode

Compound	Retention Time	Precursor	Daughter Ions	Collision Energy	Fragmentor
Zeranol d_4	3.0	325.1	281.2	20	100

7. Sample Set

Note: Each sample set must contain one QA sample/20 samples.

a. Calibration standard(s).

b. Negative Control/Blank sample.

c. Fortified Control/Recovery sample

d. Check sample (if necessary)

e. Samples.

f. Calibration standard at the appropriate level.

G. CALCULATIONS / IDENTIFICATION

1. Peak areas of analytes and internal standards are used for quantitation.

2. For analytes without internal standards, construct calibration curves by plotting the peak areas against the analyte concentrations. For analytes with internal standards, construct calibration curves by plotting the peak area rations between the analyte and the internal standard as the y-axis against the concentration of standards.

3. Calculate the regression parameters for the analyte calibration curve and interpolate the concentrations of the test samples from the regression parameters. Do not use the origin as a data regression point. Determine sample concentrations by linear regression, using the formula $Y = mx + b$ where:

   Y = either peak area or area ratio

   X = concentration of standards (ppb)

   The coefficient of correlation (r) must be ≥ 0.990.

4. The baseline as determined by automated data system analysis should be individually inspected for each standard or sample.

5. The final sample concentration (except for control and fortified samples) is correct for mass by taking into account the mass difference of the individual samples to the nominal 2 g samples use to calculate the concentrations of analyte in the calibration standards.

$$\text{Concentration in tissue (ppb)} = \frac{\text{Experimental ppb X 2}}{\text{Weight of sample (g)}}$$

6. The accuracy is calculated by dividing the concentration found in fortified samples by their theoretical concentration and expressing the results as a percentage to at least two significant figures.

$$\text{Accuracy (\%)} = \frac{\text{(ppb found) * 100}}{\text{(Theoretical ppb)}}$$

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety eyewear, protective gloves and lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Acetonitrile, Methanol, Ethyl Acetate	Flammable	Keep in well-closed containers away from ignition sources. Avoid contact or prolonged exposure to vapors. Keep away from flame or heat. Work in ventilated fume hood. Wear protective eyewear, gloves, and protective clothing.
HCl	Corrosive. Contact with skin can result in burns and skin and eye irritation.	Wear appropriate personal protective equipment to avoid contact.
Zeranol Hexestrol Megestrol MGA	Endocrine pharmacological effects (e.g. menstrual irregularities in women, gynecomastia in men.) May have androgenic and anabolic action. Suppressive effects on fertility. Teratogenic effects possible.	Wear appropriate personal protective equipment to avoid contact.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. Calibration curves should have a Coefficient of Determination (r^2) ≥ 0.990.

b. The calculated accuracy for fortified samples should be 60-110% with coefficients of variation (CV) ≤ 20%.

c. For blank, verify the absence of analytes above 5% of the recovery or sample concentration.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	2.0 ± 0.2 g
b.	Methanol wash (step F 3.)	Rinse sides of tube
c.	SPE columns	Do not allow to dry during conditioning steps

3. Intralaboratory Check Samples
   a. System, minimum contents.
   b. Frequency: One per week per analyst when samples analyzed.
   c. Records are to be maintained.
   d. Acceptability criteria.
      Refer to I. 1.
      If unacceptable values are obtained, then:
      i. Investigate following established procedures.
      ii. Take corrective action as warranted.

4. Sample Condition upon Receipt: Cold

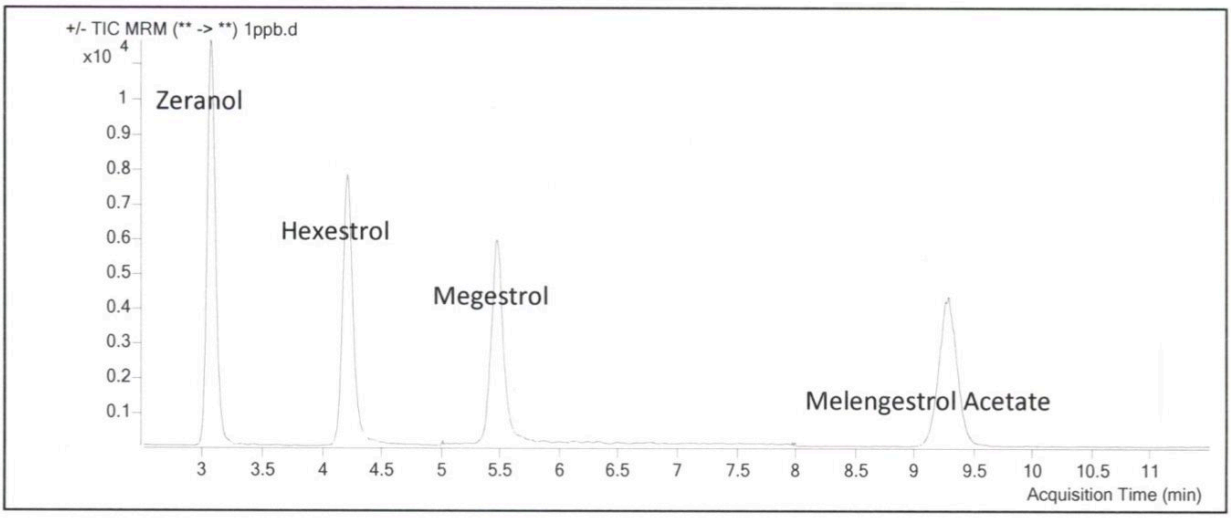
J. APPENDIX

1. References

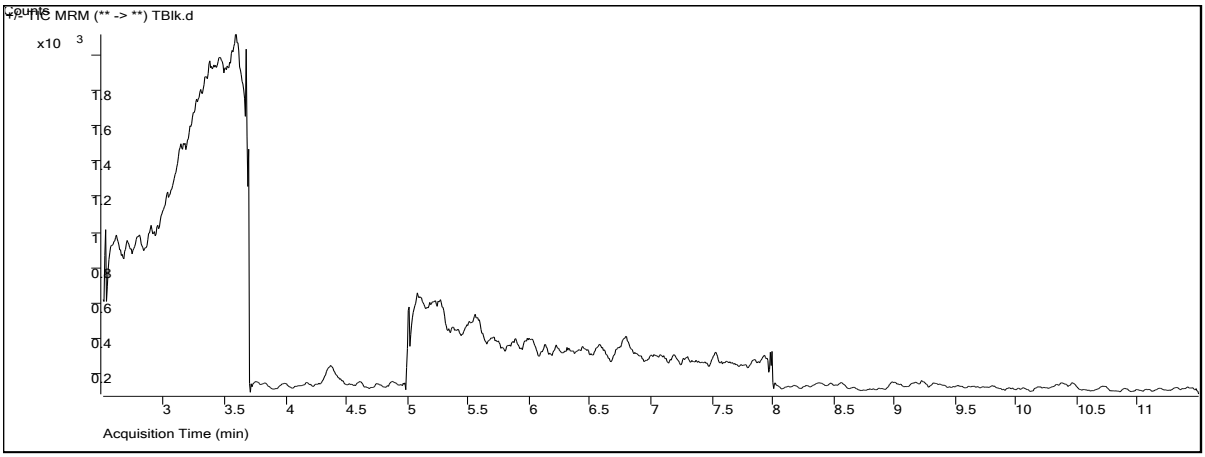
Chu, Pak-Sin and Tricia Johnson. LC-MS/MS Determination of Multiple Hormones in Beef Muscle. CVM Office of Research, Division of Residue Chemistry, FDA. November 2012.

2. Chromatograms/spectra

a. 1 ppb std



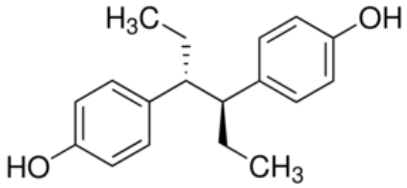
b. Blank sample



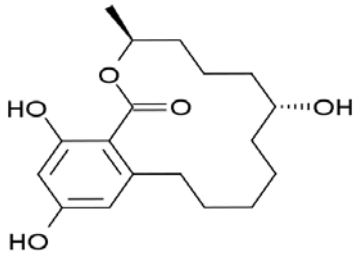
3. Minimum Level of Applicability for all analytes: 1 ppb

4. Structures of Hormones

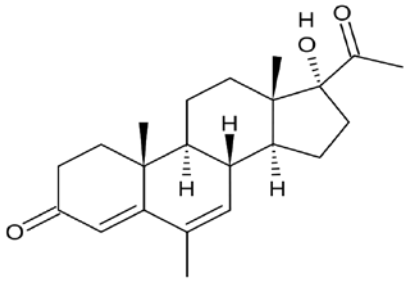
a. Hexestrol



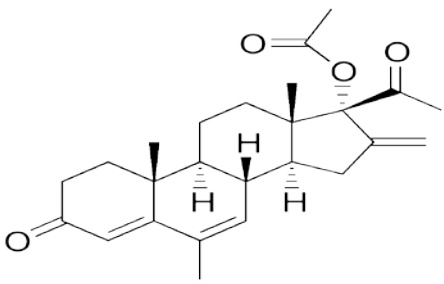
b. Zeranol



c. Megestrol



d. Melengestrol Acetate



5. Table of Fortification of Tissues

Sample	Tissue (g)	Pipette 100 µL of:	µL of working IS (0.04 ng/µL)*	IS Conc (ppb)*	Nominal Hormone Conc (ppb)
Negative Control	2	None	50	1.00	0
Fortified 1.0 ppb	2	AHORM#2(0.0100 ng/µL)	50	1.00	0.500
Fortified 2.0 ppb	2	AHORM#3(0.0200 ng/µL	50	1.00	1.00
Fortified 4.0 ppb	2	AHORM#4(0.0400 ng/µL	50	1.00	2.00

*** Optional**

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff.